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Testicular Leukemia Inhibitory Factor (LIF) and LIF Receptor Mediate Phosphorylation of Signal Transducers and Activators of Transcription (STAT)-3 and STAT-1 and Induce c-*fos* Transcription and Activator Protein-1 Activation in Rat Sertoli But Not Germ Cells*

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ABSTRACT

Increasing amounts of evidence suggest noninflammatory roles for growth factor and cytokines in development and differentiation. Leukemia inhibitory factor (LIF) belongs to a gp130 pleiotropic family of growth factors that has recently been shown to enhance the survival of rat testicular gonocytes and Sertoli cells. In this study, we show the expression of gp130 and LIF messenger RNAs (mRNAs) in the somatic (the Sertoli and Leydig cells) and specific germ cells (spermatogonia, pachytene, round, and elongated spermatids) of rodent testis, suggestive of cell-specific LIF-mediated functions. LIF receptor mRNA was demonstrated in rat somatic cells, rat elongating spermatids, and all of the mouse germ cells. In addition, we characterized the effects of LIF on the signal transducers and activators of transcription (STAT)-3 and STAT-1, c-*fos* gene expression, and activator protein-1 regulation in primary rat Sertoli cells. Electrophoretic mobility shift assay and Western blot analysis demonstrated that LIF translocates STAT-3 (and to a lesser extent STAT-1) transcription factor(s) to the nucleus within 2 min of exposure in a tyrosine but not serine/threonine phosphorylation-dependent pathway. Quantitative solution hybridization analysis revealed a transient increase in c-*fos* mRNA levels by 20-fold following 30–45 min of LIF treatment, an effect that was inhibited by the tyrosine, as well as serine/threonine kinase inhibitors, genistein, and H7. Subsequently, LIF treatment of the Sertoli cells increased nuclear activator protein-1 binding proteins at 2 h after its addition, an effect that was also sensitive to genistein and H7 pretreatments. In contrast, LIF treatment of primary rat germ cells did not alter c-*fos* mRNA levels. Species specificity in the expression of LIF receptor as well as ligand binding may play a role in LIF signaling in these germ cells. Thus, using a primary Sertoli cell model, we demonstrated that the testicular LIF signaling pathway is contingent on the phosphorylation of latent transcription factors. Our data are consistent with LIF-mediated signaling events involving both somatic and germ cells during spermatogenesis. (*Endocrinology* **139:** 1883–1890, 1998)

LEUKEMIA inhibitory factor (LIF), a multifunctional member of the gp130 cytokine family, is structurally and functionally related to interleukin-6 (IL-6), oncostatin M, and ciliary neurotrophic factor. These related cytokines bind to specific cell surface receptors that are coupled to a common gp130 signal transducing receptor component in diverse cell types and induce the homodimerization of the gp130 protein with the IL-6 receptor chain (in the case of IL-6) or heterodimerization of gp130 and the LIF receptor component (in the case of LIF, ciliary neurotrophic factor, and oncostatin M) (1–4). In response to stimulation by these cytokines, rapid intracellular tyrosine phosphorylation of the gp130 protein, Janus kinase (JAK)1, JAK2, and TYK2 kinases occurs, which activates the signal transducers and activators of transcription (STAT)-3/STAT-1 signal transduction pathway, and induces the differential transcriptional regulation of responsive genes, including the immediate early genes *jun*B and c-*fos* (5–9).

Although the effects of cytokines have primarily been shown in hematopoietic and hepatic cell lines, their presence and regulation in various cell types is consistent with physiological functions in noninflammatory conditions. LIF was originally shown to induce terminal differentiation of the immortalized M1 myeloid leukemia cells to a macrophage phenotype (10, 11). LIF is synthesized by a variety of cells and has a broad range of biological activities, including stimulation of acute-phase proteins in hepatocytes, hematopoietic cell development, neuronal development, differentiation, inflammation, and pregnancy, as well as embryo development (2, 12–16). In testes, although the localization of LIF and its receptor (LIFR), to our knowledge, has not yet been reported, the addition of LIF to cocultures enhances the *in vitro* survival of both primary Sertoli cells and proliferating gonocytes without affecting cell proliferation (17).

Studies from our laboratory have shown the expression and regulation of IL-6 and its receptor in primary Sertoli and Leydig cells (18, 19). Recently, we demonstrated that two testicular cytokines, IL-6 and interferon (IFN)-γ, differentially activate STAT-3 and STAT-1 transcription factors and induce c-*fos* gene expression in Sertoli cells, findings consistent with the presence of a functional cytokine-activated signal transduction pathway in the testis (20). In the present study, we establish the expression of the messenger RNAs (mRNAs) for LIF and its receptor chain components in both

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somatic and germ cells and identify a mechanism for LIF regulation of testicular STAT proteins, c-*fos* gene expression, and activator protein-1 (AP-1) activation using primary Sertoli cells as a model system.

Materials and Methods

Primary testicular cell preparations

Specific rat somatic (adult and immature Sertoli and Leydig cells) and various mouse and rat germ cells (spermatogonia, pachytene spermatocytes, and round and elongated spermatids) were isolated as described before (18, 19). Primary cultures of rat Sertoli cells were isolated and purified from 18-day-old Sprague-Dawley rats (Charles River, Kingston, NY) and incubated at 34 C at a density of 1×10^7 cells per 100-mm polystyrene dish in phenol red-, serum-, and endotoxin-free DME/F-12 medium (Irvine Scientific, Santa Ana, CA) as described previously (18, 19). The medium was supplemented with 2.5 μg/ml bovine insulin (Sigma, St. Louis, MO), 1 μg/ml transferrin (Calbiochem, La Jolla, CA), and 10 μg/ml bacitracin (Sigma). On day 3 *in vitro*, following the addition of specific factors, RNA or nuclear extracts were isolated at the indicated times. Duplicate or triplicate culture dishes were used for each drug treatment and were repeated at least once. The mean (± SEM) of all the repeats were shown for RNA analysis. Recombinant murine LIF (R & D Systems, Minneapolis, MN) was dissolved in 0.1% BSA as a 200× (10 μg/ml) stock solution. Matched aliquots of 0.1% BSA were used in control cultures.

Procedures involving the use of animals strictly followed the Guidelines for the Care and Use of Laboratory Animals set forth by the NIH.

Analysis of mRNA levels

Total RNA was extracted using the Trizol reagent (Life Sciences, Gaithersburg, MD). Riboprobes were prepared from plasmids containing complementary DNAs for rat c-*fos* (specific activity = 6.6×10^8 dpm/μg) and human 18S ribosomal RNA (rRNA) (specific activity = 1.0×10^7 dpm/μg) (21). For solution hybridization assays, duplicates of total RNA extracts (20–30 μg) were hybridized in 30 μl buffer (10 mM EDTA, 0.3 M NaCl, 0.5% SDS, and 10 mM N-Tris[hydroxymethyl)-methyl-2-amino-ethanesulfonic acid, pH 7.4) containing ^{32}P riboprobe, 150,000 dpm for the c-*fos* riboprobe or 80,000 dpm for the 18S riboprobe, respectively, for 4 h at 75 C. After hybridization, 300 μl 0.3 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4, containing 40 μg/ml ribonuclease (RNase) A and 2 μg/ml RNase T1 was added to each tube, and the samples were incubated at 30 C for 1 h. The samples were precipitated with 1 ml 5% TCA and 0.75% sodium pyrophosphate and one drop of 0.5% BSA and were collected onto glass fiber filter paper (Brandel, Gaithersburg, MD) using a 24-place cell harvester. The filters were counted by liquid scintillation in 5 ml hydrofluor scintillation solution (National Diagnostics, Manville, NJ). Comparison was made with standard calibration curves to quantify the c-*fos* mRNA transcript and the 18S rRNA levels. c-*fos* mRNA levels were then normalized to the level of control samples (1 pg/μg 18S rRNA). Northern blot analysis was performed using 20 μg total RNA by the glyoxal method (20).

Oligonucleotide primers and RT-PCR amplification

Rat LIF primers were 5′-CAATGCCCTCTTTATTTCCTATTACACAGC-3′ and 5′-GGGGACACAGGGCACATCCACATGGCCCAC-3′ (16). These primers are derived from exon 2 and exon 3 sequences and detect both splice variants of LIF. Because rat LIF receptor sequences were not available, mouse LIF receptor primers were designed: 5′-GAAAACTGTAAGGCGCTACA-3′ and 5′-CCAAGTGTTTACATTGGC-3′ (3, 22). These primers are from different exons to eliminate detecting contaminating genomic DNAs. One microgram total RNA was reversed transcribed using GeneAmp kit (Perkin-Elmer, Norwalk, CT) according to manufacturer's directions. Two microliters of RT reaction products were used in a 50 μl PCR reaction that contained 1.5 μCi [^{32}P]deoxycytidine triphosphate (3000 Ci/mmol, Amersham, Arlington Heights, IL) to visualize the products. PCR amplification of complementary DNAs for LIF receptor were done for 35 cycles at 1 min at 95 C, 1 min at 48 C, and 1 min at 72 C, whereas for LIF, it was done for 35 cycles at 1 min at 95 C, 1 min at 55 C, and 1 min at 72 C. Ten microliters of PCR products were analyzed on a 5% polyacrylamide gel and autoradiographed. The PCR products were confirmed by sequencing analysis. RT-PCR products for LIFR from immature Sertoli cells showed 79% and 86% homology when compared with published LIFR sequences for human (emb X61615 HSLIFR *H. sapiens* mRNA for leukemia inhibitory factor receptor) and mouse (dbj D26177 MUSDFLR Mouse mRNA for D-factor/LIF receptor), respectively. The following mouse primers were used to analyze gp130 mRNA:5′-GCAGCAGGTTTCAGATCACA-3′ and 5′-CCCAGGTGTGACTTTGTCCT-3′. The amplification of the S16 ribosomal gene was used as an internal control with the following primers: 5′-AAGTCTTCGGACGCAAGAAA-3′ and 5′-GACAAGACGAAGACCCGTT-3′ (23).

Nuclear extract preparation, electrophoretic mobility shift assay (EMSA), and Western analysis

Nuclear extracts were prepared from unstimulated or stimulated Sertoli cells as described before (24, 25). The sequence of m67 oligo is 5′ CATTTCCCGTAAATCGTCGA 3′ (26). The complementary oligos were annealed in 100 mM Tris-HCl (pH 8.0) and 50 mM $MgCl_2$ over a period of 3 h. The AP-1 oligo was obtain from Promega Corp. (Madison, WI). The oligos were end labeled with T4 polynucleotide kinase and [^{32}-gamma]ATP. Nuclear extracts (2–10 μg) were incubated in a final volume of 12 μl in 20 mM HEPES (7.9), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, and 4% Ficoll for 20 min at room temperature with the probe [150,000–200,000 cpm, 1 ng for m67 and *sis*-inducible element (SIE), and 50,000 cpm for AP-1]. Antisera to STAT-3 protein (kindly provided by J. E. Darnell, Jr., Rockefeller University) were preincubated for 10 min before addition of the probe. The reaction products were fractionated on a 4% nondenaturing polyacrylamide gel (acrylamide/bis, 29:1) in 0.25% Tris-borate-EDTA that was prerun for 30 min at room temperature. For Western analysis, 500 μg of nuclear extracts were immunoprecipitated with antiphosphotyrosine-agarose (Transduction Laboratories, Lexington, KY) for 2 h at 4 C and run on a 7.5% SDS/PAGE, blotted to nitrocellulose membrane, and probed with an anti-STAT-3 antiserum (1:1000). The bound antibodies were detected using an epichemiluminescence kit (Amersham).

Results

Distribution of gp130, LIFR, and LIF in freshly isolated testicular cells

We used RT-PCR analyses to establish the distribution of gp130, LIF, and LIFR mRNAs in different purified cell types of the testes, including rat immature and adult somatic cells (Fig. 1, lanes 4–7), rat germ cells (Fig. 1, lanes 8–11), and mouse germ cells (Fig. 1, lanes 12–15). RT-PCR analysis of total RNA using a primer set for gp130 signaling chain detected its mRNA in both the somatic and specific germ cells (Fig. 1, *top panel*). Because rat LIFR sequences are not available, we designed two sets of primers using the published mouse LIF receptor sequences. RT-PCR of total RNA from different cells showed that although all of the somatic cells express the LIFR (Fig. 1, LIF receptor, *middle panel*, lanes 4–7), in elutriator-purified germ cells from rat testis, LIFR mRNA was detected only in the elongated spermatids. Figure 1, *middle panel*, illustrates that LIF mRNA is expressed in both the immature and adult somatic cells, as well as all of the germ cells (lanes 4–11). Sequence analysis of the RT-PCR products from immature Sertoli cells (lane 5) confirmed that the PCR products were LIF and LIFR. Sequencing of the RT-PCR products for LIFR from immature Sertoli cells showed 79% and 86% homology when compared with published LIFR sequences for human and mouse, respectively. Similar to data with rat RNA samples, RT-PCR analysis of freshly isolated mouse germ cells showed the presence of gp130 and LIF mRNAs in all of the cells (Fig. 1, lanes 12–15).

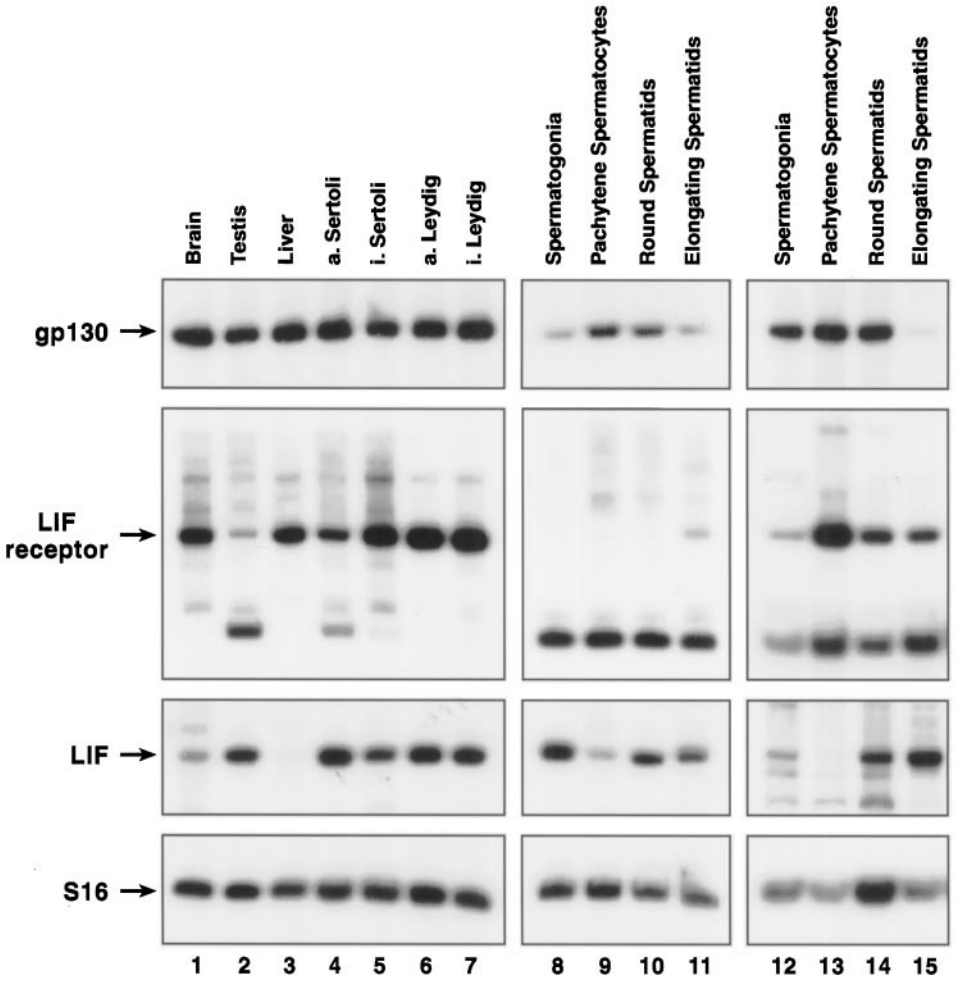

FIG. 1. Distribution of gp130, LIF, and LIFR in different testicular cells by RT-PCR. RT-PCR products from rat (lanes 1–11) and mouse (lanes 12–15) tissues were analyzed using primers for gp130 (*top panel*), LIFR and LIF (*middle panels*), and S16 control primers (*bottom panel*). For Sertoli and Leydig samples: a, adult; i, immature rats.

In contrast to the findings using rat germ cell RNA, LIFR was readily detected in mouse germ cell RNA extracts (Fig. 1, lanes 12–15). Interestingly the highest relative amounts of LIFR were observed in pachytene spermatocytes, which had a barely detectable signal for LIF mRNA. A set of primers for an internal control, S16 mRNA, showed comparable RNA levels in different cell preparations (Fig. 1, *bottom panel*).

LIF activation of STAT-3 and STAT-1 proteins in immature Sertoli cells

We selected m67, a synthetic high-affinity STAT binding oligonucleotide that is derived from the c-*fos* SIE, to study the activation of STAT DNA binding using nuclear extracts from primary Sertoli cells treated with mouse LIF. Figure 2 illustrates that LIF rapidly induces nuclear STAT-3 DNA binding as early as 2 min following its addition (lane 2). An anti-STAT-3 antiserum supershifted this complex (lane 8), and the addition of 5- or 10-fold unlabeled m67 oligo completely abolished STAT-m67 interactions (lanes 9 and 10), indicating further specificity of this DNA binding. LIF also activated some STAT-1 protein to bind to the m67 oligo after 10 min of LIF treatment (lane 4), which was supershifted with anti-STAT-1 antisera (data not shown).

LIF activation of STAT proteins requires tyrosine but not serine/threonine phosphorylation

We recently demonstrated IL-6-mediated tyrosine and serine/threonine phosphorylation events in Sertoli cells (20). In other cell types, Western blot analyses revealed that during LIF stimulation a secondary serine/threonine phosphorylation occurs rapidly after primary tyrosine phosphorylation events (8, 27, 28). We next determined the effects of genistein, a tyrosine kinase inhibitor, and H7, a serine/threonine kinase inhibitor, on LIF stimulation of Sertoli cells using EMSA (Fig. 3A). EMSA in Fig. 3A demonstrates that a 3-h preincubation of Sertoli cells with genistein (100 μg/ml) before the addition of LIF for 2, 5, 10, or 15 min inhibits STAT-3 DNA binding. In contrast, pretreatment with H7 had no effect on this m67 oligonucleotide binding at these times, indicating that tyrosine but not serine/threonine phosphorylation events are required for LIF-mediated STAT-3 translocation to the nucleus and DNA binding (Fig. 3A, lanes 4–15). Genistein or H7 alone did not activate STAT proteins (lanes 2 and 3). When these Sertoli cell nuclear extracts were immunoprecipitated with antiphosphotyrosine antiserum, Western blot analysis with anti-STAT-3 antiserum showed an immediate tyrosine phosphorylation of STAT-3 protein

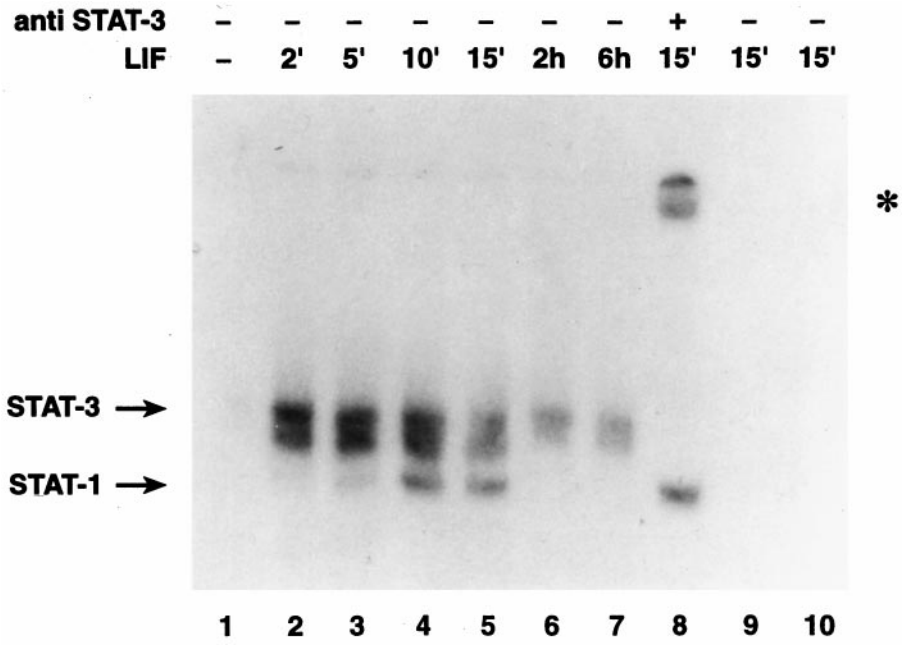

FIG. 2. Cytokine activation of STAT proteins. Sertoli cells were treated on day 3 with vehicle (lane 1) or LIF (lanes 2–10) for indicated times, and nuclear extracts prepared for EMSA using m67 oligonucleotide as probe. In lane 8, a 1:100 dilution of STAT-3 antiserum supershifted STAT-3-m67 complex, whereas addition of 5- or 10-fold unlabeled m67 diminished binding. *, Indicates shifted STAT-3 protein.

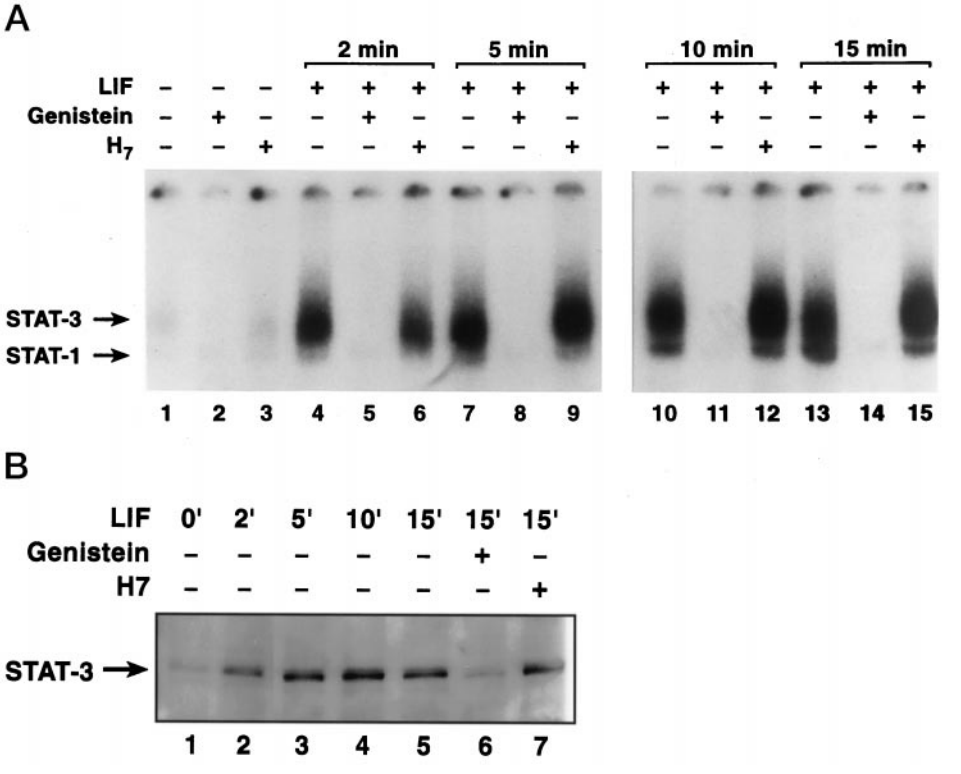

FIG. 3. LIF activation of STAT-3 and STAT-1 proteins requires tyrosine phosphorylation. A, Sertoli cells were treated on day 3 with vehicle (lane 1); genistein for 3 h (lane 2); H7 for 1 h (lane 3); LIF for 2, 5, 10, and 15 min (lanes 4–15); LIF plus genistein (lanes 5, 8, 11, and 14); or LIF plus H7 (lanes 6, 9, 12, and 15), and nuclear extracts were used in EMSA with m67 oligonucleotide. B, In a Western blot analysis, Sertoli cell nuclear extracts from LIF time course were immunoprecipitated with phosphotyrosine, run on a 7.5% SDS/PAGE, and probed with anti-STAT-3 antiserum (1:1000). Bound antibodies were detected by epichemiluminescence.

induced by LIF, an effect that was also inhibited by genistein (Fig. 3B).

LIF induction of endogenous c-fos gene expression and AP-1 binding through tyrosine and serine/threonine phosphorylation events

We used Northern blot analysis and a quantitative solution hybridization assay to measure Sertoli c-*fos* mRNA levels after LIF treatment. In Fig. 4A a representative Northern blot (*top panel*) and solution hybridization (*bottom panel*) show that LIF rapidly and transiently activated c-*fos* gene expression after 15 min of treatment with maximal induction (20-fold) at 30–45 min. Furthermore, EMSA using Sertoli cell nuclear extracts showed increasing amounts of AP-1 proteins bound to the AP-1 oligo after 2 h of LIF treatment (Fig. 4B, lane 3), whereas the addition of 5- or 10-fold unlabeled AP-1 oligonucleotide (lanes 5 and 6) reduced this DNA binding.

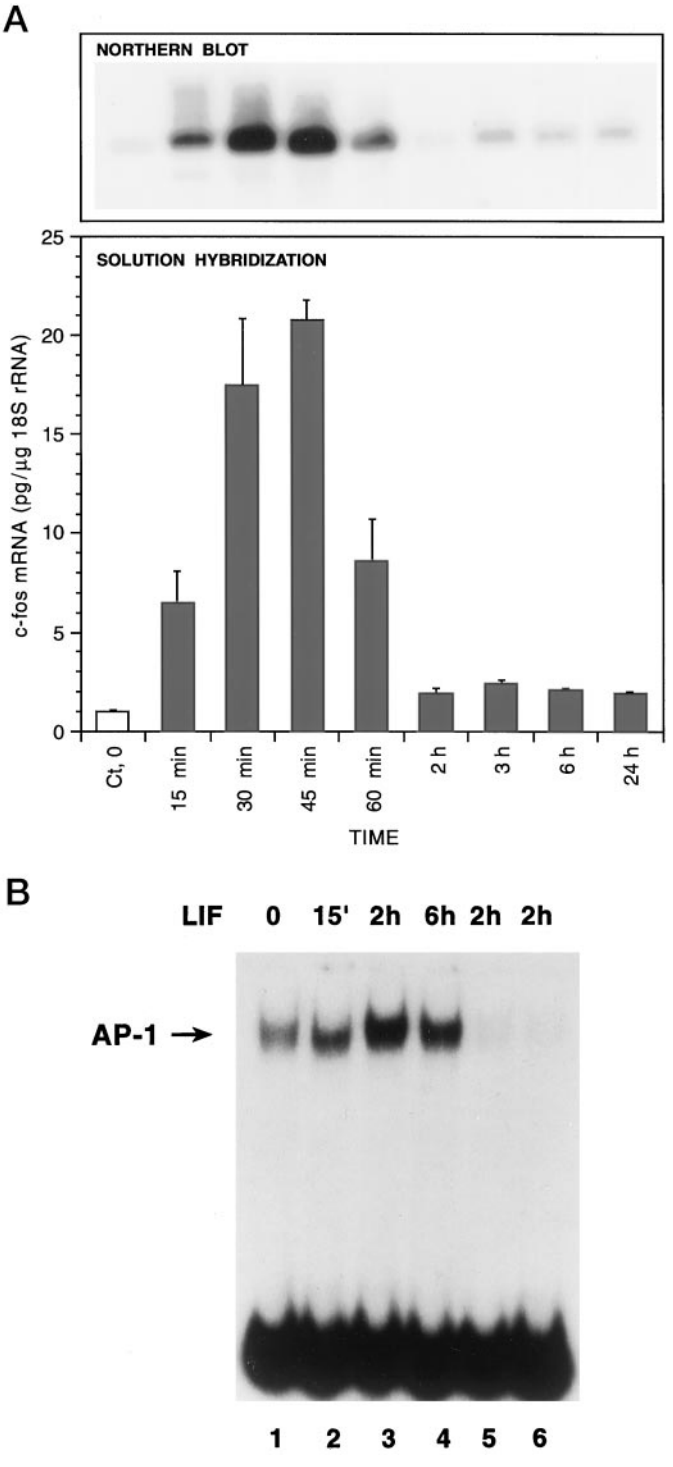

FIG. 4. Time course of activation of c-*fos* transcription and AP-1 nuclear factors by LIF. Sertoli cells on day 3 were untreated (lane 1) or treated with LIF for indicated times. A, Northern blot (*top panel*) and solution hybridization (*bottom panel*) assays were used to measure c-*fos* mRNA levels in total Sertoli RNA extracts. Mean (± SEM) of c-*fos* mRNA level is normalized to that of control cells at 0 time (*open bar*, 1 pg/μg 18S rRNA) is shown. B, EMSA was used to detect increasing AP-1 binding to AP-1 oligo after 2 h (lane 3) or 6 h (lane 4) of LIF. Lanes 5 and 6 show competition reactions with unlabeled AP-1 oligo (5-fold and 10-fold, respectively).

Next we determined the LIF regulation of the c-*fos* transcription in the presence or absence of genistein and H7. Figure 5A shows that the LIF-induced increase in steady state c-*fos* mRNA levels was diminished to 5-fold over the basal levels by genistein, whereas H7 completely abolished LIF

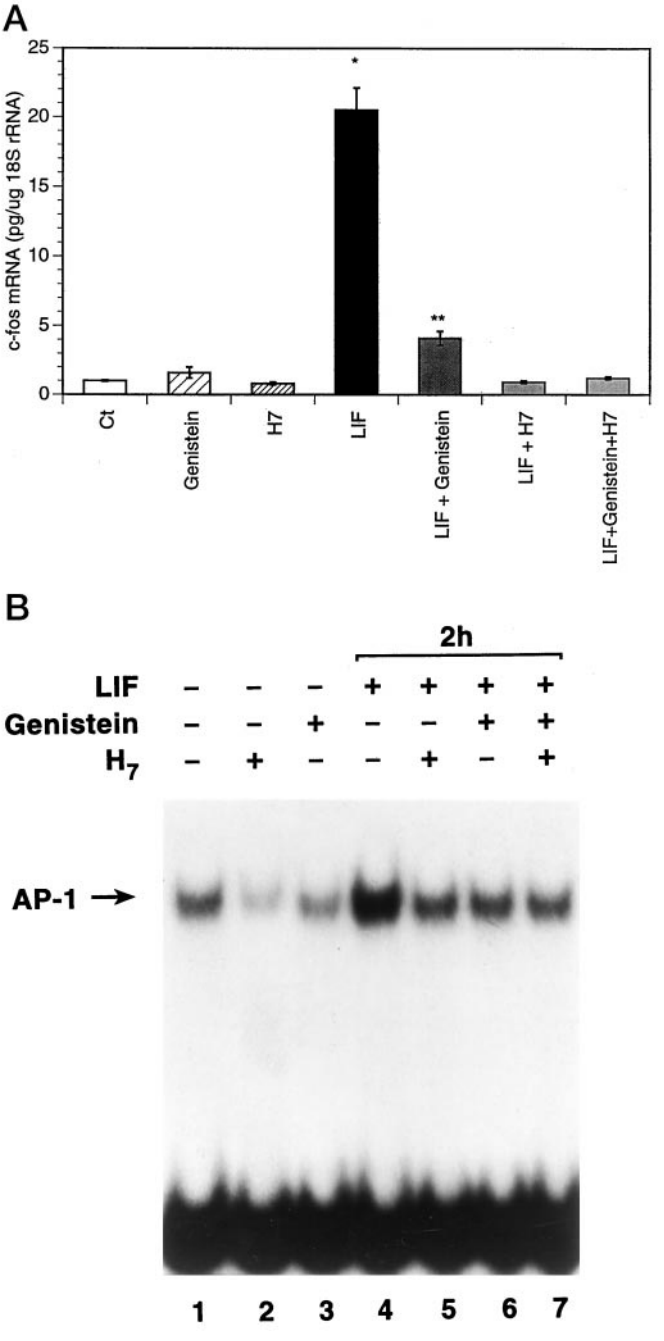

FIG. 5. Activation of c-*fos* gene expression and AP-1 binding by LIF requires tyrosine and serine/threonine phosphorylation. On day 3 of culture, primary Sertoli cells were pretreated with genistein for 3 h, H7 for 1 h, LIF for 45 min (A) or 2 h (B), LIF plus genistein, LIF plus H7, or LIF plus genistein plus H7. A, Total RNA samples were subjected to solution hybridization analysis for c-*fos* and 18S rRNA transcripts and mean (± SEM) values of c-*fos* mRNA levels were normalized to those of control cells (Ct) (1 pg/μg 18s rRNA). *, **, Indicate significant changes compared with control cells. B, EMSA using Sertoli nuclear extracts and AP-1 oligo.

induction of c-*fos* to that of control levels. To study the effects of the kinase inhibitors on LIF induction of AP-1 proteins, we next pretreated the Sertoli cells with genistein (100 μg/ml, 3 h) and/or H7 (100 μM, 1 h) followed by the addition of LIF for 2 h. Figure 5B illustrates that both genistein and H7 inhibit the induction of AP-1 protein binding to the AP-1 oligonucleotide subsequent to a 2-h LIF treatment (compare lane 4 to lanes 5–7).

We next studied the c-*fos* mRNA levels after LIF treatment of specific rat germ cells. Primary rat spermatogonia, pachytene spermatocytes, and round and elongating spermatids were purified and cultured for 4 h before addition of mouse LIF. In contrast to the Sertoli cells, the germ cell c-*fos* mRNA levels did not change after 45 min of LIF treatment (data not shown).

Discussion

In this report we showed the presence of LIF, LIFR, and gp130 mRNAs in specific testicular cell types. Furthermore, we identified and characterized an immediate intracellular signaling pathway for LIF in the testis, using primary Sertoli cells to demonstrate STAT protein phosphorylation and DNA binding, c-*fos* gene expression, and AP-1 induction. Using a somatic cell model system, our studies demonstrated that testicular LIF interacts with receptor chains in target cells and has the potential to result in sequential phosphorylation and translocation of latent cytoplasmic STAT-3 (and some STAT-1) protein(s) to the nucleus (by 2 min), to increase c-*fos* mRNA levels by 20-fold (by 30 min), and to increase the amounts of AP-1 binding proteins (by 2 h). Although STAT-m67 interaction is genistein-sensitive, induction of c-*fos* transcription and AP-1 activation is responsive to both genistein and H7 inhibition.

In the testis, LIF exposure increases the survival of Sertoli cells and proliferating gonocytes (17). Together with stem cell factor, LIF treatment promotes survival and proliferation of mouse primordial germ cells while inhibiting their apoptosis (29, 30). Our RT-PCR analyses of gp130 and LIF mRNA show their expression both in immature and adult rat somatic cells and specific rat and mouse germ cells. In contrast to gp130 and LIF, LIFR mRNA is expressed in somatic cells with comparatively small amounts in rat elongating spermatids. Taken together with our findings on STAT-3 activation, LIF may therefore function as an autocrine/paracrine factor regulating spermatogenesis mediated by its effects on the Sertoli cells. Alternatively, although we used two different sets of LIF receptor primers derived from the mouse sequences, it is possible that the rat germ cells express a different splice variant and/or sequence variations in LIFR mRNA that is not detectable by our primers. Should rat germ cells produce LIFR protein, they may represent a direct functional target for somatic cell-produced LIF. Species specificity in the expression of LIFR as well as ligand binding may play a role in LIF signaling in germ cells.

LIF and the other members of the gp130 protein family have been shown, in several established clonal cell lines, to induce in a sequential fashion, a progressive tyrosine- and serine/threonine-dependent phosphorylation of STAT-3 protein with both a primary tyrosine and a secondary serine/threonine phosphorylation step (8, 27, 28). Although both events induce nuclear translocation, the former induces *in vitro* DNA binding activities, and the latter is correlated with maximal transactivation and the formation of stable STAT-DNA complexes (28). Our EMSA analysis of Sertoli cell nuclear extracts shows that during 2–15 min LIF exposure, a time course when ongoing phosphorylation of STAT-3 occurs, the tyrosine kinase inhibitor, genistein, diminished the activation of STAT-3 and its interaction with the m67 oligonucleotide. Although the serine/threonine kinase inhibitor, H7, has been shown to selectively abolish binding of STAT-3 homodimers in a cell type-dependent manner (28), in primary Sertoli cell nuclear extracts the high-affinity m67-STAT-3 (or -STAT-1) complexes were not affected by H7. Because H7 did not cause a change in the migration or the abundance of STAT-3 protein, at present it is uncertain whether a secondary form of STAT-3 protein, due to the serine phosphorylation that is observed in immortalized cell lines (8, 27, 28), also occurs in primary Sertoli cells.

Intracellular signaling by gp130-sharing cytokines is mediated by the STAT protein(s), resulting in their induction and transcriptional activation of several targeted immediate early genes (24, 31). Specific DNA response elements have been mapped in c-*fos* and *jun*B promoter regions, which contain sequences derived from the reported IFN-γ activation site TTCCNNNAA and bind STAT proteins *in vitro* (7, 31, 32). Transactivation of these genes appears to depend on the STAT-DNA interactions and requires phosphorylation by distinct protein kinases (7, 8, 20). *jun*B gene expression is distinctively induced in several hematopoietic and hepatic cell lines in response to cytokines and growth factors and requires phosphorylation of STAT proteins at specific tyrosine and serine residues (8). In primary Sertoli cells, our data indicate that testicular cytokines (such as LIF, IL-6, and IFN-γ) differentially activate STAT proteins to bind to the c-*fos* SIE and induce immediate early gene expression at transcriptional levels. These effects are also dependent on tyrosine and serine phosphorylation pathways (this study and Refs. 20 and 32a). Several other members of the STAT proteins, including STAT-1α, STAT-4, and STAT-5, contain a conserved carboxy-terminal amino acid sequence (XPXSP) that contains serine phosphorylation sites important for maximal transcriptional activity (33). This C-terminal transactivation domain is missing in STAT-1β, and although STAT-1β is shown to be tyrosine phosphorylated and binds to DNA elements, it cannot restore IFN-γ induction of U3A cells deficient in STAT-1α and STAT-1β. Thus, specific serine phosphorylation of STATs is obligatory for maximal transactivation of responsive genes. Because other major signaling pathways such as mitogen-activated protein, cAMP response element binding protein, and protein kinase C are also regulated by increases in serine kinase activities and may further enhance transactivation (34, 35), it is possible that concomitant activation of these pathways will modulate LIF-mediated gene expression in testicular cells.

Stimulation of the AP-1 family of transcription factors is a crucial first step in response to various extracellular factors that play important roles in signal transduction of cellular proliferation and differentiation (36, 37). Increasing amounts of evidence suggest that c-*fos* is directly involved in the

regulation of testicular function. FSH regulates the Sertoli cell functions that are mediated through the c-*fos*, *jun*B, and c-*jun* messages (38, 39). In addition, a paracrine factor, PModS, secreted by mesenchymal peritubular myoid cells, enhances the differentiation of Sertoli cells mediated by c-*fos* (40). When primary Sertoli cells are cultured on Matrigel (Becton Dickinson, Franklin Lakes, NJ) *in vitro*, c-*fos* expression is increased, whereas the addition of antisense oligonucleotides to c-*fos*, which decreases c-fos protein, inhibited the attachment and spreading of the cells (41). c-*fos* and *jun*B genes contain a bipartite DNA binding domain that consists of a basic leucine zipper region. These proteins regulate target genes by binding as homo- or heterodimers to the AP-1 elements (42). Furthermore, the regulation of the c-*fos* gene itself by two factors, the AP-1 and the ternary, has been shown to be mediated by serine phosphorylation events (43, 44). Our data indicate that LIF increases AP-1 binding by two pathways: first, the increase in c-*fos* transcription, which in turn increases c-fos protein, and second, an increase in phosphorylation of the existing AP-1 proteins by other signaling pathways (such as protein kinase A) that increases the effectiveness of latent AP-1 transcription factors. Because we show that tyrosine and serine/threonine phosphorylation inhibitors reduce both LIF-activated c-*fos* transcription and the level of AP-1 proteins, both of these pathways may contribute to AP-1 activation.

These findings and other recent studies from our laboratory have shown for the first time that IL-6, LIF, and their receptors are expressed in different cells of the testes and, using a Sertoli cell culture model, provide evidence that a functional testicular cytokine signaling pathway exists *in vivo* (18–20, 45). Thus, primary Sertoli cultures are a valuable system for dissecting the molecular mechanisms involved in the transduction of signals from the testicular receptor to the regulatory elements of several responsive genes under noninflammatory autocrine/paracrine states. Additional studies will be needed to clarify the functional role of LIF, LIFR, and gp130 in germ cell signaling. Our findings suggest that additional cofactors may be required to facilitate LIF signaling within the tubules. Further studies are currently in progress to analyze downstream genes whose expressions are affected by testicular cytokines.

Acknowledgments

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