Isolation and partial amino acid sequence analysis of human ovarian steroidogenesis-inducing protein: A novel protein that belongs to the immunoglobulin super family

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Summary

It is now well established that local factors acting via paracrine and autocrine mechanisms play important roles in mediating the effects of luteinizing hormone and follicle-stimulating hormone in the gonads. However, attempts to purify and characterize gonad-specific factors have had limited success. We have previously characterized a protein, steroidgenesis-inducing protein (SIP), in human ovaries which exerts major effects on steroidogenesis and proliferation of testicular and ovarian cells. In the present study, we have purified SIP to homogeneity. N-terminal sequence of SIP exhibited significant homology to p205, a protein which has been purified from the synovial fluid of rheumatoid arthritis patients. Antibodies raised against p205 cross-reacted with SIP found in follicular fluid and in all purified fractions of SIP. Furthermore, the immunoreactive protein isolated from hFF by immunoaffinity chromatography exhibited SIP bioactivity and demonstrated 100% homology with the purified SIP. Immunoreactive SIP was also present in conditioned media from primary cultures of human granulosa cells. We conclude that SIP is a novel member of the immunoglobulin superfamily of proteins, which is secreted by ovarian granulosa cells and may play an important role in follicular development and luteal function in the human ovary.

Introduction

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play major roles in the development and maintenance of function in the gonads (1). FSH acts to regulate proliferation and differentiation of ovarian granulosa cells and stimulates aromatase activity, which is required for biosynthesis of estrogens from androgen substrates (2,3). In the testis FSH regulates the proliferation and differentiation of Sertoli cells and is essential for the initiation of

spermatogenesis (4,5). LH stimulates ovarian theca cells to produce androgens, induces ovulation, and stimulates progesterone production in the luteal cells (6). In the testis, LH is required for proliferation, differentiation, and testosterone production of Leydig cells (7). Because of the many and varied effects of these two hormones in the gonads, it is commonly accepted that locally produced factors modulate the effects of LH and FSH via paracrine and autocrine mechanisms (8-14). Such factors include steroid hormones, growth factors, cytokines, inhibin, activin, small peptides such as corticotropin releasing hormone (CRH) and growth hormone releasing hormone (GHRH), along with other, hitherto unidentified gonadal proteins (6,9,15-23).

We have previously reported the partial purification of a multi-functional protein from human ovarian follicular fluid (hFF) obtained from pre-ovulatory follicles, which exhibits potent steroidogenic and mitogenic properties. This protein was named steroidogenesis-inducing protein (SIP) due to its major stimulatory effects on *in vitro* steroid production by Leydig cells from several species, human granulosa-lutein cells, and rat adrenal cells (24,25). The stimulatory effect of SIP on steroidogenesis is exerted at the level of conversion of cholesterol to pregnenolone in the mitochondria, a step which is common to all steroidogenic cells (25,26). However, unlike tropic hormones, SIP exerts these effects on steroidogenesis in a cAMP-independent manner (26,27). Later studies demonstrated that SIP also exerts dramatic stimulatory effects on *in vitro* DNA synthesis in immature rat and porcine Leydig cells, rat granulosa cells, and several cell lines derived from ovarian epithelial carcinomas (28-31). We have also shown that the steroidogenic and mitogenic effects of SIP on target cells may be mediated via a tyrosine kinase signaling pathway (32). It was also observed in previous studies

that human granulosa-lutein cells secrete biologically active SIP *in vitro*. Initial characterization showed that SIP exhibited a molecular weight between 50 and 60 kDa and a pI value of 4.8 (25).

Our previous studies suggest that SIP may play a significant role in the autocrine and paracrine regulation of development and function of gonads, and that it is distinct from other growth factors and cytokines previously known to influence gonadal cells. Furthermore, SIP may also be involved in the development and etiology of human ovarian epithelial cancers (31). However, further studies on its biological effects, mechanism of action, cellular origin, and hormonal regulation have been hampered by the lack of information on the biochemical and molecular identity of SIP. Our goal in the present study was to further characterize SIP by purification of the active protein to homogeneity and subsequent determination of its primary structure. The results of this study indicate that SIP is a novel gonadal protein which belongs to the immunoglobulin superfamily of proteins and exhibits partial homology with another novel protein isolated from the synovial fluid of rheumatoid arthritis patients (33).

Experimental Procedures

Purification of SIP: Human follicular fluid (hFF) was obtained at the time of egg retrieval from women participating in the *in vitro* fertilization (IVF) programs at East General Hospital (Toronto, Ontario, Canada) and Texas Tech University Health Sciences Center (Lubbock, TX). These women had been treated with human menopausal gonadotropin (hMG) and human chorionic gonadotropin (hCG) to induce follicular development and ovulation, respectively. Pooled, cell-free hFF was heated at 60°C for 15 min to inactivate possible residual proteolytic enzymes and then centrifuged at 1500xg for 10 min. This heating step had negligible effects on SIP bioactivity. hFF proteins were then precipitated with 80% ammonium sulfate at 4°C

overnight. Precipitates were dissolved in 15mM Tris-HCl (pH 7.2) and dialyzed for 48 hrs, followed by precipitation with 10 volumes of acetone to remove remaining steroids. Precipitates were re-dissolved in Tris-HCl buffer. The hFF proteins were then subjected to gel chromatography on a Sephacryl S-200 column (Pharmacia; dimensions 2.5 x 80 cm) as described previously (25). Pooled bioactive fractions were concentrated using Centriprep centrifugal concentrators (Amicon; MW cut of 10,000) and further purified by Cibacron-Blue affinity chromatography (Blue Sepharose, Pharmacia; column dimensions 1.0 x 30 cm). Proteins were loaded on the column dissolved in Tris-HCl buffer (pH 7.2), and the unbound proteins were eluted in the initial buffer. The proteins which bound to the Blue Sepharose column were eluted in two steps: 20 mM Tris-HCl buffer (pH 7.2), containing 0.15 M NaCl, was used to elute the first fraction of the bound proteins, while the rest of the bound proteins were eluted using the above buffer containing 2M NaCl. SIP bioactivity was present in both bound fractions. However, the fraction eluted with 0.15 M NaCl was used for further purification because of relatively higher amounts of human serum albumin (hSA) in fractions eluted with 2 M NaCl Tris-HCl buffer. After concentration by Amicon filtration as described above, the pooled bioactive fractions were then applied to a Heparin Sepharose column (Pharmacia; dimensions 1 x 1.5 cm) to remove possible heparin-binding proteins such as those of the EGF and FGF family, and β_2-macroglobulin. SIP bioactivity was eluted in the unbound protein fractions, which were pooled and concentrated. These fractions still contained a significant amount of hSA, which was removed by an additional chromatography on Blue Sepharose. SIP preparations were applied on a small Blue Sepharose column (1 x 5 cm) in Tris-HCl buffer containing 0.15 M NaCl. SIP was eluted in the unbound fractions while hSA and other remaining bound proteins were eluted with Tris-HCl buffer containing 2 M NaCl. In some experiments chromatofocusing was used to

purify SIP using a PBE-94 column (1 x 1.5 cm; Pharmacia). This technique proved to be very useful for complete removal of hSA from partially purified SIP preparation (24). Proteins were loaded onto the PBE column in 0.25 M triethyleneamine buffer (pH 9.4). SIP bioactivity was eluted in the unbound fraction. The bulk of bound proteins including hSA were then eluted with Tris-HCl buffer (pH 6.0). The unbound protein fractions were concentrated and equilibrated into 20 mM Tris-HCl buffer (pH 7.2) by Sephadex G25 chromatography (NAP 10; Pharmacia). The purified SIP preparations were mixed with 2x Lammeli's sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.05% Bromophenol blue) and subjected to SDS-PAGE in 8% gels and the protein bands were identified by Coomassie blue or silver staining. To assess which of the protein band(s) exhibited SIP bioactivity, the purified SIP fractions were run on 8% SDS-PAGE gels, the protein bands were visualized by 0.25 M KCl and the visible bands were excised from the gel. Bioactivity was determined after removal of SDS and renaturation of proteins (34). The protein band corresponding to the one exhibiting SIP bioactivity was used for N-terminal amino acid sequence analysis by an automated amino acid sequencer (Protein sequencer, 477 A; Applied Biosystems, Wurlington, CA) after separation on SDS-PAGE and transfer to PVDF membrane (Millipore).

Determination of SIP Bioactivity: SIP bioactivity was determined by a bioassay based on its stimulation of DNA synthesis in immature rat Leydig cells. Leydig cells were isolated from10 day old rats and cultured as described previously (29,35). After 48 hrs of incubation, cells were washed and incubated with fresh media containing the appropriate SIP fractions. After 18 hrs of incubation, the cells were washed, and fresh medium containing 1 μCi/ml of [^{3}H] thymidine (90 Ci/mmol; Amersham) was added. The cells were incubated for an additional 4 hrs. Cells were

then washed and lysed by sonication, and incorporation of the [^{3}H] thymidine into DNA was determined as described previously (29,35).

The SIP preparations at various stages of purification were also analyzed for their steroidogenic effects on rat Leydig cells (24,25) and mitogenic effects on a cell line (SKA cells) derived from ovarian epithelial cancer cells (31).

Western Blotting of SIP: The SIP fractions obtained after different steps of purification, cellular extracts, and concentrated conditioned media were separated by SDS-PAGE on 8% gels and transferred to PVDF membranes as described previously (36). PVDF membranes were blocked overnight in 50mM Tris-HCl, pH 7.5, containing 0.15M NaCl, 0.05% Tween 20, and 4% fat free skimmed milk. Membranes were then incubated with polyclonal antiserum (1:1000) raised against a 40 kDa fragment of p205 (41) for 1 hr at room temperature, washed, and then incubated with anti-rabbit immunoglobulins coupled to horseradish peroxidase (1:20000; Promega). After washing, the membranes were incubated in enhanced chemiluminescence (ECL) mixture (Supersignal, Pierce) for 1 min, exposed to x-ray film, and developed by autoradiography.

Cellular Origin of SIP: We have previously shown that SIP-like bioactivity is present in conditioned media obtained from *in vitro* cultures of human granulosa-lutein cells (25). To confirm these findings, we determined the presence of immunoreactive SIP in conditioned media from human granulosa-lutein cell (HGLC) cultures. HGLC were obtained from IVF patients at the time of egg retrieval. HGLC were prepared for culture after the techniques of Tapanainen *et al* (37). Follicular aspirates from a single individual were pooled and transferred to 50 ml centrifuge tubes and centrifuged at 300xg for 10 min. The supernatant was discarded and the pellet resuspended in 5 ml phosphate buffered saline (PBS) containing 0.1% collagenase-dispase (Sigma Chemical; St. Louis, MO) and incubated 30 min at 37°C. The suspension was then

transferred to a 15 ml centrifuge tube containing 1 ml of fetal calf serum (Irvine Scientific; Irvine, CA) and centrifuged for 5 min at 300xg. The cell pellet was resuspended in 5 ml of medium 199 (Irvine Scientific) and carefully layered over 5 ml of Ficoll (Sigma Chemical). The HGLC were separated from the other cell types by centrifugation at 600xg for 10 min. The HGLC migrated only to the media/Ficoll interface, while the other cell types (red blood cells) collected at the bottom of the tube. The HGLC layer was removed, resuspended in fresh medium 199 and centrifuged at 600xg. The cell pellet was then resuspended in fresh Medium 199 (containing 5% human serum, 100 mU hMG, and antibiotics) to a density of 50,000 cells/ml and cultured in 24-well plates. The conditioned media from multiple cultures from eight patients were collected after 24 hr, concentrated (~100 fold), and analyzed for SIP by Western blotting. In parallel experiments, a human granulosa cell line (HGCL5), obtained from Dr. W. Rainey (Dallas, TX; (38)) was cultured in DMEM:Ham's F-12 medium (1:1; Sigma) containing 5% horse serum (HyClone), 5% FBS (HyClone), and 1% ITS (insulin 6.5 mg/l, transferrin 6.25 mg/l, selenius acid 6.25 mg/l, BSA 1.25 g/l, linoleic acid 5.35 mg/l; Collaborative Biomedical Products) as described by Rainey *et al* (38). At 60-80% confluence, the media were removed, and HGCL5 were cultured in serum-free media in the presence or absence of dibutyryl cAMP (40 μM; Sigma Chemicals) or forskolin (0.2 mM; Biomol Research Labs, Plymouth Meeting, PA) for 48 hours (38). The conditioned media were collected and analyzed for SIP immunoreactivity after concentration as described above.

Immature rat Sertoli cells were isolated and cultured as described previously (39). The conditioned media were collected, concentrated (100X) by Amicon filtration and analyzed for SIP immunoreactivity as described above.

Affinity Chromatography of SIP using p205 antibodies: Antibodies raised against the 40 kDa fragment of p205 protein were first purified on a Protein G-Sepharose column (Pharmacia; HiTrap3) using Fast Protein Liquid Chromatography (FPLC; Pharmacia). The Protein G-Sepharose column was washed three times with 20mM sodium phosphate buffer (pH 7.0). One ml of the antiserum (K180) was added to the column and run at a flow rate of 1 ml/min. The unbound proteins were eluted with 5 ml of sodium phosphate buffer. Bound IgG fraction was then eluted with 5 ml of 0.1M glycine-HCl buffer (pH 2.7). The IgG fractions were equilibrated by Sephadex G-25 chromatography and stored at 4°C in Tris-HCl buffer (pH 9.0). The purified IgG fractions were coupled to carbonydiimidazole (CDI)-activated agarose by the procedure described by Ulbricht *et al* (40). Briefly, 5 ml CDI-activated agarose (Sigma) was washed several times in 0.1M sodium bicarbonate, 0.5M NaCl (pH 8.5) buffer. The resulting matrix was incubated with the purified antibody (6 mg/ml) for 22 hrs with shaking at 4°C. Immunoabsorbant was washed successively with sodium bicarbonate and 1M ethanolamine (pH 8.5) buffer for 2 hrs to block any residual unreacted active groups. Beads were then washed in 0.3M glycine-HCl buffer (pH 2.8) for 2 hrs to disrupt any antibody-antibody interactions. Beads were placed in a column (BioRad; 1 x 1.5 cm) and washed in PBS (pH 7.2). Concentrated SIP fractions obtained after Sephacryl S-200 chromatography were applied to the affinity column, and the unbound proteins were eluted with 20 mM Tris-HCl buffer (pH 7.2), while the bound fractions were eluted by 0.3M glycine-Tris buffer (pH 2.8). The fractions eluted with the latter buffer were applied to Sephadex G25 columns (NAP-10) in order to equilibrate with the 20mM Tris-HCl (pH 7.2) buffer. Samples were then concentrated by Amicon filtration and subjected to SDS-PAGE for Western blotting or visualization of the proteins by silver staining. Pooled,

concentrated antibody-bound fractions were also analyzed for SIP bioactivity using DNA synthesis in immature rat Leydig cells as described above.

Results

We have previously shown that SIP bioactivity can be recovered after purification of hFF by several chromatographic and isoelectrofocusing procedures and that this activity is associated with a protein with a molecular weight between 50 and 60 kDa and a pI value of 4.8 to 4.9 (25,41). Based on these studies, we devised a protocol for purification of SIP which consisted of ammonium sulfate and acetone precipitation of hFF proteins, gel chromatography on Sephacryl S-200 column followed by affinity chromatography on Blue-Sepharose columns. The bioactive fractions from Blue Sepharose chromatography were further purified by either Heparin Sepharose affinity chromatography or by chromatofocusing on PBE resins. In both of these procedures, SIP bioactivity eluted in the unbound fractions.

Fig.1 shows SDS-PAGE analysis of hFF proteins after ammonium sulfate precipitation, Sephacryl S-200 chromatography, and affinity chromatography on Blue Sepharose and Heparin Sepharose (left panel). The fractions obtained after Heparin chromatography contained a protein band (~45 kDa; lane 5) in addition to other protein bands at 66 and 80 kDa. On the other hand, when SIP fractions obtained after Blue Sepharose chromatography were purified by chromatofocusing, the bioactive fractions exhibited a single protein band at approximately 50 kDa (Fig. 1, right panel; lane 3). Based on earlier studies on the physico-chemical properties of SIP (25, 40), both the 45 and 50 kDa (Fig. 1; arrows) proteins were assumed to be SIP. The bioactivity of SIP in various purification fractions was determined using three different bioassays

based upon stimulation of steroidogenesis in rat Leydig cells (25) and stimulation of DNA synthesis in immature rat Leydig cells (29) and a human ovarian cancer cell line (SKA cells;(31).

To remove hSA from SIP fractions obtained after Heparin Sepharose affinity chromatography, we used additional purification on a smaller Blue Sepharose column. As shown in Fig. 2a, significant amounts of hSA were removed by Blue Sepharose chromatography from SIP fractions, and the SIP bioactivity was unaffected after removal of hSA from SIP fractions (Fig. 2b). Two protein bands (80 and 45 kDa; Lane B, Fig. 2a) were visible after Coomassie Blue staining of the purified SIP fraction. To identify which of these proteins exhibited SIP bioactivity, the proteins separated on SDS-PAGE were stained with 0.25M KCl and the visible bands (Fig. 2c) were excised, extracted, renatured, and tested for SIP bioactivity. The SIP bioactivity of the renatured proteins was determined based on its stimulation of DNA synthesis in immature rat Leydig cells and is shown in Fig. 2d. The protein band recovered at ~45 kDa exhibited *in vitro* SIP activity after elution and renaturation. Segments excised from other regions of the gel (80 kDa band and other unstained areas potentially containing non-Coomassie-binding proteins) and processed in parallel showed no *in vitro* SIP bioactivity. The high (80 kDa) protein band was sequenced and found to be human transferrin. Purified human serum transferrin does not exhibit any SIP bioactivity (data not shown).

The protein band (~45 kDa) exhibiting SIP bioactivity was used for determination of N-terminal amino acid sequence. The amino acid sequence was found to be **DVNGGGATLPQPLYQTA**. BLAST analysis revealed that this peptide fragment exhibited 94% homology to the N-terminus of a 40 kDa fragment of human p205 (Accession # SNSP_HUMAN_3) and 88% homology with a sequence obtained from a bacterial protein (periplasmic [Fe]-hydrogenase; Accession # AAA91808). Human p205 is a novel protein

isolated from synovial fluid of arthritis patients that stimulates T-lymphocytes from these patients to proliferate (33).

As described above, an alternative purification protocol, employing chromatofocusing on PBE-94 columns as the final purification step, resulted in the purification of a protein with a slightly higher molecular weight of ~50 kDa (Fig. 1; right panel). To investigate whether this protein band is different from the SIP purified and sequenced above, we purified SIP bioactivity by ammonium sulfate precipitation, gel chromatography on Sephacryl S-200, Blue Sepharose affinity chromatography followed by chromatofocusing on PBE columns. As shown in Fig. 3, the unbound fractions collected after chromatofocusing (PBE-UB) which exhibited SIP bioactivity contained 2 major protein bands after separation on SDS-PAGE. These bands exhibited molecular weights of 80 kDa and 50 kDa. The two bands were excised from the gel and renatured to determine the SIP bioactivity; the activity was associated with the 50 kDa protein band. The N-terminal amino acid sequence of this band was found to be **EVQLVESGA**. BLAST analysis revealed that this fragment exhibited significant homology to rheumatoid factors and IgG heavy chain VIII domain (Accession # PL0122). However, several experiments using binding to protein A Sepharose or anti-human IgG antibodies showed that SIP is distinct from human immunoglobulins (data not shown). Further, identity of this sequence exists with the N-terminus of the 70 kDa fragment of p205 (Table 1).

To investigate whether SIP and the p205 proteins were related to each other, we used anti-p205 antibodies (33,42) for Western blotting of SIP. As shown in Fig. 4A, the anti-p205 antibody (K180) detected immunoreactive bands with an apparent molecular weight of ~50 kDa in hFF, granulosa cell conditioned medium, and in the ascites fluid from ovarian cancer patients.

All these preparations have previously been shown to contain SIP bioactivity. Interestingly, no immunoreactivity was detected from pooled sera from the same patients who donated the hFF.

When analyzed by Western blot analysis using anti-p205 antibodies, a similar 50 kDa immunoreactive band was present in SIP fractions obtained after purification of hFF by various procedures (Fig. 4B). A comparison of Western blotting of SIP purified by Heparin Sepharose chromatography and chromatofocusing on PBE indicated that while the 50 kDa band was present in both preparations, an additional band (45 kDa) was observed in Heparin Sepharose fraction.

To confirm the identity of the immunoreactive band in hFF, we purified SIP by immunoaffinity chromatography using the above antiserum. Anti-p205 antibodies (K180) were coupled to Sepharose beads, and partially purified SIP preparations were applied to the immunoaffinity column. SDS-PAGE and silver staining revealed a single ~50 kDa protein band in the antibody-bound fractions (Fig. 5; top left panel). Western blotting with the same antibody confirmed this band to exhibit immunoreactivity (Fig. 5; bottom left panel; lane 7). When pooled and concentrated, the fractions containing the antibody bound proteins exhibited SIP bioactivity as determined by stimulation of DNA synthesis in immature rat Leydig cells (Fig. 5; right panel). Finally, the N-terminal amino acid sequence analysis of this band yielded 100% homology with the sequence of the 50 kDa isoform of purified SIP.

We have previously shown that human granulosa-lutein cells and rat Sertoli cells secrete SIP-like bioactivity in culture (25). To confirm the identity of this activity with SIP, we analyzed conditioned media from primary cultures of human granulosa-lutein cells, from a human granulosa cell line and from immature rat Sertoli cells. An immunoreactive band at ~50 kDa was observed in conditioned media obtained from both primary granulosa-lutein cells and from the granulosa cell line while a faint band was observed in conditioned media from rat

Sertoli cells (Fig. 6; left panel). These results indicate that granulosa-lutein cells may be the primary source of SIP in the human ovary while Sertoli cells may secrete a similar protein in the testis. Culture medium containing 5% human serum or 5% horse serum plus 5% FBS (used for granulosa cell cultures) when concentrated and analyzed in parallel with the granulosa cell conditioned media exhibited no SIP immunoreactivity (data not shown). When human granulosa cells (HGL5) were cultured in the presence of dbcAMP or forskolin for 48 hours and the conditioned media was analyzed for SIP immunoreactivity, there was a significant increase in the intensity of SIP band compared to that in the untreated cells (Fig. 6, right panel).

Discussion

We have purified steroidogenesis-inducing protein (SIP) from human ovarian follicular fluid to homogeneity and have determined its partial amino acid sequence. Results of these studies show that this multi-functional ovarian factor is a novel protein, which belongs to the immunoglobulin super family of proteins. Furthermore, bioactive and immunoreactive SIP is secreted by human ovarian granulosa cells *in vitro*.

Many studies in the last two decades have indicated the presence of factors in ovarian and testicular secretions, which exert major effects on *in vitro* proliferation and function of gonadal cells. The factors which have been identified and partially characterized in the gonads include, oocyte maturation inhibitor, inhibitors of LH and FSH binding, factors which inhibit or enhance steroidogenesis in testicular and ovarian cells, interleukin-1-like factors, and stimulator(s) and inhibitor(s) of luteinization (8,10,14,18,43-46). The ultimate objective of such studies has been to identify and characterize novel gonad-specific factors that play significant physiological roles in the paracrine and autocrine regulation of development and function of gonadal cells. These

studies have, however, had only limited success due to inherent problems associated with such investigations. To identify and purify gonad-specific factors, ovarian follicular fluid, rete testis fluid, testicular interstitial fluid, and conditioned media collected from *in vitro* cultures of gonadal cells have been used. These materials contain a variety of regulatory factors in very low concentrations, hence making it very difficult to purify these factors. The secretion of such substances and their relative concentrations in these materials vary depending on the endocrine and functional status of the gonadal cells, further restricting the efforts at purification. Further, the majority of bioassays based on *in vitro* incubations and culture of gonadal cells used for identification and purification of gonadal factors are relatively cumbersome and their physiological relevance is questionable. Finally, the possible interactions and synergism of active compounds with gonadotropins and other local factors to exert a specific biological effect and modification of these compounds in gonadal secretions and conditioned media, increase the complexity of these studies.

Several laboratories have reported on the presence of factors in Sertoli cell conditioned media, testicular interstitial fluid, and ovarian follicular fluid that stimulate and/or inhibit steroidogenesis (47-55). Preliminary characterization suggested that most of these factors are proteins; however, the majority of these proteins have not been purified and their biochemical structure remains unknown. It has also been suggested that the steroidogenic effects of testicular interstitial fluid are caused by albumin which is present in these preparations (56,57). Only one gonadal protein with steroidogenic effects has been identified and characterized; a 70 kDa complex of tissue inhibitor of metaloproteinase-1 (TIMP-1) and procathepsin L has recently been purified from Sertoli cell conditioned medium (55). This complex has been shown to stimulate steroidogenesis in testicular Leydig cells and ovarian granulosa cells in a dose-dependent and

cAMP-independent manner. While FSH-induced TIMP-1 (28 kD) is responsible for the steroidogenic activity, the 38 kDa (procathepsin L) protein is indispensable for maximal activity.

SIP was identified several years ago in human ovarian follicular fluid, obtained from pre-ovulatory follicles, on the basis of its major stimulatory effects on steroid production in ovarian, testicular, and adrenal cells (24,25). Later studies revealed that partially purified SIP preparations caused significant stimulation of *in vitro* proliferation of immature Leydig cells, rat granulosa cells, and several cell lines derived from human ovarian epithelial cell carcinomas (28-31,58). These bioactivities were associated with trypsin- and heat-sensitive fractions of hFF. The active compound in these crude fractions exhibited a molecular mass between 50 and 60 kDa and a pI value between 4.8 and 4.9. Whether all of the biological effects of SIP preparations were associated with the same protein remained unknown.

In previous studies, we were able to achieve significant purification (>2000 fold) of SIP using several protein fractionation procedures (25). The results of these studies indicated that SIP activity is associated with a protein which co-purifies with hSA in several chromatographic procedures, but SIP activity is neither associated with hSA nor a protein bound to hSA. The very low amounts and instability of purified SIP during storage made it impossible to further characterize this protein in these studies. Such problems were compounded by changes in the physico-chemical properties and loss of bioactivity of SIP in procedures employing low salt buffers. Using an improved purification protocol, we have been able to purify sufficient amounts of biologically active SIP to homogeneity in the current study and have been able to determine partial N-terminal amino acid sequence of the purified protein. Initial purification procedures yielded a ~45 kDa protein, while an alternative procedure yielded a SIP band of higher molecular weight (~50 kDa). The N-terminal sequence of the smaller SIP isoform demonstrated significant

homology to that of a 40 kDa fragment of p205, while the sequence of high molecular weight SIP isoform demonstrated homology with 70 kDa subunit of p205 indicating that both sequences obtained for SIP belong to the same protein. Furthermore, antibodies raised against 40 kDa fragment of p205 cross-reacted with both isoforms of SIP. Subsequent immunoaffinity chromatography with the anti-p205 antibodies allowed us to isolate a ~50 kDa protein from partially purified hFF fractions. This protein also exhibited SIP bioactivity causing stimulation of DNA synthesis in immature rat Leydig cells. Furthermore, the N-terminal amino acid sequence of this protein matched with that obtained for the purified 50 kDa SIP band. Since both SIP sequences appear to be present in p205, we concluded that partial cleavage of 50 kDa protein during chromatography (Heparin affinity chromatography) results in the formation of a 45 kDa fragment. Further studies are underway to understand the mechanism of this cleavage and its effects on SIP bioactivity. Although our results suggest that SIP displays significant homology with p205, we assume that the two proteins are not identical on the basis of significant differences in the physico-chemical properties. Further studies aimed at cloning of one or both of these proteins should provide definitive information on the relation of the two proteins to each other and to the other members of immunoglobulin super family of proteins.

Our previous studies have shown that while SIP activity is present in high amounts in the hFF, very little, if any, activity was observed in serum samples collected from the same patients at the time of egg retrieval (24). These results suggested that SIP is predominantly produced by ovarian cells and subsequently accumulates in the follicular fluid. This notion was strengthened by our *in vitro* studies which showed that human granulosa-lutein cells isolated from the hFF of IVF patients secreted SIP-like bioactivity in culture (25). In the current study, we confirmed that SIP immunoreactivity is absent in serum and that significant amounts of SIP are present in the

conditioned media from human granulosa-lutein cells and from a human granulosa cell line (HGL5) using an anti-p205 antiserum. The immunoreactive protein band found in granulosa cell conditioned media was identical to that found after Western blotting of SIP in hFF fractions. These results confirm that SIP found in hFF is indeed produced by the granulosa cells in the preovulatory follicle; however, the possibility that other ovarian cells may also secrete SIP *in vivo* has not been ruled out. Since SIP exerts significant effects on testicular and adrenal cells, it is possible that SIP is produced in these and other tissues. Presence of immunoreactive SIP in conditioned media from rat Sertoli cells indicates that these cells may be the source of SIP in the rat testis. However, this possibility and a possible physiological role of SIP in the testis have not been pursued further.

The secretion of SIP by *in vitro* cultures of human granulosa cell line and by human granulosa-lutein cells indicates that differentiating granulosa cells and luteal cells may secrete this protein *in vivo* during follicular development and in the corpus luteum. In the present study we observed that forskolin and dbcAMP caused a significant stimulation of SIP secretion in the granulosa cell line. Since gonadotropin effects on granulosa and luteal cells are mediated by cAMP, it is plausible to conclude that secretion of SIP may be regulated by gonadotropins *in vivo*. However, whether the secretion of SIP is regulated by FSH or LH or both tropic hormones in ovarian cells remains be investigated.

We have previously shown that purified preparations of SIP stimulate proliferation of several cell lines obtained from human ovarian epithelial carcinomas (31) and have proposed that SIP might play a role in the development and maintenance of ovarian cancers. These ovarian cancers, however, develop mostly in postmenopausal women who do not ovulate or when the development of ovarian follicles does not take place. However, the number of ovulations and

induction of follicular development by fertility drugs have been found to be the major risk factors in the development of ovarian cancers (59,60). We hypothesize that SIP may be involved in the rapid proliferation of ovarian epithelial cells which takes place after ovulation to repair the ovulation wound (61). This effect of SIP on epithelial cells in combination with other factors may, therefore, contribute to the malignant transformation of these cells. We have recently found high amounts of SIP bioactivity and immunoreactivity in ascites fluid from ovarian cancer patients (Reddy et al., unpublished data), and it is tempting to speculate that the cancer cells may produce SIP which serves as an autocrine regulators of cell proliferation. Further studies are underway to critically evaluate this possibility.

In conclusion, the results of the present study indicate that SIP is a novel member of the immunoglobulin superfamily of proteins and represents a potent regulator in the normal function of gonadal cells.

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Legends to Figures

Fig.1. Purification of SIP from human ovarian follicular fluid. Human follicular fluid proteins (hFF) were purified sequentially by ammonium sulfate precipitation (AS), gel chromatography on Sephacryl S-200 (S200), Blue Sepharose (BS) and Heparin Sepharose (Hep) affinity chromatography. SDS-PAGE in 8% gels was performed using SIP preparations at each step of purification as shown in the left panel. The right panel shows the differences in the SIP preparations (arrows) obtained after Heparin Sepharose (Hep) affinity chromatography or chromatofocusing (PBE) of SIP preparation obtained after Blue Sepharose chromatography. The molecular weight standards are depicted on the left side of the figure. Equal amounts of total proteins (50 μg) were loaded in each lane and the proteins were visualized by silver staining.

Fig. 2a. SDS-PAGE (8%) analysis of SIP fractions obtained after Heparin-Sepharose affinity chromatography (A) after further purification on small columns of Blue Sepharose chromatography. B: Unbound fraction, C: Bound fractions containing the bulk of human serum albumin. Equal volumes of concentrated proteins were applied in each lane and the proteins were visualized by Coomassie blue staining.

2b. Biological activity of SIP preparations depicted in Fig. 2a, as determined by the stimulation of DNA synthesis in immature rat Leydig cells. The preparations obtained after Heparin-Sepharose chromatography (A) and the unbound fractions from Blue Sepharose column (B) contained significant SIP activity, while the Blue-Sepharose bound fractions (hSA) contained very little biological activity.

2c. Purified SIP preparation (B) from Fig. 2a. The gel pieces containing visible protein bands (2, 4) and regions containing residual hSA (3) or no protein bands (1) were excised. The proteins were eluted from gel pieces and analyzed for SIP activity.

2d. SIP activity in the renatured proteins eluted from SDS-PAGE gel pieces (Fig. 2c) as determined by the stimulation of DNA synthesis in immature rat Leydig cells. Further details of these procedures are given in the Materials and Methods.

Fig. 3a. SDS-PAGE analysis of SIP preparations obtained after ammonium sulfate precipitation (AS), Sephacryl S-200 gel chromatography (S200), Blue Sepharose affinity chromatography (BS-UB and -B), and chromatofocusing on PBE (PBE-UB and –B). Two protein bands exhibiting molecular weights of 80 and 50 kDa were visible in the unbound fractions obtained from chromatofocusing on PBE. The low molecular weight band (SIP) and high molecular weight band (Cont) were excised from the gel, renatured, and analyzed for SIP bioactivity as described in legend to Fig. 2.

3b. Bioactivity of SIP in proteins eluted from SDS-PAGE gel (Cont and SIP) as determined by stimulation of DNA synthesis in immature rat Leydig cells. NC: cells cultured in serum-free medium. EB: cells cultured in elution buffer used for extraction of proteins from gel. S200: SIP preparation obtained after Sephacryl S-200 gel chromatography, used as a positive control. While the 50 kDa protein band caused significant stimulation of DNA synthesis, the 80 kDa protein band had no SIP activity.

Fig. 4. Western blot analysis of SIP. A: conditioned media from human granulosa-lutein cells (GC), human follicular fluid (hFF), ascites fluid from ovarian cancer patients (AF), and pooled

serum from IVF patients (Serum) were analyzed by Western blot analysis using antibodies raised against a 40 kDa fragment of p205. B: Western blot analysis of SIP in human follicular fluid (hFF) and in SIP preparations obtained after ammonium sulfate precipitation (AS), Sephacryl S-200 gel chromatography (S200), and Blue Sepharose affinity chromatography (BS). C: Western blot analysis of SIP in purified preparations obtained after Heparin affinity chromatography (Heparin) or chromatofocusing (PBE).

Fig. 5. Purification of SIP from Sephacryl S-200 fractions by immunoaffinity chromatography. The proteins eluted in the unbound fractions (1-5) and in antibody-bound fractions (6-9) were separated on SDS-PAGE. Proteins were visualized by silver staining (top left panel). These fractions were also analyzed by Western blotting using anti-p205 antibodies (lower left panel). A single protein exhibiting a molecular weight of approximately 50 kDa was found in the antibody-bound fractions (fraction 7). The same protein band was also detected in the same fraction by Western blot analysis. The fraction 7 was concentrated by Amicon filtration and analyzed for SIP bioactivity (right panel). This fraction caused significant stimulation of DNA synthesis in immature rat Leydig cells. FBS: cells stimulated with 5% fetal bovine serum were used as a positive control.

Fig. 6. Left panel: Western blot analysis of SIP in conditioned media from human granulosa-lutein cells (PGC), from human granulosa cell line (HGL5), and from rat Sertoli cells (SC). Right panel: Effects of dibutyryl cAMP and forskolin on secretion of SIP in human granulosa cell line (HGL5). The cells were cultured for 48 hours in the presence of various agonists, the

conditioned media were collected, concentrated, and analyzed by Western blot analysis. PGC: conditioned media from human granulosa-lutein cells used as a positive control.

Table 1. Comparison of amino acid sequences of SIP and p205

SIP	50 kDa fragment	EVQLVESG
p205	70 kDa fragment	XVQLVE
SIP	45 kDa fragment	DVNGGGATLPQPLYQTA
p205	40 kDa fragment	DINGGGATLPQPLYQT

Fig. 1

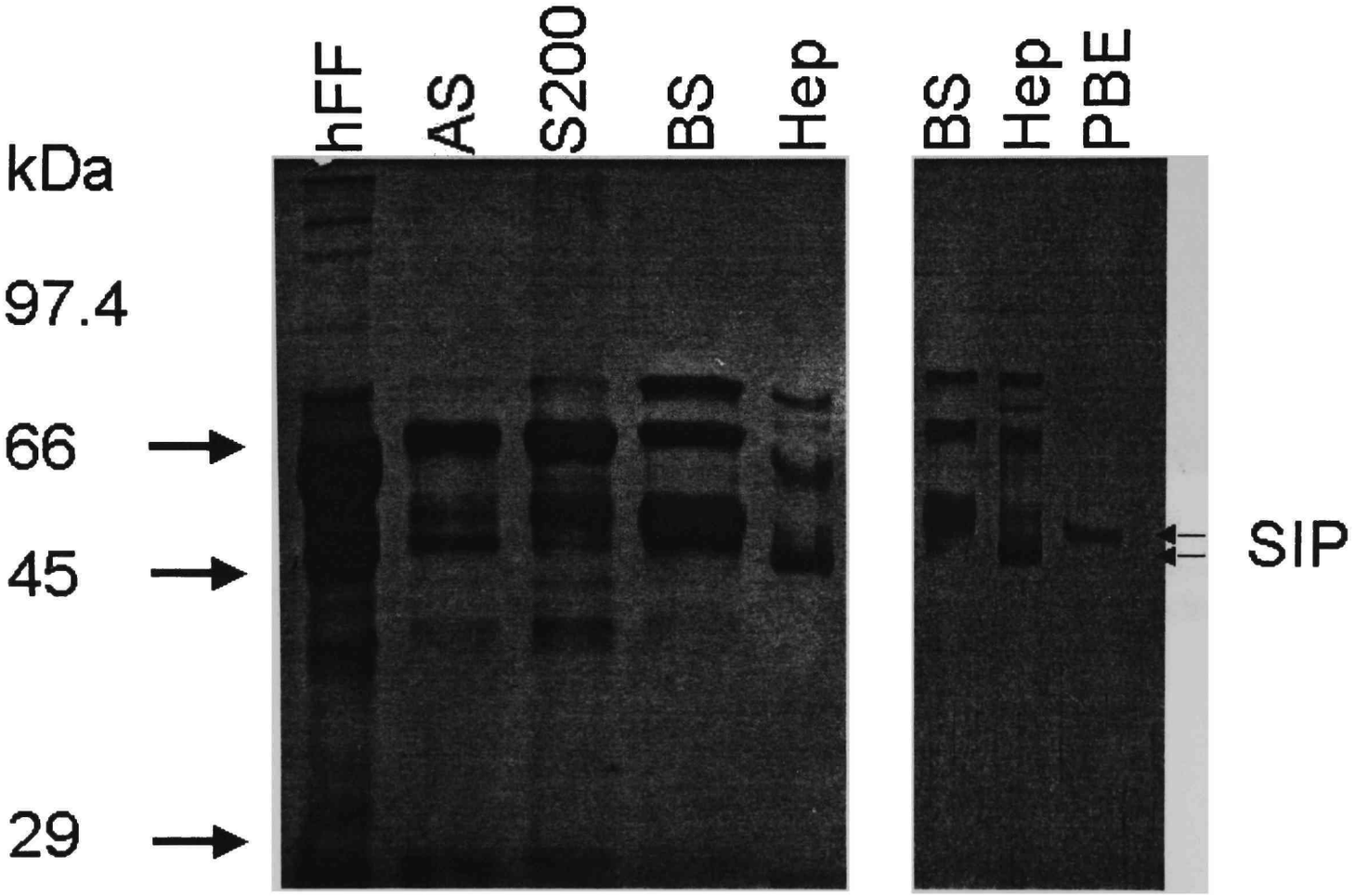
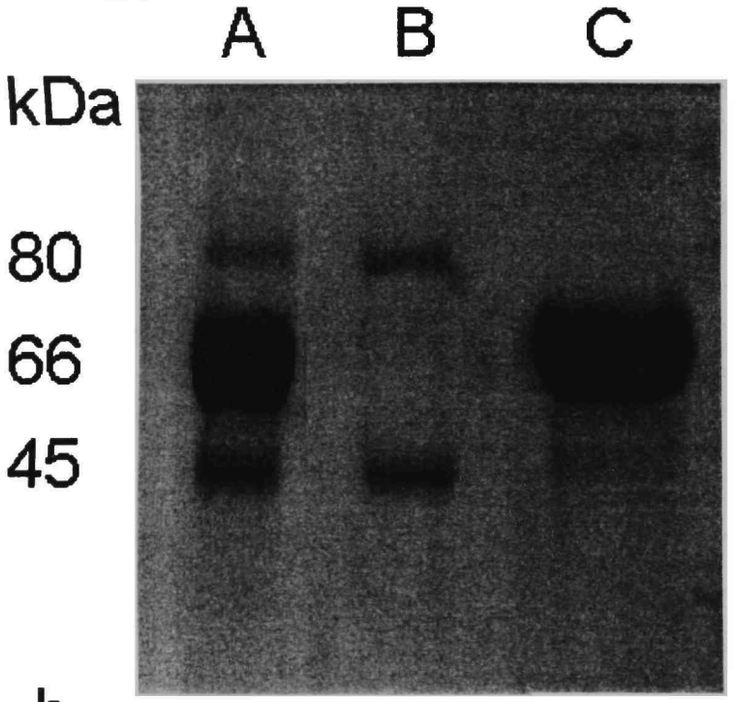
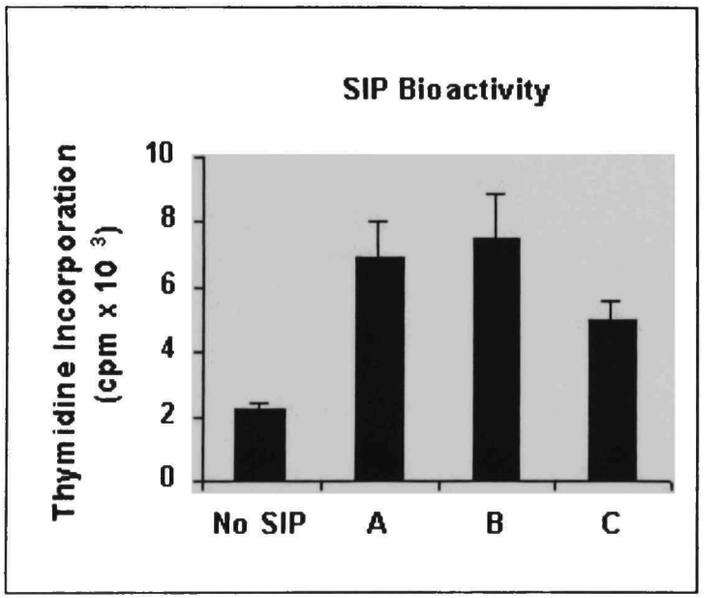


Fig. 2

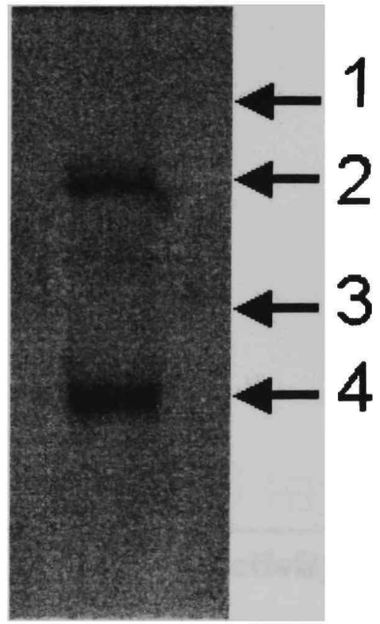
a.


A
B
C
kDa
80
66
45

b.


SIP Bioactivity
Thymidine Incorporation (cpm x 10 3)
10
8
6
4
2
0
No SIP
A
B
C

c.


1
2
3
4

d.

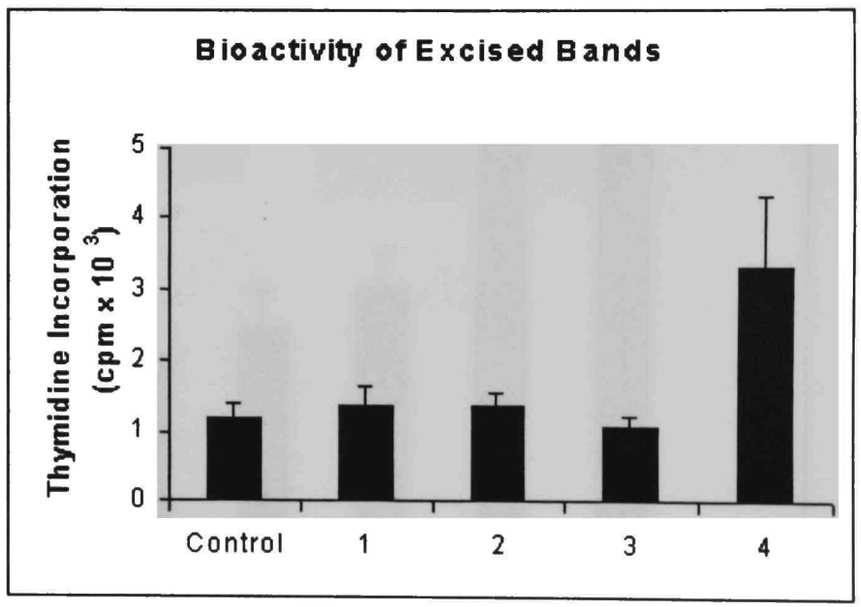

Bioactivity of Excised Bands
Thymidine Incorporation (cpm x 10 3)
5
4
3
2
1
0
Control
1
2
3
4

Fig. 3

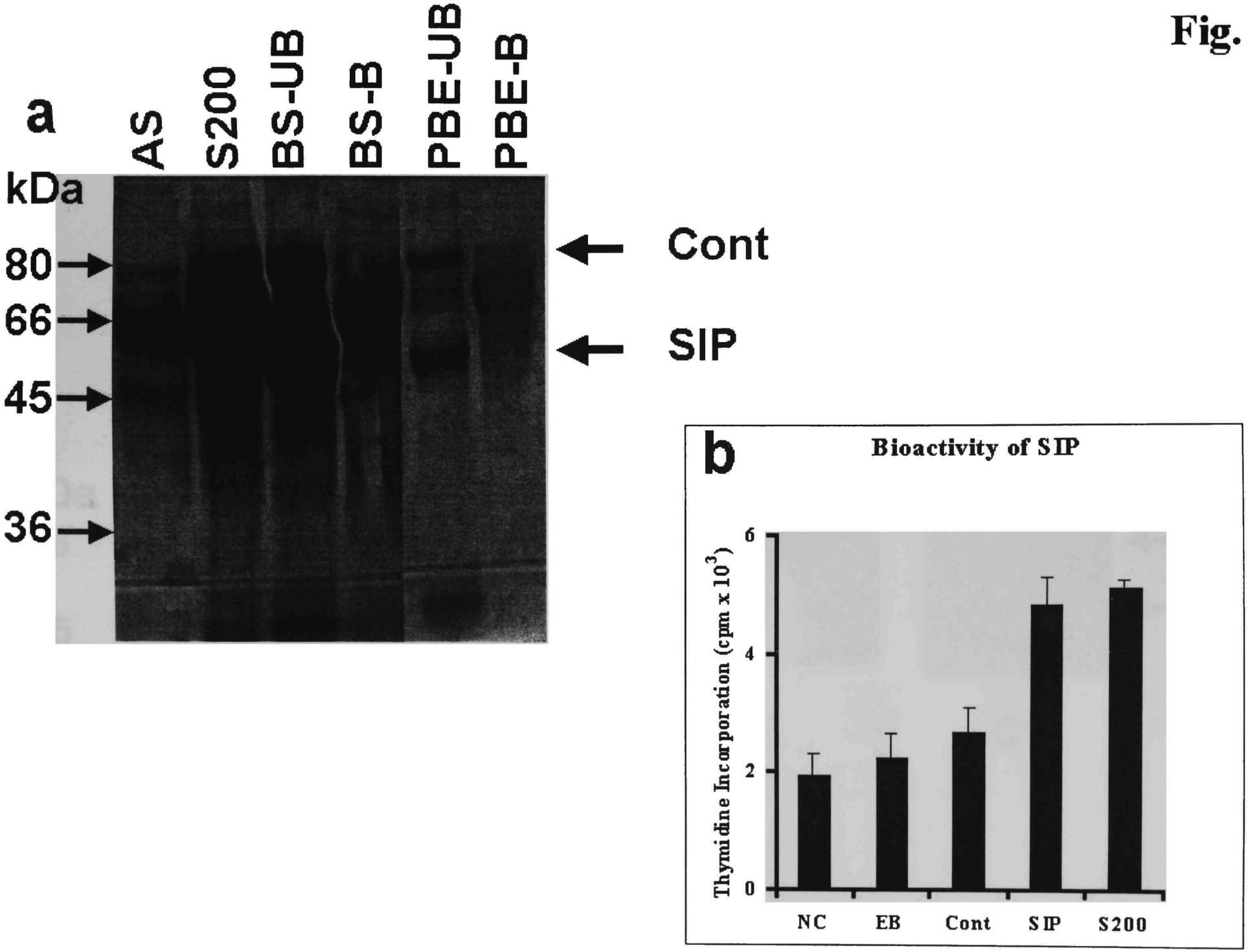

a
AS
S200
BS-UB
BS-B
PBE-UB
PBE-B
kDa
80
66
45
36
Cont
SIP
b
Bioactivity of SIP
Thymidine Incorporation (cpm x 10^3)
6
4
2
0
NC
EB
Cont
SIP
S200

Fig. 4

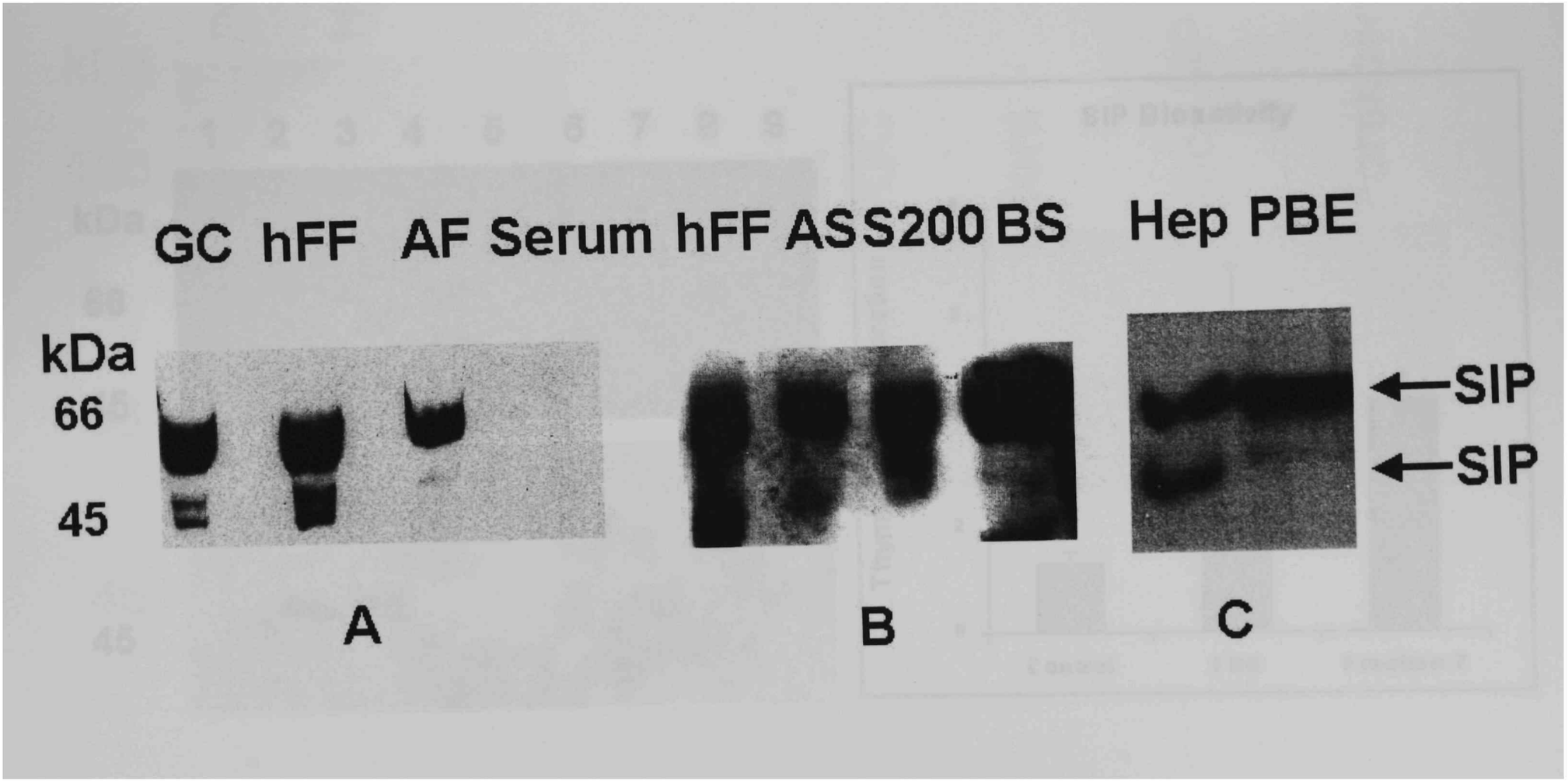
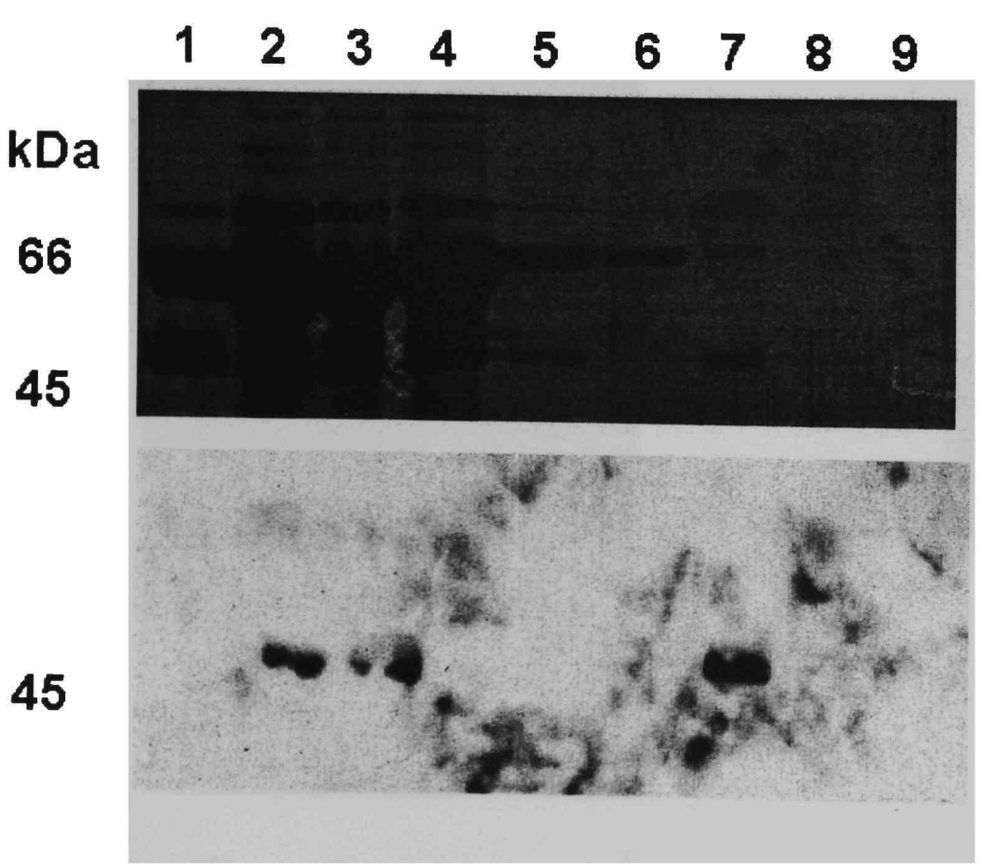


Fig. 5


1 2 3 4 5 6 7 8 9
kDa
66
45
45

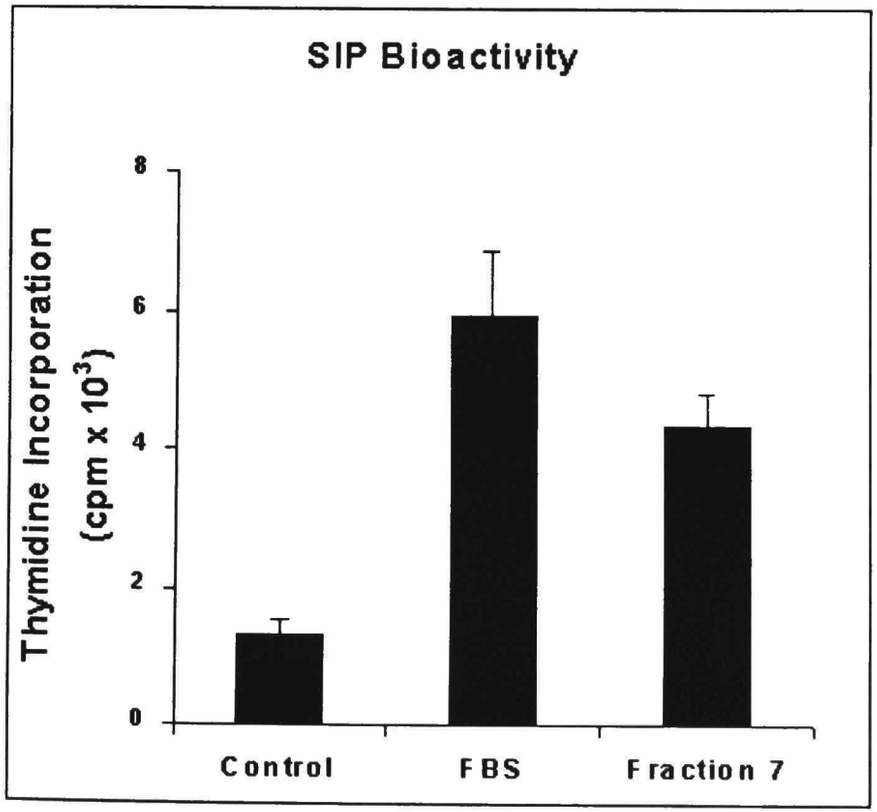
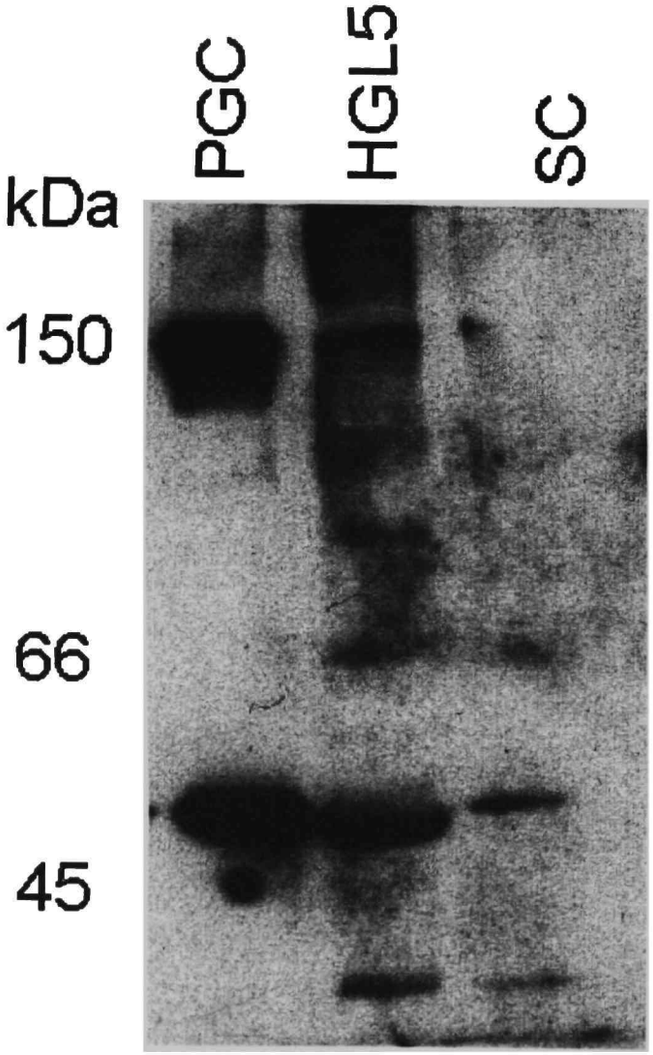
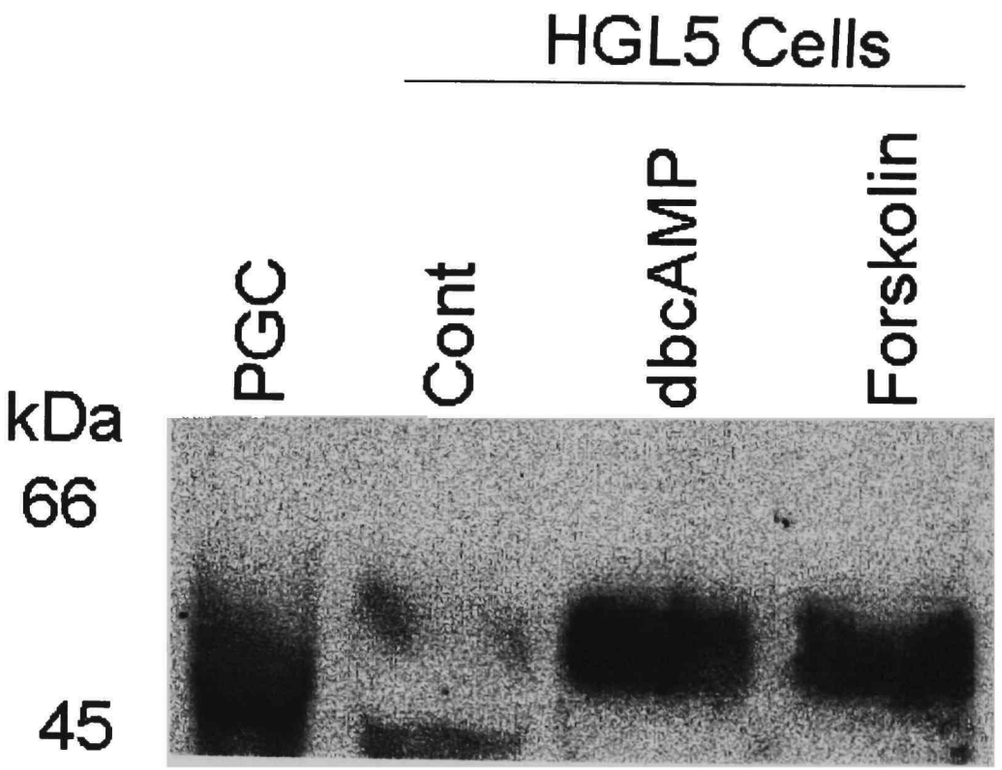

SIP Bioactivity
Thymidine Incorporation
(cpm x 10³)
8
6
4
2
0
Control
FBS
Fraction 7

Fig. 6


PGC
HGL5
SC
kDa
150
66
45


HGL5 Cells
PGC
Cont
dbcAMP
Forskolin
kDa
66
45