Molecular Cloning, Sequence Analysis, Expression, and Tissue Distribution of Suppressin, a Novel Suppressor of Cell Cycle Entry*

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Suppressin (SPN) is an inhibitor of cell proliferation that was originally identified and purified to homogeneity from bovine pituitaries (LeBoeuf, R. D., Burns, J. N., Bost, K. L., and Blalock, J. E. (1990) J. Biol. Chem. 265, 158–165). In this report we have cloned the full-length cDNA encoding rat SPN and have identified the tissue distribution of SPN expression. The cDNA of SPN is 1882 nucleotides with a 1488-base coding region and 55 and 339 nucleotides of 5'- and 3'-untranslated sequences, respectively. Northern gel analysis of rat pituitary mRNA showed a single hybridizing species at ~2 kilobases. Sequence analyses showed that the nucleotide and deduced amino acid sequences of SPN are novel and unrelated to any known vertebrate inhibitors of proliferation. However, the deduced amino acid sequence of SPN contains two domains that have extensive sequence identity with a recently cloned transcription activator in Drosophila, deformed epidermal autoregulatory factor-1 (DEAF-1, see Gross, C. T., and McGinnis, W. (1996) EMBO J. 15, 1961–1970) suggesting that SPN represents a vertebrate cognate of deformed epidermal autoregulatory factor-1. Reverse transcriptase-polymerase chain reaction and immunohistochemical analyses showed that the SPN mRNA and the SPN protein are expressed in every tissue examined including testis, spleen, skeletal muscle, liver, kidney, heart, and brain suggesting that SPN may be involved in the control of proliferation in a variety of cell types.

Suppressin (SPN) is a 63-kDa monomeric protein identified and purified to homogeneity from the bovine pituitary based on its ability to inhibit mitogen-stimulated murine splenocyte proliferation (1). Initial immunologic and species-specificity studies indicated that SPN was a structurally conserved molecule. Specifically, (i) anti-SPN antibodies prepared against purified bovine SPN cross-reacted with human, mouse, and rat SPN (1–4), and (ii) purified bovine SPN was active on mouse, rat, and human cells. Other cross-species activities could also be shown for SPN (e.g. human SPN was active on rat cells). In the rat pituitary, SPN production is restricted to five hormone-secreting cell phenotypes (somatotrophs, lactotrophs, corticotrophs, thyrotrophs, and mammomatosatrophs) in the anterior pituitary (2). The primary biological activity, inhibition of cell proliferation, has been most extensively studied in vitro in murine and human lymphocytes (1, 3). The inhibition of cell proliferation by SPN does not occur by either a cytotoxic mechanism or by increasing the rate of apoptosis (1). The results of cell cycle analyses on SPN-treated lymphocytes have shown that SPN arrests cells in G0 or early G1 stages of the cell cycle (3). Suppressin also inhibits the proliferation of tumor cells. The addition of exogenous SPN to cultures of leukemia, lymphoma, and thymoma cells and tumor cells from brain, adrenal, breast, and pituitary resulted in markedly reduced proliferation (4). The results of metabolic labeling have shown that SPN is synthesized and secreted as an active molecule by human and mouse lymphocytes and GH3 cells (1, 3). Moreover, neutralization of secreted SPN in culture supernatants by anti-SPN antibodies (Ab) increases proliferation in the absence of exogenous growth factors (6) showing that SPN acts as an autocrine/paracrine inhibitor of entry into the cell cycle. Collectively, the results of our studies show that SPN is a fundamental component of a regulatory circuit that functions to maintain cells in a nondividing state.

To understand the structure, function, and regulation of SPN, we cloned, sequenced and characterized the full-length SPN cDNA from the rat pituitary. The molecular cloning of SPN was accomplished by immunoscreening a pituitary cDNA library with an anti-SPN Ab, by DNA hybridization screening of cDNA libraries with a partial SPN cDNA, and by polymerase chain reaction (PCR) and 5'-rapid amplification of cDNA ends (RACE) using rat pituitary mRNA. The results of sequence analyses and comparisons showed that SPN is a novel vertebrate regulatory molecule. However, SPN is highly homologous to DEAF-1, a recently cloned molecule from Drosophila (7). In addition, we provide results from studies on the tissue distribution of SPN expression, on structural characteristics of SPN, and on the expression of recombinant SPN.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U99659.

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The abbreviations used are: SPN, suppressin; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; mAb, monoclonal antibody; Ab, antibody; RACE, 5'-rapid amplification of cDNA ends; bp, base pairs; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; FGF-1, fibroblast growth factor-1; EST, expression sequence-tagged; DEAF-1, deformed epidermal autoregulatory factor-1; PBMC, peripheral blood mononuclear cells; PNGase F, peptide-N'-[N-acetyl-β-glucosaminyl]-asparagine amidase.
**Molecular Cloning of Rat Suppressin**

**EXPERIMENTAL PROCEDURES**

**RNA Analysis**—Whole pituitaries were surgically removed from male Sprague-Dawley rats (Harlan) as described previously (2), and total RNA was isolated by the guanidinium thiocyanate/cesium chloride method (8). Poly(A)^+ mRNA was obtained by two rounds of chromatography using oligo(dT)-cellulose type 7 (Pharmacia Biotech Inc.) as described previously (9). Total RNA (10–20 μg) or poly(A)^+ mRNA (1–5 μg) were denatured in 50% (v/v) formamide and 2 mM formic acid, resolved by agarose-formaldehyde gel electrophoresis, and the gel was transferred to biotin (ICN, Irvine, CA) nylon membranes by capillary diffusion in 10 × SSC using standard procedures (9). Membranes were baked for 2 h at 80 °C in a vacuum oven and prehybridized and hybridized in the following buffer (0.5 M NaHPO₄, pH 7.2, 1 mM EDTA, 7% SDS, and 0.1% (w/v) bovine serum albumin). ³²P-Labeled SPN cDNA probes as follows: one that was complementary to the cDNA sequence containing the open reading frame. Both oligodeoxynucleotides were labeled with ³²P using T4 polynucleotide kinase by standard procedures (9).

**hybridized, and washed as described above except the temperature for these procedures was 45 °C. Two oligodeoxynucleotides were used as probes as follows: one that was complementary to the cDNA sequence containing the largest open reading frame (5’-TGATGGCTTCTCTGAGTAG-3’), and the complement to this oligodeoxynucleotide that is homologous to the mRNA sequence containing the open reading frame. Both oligodeoxynucleotides were labeled with ³²P using T4 polynucleotide kinase by standard procedures (9).

**cDNA Library Construction and Screening**—An oligo(dT) adaptor-primed unidirectional rat pituitary cDNA library was constructed from rat pituitary poly(A)^+ mRNA using a Uni-Zap XR/Gigapack Cloning system (Strategene, La Jolla, CA). This cDNA library was immunoscreened with a single rat pituitary poly(A)^+ mRNA containing a characterized (1) monospecific polyclonal anti-SPN Ab and Ab-positive clonal plaques detected using a BioStain Super ABC alkaline-phosphatase immunodetection kit (Biomedia, Foster City, CA). After screening, the Ab-positive clonal plaques were cloned for cloning efficiency and cDNA insert size by PCR with vector-specific primers that flank the XhoI/EcoRI cloning site in the Uni-Zap XR vector as described previously (10). The largest cDNA insert (691 bp) was direct-sequenced and then used as a probe to re-screen the rat pituitary Uni-Zap XR cDNA library. After screening, one larger clone was obtained by nucleic acid hybridization screening, with an insert of 924 bp corresponding exactly to the SPN 691-bp cDNA sequence but extended at its 5’ end. The SPN 924-bp cDNA was subcloned in M13mp18 and mp19 by standard methods (9), and the sequence of this cDNA was determined in both orientations. DNA sequencing was performed using the dideoxy method with Sequenase (U. S. Biochemical Corp.). Sequence analyses were performed with the Geneprac program (Riverside Scientific, Bainbridge Island, WA). Homology searches and protein structure analyses were performed using the GCG programs (Genetics Computer Group, Madison, WI) against all available public sequence data bases (11). After screening, the recombinant protein was purified by metal chelation chromatography (12).

**Tissue-specific Expression of SPN—Poly(A)^+ mRNA from several rat tissues was obtained commercially (CLONTECH, Palo Alto, CA). mRNAs (250 ng) from each tissue was used as template in an oligo(dT)-primed first-strand cDNA synthesis with Superscript RT (Life Technologies, Inc.) using the protocol provided by the manufacturer. Each reaction was treated with DNase I before the addition of RT and cDNA synthesis using a standard protocol (9). A replicate first-strand cDNA reaction in which RT was not added was performed for each tissue as a control to show the presence of genomic DNA. One-tenth of the first-strand cDNA synthesis reaction was used as template with a sense SPN primer (5’-GGAGGTATCGAGCATCGC-3’) and an antisense SPN primer (5’-TGATGGCTTCTCTGAGTAG-3’) that will amplify a 402-bp target sequence of the SPN cDNA. The products from each set (with and without RT) of PCR reactions were analyzed by agarose gel electrophoresis. This restriction analysis was performed on each reaction and yielded the expected restriction fragments for the SPN target sequence (data not shown).

**Immunohistochemical Methods**—Tissue sections were collected and fixed in 5% glacial acetic acid in 95% ethanol at −20 °C for 24 h before embedding in parafilm. Serial sections 4 μm thick were cut and stained on glass slides. Staining for SPN expression was performed using an anti-SPN monoclonal antibody (mAb) (3F10; Ref. 2). The specificity of this anti-SPN mAb has been previously demonstrated on intracellular SPN in rat pituitary cells (2). 3F10 binding to intracellular SPN from rat pituitary cells is specifically blocked by preincubation of the mAb with pure native SPN (2). Sections were stained in PBS containing 10% goat serum/Tween 0.05% and 2 μg/ml of anti-SPN mAb. The levels of background staining (negative controls) were deduced by omitting the sections. There was an irrelevant mAb that matched IgM (mAb 1A8, mAb Core Facility). After incubation with the mAb (1.5 h at room temperature), the slides were washed three times in PBS and incubated with PBS/Tween 0.05% containing a biotinylated rabbit anti-mouse IgM (μ-chain specific) antibody (5 μg/ml) (PharMingen, San Diego, CA). Slides were washed three times in PBS and incubated with streptavidin coupled to fluorescein isothiocyanate at the concentration of 1 μg/ml in PBS/
RESULTS AND DISCUSSION

Isolation and Analysis of cDNA Clones—A polyclonal anti-SPN antibody (Ab) prepared against purified bovine SPN (1) was used to screen a rat pituitary cDNA library. This Ab cross-reacts with bovine, human, mouse, and rat SPN (1, 3, 4).

Western Analysis—GH3 cells (ATCC, ROCKVILLE, MD) were cultured in RPMI, 10% horse serum. PBMC were cultured in RPMI, 5% fetal calf serum for 24 h. Supernatants were collected and concentrated 40 times using CENTRIPREP 30. Cells were prepared from 10^6 cells with standard procedures (7), and proteins were separated by electrophoresis using a 4–20% continuous gradient Tris-HCl acryl/bisacryl gel (Biorad) and transferred to polyvinylidene difluoride membranes (Bio-Rad) by electroblotting under 30 mV for 16 h at 4 °C. Membranes were blocked in Tris borate saline (TBS), 3% casein, 10% goat serum, washed, and then incubated with the 3F10 mAb (2.5 μg/ml) for 1.5 h at room temperature. After three washes, membranes were incubated with a biotinylated goat antibody against mouse immunoglobulin (H + L) (0.2 μg/ml), washed again 3 times and then incubated with avidin conjugated to horse peroxidase (1 μg/ml). Membranes were washed before a 60-s incubation with the enhanced chemiluminescent substrate (Amer sham Corp.) and exposed for 5 min to autoradiographic film.

Glycosylation Analysis of Native SPN—Two hundred ng of affinity purified native rat SPN was analyzed for the presence of Asn-linked oligosaccharides by digestion with the glycosidase, PNGase F, according to the protocol provided by the manufacturer (Glyko Inc., Novato, CA). A parallel reaction in which fetuin was digested with PNGase F served as a positive control for enzyme activity. PNGase F and control reactions (without PNGase F) were performed for SPN and the control glycoprotein, fetuin, and the reaction products were analyzed under reducing conditions on 10% SDS-PAGE (13), and protein bands were stained with silver (14).

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Homogeneous bovine pituitary SPN (100 ng/well), as determined by two-dimensional gel electrophoresis, was placed on COBIND microtiter wells for 2 h at 37 °C. The plate was washed, blocked with 1% casein, and then the indicated Ig fraction (25 μg/well), incubated with and without 22.5 μg of peptide for 1 h, placed on the wells in 100 μl of PBS + 0.1% Tween 20 and allowed to bind for 2 h at room temperature. The plate was then washed and a goat anti-rabbit Ig alkaline phosphatase-conjugated antibody (Sigma) was placed in the well for 2 h at room temperature. After extensive washing the substrate (p-nitrophenyl phosphate) was added to well and the A_{405} determined for each well. Results presented are the mean ± S.E. for replicate wells (n = 4).
amino acids. Preparation of new rat pituitary cDNA libraries and nucleic acid hybridization screening of these libraries failed to produce any larger SPN cDNA clones. The inability to clone larger SPN cDNAs from these libraries using the clone 12 cDNA as a probe was unexpected, because the quality (recombinant titer and average size) of all libraries used were normal, and these libraries have been used to clone cDNAs of similar size to the size of SPN cDNA. It may be that the SPN transcript contains structural features that lead to the generation of truncated transcripts or that SPN recombinants are unstable, and their proportion in the library is reduced.

To overcome this problem, PCR and 5'-RACE were used to obtain the remaining sequence of the SPN cDNA. The sequences obtained were used to generate the full-length SPN cDNA sequence and to design primers that were then used to PCR amplify from the 5'-end of the sequence through the complete ORF. This PCR product was cloned in pGEM-T, and three independent clones were sequenced in both directions to obtain the complete cDNA sequence of SPN.

Nucleotide and Predicted Amino Acid Sequence of SPN—The rat full-length SPN cDNA isolated is 1882 nucleotides (Fig. 3), and the size of this cDNA agrees with estimates of the size of the SPN mRNA obtained by Northern gel analysis. The complete SPN cDNA contains an open reading frame of 1488 nucleotides that extends from the first translation consensus sequence (15) of an initiation codon at position 56 to the first termination codon at position 1545. The entire cDNA contains 55 and 339 nucleotides of 5'- and 3'-untranslated sequences, respectively. Translation of the cDNA results in a predicted protein of 496 amino acids with a relative Mr of 53,113 (Fig. 4), which is less than the molecular mass previously observed for native SPN (~63 kDa) by SDS-PAGE analysis. Differences in the actual molecular mass of a protein and its electrophoretic mobility in SDS-PAGE analysis could be due to either post-translational modifications (e.g., glycosylation and/or phosphorylation) and/or to specific regions that bind SDS anomalously and affect electrophoretic mobility (16, 17). The results of bacterial expression studies using the SPN coding sequence (Fig. 5) suggest that the SPN protein contains structural regions that apparently bind SDS anomalously and affect its electrophoretic mobility (Fig. 5). The expression of recombinant proteins in E. coli typically leads to the production of proteins that are not modified post-translationally. Therefore, analyses of the mass of recombinant forms by SDS-PAGE reveals proteins of lower molecular weight compared with native proteins that are post-translationally modified. However, SDS-PAGE analysis of SPN produced by E. coli (Fig. 5) showed that it migrated at molecular mass higher (~63 kDa) than predicted by the deduced amino acid sequence (~53 kDa). Consistent with the idea of structural features of SPN as a major cause of anomalous electrophoretic mobility are the results from similar studies on the homologous protein DEAF-1. The predicted molecular mass of DEAF-1 from the deduced amino acid sequence is 61.5 kDa; however, SDS-PAGE analysis of both recombinant and native DEAF-1 shows that it migrates with an apparent molecular mass of approximately 85 kDa (7). Similar anomalous electrophoretic mobility has been reported for U1 70K proteins (16, 17). These proteins migrated in SDS-PAGE at a molecular mass that was about 20 kDa greater than its actual calculated molecular weight, and this anomalous mobility was shown to be due to highly charged clusters of amino acids (17). The precise reason for the decreased electrophoretic mobility of SPN is not known but may be due to the
for tyrosine kinase. These include consensus sequences for the following kinases: 1 cAMP- or cGMP-dependent protein kinase phosphorylation site (20); 13 casein kinase II phosphorylation sites (21); 6 protein kinase C phosphorylation sites (22); 2 tyrosine kinase phosphorylation sites (23); and 1 G<sub>i</sub> cyclin-dependent kinase (Cdk2) phosphorylation sites (24). Although we have not formally tested if native SPN is phosphorylated, the large number of possible phosphorylation sites suggests that phosphorylation of SPN is probable.

Lastly, Dingwall and Laskey (25) have described a consensus bipartite nuclear localization sequence that consists of two discrete clusters of basic amino acids separated by any 10 amino acids that target proteins to the nucleus. Suppressin contains such a nuclear targeting sequence (RKKENVSCPRLVKK) at amino acids 235–248 which may facilitate its translocation to the nucleus (Fig. 4). In the results of studies presented below (see “Expression of SPN in Rat Tissues”), we show that SPN is localized to the nucleus of cells from a variety of tissues. These results are consistent with the presence of a nuclear localization sequence in the primary sequence of SPN; however, they do not indicate whether this process is dependent on the indicated nuclear localization sequence or if nuclear transport of SPN occurs by other mechanisms.

**Sequence Homology of SPN with Other Proteins**—The cDNA sequence of SPN is highly related to several human and mouse cDNAs of unknown function in the NCBI GenBank non-redundant expression sequence-tagged (EST) data base. Similarities are as high as 90–98% between the cDNA of rat SPN and these EST cDNAs. We assembled a contiguous sequence from the human ESTs. Sequence comparisons between this assembled human cDNA sequence and the rat SPN cDNA showed that they were approximately 80% homologous in their coding regions indicating that it represents the human SPN cDNA sequence. Additionally, we had cloned portions of the human SPN cDNA by RT-PCR using primers designed from the rat cDNA sequence, and the sequence of these human SPN cDNA fragments were identical to those contained in the EST data base. The results of the sequence comparisons between rat and human SPN confirmed in part the results of earlier immunologic and species specificity studies which indicated that human, rat, bovine, and mouse SPN were structurally conserved (1, 3, 5).

Sequence comparisons between the cDNA of SPN and the NCBI GenBank<sup>TM</sup> EMBL, DDBJ, PDB sequence data base showed only one cDNA that was highly homologous (64%) to SPN in a 102-bp region. This cDNA belongs to a recently cloned protein CEC44f1.2 (gp Z49067). Additionally, in part of Caenorhabditis elegans (dbest R19688), a human breast protein (dbest R49909), a human nuclear phosphoprotein (26), and Caenorhabditis elegans protein CEC44FL2 (gp Z49067). Additionally, in part of the KDW domain (amino acids 135–205) SPN is 63% homologous to a sequence in the COOH-terminal region of a human lymphoid-specific SP100 homolog (U36500).

The COOH domain in SPN that is highly homologous to DEAF-1 is the MYND domain previously described in analyses of DEAF-1 (7). This region shows a conserved repeated pattern of cysteines and histidines (27). In this domain there are two
CXCC and two (C/H)XXXC regions that correspond to cysteine “knuckle” structures that are basic building blocks of many zinc finger proteins (27). In this region the position and spacing of all cysteine residues are completely conserved between DEAF-1 and SPN (Fig. 6). This conserved domain has been previously described (7) as having similarity to human myeloid translocation protein 8 (MTG8; Ref. 28), Drosophila bergy (29), rat, mouse, and C. elegans RP-8 proteins (30), putative C. elegans R06F6.4 protein (gp z 46794), Saccharomyces cerevisiae 6543.7 protein (gp Z49807), Arabidopsis thaliana protein (dbest T45013), and human brain proteins (dbest M85494 and R35199). Lastly, a recently reported human protein kinase C-binding protein RACK7 (U48251) also shows significant homology to SPN and DEAF-1 in the MYND domain.

The high degree of sequence similarity in these two structural domains between DEAF-1 and SPN suggests that they may be involved in fundamental physiological processes that are evolutionarily conserved between insects and mammals. DEAF-1 was identified and subsequently cloned based on its ability to bind specifically to a particular DNA sequence, module E of the dfd response element (7). Below we show that SPN is present in the nucleus of cells in several tissues (Fig. 8). The presence of SPN in the nucleus may indicate that SPN also binds DNA and that the mechanism of SPN function may involve its interactions with regulatory elements of certain genes that may influence transcription. The “cysteine knuckle” structure in the COOH domains of DEAF-1 and SPN have been associated with DNA binding in other proteins (27) but may only be considered a likely possible region for DNA binding because earlier studies on DEAF-1 showed that this region does not serve a direct DNA binding function (7).

Expression of SPN in Rat Tissues—The above results show that SPN is a structurally conserved molecule across several metazoan groups. Another important question is whether within an organism SPN plays a restricted role and functions in only certain cell lineages to regulate proliferation or, alternatively, it inhibits proliferation in a variety of cells and tissues. The majority of our studies on SPN have focused on its expression and activity in pituitary cells and in lymphocytes. To determine if SPN was expressed by other cell types, we performed RT-PCR analysis on mRNA from selected tissues in the rat. The results of this analysis showed that expression of SPN is quite broad, and SPN expression was observed in every tissue examined. Specifically, SPN expression was observed in testis, spleen, skeletal muscle, liver, kidney, heart, and brain (Fig. 7).

We analyzed selected rat tissues to determine if the above pattern of SPN gene expression is mirrored by the presence of the SPN protein in these tissues. The results of immunohistochemical analysis of selected tissues for the presence of SPN showed that SPN was present in all tissues examined. Specifically, strong SPN staining was observed in brain, heart, liver, kidney, spleen, lung, and intestinal tissues (Fig. 8). Other tissues in which SPN staining was observed include ovary, thymus, peyer patches, and adrenal (data not shown). As we had previously observed with studies of the pituitary, a large number of the cells in a tissue contained SPN; however, not every cell in a tissue contained SPN. The presence of cells that do not contain SPN is most clearly shown in sections from the spleen where the intensity of SPN immunostaining in the cortex area was stronger than in the germinal centers (Fig. 8C). The subcellular staining of SPN in most tissues was primarily nuclear with relatively reduced cytoplasmic staining. For example in the heart, multiple small nuclei can easily be identified and stain strongly for the presence of SPN (Fig. 8D), whereas SPN is essentially absent from the cytoplasm. Simi-
larly, anti-SPN staining in the brain was most intense in the nucleus of neurons and greatly diminished in neuronal fibers (Fig. 8E). The subnuclear pattern of anti-SPN staining was most intense along the inside of the nuclear envelope (Fig. 8A), a heterochromatin containing region. These results extend our earlier results on pituitary and immune cells and suggest that SPN may be involved in regulating the proliferation of different cell types in many tissues.

The cellular distribution of SPN staining within the tissues examined was very similar to the pattern observed previously in studies of the rat pituitary (2). In the pituitary, SPN staining was observed only in the anterior pituitary and was restricted to five hormone-secreting cell types. Interestingly, the percentage of SPN-staining cells within certain hormone secreting cell types varied from animal to animal. For example, in lactotrophs the percentage of SPN-staining cells ranged from 29 to 65% and was negatively correlated with the amount of prolactin secreted (2). These results may indicate that the physiological state of the animal or of a tissue may influence whether cells contain SPN or not. For example, certain regions of a tissue may have cells that are actively dividing, and in this area one might expect that these cells may have a different SPN status from cells that are not dividing. Alternatively, SPN may arrest proliferation and participate in maintenance of the differentiated state, and in this case one might find SPN only in these cells. Support for the above hypothesis is seen in the staining for SPN in the intestinal epithelium (Fig. 8G). Anti-SPN staining was most intense in epithelial cells of the microvilli, whereas anti-SPN staining in actively proliferating cells in the crypts of the intestine was greatly reduced. These explanations are only two of several possibilities of why certain cells in a tissue may contain intracellular SPN. It should be noted that the methods used in these studies do not differentiate between cells that are SPN producers and cells that have taken up SPN from extracellular sources. It is likely that both of these processes contribute to the observed distribution pattern of SPN in cells of a tissue.

Previously, we have shown that SPN was synthesized and secreted as an active molecule by a rat pituitary cell line (1) and primary murine splenocytes (5). Subsequent studies showed that in the rat pituitary SPN is found primarily in secretory cells (2) which was consistent with the observation that SPN is secreted. Additionally, we have observed that SPN accumulates in the nucleus and cytoplasm of human peripheral blood mononuclear cells (data not shown). The results presented above now show that SPN is also present in the cytoplasm and nucleus of cells from a wide variety of tissues. It may be that different forms of SPN exist in the extracellular and subcellu-
nuclear compartments. Recent studies have shown that there are different forms of the BRCA1 gene product, full-length BRCA1 (230 kDa), and a splice variant BRCA1 delta 11b (110 kDa), that had the same apparent molecular mass (Fig. 9). We concluded by incubation of the membrane with a biotinylated anti-mouse antibody followed by incubation with an avidin-horse peroxidase complex and development with enhanced chemiluminescent substrate. The results of this analysis showed that SPN contains a nuclear localization sequence and that it is translocated in the nucleus and cytoplasm, respectively (31). Northern gel analysis of rat pituitary RNA (Fig. 1) and human peripheral blood mononuclear RNA showed that only a single SPN transcript was present suggesting that the accumulation of SPN in the nucleus, cytoplasm, and extracellular space is not the result of SPN splice variants in these cells. Furthermore, supporting evidence for this conclusion was provided by Western blot analysis of SPN in a secreted preparation from an extracellular extract containing cytosolic and nuclear proteins. The results of this analysis showed that SPN was present in both preparations as a single protein species that had the same apparent molecular mass (Fig. 9). We conclude from these studies that significant differences in the assembly of SPN gene do not produce markedly different sized proteins that preferentially accumulate in subcellular and extracellular compartments in immune and pituitary cells. It may be that SPN is post-translationally modified, and these changes are important in directing the trafficking of SPN. Phosphorylation and/or dephosphorylation would appear to be a likely candidate since the primary sequence of SPN contains 23 potential phosphorylation sites. Currently, we are investigating this possibility.

The completion of the molecular cloning of SPN provides a foundation for future studies on SPN that focus on determining how and if the signal of this negative regulator of proliferation is integrated into downstream cell cycle effector circuits. The relatedness of SPN to DEAF-1 and its broad tissue expression suggests that SPN is an evolutionarily conserved negative regulator of proliferation that may be implicated in the proliferative control circuits for many tissues. What is the mechanism(s) of action of SPN? Originally, with the results showing secretion of SPN, we assumed that the SPN negative signal would most likely be transduced through a cell-surface receptor to influence or perturb selected second messenger systems and ultimately gene expression. The result presented here showing nuclear accumulation of SPN and a structural motif within its sequence consistent with DNA binding suggests that models of SPNs' mechanism of action need to be expanded to consider the possibility of direct effects on gene expression. Secretion and translocation of a proliferation molecule are not unprecedented in that there are other examples of proliferation control molecules that possess these properties (see Ref. 32 for review). For example, the results from recent studies on fibroblast growth factor-1 (FGF-1) have shown that this secreted molecule also contains a nuclear localization sequence and that it is translocated to the nucleus (33). Deletion of the FGF-1 nuclear localization sequence results in a molecule that is not mitogenic in vitro showing that the nuclear localization sequence of FGF-1 is directly involved in FGF-1-induced mitogenic signaling (33). The above results on FGF-1 indicate that there are alternative proliferation signaling pathways that involve nuclear transport and transcriptional activation by the extracellular stimuli. A similar mechanism of action may exist for SPN, although with SPN the signal generated would be expected to be inhibitory.

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Fig. 9. Western analysis of secreted and intracellular SPN. Western analysis of lysates obtained from PBMC using mAb 3F10. Proteins from PBMC intracellular extracts (lane a), PBMC culture supernatant (lane b), and GH3 culture supernatant (lane c) were separated by electrophoresis in a 4–20% gradient gel. Western blots were immunostained with 2.5 μg/ml 3F10. Binding of the 3F10 was detected by incubation of the membrane with a biotinylated anti-mouse antibody followed by incubation with an avidin-horse peroxidase complex and development with enhanced chemiluminescent substrate. The single band detected is at a molecular mass of ~63 kDa.