Journal of Andrology, Vol. 21, No. 2, March/April 2000

Identification and Regulation of Receptor Tyrosine Kinases Rse and Mer and Their Ligand Gas6 in Testicular Somatic Cells

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ABSTRACT: Receptor tyrosine kinases act to convey extracellular signals to intracellular signaling pathways and ultimately control cell proliferation and differentiation. Rse, Axl, and Mer belong to a newly identified family of cell adhesion molecule–related receptor tyrosine kinase. They bind the vitamin K-dependent protein growth arrest–specific gene 6 (Gas6), which is also structurally related to the anticoagulation factor Protein S. The aim of this study is to investigate the possible role of Rse/Axl/Mer tyrosine kinase receptors and their ligand in regulating testicular functions. Gene expression of Rse, Axl, Mer, and Gas6 in the testis was studied by reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis. The results indicated that receptors Rse and Mer and the ligand Gas6 were expressed in the rat endothelial cell line (TR1), mouse Leydig cell line (TM3), rat peritubular myoid cell line (TRM), mouse Sertoli cell line (TM4), and primary rat Sertoli cells. Axl was not expressed in the testicular somatic cells by RT-PCR or Northern blot analysis. The highest level of expression of Gas6 messenger RNA (mRNA) was observed in the Sertoli cells, and its expression was responsive to the addition of forskolin in vitro. The effects of serum, insulin, and transferrin on Gas6 expression by TM4 cells were examined. It was shown that they all exhibited an up-regulating effect on Gas6 expression. The forskolin-stimulated Gas6 expression was accompanied by an increase in tyrosine phosphorylation of the Rse receptor in vitro, suggesting that Gas6 may exhibit an autocrine effect in the Sertoli cells through multiple tyrosine kinase receptors. Our studies so far have demonstrated that tyrosine kinase receptors Rse and Mer and their ligand Gas6 are widely expressed in the testicular somatic cell lines and may play a marked role in promoting testicular cell survival.

Key words: Sertoli cells, tyrosine phosphorylation, testicular cell lines.

J Androl 2000;21:291–302

Receptor tyrosine kinases (RTKs) are cell surface receptors that have intrinsic protein tyrosine kinase activity. They are responsible for the coordination of cell-cell communication in all multicellular eukaryotic organisms (Van der Geer et al, 1994). Binding of the extracellular portion of an RTK to its cognate polypeptide ligand initiates receptor dimerization or oligomerization. This leads to activation of the intracellular protein tyrosine kinase domain of the receptor, resulting in autophosphorylation as well as cytoplasmic substrate phosphorylation. Specific signals generated by RTK receptor bindings can elicit a great diversity of cellular responses, including cell growth and proliferation, or growth arrest and cell differentiation. The key role of these signal transduction pathways is underscored by the broad range of developmental abnormalities that result from alterations in the RTKs (Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992; Van der Geer et al, 1994).

Rse, Axl, and Mer belong to a newly identified family of cell adhesion molecule–related RTKs. Rse also is known as Tyro 3, Sky, Brt, and Tif (Dai et al, 1994; Fujimoto and Yamamoto, 1994; Lai et al, 1994; Mark et al, 1994; Ohashi et al, 1994); Axl also is known as UFO and Ark (Janssen et al, 1991; Rescigno et al, 1991; Bellosta et al, 1995); and Mer is a putative mammalian homologue of chicken c-Eyk (Graham et al, 1994, 1995; Jia and Hanafusa, 1994). Receptors in the Rse/Axl/Mer family share high-sequence homologic features with each other. Their extracellular region, which is composed of 2 immunoglobulinlike (Ig) repeats followed by 2 membrane proximal fibronectin type III (Fn-III) repeats, contains more than 30% amino acid identity. Their intracellular region, which consists of the transmembrane domain and catalytic kinase domain, contains approximately 60% amino acid identity. This similar combination of structural motifs within the extracellular region of Rse, Axl, and Mer is also observed in neural cell adhesion molecules (N-CAM), the receptor-type protein tyrosine phosphatase (PTP), and the putative tumor suppressor gene product deleted in colorectal carcinoma (DCC; Van der Geer et al, 1994).

Supported in part by grants from the Hong Kong Research Grant Council (HKU7235/97M), University of Hong Kong (CRCG) and the International Consortium for Male Contraception (B97.064P–9).

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Received for publication July 26, 1999; accepted for publication September 30, 1999.

Growth arrest–specific gene 6 (Gas6) was initially cloned from serum-starved 3T3 fibroblasts (Manfioletti et al, 1993). It was able to induce cell cycle re-entry of serum-starved 3T3 cells and to efficiently prevent the apoptotic cell death caused by complete serum removal (Goruppi et al, 1996). Subsequently, Gas6 was identified as a common ligand for the Rse/Axl/Mer RTK family (Godowski et al, 1995; Varnum et al, 1995; Chen et al, 1997). The protein encoded by Gas6 is a 75-kd molecule, containing 678 amino acids. It shares 46% amino acid identity and a similar domain organization with Protein S, which is an abundant serum protein and a negative regulator of the coagulation cascade (Dahlback et al, 1986). The C-terminal portion of Gas6 is similar in structure to that of the sex hormone–binding globulin (SHBG; Gershagen et al, 1987) and contains tandem globular (G) domains. It has been shown that the G domains of Gas6 are sufficient to bind with high affinity to Rse or Axl and to activate receptor phosphorylation with a specific activity similar to that of the full-length molecule (Mark et al, 1996). Recent studies have demonstrated that Gas6 can block apoptosis induced by growth arrest in rat vascular smooth muscle cells (Nakano et al, 1996) and stimulate human Schwann cell growth and proliferation (Li et al, 1996).

The function of the Rse/Axl/Mer family receptors and their ligand Gas6 is not known. Although they are found in a variety of tissues, a recent study with gene knockout mice showed that null mutation in all 3 receptors imposed no significant detrimental effects to most tissues and organs except the adult male gonads (Lu et al, 1999). This finding suggests that the Rse/Axl/Mer receptors are essential regulators of spermatogenesis. By use of cell cultures in the present study, we confirmed the expression of Rse and Mer and their ligand Gas6 in various testicular somatic cell types. We also detected an increase in tyrosine phosphorylation of Rse receptor in mouse Sertoli cells (TM4) in response to the addition of forskolin in vitro. These results suggest that Gas6 may exhibit paracrine or autocrine activity in regulating testicular functions. Testis is an organ composed of multiple cell types, with stem cells differentiating to spermatids in a cyclic fashion throughout adult life. Gas6 and Rse are newly identified growth factors and tyrosine receptor kinase pair. Our findings have provided an initial step toward understanding their possible role in autocrine and paracrine regulation in the testis.

Materials and Methods

Cell Lines

The clonal cell lines used were derived from primary cultures of normal testicular tissue or testicular tumor. The rat testicular endothelial cells (TR1), mouse Leydig cells (TM3), normal immature mouse Sertoli cells (TM4), and and rat peritubular myoid cells isolated from a Sertoli cell tumor (TRM) lines have been previously described (Mather 1980; Mather et al, 1982; Mather and Philips, 1984a). In brief, the rat ovarian granulosa (ROG) cell line was derived from primary culture of cells obtained from normal ovarian follicles after 14 days (Li et al, 1997).

Cell Culture and Treatment

Testicular cell lines were grown in a 1:1 (vol/vol) mixture of Ham F12 nutrient mixture (Sigma Chemical, St Louis, Mo) and Dulbecco's modified Eagle medium (Sigma) supplemented with 1.2 g/L sodium bicarbonate, 15 mmol/L Hepes buffer, and 20 mg/L gentamycin (Gibco Research Laboratories, Grand Island, NY; F12/DMEM). Stock cultures were maintained in F12/DMEM supplemented with 5% (vol/vol) fetal bovine serum (FBS; Gibco). In serum-free cultures, epidermal growth factor (EGF; 5 ng/mL; Sigma), insulin (10 μg/mL; Gibco), and transferrin (5 μg/mL; Gibco) were added to F12/DMEM. Cells from exponentially growing stock cultures were removed from the plate with trypsin (Gibco), washed with F12/DMEM, and plated in 100-mm tissue culture dishes. Stock culture was split at a ratio of 1:200 or 1:50 and subcultured every 3 to 7 days when the culture became subconfluent. ROG cells were carried in hormone-supplemented, serum-free medium as previously described (Li et al, 1997) with a 1:3 split ratio.

Cell numbers were obtained before RNA extraction by removing the cells from the plate with trypsin, neutralizing with an equal volume of serum-supplemented medium, and being counted by hemocytometer. All growth factors were added, at final concentration, directly to the tissue culture medium. Cells were incubated at 37°C in 5% CO_2 (vol/vol), 95% air (vol/vol), and saturated humidity. To study the effect of forskolin on the expression of Gas6, TM4 cells were grown in 100-mm tissue culture dishes in serum-supplemented media until 80% confluent. They were then washed twice with phosphate-buffered saline solution and incubated in serum-free media supplemented with insulin, transferrin, EGF, and 20 μmol/L forskolin for the indicated times. At the end of incubation, cell viability was checked (>95%) before they were lysed for total RNA or protein extraction. All media are prepared in the laboratory from powdered formulations and used within 2 weeks of preparation.

Primary Sertoli Cells

Primary Sertoli cells were obtained from 20-day-old male Sprague-Dawley rats and prepared by sequential enzymatic treatments as previously described (Chung et al, 1998). Isolated cells were plated on Matrigel (diluted 1:7 with F12/DMEM) at a density of 7.5×10^6 cells/cm^2 in F12/DMEM supplemented with insulin, human transferrin, and EGF. Cells were incubated at 35°C in a humidified atmosphere of 95% air and 5% CO_2. To obtain Sertoli cells with a purity greater than 95%, cultures were hypotonically treated after 48 hours with 20 mmol/L Tris-HCl, pH 7.4, at 22°C for 2.5 minutes to lyse the contaminating germ cells (Galdieri et al, 1981) and then washed twice with F12/DMEM. Media were replaced every 24 hours, and cells were cultured for an additional 7 days. These cells were then used for the RNA extraction. Under these cell density and culturing con-

ditions, it has been established in our laboratory that specialized inter–Sertoli cell junctions were formed by day 4 that mimic the in vivo situation in the seminiferous epithelium (Chung et al, 1999).

Reverse Transcriptase–Polymerase Chain Reaction and Southern Blot Analysis

To detect the expression of tyrosine kinase receptors Rse, Axl, and Mer and their ligand Gas6 in clonal cell lines of the testis, reverse transcriptase-polymerase chain reactions (RT-PCRs) were performed. The primers used were as follows: 5′-TGTCTGCGAATGGAACTGGAGAAC-3′ (Rse, sense) and 5′-CAGGCTGTTGCTACCCTCCCTTACT-3′ (Rse, antisense), which generated a 663-bp Rse PCR product; 5′-CCTTTACTTGCTTGCTTCGGAACT-3′ (Axl, sense) and 5′-TCTATGTCGCTTGAGGCAATGATGT-3′ (Axl, antisense), which generated an 850-bp Axl PCR product; 5′-GCAGGGACTTACAAAGAGCTTTCT-3′ (Mer, sense) and 5′-AGCCGAGGATGATGAACATAGAGT-3′ (Mer, antisense), which generated a 238-bp Mer PCR product; 5′-CGGCATTCCCTTCAAGGAGAGT-3′ (Gas6, sense) and 5′ CTCAACTGCCAGGACCACCAACT-3′ (Gas6, antisense), which generated a 397-bp Gas6 PCR product; 5′-TCACCGAGGCCCCTCTGAACCCTA-3′ (β-actin, sense) and 5′-GGCAGTAATCTCCTTCTGCATCCT-3′ (β-actin, antisense), which generated a 644-bp β-actin product; and 5′-TCCGCTGCAGTCCGTTCAAGTCTT-3′ (S16, sense) and 5′-GCCAAACTTCTTGGATTCGCAGCG-3′ (S16, antisense), which generated a 384-bp S16 product. The cytoskeletal protein β-actin or ribosomal protein S16 was used as an endogenous control and coamplified in the PCR reactions. Five micrograms of total RNA was used for each sample. RT-PCRs were performed by Ez rTth RNA core kit (Perkin Elmer/Applied Biosystems, Foster City, Calif) with the following parameters: reverse transcription at 50°C for 5 minutes, 55°C for 5 minutes, and 60°C for 10 minutes; denaturation at 94°C for 2 minutes and annealing at 60°C for 15 seconds; and extension at 72°C for 15 seconds. A total of 35 to 40 cycles were performed, which were followed by a 15-minute extension at 72°C. With these conditions, the amplifications of the target gene and endogenous gene were in the linear range as demonstrated in a series of preliminary experiments by removing samples for analysis at cycles 25, 30, 35, 38, and 42. A 10-μL aliquot from each amplified sample tube was resolved in 1.5% agarose gels in 0.5× 45 mmol/L Tris, 45 mmol/L boric acid, 1 mmol/L EDTA (ethylenediaminetetraacetic acid), pH 8.0 at 22°C and visualized with ethidium bromide. The authenticity of the target gene PCR products were confirmed by direct nucleotide sequencing (for Mer) and Southern blot analyses (for Rse, Axl, and Gas6). Nucleotide sequencing was performed by the chain termination method with an ALF automated sequencer (Pharmacia LKB Biotechnology, Uppsala, Sweden) and Autoread Sequencing Kit (Pharmacia). In Southern blot analysis, PCR products resolved in agarose gel were transferred by capillary blotting to a nylon membrane (Hybond-N, Amersham Life Science, Buckinghamshire, UK) and hybridized at 65°C in hybridization solution containing Rse, Axl, or Gas cDNA labeled with [α-^{32}P]-deoxycytidine triphosphate (dCTP) by random priming as previously described (Lee et al, 1997). Radioactivity of the hybridized PCR fragments was visualized by autoradiography with Kodak X-OMAT AR films (Eastman Kodak, Rochester, NY).

Northern Blot Analysis

Northern blot analysis was performed as previously described (Lee et al, 1997). In brief, total RNA was isolated from the clonal cell lines with RNAgent Total RNA Isolation System (Promega, Madison, Wis). The quality and concentration of RNA were determined by gel electrophoresis and spectrophotometry at 260 nm. Total RNA (25 μg/sample) was resolved by 1% agarose-formaldehyde gel electrophoresis and transferred by capillary blotting to a nylon membrane (Hybond-N, Amersham Life Science). RNA was cross-linked by exposure to ultraviolet light (Ultraviolet Crosslinker, Amersham Life Science), and the blotted membranes were prehybridized for 5 hours in 50% deionized formamide at 42°C. Membranes were hybridized under the same conditions for 24 hours in fresh hybridization solution containing a α-^{32}P-labeled Rse, Axl, or Gas6 riboprobe synthesized by the Riboprobe In Vitro Transcription System (Promega). The membranes were then washed 3 times for 10 minutes each with 2× 0.3 mol/L sodium chloride and 30 mmol/L sodium citrate (SSC, pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature and then washed 2 times for 20 minutes each with 1× SSC containing 0.1% SDS at 65°C. The same blot was washed and reprobed with a α-^{32}P-labeled β-actin cDNA probe to confirm the integrity of the RNA samples. Radioactivity of hybridized mRNA species was measured by phosphor-imaging scanning (STORM 860, Molecular Dynamics, Sunnyvale, Calif) and visualized by autoradiography with double intensifying screen at −80°C with Kodak X-OMAT AR films for 4 hours to 7 days. The time was dependant on the abundance of the target mRNA.

Detection of Tyrosine Phosphorylation

Cells removed from culture were lysed on ice with 1 mL of lysis buffer (20 mmol/L Tris-HCl, 135 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L sodium vanadate, 1 mmol/L sodium molybdate, 2 mmol/L EDTA, 1% Nonidet P-40 (vol/vol), 1 mmol/L phenylmethylsulfonyl fluoride; pH 7.4). Cell lysates were clarified by centrifuging at 14 000 × g at 4°C for 10 minutes. Immunoprecipitation was performed by incubating cell lysates with antiphosphotyrosine antibody (1:500; Sigma) at 4°C overnight and subsequently incubating cell lysates with protein G-Sepharose (Sigma) for 1 hour at room temperature. Immunocomplexes were resolved by SDS-polyacrylamide gel electrophoresis (4% T stacking and 10% T resolving) and transferred to nitrocellulose membranes. Immunoblotting was performed by incubating the membranes with goat anti-Rse polyclonal antibody (against a synthetic peptide containing the last 20 amino acids of the Rse C-terminus; 1:500, sc-1095, Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) for 2 hours at room temperature. The immunocomplexes on the blotted membranes were visualized by incubation for 30 minutes at room temperature with anti-goat alkaline phosphatase conjugate (1:1000) and then incubated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The bands were scanned with laser densitometer (GS-300, Bio-Rad).

Statistical Analysis

The data were expressed as mean ± SEM for 3 independent experiments. Differences between means in compared groups were evaluated by Student's *t* test with the Instat Graph Pad Software (GraphPad Inc, San Diego, Calif).

Results

RT-PCR Analysis Showed That Tyrosine Kinase Receptors Rse and Mer and Ligand Gas6 Were Widely Expressed in the Testis

RT-PCR was used to assess whether tyrosine kinase receptors Rse, Axl and Mer and their ligand Gas6 were expressed in the testicular somatic cell lines. A 663-bp PCR product corresponding to the expected size of Rse, a 397-bp PCR product corresponding to the expected size of Gas6, and a 238-bp PCR product corresponding to the expected size of Mer (Figure 1A) were found in TR1, TM3, TM4, and TRM cells. ROG cells served as a positive control for Rse. The authenticity of PCR fragments was confirmed either by subsequent Southern blot analysis (for Rse and Gas6, Figure 1B) or by direct nucleotide sequencing of the PCR fragment (for Mer, data not shown). Figure 1 illustrates tyrosine kinase receptors Rse and Mer and their ligand Gas6 were widely expressed in the testicular somatic cell lines. However, Axl mRNA could not be detected in any of the cell types studied by RT-PCR or by Southern blot analysis (data not shown).

Northern Blot Analysis Reveals That Rse and Gas6 mRNAs Were Expressed in the Testis

We further characterized the expression of Rse and Gas6 by Northern blot analysis of total RNA extracted from various testicular cell lines. When 25 μg of total RNA from various cell cultures was analyzed by Northern blotting, a single predominant band of 3.8-kilobase (kb) Rse mRNA was detected in all of the testicular cell types studied (Figure 2A and B). The highest amount of hybridization was detected in TR1 cells when hybridization was measured by phosphor-imaging scanning and normalized against β-actin (Figure 2C). Similarly, a 2.9-kb Gas6 mRNA was detected in all of the cell types examined, and was most abundantly expressed in the TM4 cell line (Figure 2A through C). Figure 2 illustrates the widespread distribution of Rse and Gas6 mRNAs in the testis. However, Axl mRNA level was not detectable in the testicular cell lines by Northern blot analysis (data not shown).

Gas6 Is Expressed in Primary Sertoli Cells

Because the highest level of Gas6 mRNA was detected in the TM4 cells, we sought to examine whether Gas6 mRNA was detected in the primary Sertoli cells. When primary rat Sertoli cells cultured at 0.75×10^6 cells/cm^2 were terminated at various times between day 0 and day 7 and examined for Gas6 mRNA expression by RT-PCR, it was found that there was a constant expression of Gas6 mRNA (Figure 3). It has been reported that inter-Sertoli cell specialized junctions are known to form by day 4 under these culture conditions (Galdieri et al, 1981). These results demonstrate that Gas6 is expressed in primary Sertoli cells, and its mRNA expression level remains constant throughout the culturing periods.

Insulin and Transferrin Have an Up-Regulating Effect on the Expression of Gas6 in Mouse Sertoli Cells

Because it was found that Sertoli cells expressed the highest level of Gas6 among other cell types in the testis, we next sought to demonstrate the regulation of Gas6 expression, and we used TM4 cell line as an in vitro model. The effect of insulin and transferrin on the regulation of Gas6 mRNA expression in TM4 cells was studied. TM4 cells were cultured in serum-free media without or with different concentrations of insulin or transferrin for 20 hours to 2 days until 80% confluency when the cells were analyzed for Gas6 expression by Northern blotting. It was noted that addition of insulin and transferrin to cultures promoted cell proliferation to attain confluency. Even in the absence of insulin or transferrin alone, the cells cultured in serum-free medium abundantly expressed the 2.9-kb Gas6 mRNA. The level of expression was significantly increased at 10 ng/mL of insulin and remained constant up to a concentration of 5 μg/mL of insulin (Figure 4). A similar pattern was seen for transferrin, with a significant increase of Gas6 expression at concentrations of 500 ng/mL to 5 μg/mL of transferrin (Figure 4).

Expression of Gas6 mRNA is Regulated by Forskolin

Gas6 mRNA was expressed in the TM4 cells. Because follicle-stimulating hormone (FSH) is thought to be the

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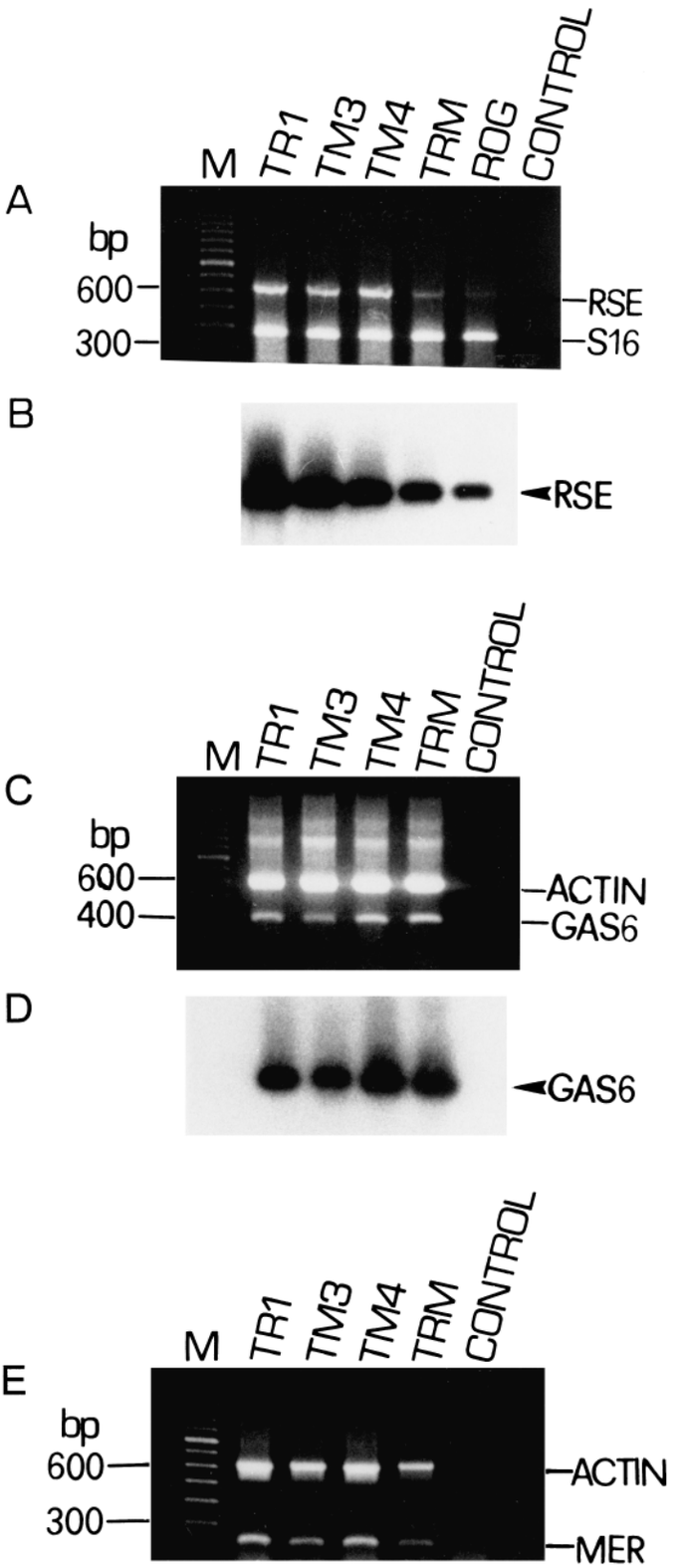
Figure 1. Rse/Mer and Gas6 expression in clonal cell lines examined by reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR reaction was performed with total RNA from each of the cell lines cultured in serum-supplemented medium. RT-PCR detection of Rse coamplified with S16 ribosomal protein primers (**A**) to give a 663-bp Rse and 384-bp S16 fragments. The same gel was transferred onto nylon membrane and was hybridized **(B)** with α-^{32}P-labeled Rse complementary DNA (cDNA) at 42°C overnight. RT-PCR detection of growth arrest–specific gene 6 (Gas6) coamplified with β-actin primers **(C)**to give a 397-bp Gas6 and 644-bp β-actin fragments. The gel from RT-PCR detection of Gas6 was transferred onto nylon membrane **(D)** and was hybridized with α-^{32}P-labeled Gas6 cDNA. RT-PCR detection of Mer **(E)** coamplified with β-actin primers to give a 238-bp Mer and 644-bp β-actin fragments. Each diagram shows typical gel picture from 3 sets of independent experiments. M indicates DNA size markers; C, control RT-PCR with no reverse transcriptase added.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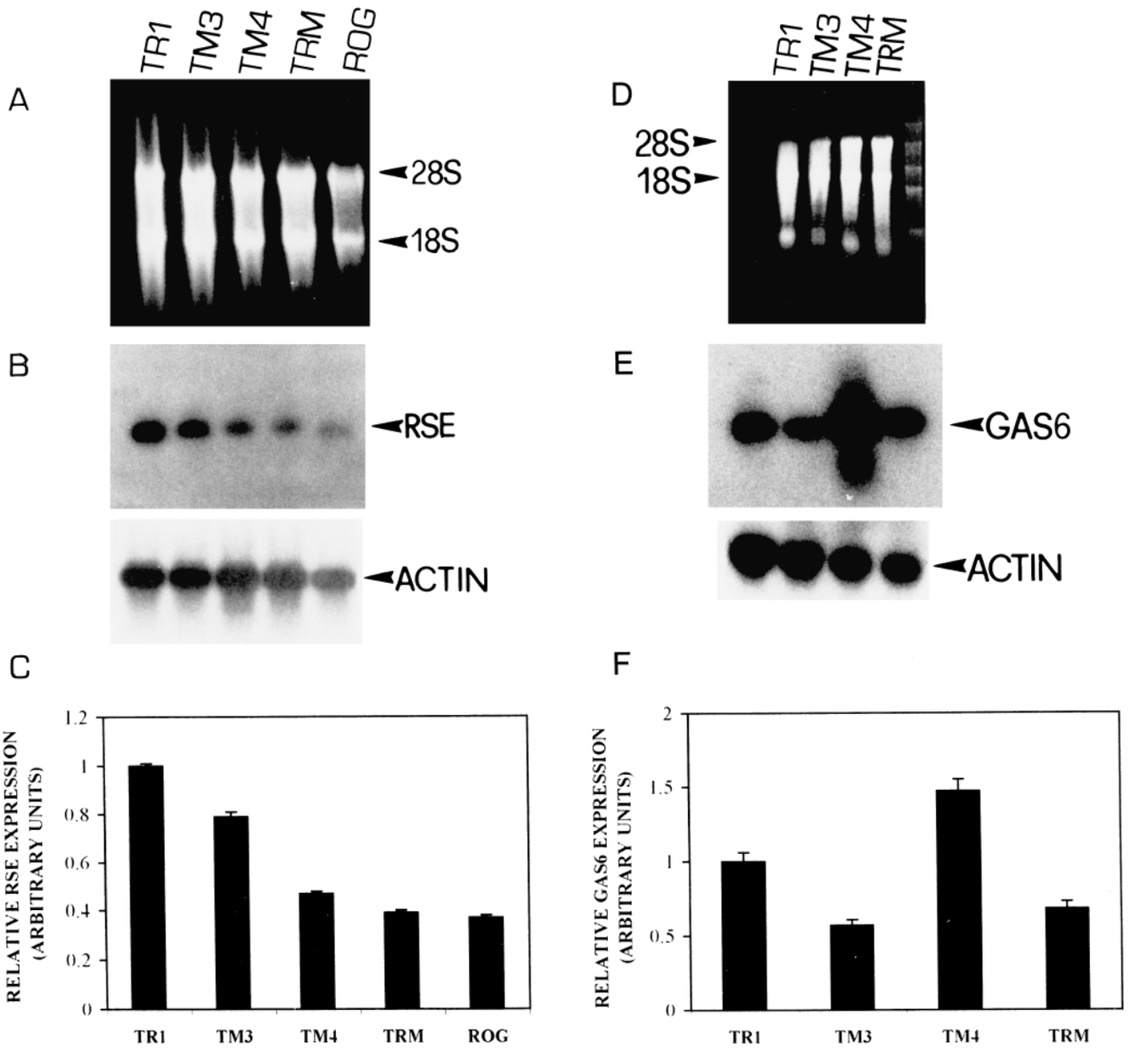

Figure 2. Northern blot analysis of Rse **(A–C)** and Gas6 **(D–F)** expression in clonal cell lines. Total RNAs were extracted from various cells cultured, resolved, and stained **(A, D)**. The gels were hybridized with either an α-^{32}P-labeled Rse riboprobe **(B)** or an α-^{32}P-labeled Gas6 riboprobe **(E)**. A 3.8-kilobase (kb) Rse RNA and 2.9-kb Gas6 RNA were detected after 3 days and 2 days of autoradiographic exposure at −80°C, respectively. The blots were washed and reprobed with α-^{32}P-labeled β-actin cDNA **(B, E)**. The graphs show Rse **(C)** and Gas6 **(F)** expression in Northern blots quantified by phosphor-imaging. Results are presented as mean ± SEM from 3 independent experiments. Arrows indicate the position of 18S and 28S ribosomal RNA subunits.

hormone that most influences the development and physiologic features of the seminiferous epithelium, where those cells are originally located, we next sought to examine the effect of the cyclic adenosine monophosphate (cAMP) stimulator forskolin on Gas6 expression by TM4 cells. Cells were cultured in serum-free medium supplemented with insulin, transferrin, and EGF in the presence or absence of 20 μmol/L of forskolin for 22 hours. Gas6 expression was detected by Northern blot analysis. It was shown that the level of Gas6 mRNA expression in TM4 cells was markedly increased when the cells were cultured with forskolin (Figure 5A and B). This stimulation of Gas6 expression by forskolin on TM4 cells was further confirmed by time course studies in serum-free and serum-supplemented cultures. In such experiments, cells were split from the same stock culture, incubated in the presence or absence of 20 μmol/L of forskolin and with or without serum in the same multiwell plate. Cells were

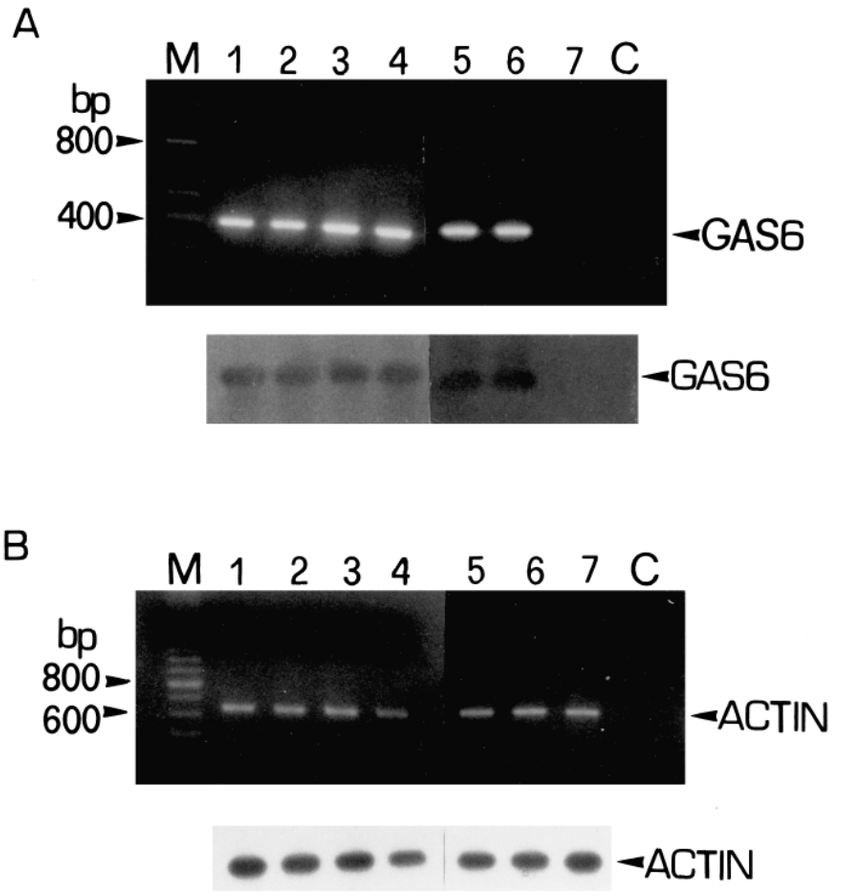

Figure 3. RT-PCR analysis of Gas6 expression in primary Sertoli cell culture. Total RNA extracted from cells were reverse transcribed and subsequently amplified with either specific Gas6 primers **(A)** to give a 397-bp fragment or β-actin primers **(B)** to give a 644-bp fragment. PCR products were resolved on gel and stained. The authenticity of the PCR fragments was confirmed by transferring the same gel to nylon membrane and hybridizing with either α-^{32}P-labeled Gas6 or β-actin cDNA at 42°C overnight **(Lower Panels A and B)**. This experiment was repeated 3 times with identical results with different culture preparations. Lanes 1 through 6 indicate 0, 1, 2, 3, 5, and 7 days in culture, respectively; lane 7, rat ovarian granulosa (ROG) cells; lane C, control RT-PCR with no reverse transcriptase added; and M, DNA size markers.

removed at same intervals for analysis of Gas6 expression by Northern blotting. It was shown that in serum-supplemented culture, Gas6 mRNA expression was markedly increased 20 hours after forskolin was added and was maintained there until 50 hours after forskolin addition (Figure 5C). In serum-free culture, Gas6 mRNA level was gradually increased with addition of forskolin, and it reached its highest level at 50 hours (Figure 5D).

Rse Is Phosphorylated in Response to Forskolin In Vitro

Gas6 has been shown to bind to the Rse/Axl/Mer receptor tyrosine kinases and to promote proliferation or differentiation in a number of cell types (Godowski et al, 1995; Varnum et al, 1995; Goruppi et al, 1996; Li et al, 1996). Our results have indicated that forskolin has an up-regulating effect on Gas6 expression by TM4 cells, and TM4 cells express Rse receptor. We therefore sought to determine whether forskolin would enhance Rse phosphorylation in TM4 cells. TM4 cells cultured in the presence of 20 μmol/L of forskolin were removed from serum-free culture at different times. They were homogenized and subjected to immunoprecipitation and immunoblotting by antiphosphorylated tyrosine antibody and anti-Rse antibody, respectively. Immunoblotting with anti-Rse antibody revealed a 140-kd band of the Rse receptor. The results from experiments with immunoprecipitation and immunoblotting indicated that there was a notable increase in tyrosine phosphorylation of the Rse receptors when forskolin was added (Figure 6A and C). Experiments with immunoblotting alone did not show a dramatic change in Rse receptor expression, indicating that the increase in tyrosine phosphorylation was not caused by an increase in Rse receptor number (Figure 6B). These results illustrate that forskolin enhances tyrosine phos-

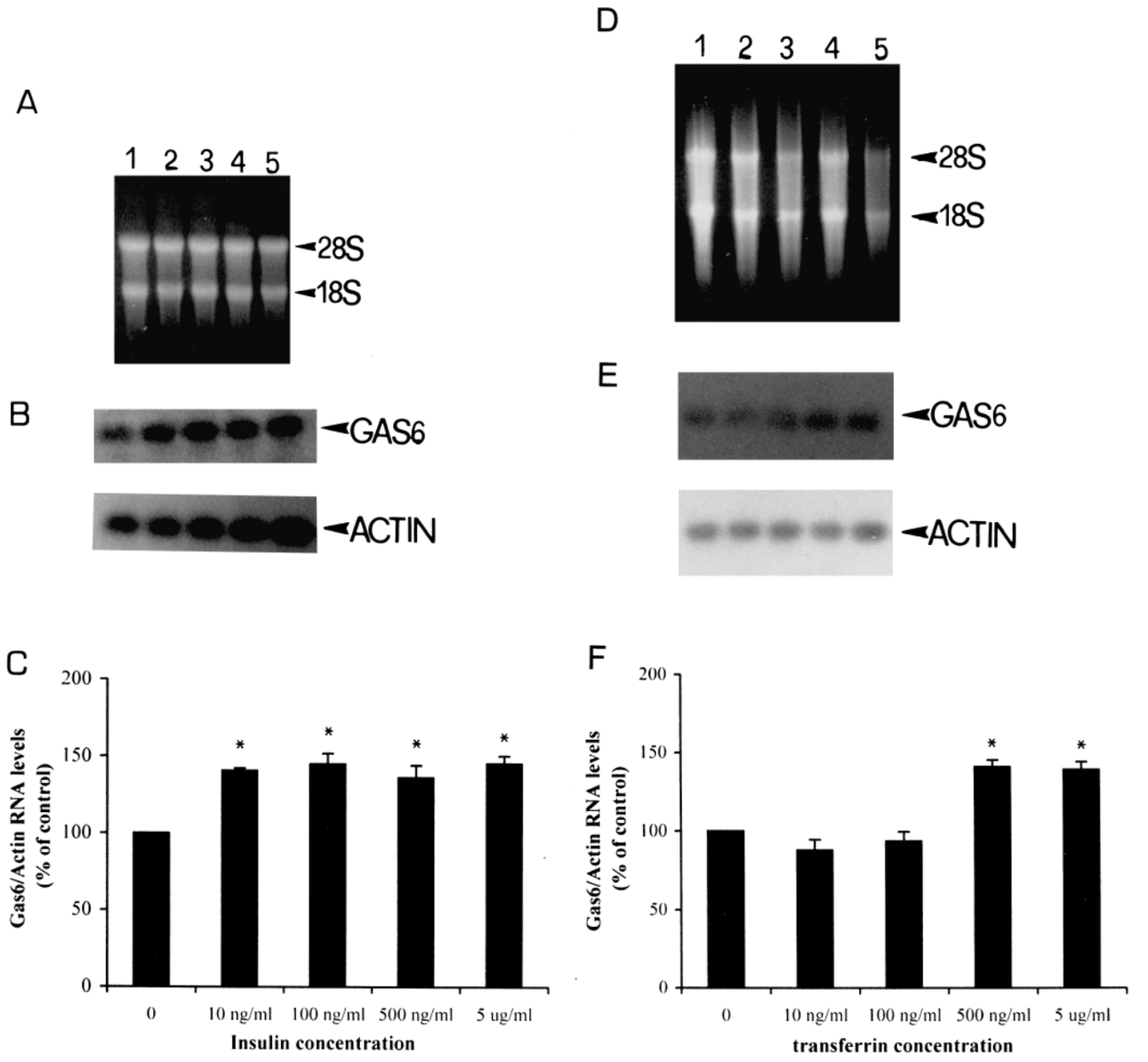

Figure 4. Northern blot analysis of the effect of insulin **(A–C)** and transferrin **(D–F)** on Gas6 expression in mouse Sertoli (TM4) cells cultured in serum-free medium. Total RNA was extracted from TM4 cells cultured in various concentrations of insulin **(A)** or transferrin **(D)**. Gels were transferred onto nylon membranes and hybridized **(B**, **E)** overnight. A 2.9-kb Gas6 RNA was detected after 1 day of autoradiographic exposure at −80°C. The blots were washed and reprobed. The graphs **(C**, **F)** show Gas6 expression in Northern blots measured by phosphor-imaging. The amount of Gas6 expression was normalized to β-actin expression detected in the same application. Results are presented as mean ± SEM from 3 independent cell culture experiments. Lanes 1 through 5 indicate 0, 10, 100, 500, and 5000 ng/mL insulin **(A)** or transferrin **(B)**, respectively; arrows, the position of 18S and 28S ribosomal RNA subunits; *, significantly different from control without the addition of insulin or transferrin, $P < .02$.

phorylation of Rse receptor in mouse Sertoli cells. Although a dramatic increase in Gas6 mRNA level was detected 20 hours after forskolin addition to the serum-free culture (Figure 5D), a measurable increase of Rse tyrosine phosphorylation was not observed until 50 hours after forskolin addition (Figure 6A and C). This lag between Gas6 mRNA expression and tyrosine phosphorylation suggests that there are other unknown factors that may regulate and delay Gas6 protein synthesis and secretion and its binding onto the cell surface receptors.

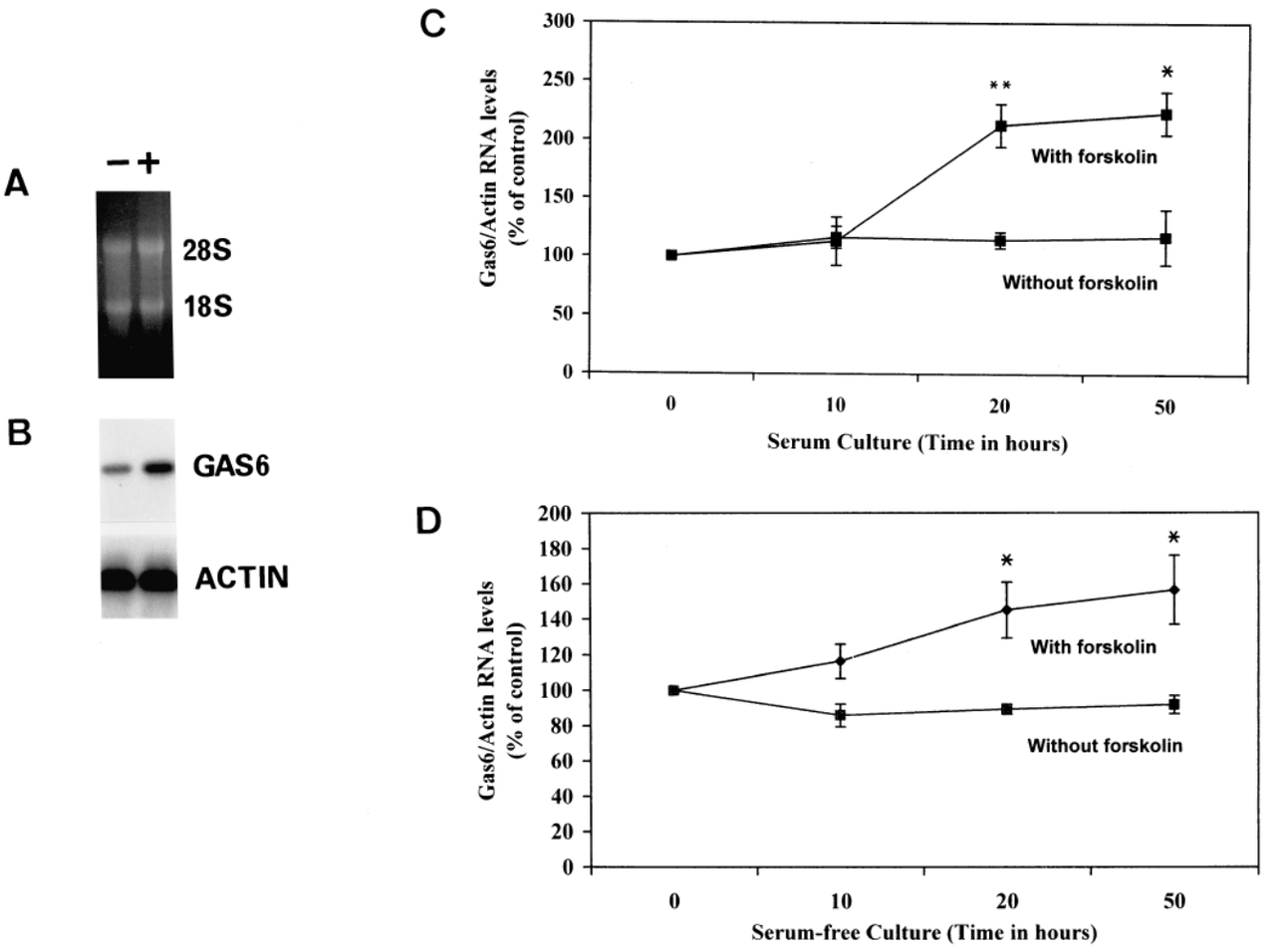

Figure 5. Northern blot analysis of the effect of forskolin on Gas6 expression in TM4 cells. Total RNA was extracted from cells cultured in the absence or presence of forskolin **(A)**. All culture media contained epidermal growth factor (EGF), insulin, and transferrin. RNA was resolved onto 1% agarose-formaldehyde gel and stained. The same gel was transferred onto nylon membrane and hybridized **(B)** overnight. A 2.9-kb Gas6 RNA was detected after 1 day of autoradiographic exposure at −80°C. The same blot was washed and reprobed with α-^{32}P-labeled β-actin cDNA. Time course studies of the effect of forskolin on Gas6 expression in TM4 cells cultured in serum-supplemented medium **(C)** and serum-free medium **(D)**. The graphs show Gas6 expression in Northern blots measured by phosphor-imaging. The amount of Gas6 expression was normalized to β-actin expression detected in the same application. Results are presented as mean ± SEM from 2 independent cell culture experiments. Arrows indicate the position of 18S and 28S ribosomal RNA subunits; * and **, significantly different from the respective control without forskolin, $P < .05$ and $P < .01$, respectively.

Discussion

RTKs are proteins that transmit signals from the extracellular environment into the cell cytoplasm following the binding of peptide growth factors (Ullrich and Schlessinger, 1990; Van der Geer et al, 1994). Interactions involving these molecules are critical in regulating cell survival, proliferation, and differentiation. The mammalian testis is a paracrine organ composed of multiple cell types functioning together to maintain androgen production and germ cell development. It is a complex organ consisting of Leydig cells, Sertoli cells, myoid cells, and germ cells as the major distinct cell types. The seminiferous epithelium of the testis is sequestered from the systemic blood circulation and its hormonal influence. It is not surprising that a variety of gonadal peptides are expressed and serve as paracrine regulators of testicular function in this compartment. In view of the difficulties obtaining experimental systems to study each primary cell type individually, testicular clonal cell lines were used as models in the present study. We have demonstrated that of the 3 RTKs studied, Rse and Mer mRNAs were widely expressed in these cell lines.

The tyrosine kinase receptors, Rse, Axl, and Mer, were once regarded as ''orphan'' receptors. Recently, however, the ligands that bind and activate these receptors have been identified. By use of techniques of receptor-based detection and affinity purification, recent studies have identified that the vitamin K–dependent protein Gas6 is a ligand for Rse (Godowski et al, 1995), Axl (Varnum et

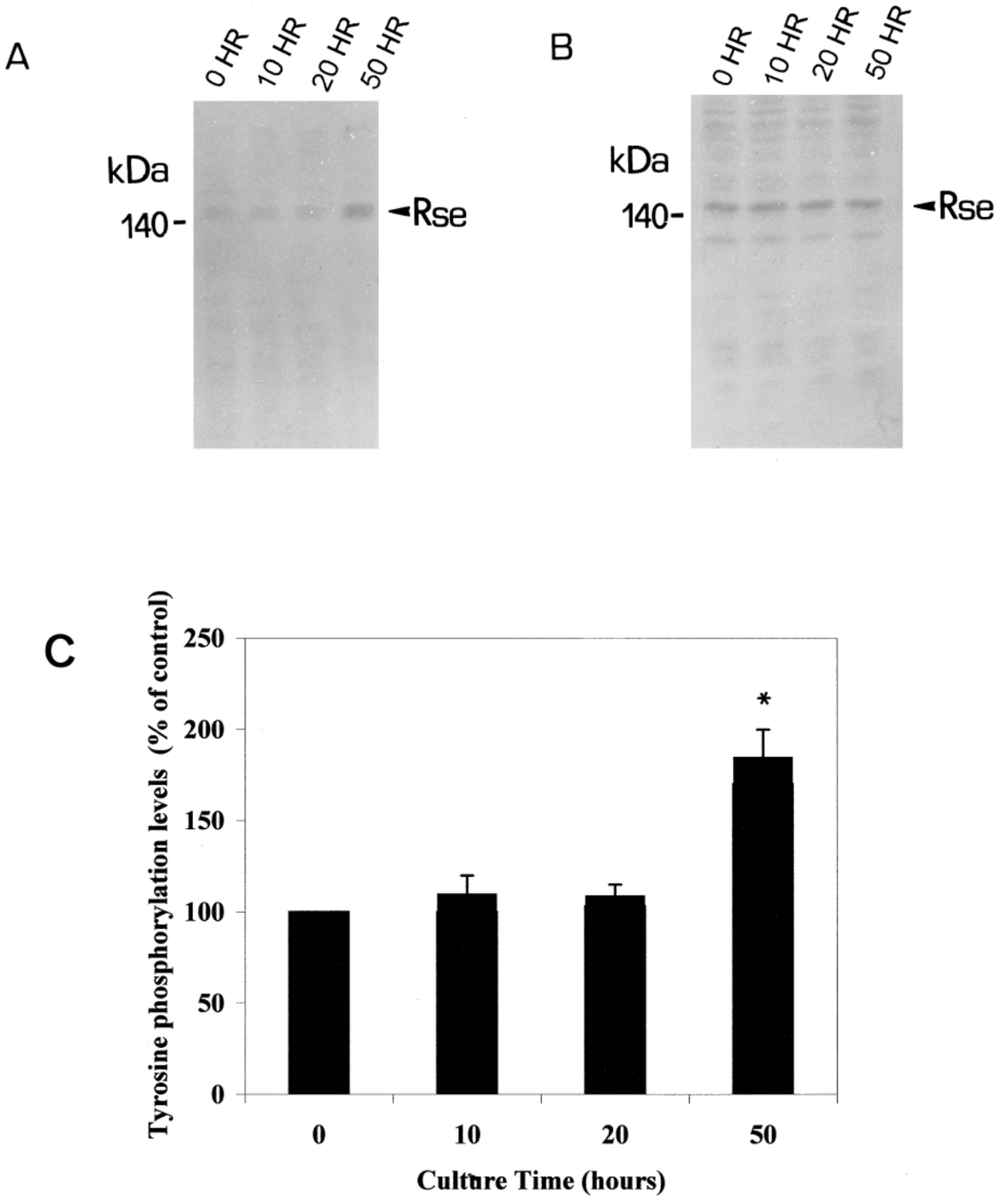

Figure 6. Forskolin activation of Rse receptor tyrosine kinase phosphorylation in TM4 cells. Cells were incubated with 20 μmol/L forskolin in serum-free media supplemented with insulin, transferrin, and EGF for time indicated. Cell lysates **(A)** were prepared, immunoprecipitated with mouse anti-phosphotyrosine antibody, and immunoblotted with goat anti-Rse polyclonal antibody. Cell lysates **(B)** were immunoblotted with goat anti-Rse polyclonal antibody directly without immunoprecipitation with anti-phosphotyrosine antibody. The graph shows Rse tyrosine kinase phosphorylation **(C)** from cell lysates that were immunoprecipitaed. Phosphorylation was measured by laser densitometry. Results are presented as mean ± SEM from 3 experiments. * indicates significantly different from cell cultures at Time 0, $P < .05$.

al, 1995), and Mer (Chen et al, 1997). The fact that both the ligand and receptors are highly expressed in the nervous system suggests that RTKs play an important role in regulating neuronal function (Mark et al, 1994; Stitt et al, 1995; Li et al, 1996). In a recent study of gene knockout mice, Lu et al (1999) demonstrated that depletion of Rse, Axl, and Mer markedly impairs male fertility by disruption of spermatogenesis. This depletion does not affect

the accessory sex glands or sexual behavior, indicating that those receptors are playing exclusively important roles in paracrine actions in the testis. By in situ hybridization, they also found that Rse, Axl, Mer, and Gas6 were expressed in the Sertoli cells, and that Mer and Gas6 were also detectable in the Leydig cells. In our study, we have extended this discovery to testicular somatic cell lines and have shown that Rse, Mer, and Gas6 are expressed in the TM4 and TM3 cell lines. We have also shown that the expression of Gas6 in TM4 cells is enhanced by the addition of forskolin, insulin, and transferrin to the cell cultures. This indicates that those cell lines, particularly the TM4 cells, are important for studying the function of Rse family receptors in regulating spermatogenesis in the testis. TM4 cells originate from immature testes of normal 11- to 13-day-old BALB/c mice (Mather 1980). This may be the reason why Axl was not detected in our studies; it was detected in Lu et al (1999), where more mature animals (5 and 18 weeks) were used.

Sertoli cells are the dominant somatic cells in the seminiferous tubules and nurse cells for spermatogenesis. From our data, Gas6 mRNA was more prominently expressed in the TM4 cells than in other cell types, and its expression showed a time-dependent increase in response to the addition of forskolin in vitro. Forskolin has been shown to be an adenylate cyclase activator and shares the same signaling pathway as FSH in elevating intracellular cAMP level, and TM4 cells bear FSH receptors (Mather 1980; Mather and Philips, 1984a). We have extended the observation by demonstrating a steady expression of Gas6 mRNA in primary rat Sertoli cell cultures. Gas6 is a potential growth factor that stimulates human Schwann cell proliferation through the Axl-Rse tyrosine kinase receptors (Li et al, 1996). Our results indicate that Gas6 expression by Sertoli cells in vitro is enhanced by forskolin. This forskolin-stimulated Gas6 expression was accompanied by an increase in Rse phosphorylation. It is likely that the Sertoli cells respond to hormonal signals (FSH) through the cAMP cascade to stimulate Gas6 production, which is also under the influence of insulin and transferrin. The Gas6 secreted from cells binds onto their own Rse receptors to trigger the tyrosine phosphorylation. This autocrine activity of the RTK system has precedence. Gas6 has been shown to potentiate thrombin-induced cell proliferation and apoptosis of rat vascular smooth muscle cells, and rat vascular smooth muscle cells produce Gas6 (Nakano et al, 1995, 1996).

Gas6/Ark (Axl) signaling has been shown to inhibit apoptosis in murine gonadotropin-releasing hormone (GnRH) neuronal cells via pathways of the extracellular signal-regulated kinase (ERK) and the serine-threonine kinase, Akt (Allen et al, 1999). Although we did not detect Axl expression in the testicular somatic cell lines, the presence of Rse and Mer may exhibit similar roles to promote cell survival. It is shown in the present study that insulin, transferrin, and FSH (foskolin), which are essential supplements to promote Sertoli cell survival in serum-starved cultures (Mather et al, 1982; Mather and Philips, 1984b), enhance Gas6 expression.

In summary, we have presented data showing that the tyrosine kinase receptors Rse and Mer (but not Axl) and the growth factor Gas6 are widely expressed in the testicular somatic cell lines. Our experiments demonstrated a time-dependent increase in Gas6 expression and Rse tyrosine phosphorylation with the addition of forskolin in vitro, which suggests that Gas6 may exhibit an autocrine effect through multiple tyrosine kinase receptors in regulating testicular function. Further studies are required to identify the signaling pathway involved in the interaction between Rse/Mer receptor tyrosine kinases and their ligands. There will undoubtedly be continued rapid progress in this area, and seemingly, TM4 is a good cell line model for further studies in this field.

References

Allen MP, Zeng C, Schneider K, Xiong X, Meintzer MK, Bellosta P, Basilico C, Varnum B, Heidenreich KA, Wierman ME. Growth arrest-specific gene 6 (Gas6)/adhesion related kinase (Ark) signaling promotes gonadotropin-releasing hormone neuronal survival via extracellular signal-regulated kinase (ERK) and Akt. *Mol Endocrinol.* 1999;13:191–201.

Bellosta P, Costa M, Lin DA, Basilico C. The receptor tyrosine kinase Ark mediates cell aggregation by homophilic binding. *Mol Cell Biol.* 1995;15:614–625.

Chen J, Carey K, Godowski PJ. Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. *Oncogene.* 1997;14:2033–2039.

Chung SSW, Lee WM, Cheng CY. A study on the formation of specialized inter-Sertoli cell junctions in vitro. *J Cell Physiol.* 1999;181:258–272.

Chung SSW, Mo MY, Lee WM, Cheng CY. Rat testicular N-cadherin: its complementary deoxyribonucleic acid cloning and regulation. *Endocrinology.* 1998;139:1853–1862.

Dai W, Pan H, Hassanain H, Gupta SL, Murphy MJ, Jr. Molecular cloning of a novel receptor tyrosine kinase, tif, highly expressed in human ovary and testis. *Oncogene.* 1994;9:975–979.

Dahlback BA, Lundwall A, Stenflo J. Localization of thrombin cleavage sites in the amino-terminal region of bovine protein S. *J Biol Chem.* 1986;261:5111–5115.

Fujimoto J, Yamamoto T. Brt, a mouse gene encoding a novel receptor-type protein-tyrosine kinase, is preferentially expressed in the brain. *Oncogene.* 1994;9:693–698.

Galdieri M, Ziparo E, Palombi F, Russo MA, Stefanini M. Pure Sertoli cell cultures: a new model for the study of somatic-germ cell interactions. *J Androl.* 1981;5:249–259.

Gershagen S, Fernlund P, Lundwall A. A cDNA coding for human sex hormone binding globulin. *FEBS Lett.* 1987;220:129–135.

Godowski PJ, Mark MR, Chen J, Sadick MD, Raab H, Hammonds RG. Reevaluation of the role of protein S and Gas6 as ligand for the receptor tyrosine kinase Rse/Tyro 3. *Cell.* 1995;82:355–358.

Goruppi S, Ruaro E, Schneider C. Gas6, the ligand of Axl tyrosine kinase receptor has mitogenic and survival activities for serum starved NIH3T3 fibroblasts. *Oncogene.* 1996;12:417–480.

Graham DK, Bowman GW, Dawson TL. Cloning and developmental expression analysis of the murine c-mer tyrosine kinase. *Oncogene.* 1995;10:2349–2359.

Graham DK, Dawson TL, Mullaney DL. Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. *Cell Growth Differ.* 1994;5:647–657.

Janssen JWG, Schulz AS, Steenvoorden ACM, Schmidberger M, Strehl S, Ambros PF, Bartram CR. A novel putative tyrosine kinase receptor with oncogenic potential. *Oncogene.* 1991;6:2113–2120.

Jia R, Hanafusa H. The proto-oncogene of v-eyk (*v-eyk*) is a novel receptor-type protein tyrosine kinase with extracellular Ig/FN-III domains. *J Biol Chem.* 1994;269:1839–1844.

Lai C, Gore M, Lemke G. Structure, expression, and activity of Tyro 3, a neural adhesion-related receptor tyrosine kinase. *Oncogene.* 1994; 9:2567–2578.

Lee WM, Wong AST, Tu AWK, Cheung C-H, Li JCH, Hammond CL. Rabbit sex hormone binding globulin: primary structure, tissue expression, and structure/function analyses by expression in Escherichia coli. *J Endocrinol.* 1997;153:373–384.

Li R, Chen J, Hammonds G, Philips H, Armanini M, Wood P, Bunge R, Godowsky PJ, Sliwkowski MX, Mather JP. Identification of Gas6 as a growth factor for human Schwann cells. *J Neurosci.* 1996;16:2012–2019.

Li R, Phillips DM, Moore A, Mather JP. Follicle-stimulating hormone induces terminal differentiation in a predifferentiated rat granulosa cell line (ROG). *Endocrinology.* 1997;138:2648–2657.

Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagranda F, Lai C, Skinner MK, Klein R, Matsushima GK, Earp HS, Goff SP, Lemke G. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. *Nature.* 1999;398:723–728.

Manfioletti G, Brancolini C, Avanzi G, Schneider C. The protein encoded by a growth arrest-specific gene (Gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Mol Cell Biol.* 1993;13:4976–4985.

Mark MR, Scadden DT, Wang Z, Gu Q, Goddard A, Godowski PJ. Rse, a novel receptor-type tyrosine kinase with homology to Axl/Ufo, is expressed at high level in the brain. *J Biol Chem.* 1994;269:10720–10728.

Mark MR, Chen J, Hammonds RG, Sadick M, Godowski PJ. Characterization of Gas6, a member of the superfamily of G domain-containing proteins, as a ligand for Rse and Axl. *J Biol Chem.* 1996;271:9785–9789.

Mather JP. Establishment and characterization of two distinct mouse testicular epithelial cell lines. *Biol Reprod.* 1980;23:243–252.

Mather JP, Phillips DM. Establishment of a peritubular myoid-like cell line and interaction between established testicular cell lines in culture. *J Ultrastruct Res.* 1984a;87:263–274.

Mather JP, Phillips DM. Primary culture of testicular somatic cells. In: *Methods for Serum-Free Culture of Cells of the Endocrine Systems.* New York: Alan R Liss Inc; 1984b:29–45.

Mather JP, Zhuang L-Z, Perez-Infante V, Phillips DM. Culture of testicular cells in hormone-supplemented serum-free medium. *Ann NY Acad Sci* 1982;383:44–68.

Nakano T, Higashino K, Kikuchi N, Kishino J, Nomura K, Fujita H, Ohara O, Arita H. Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factors for Ca(2+)-mobilizing growth factors. *J Biol Chem.* 1995;270:5702–5705.

Nakano T, Kawamoto K, Higashino K, Arita H. Prevention of growth arrest-induced cell death of vascular smooth muscle cells by a product of growth arrest-specific gene, gas6. *FEBS Lett.* 1996;387:78–80.

Ohashi K, Mizuno K, Kuma K, Miyata T, Nakamura T. Cloning of the cDNA for a novel receptor tyrosine kinase, Sky, predominantly expressed in brain. *Oncogene.* 1994;9:699–705.

Rescigno J, Mansukhani A, Basilico C. A putative receptor tyrosine kinase with unique structural topology. *Oncogene.* 1991;6:1909–1913.

Schlessinger J, Ullrich A. Growth factor signaling by receptor tyrosine kinases. *Neuron.* 1992;9:383–391.

Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, Mattsson K, Fisher J, Gies DR, Jones PF, Masiakowski P, Ryan TE, Tobkes NJ, Chen DH, DiStefano PS, Long GL, Basilico C, Goldfarb MP, Lemke G, Glass DJ, Yancopoulos GD. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell.* 1995;80:661–670.

Ullrich A. Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell.* 1990;61:203–212.

Van der Geer P, Hunter T, Lindberg RA. Receptor tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol.* 1994;10:251–337.

Varnum BC, Young C, Elliott G, Garcia A, Bartley TD, Fridell YW, Hunt RW, Trail G, Clogstone C, Tosos RJ, Yanagihara D, Bennett L, Sylber M, Merewether LA, Tseng A, Escobar E, Liu ET, Yamane HK. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. *Nature.* 1995;373:623–626.