BIOLOGY OF REPRODUCTION **64,** 1566–1574 (2001)

Activation of the Akt/Protein Kinase B Signaling Pathway Is Associated with Granulosa Cell Survival[1]

A.L. Johnson,[2] J.T. Bridgham, and J.A. Swenson

Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556

ABSTRACT

Follicles from the hen ovary that have been selected into the preovulatory hierarchy are committed to ovulation and rarely become atretic under normal physiological conditions. In part, this is attributed to the resistance of the granulosa layer to apoptosis. The present studies were conducted to evaluate the role of the phosphatidylinositol (PI) 3-kinase/Akt signaling pathway in hen granulosa cell survival and, by implication, follicle viability. Cloning of the chicken akt2 homologue revealed a high degree of amino acid homology to its mammalian counterparts within the catalytic domain, plus complete conservation of the putative Thr^{308} and Ser^{474} phosphorylation sites. Treatment of granulosa cells from the three largest preovulatory follicles with insulin-like growth factor (IGF)-I and, to a lesser extent, transforming growth factor (TGF)-α induces rapid phosphorylation of Akt, and such phosphorylation is effectively blocked by the PI 3-kinase-inhibitor LY294006. Serum withdrawal from cultured cells for 33–44 h initiates oligonucleosome formation, an indicator of apoptotic cell death, whereas cotreatment with IGF-I prevents this effect. Moreover, treatment of cultured cells for 20 h with LY294006 induces apoptosis. The potential for nonspecific cell toxicity following LY294006 treatment is considered unlikely because of the ability of either LH or 8-bromo cAMP cotreatment to block LY294006-induced cell death. Finally, both IGF-I and TGF-α also activate mitogen-activated protein (MAP) kinase signaling, at least in part, through the phosphorylation of Erk. However, treatment with neither U0126 nor PD98059 (inhibitors of MAP kinase kinase) induced cell death in cultured granulosa cells, despite the ability of each inhibitor to effectively block Erk phosphorylation. Taken together, these results provide evidence for a role of the Akt signaling pathway in promoting cell survival within the preovulatory follicle granulosa layer. In addition, the data indicate the importance of an alternative survival pathway mediated via gonadotropins and protein kinase A independent of Akt signaling.

apoptosis, follicular development, growth factors, ovary, signal transduction

INTRODUCTION

A majority of primordial and developing ovarian follicles in both avian and mammalian species are eliminated on a continuous basis throughout the female's reproductive life via follicle atresia [1]. In the hen, atresia is most prevalent in undifferentiated, prehierarchal (<9 mm) follicles, whereas follicles selected into the preovulatory hierarchy rarely undergo atresia and are, under normal physiological conditions, destined for ovulation [2]. A number of recent studies have demonstrated that apoptotic cell death is the mechanism underlying the process of atresia, and that the majority of cells undergoing apoptosis during early atresia are of granulosa cell origin [3, 4]. Numerous studies have shown that the rate of granulosa cell apoptosis from vertebrate ovarian follicles can be attenuated by local ovarian growth factors such as insulin-like growth factor (IGF)-I, basic fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor (TGF)-α [1, 3, 4]. To date, however, detailed cell signaling pathways and the transcriptional factors activated by such growth factors that normally maintain the viability of granulosa cells remain largely unknown.

The Akt/protein kinase B subfamily of protein kinases consists of at least three isoforms (Akt1 [α], Akt2 [β], and Akt3 [γ]) and is activated in response to stimulation with a variety of growth factors [5–7]. Such activation is correlated with cell survival in a wide variety of cells, including those of epithelial, mesenchymal, and neuronal origin [8, 9]. Conserved primary sequences within this group of kinases include an NH2-terminal plecstrin homology (PH) domain, a central kinase domain, and a serine/threonine-rich C-terminal regulatory domain [7]. Optimal activation of Akt2 initially requires association of phosphatidylinositol(3,4,5)trisphosphate ($PI[3,4,5]P_3$) with the PH domain, followed by phosphorylation of Ser^{474} by the recently identified phosphoinositide-dependent kinase PDK1 [7, 10]. The identity of the kinase responsible for the subsequent phosphorylation of Thr^{309} and, thus, full potentiation of Akt activity remains to be conclusively established [11]. Included among the potential downstream physiological substrates from a variety of cell types targeted specifically by activated Akt and linked to cell survival are Bad, caspase-9, NF-κB, Forkhead family members, and the cAMP response element-binding protein CREB [9, 12–15].

Aside from cell survival, activation of Akt2 has also been associated with the regulation of cell-cycle progression, whereas its overabundance can induce cell transformation [16, 17]. Amplification of Akt2 is observed in 10%–20% of human ovarian and pancreatic cancers and, to a lesser extent, in breast cancer [18–20]. Although Akt3 overexpression has been documented in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer cell lines [21], consistent alterations of Akt1 or Akt3 expression or function have not been observed in any human malignancy. Thus, to date, only Akt2 appears to play a significant role in tumor transformation and enhanced aggressiveness [22].

Recent work with hen ovarian follicles has identified two populations of granulosa cells that are differentially sensitive to apoptosis induced by tropic hormone deprivation. For instance, granulosa cells from follicles that inherently exhibit a high incidence of atresia in vivo (e.g., prehierarchal follicles) are susceptible to apoptotic cell death in vi-

[1]Supported by USDA 99-35203-7736 and NIH HD36095. Genbank accession number for the chicken akt2 cDNA is AF181260.
[2]Correspondence: A.L. Johnson, Department of Biological Sciences, P.O. Box 369, University of Notre Dame, Notre Dame, IN 46556. FAX: 219 631 7413; e-mail: johnson.128@nd.edu

Received: 9 October 2000.
First decision: 21 November 2000.
Accepted: 5 December 2000.

ISSN: 0006-3363. http://www.biolreprod.org

tro; by contrast, granulosa cells from preovulatory follicles are comparatively resistant to apoptosis [4, 23]. Extracellular ligands such as FSH, vasoactive intestinal peptide, and TGF-α can attenuate the progression of apoptotic cell death in apoptosis-sensitive hen granulosa cells [4, 24], but less is known about the factors and signaling pathways that maintain the viability of granulosa cells in preovulatory follicles.

To date, only limited information is available regarding the importance of signaling via Akt in granulosa cell survival among vertebrate species [25], and in the chicken, only the homologue corresponding to mammalian Akt1 has been characterized (Genbank accession no. AF039943). Therefore, the objectives of the present studies were to identify and characterize expression of Akt2 in the hen granulosa cell model system and to assess the relationship between activation of the Akt signaling pathway and granulosa cell viability in vitro.

MATERIALS AND METHODS

Animals and Reagents

Single-comb, white Leghorn hens (H&H Poultry, Portland, IN) from 25–35 wk of age and laying in regular sequences of at least five to six eggs were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layeena Mash; Purina Mills, St. Louis, MO) and water, and were exposed to a photoperiod of 15L:9D, with lights-on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h before a midsequence ovulation by cervical dislocation. All procedures described were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Affinity-purified Akt2 polyclonal antiserum and monoclonal phospho-Erk1/2 antibody were obtained from Upstate Biotechnology (Lake Placid, NY). The Akt2 antiserum was generated against a synthetic peptide that shares 66% identity with the deduced chicken Akt2 sequence and contains two potential epitope regions of three and five consecutive amino acids. On the other hand, the immunogen contains only 4 of 15 amino acids found within the chicken Akt1 deduced amino acid sequence, with no two consecutive identical residues. The affinity-purified polyclonal antiphospho-Akt serum was from New England BioLabs (Beverly, MA), and the immunogen used to generate this antiserum is identical to the deduced chicken Akt sequence. The phospho-Akt antibody detects the phosphorylated Ser^{474} of Akt2 and equivalent phosphorylation sites present within Akt1 and Akt3. The α-tubulin monoclonal antiserum used for standardization was from Sigma (St. Louis, MO). Recombinant human IGF-I, TGF-α, basic FGF, and platelet derived growth factor-AB (PDGF) were obtained from PeproTech (Rocky Hill, NJ). Ovine LH (lot 26) was from the National Hormone and Pituitary Program, and 8-bromo-cAMP was from Sigma. The PI 3-kinase-inhibitor LY294002 (prepared in dimethyl sulfoxide [DMSO] as a 10 mM stock solution) and the mitogen-activated protein (MAP) kinase kinase (MEK)-inhibitors U0126 (prepared in DMSO as a 2.3 mM stock solution) and PD98059 (prepared in DMSO as a 20 mM stock solution) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Each inhibitor was stored at −20°C until use. A 100× insulin (1 g/L)-transferrin (0.55 g/L)-selenium (0.67 mg/L) (ITS) supplement and fetal bovine serum (FBS) were purchased from Gibco-BRL (Rockville, MD).

Isolation and Characterization of Chicken akt2 Partial cDNA

Chicken akt2 nucleotide sequence was generated using nested polymerase chain reactions (PCR). Initial reactions utilized one primer (akt2-F1) based on the human akt2 sequence (Genbank accession no. M95936; 5′-CGC TAC TAC GCC ATG AAG ATC C-3′, corresponding to nucleotides 614–635) together with an adaptor primer (AP1) and adaptor-ligated cDNA template derived from hen granulosa cell poly(A)$^+$ RNA (Marathon cDNA amplification kit; Clontech, Palo Alto, CA). Amplification was carried out with 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. An aliquot of this reaction product was reamplified using two internal primers based on human akt2 sequence (akt2-F2: 5′-TGC AAA GAG GGC ATC AGT G-3′, nucleotides 977–995; akt2-B2: 5′-GGA CGT GAC CTG AGG TTT GAA G-3′, nucleotides 1382–1361). Amplification conditions were as described above, with the exception of a 57°C annealing temperature. Products were ligated into pCR2.1 TA cloning vector, then transfected into INVαF′ cells (Invitrogen, San Diego, CA) and subsequently sequenced using dideoxychain-termination method with the Sequenase v.2.0 kit (Amersham Pharmacia Biotech, Piscataway, NJ). The 405-base pair product was determined to be homologous to human akt2 and was isolated for use as a template for Northern probes (see below).

Further sequence of the chicken Akt2 was generated by 3′- and 5′-rapid amplification of cDNA ends (RACE) reactions (Marathon cDNA Amplification Kit). The original akt2-F1-AP1 products from above were reamplified using the human akt2-F2 and adaptor primer 2 (AP2) to give the 3′-RACE products using the PCR conditions described above. Finally, 5′-RACE reactions with chicken-specific nested primers were done first with GSP1 (5′-TGC ACC ACG TCC TGC CAG TTG-3′, corresponding to human akt2 nucleotides 1342–1322) and AP1, followed by GSP2 (5′-CCG TAG TCG TTG TCC TCC AGC AC-3′, human akt2 nucleotides 1069–1047) and AP2. These 5′-RACE reactions were performed using touchdown PCR conditions, first with annealing at 72°C and then followed by 68°C annealing temperatures. All products were amplified and fully sequenced in both directions as described above.

Tissue Collection

Ovarian tissues collected for analysis of akt2 mRNA or Akt1 and Akt2 protein expression included granulosa and theca tissue from the largest (F1), second-largest (F2), and third-largest (F3) preovulatory follicles; follicles recently selected into the follicle hierarchy (9–12 mm); prehierarchal (6–8 and 3–5 mm) follicles; and ovarian stromal tissue. Other tissues collected included spleen, bone marrow, pituitary, cerebrum, oviduct, heart, and liver, which were prepared as previously described [26, 27].

Northern Blot Analysis of akt2 mRNA

Levels of akt2 mRNA were evaluated in the various hen tissues using the homologous 405-nt cDNA probe described above, together with highly stringent hybridization conditions as previously outlined [28]. Briefly, total cellular RNA from each tissue and stage of follicular development was isolated using Trizol Reagent (Gibco-BRL). Ten to 15 μg

of total cellular RNA were electrophoresed on 1% w/v agarose gels in the presence of formaldehyde, then transferred to nitrocellulose Nitro ME membranes (Micron Separations, Inc., Westborough, MA) by capillary action overnight. The akt2 cDNA probe was labeled by the random-prime labeling method using the Megaprime DNA labeling System (Amersham) and ^{32}P-deoxycytidine 5′-triphosphate (3000 Ci/mmol; Amersham). Blots were prehybridized for 30 min at 60°C and subsequently hybridized overnight at 60°C. Membranes were exposed to phosphorimage plates for 7–10 days, then analyzed using a Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). Blots were routinely rehybridized to a random-primed chicken 18S ribosomal RNA cDNA probe to standardize for equal loading of RNA samples.

Granulosa Cell Cultures

Pure populations of granulosa cells from preovulatory (F1, F2, and F3) or prehierarchal (6–8-mm) follicles were harvested from each hen, combined within their respective group, and dispersed in 0.3% w/v type 2 collagenase (Worthington, Freehold, NJ) in Medium (M) 199/Hepes supplemented with Hanks salts (Gibco-BRL). An aliquot of freshly dispersed cells was routinely frozen for use as a reference (T_0) point. The remainder of cells were plated in six-well polystyrene culture plates (Falcon 3046; Fisher Scientific, Chicago, IL) at a density of approximately 1.5×10^6 cells/well in 1 ml of M199-Hepes plus 1 ml of Dulbecco modified Eagle medium (Gibco-BRL).

In the first set of culture experiments, granulosa cells from prehierarchal or preovulatory follicles were plated in the presence of 2.5% v/v FBS, with or without IGF-I (50 ng/ml) or LH (100 ng/ml), for 9 or 20 h. The medium was removed, and plated cells were then collected for the analysis of Akt2 protein.

In the next set of experiments, preovulatory follicle granulosa cells were precultured in the absence of FBS for 6 h, then treated with 0, 1, 10, 50, or 100 ng IGF-I/ml for 20 min or with 50 ng IGF-I/ml for 0, 5, 20, 120 min, or 16 h. The effect of various growth factors or medium supplements was evaluated by preculturing cells for 6 h in the absence of FBS, then treating for 20 min with IGF-I (50 ng/ml), TGF-α (50 ng/ml), FGF (50 ng/ml), PDGF (50 ng/ml), LH (100 ng/ml), 8-br-cAMP (1 mM), FBS (2.5% v/v), or ITS (1× strength). Cells were collected and processed for analysis of phosphorylated Akt (Akt-P) and total Akt2 protein.

To test the cell survival effects of IGF-I, preovulatory follicle granulosa cells were cultured in the absence of FBS or in the presence of 2.5% v/v FBS, with or without IGF-I (100 ng/ml), for 33 or 44 h, with a supplemental treatment of IGF-I added to appropriate wells after 24 h. Media plus cells were collected, and cells were then pelleted by centrifugation at $200 \times g$ for 5 min. The pellets were subsequently collected and frozen at −70°C until DNA was prepared for the evaluation of oligonucleosome formation.

The final set of experiments was designed to compare the effects of the PI 3-kinase and MAP kinase signaling pathways on granulosa cell viability. First, specificity of the PI 3-kinase-inhibitor LY294006 was evaluated by preculturing preovulatory follicle granulosa cells for 6 h in the absence of FBS. Cells were subsequently pretreated for 1 h in the absence or the presence of LY294002 (50 μM), then treated for 20 min with or without IGF-I (50 ng/ml) or TGF-α (50 ng/ml). Cells were collected and the protein analyzed for levels of Akt-P and phosphorylated Erk (Erk-P). Identical experiments were conducted, except that the MEK-inhibitors U0126 (50 μM) or PD98059 (50 μM) were substituted for LY294006. Finally, the effects of each inhibitor on granulosa cell viability were evaluated by culturing cells in the absence or the presence of LY294006, U0126, or PD98059 for 20 h. Treatments with either LH (100 ng/ml) or 8-bromo-cAMP (1 mM) were included to evaluate nonspecific effects of LY294006 on cell viability. Cells plus media were collected, and the DNA was analyzed for oligonucleosome formation.

Western Blot Analysis

Granulosa and all other tissues were homogenized in lysis buffer (1.7 mM sodium monophosphate, 17 mM sodium diphosphate, 1% v/v Triton X-100, 0.1% SDS, 0.1% w/v sodium azide, and 0.1 M NaCl) containing aprotinin (10 μg/ml), pepstatin (10 μg/ml), phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μg/ml), and sodium orthovanadate (0.1 M), then centrifuged at $12\,000 \times g$ at room temperature (RT) for 10 min as previously described [28]. The supernatant was collected and protein quantified using the Bio-Rad DC Protein Assay kit (Hercules, CA). Protein samples were subsequently diluted with sample buffer, heated at 70°C for 10 min, then centrifuged at $12\,000 \times g$ for 2 min. Proteins were separated on a 10% w/v SDS-PAGE gel before transferring to a nitrocellulose (Micron Separations) or polyvinylidene fluoride (Millipore, Bedford, MA) membrane.

Membranes were blocked for 1 h at RT in Western blocking solution (5% w/v nonfat dry milk and TBST [10 mM Tris, pH 8.0; 100 mM sodium chloride; 0.1% v/v Tween 20). Antibodies for Akt2, phospho-Akt, and phospho-Erk were diluted to 1:1000 in blocking solution. Each diluted antiserum was incubated with membranes at 4°C overnight, and membranes were then washed (three times for 10 min) in blocking solution. Goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL), goat anti-mouse (Pierce), or donkey anti-sheep IgG (Sigma) coupled to horseradish peroxidase were diluted 1:10 000 in blocking solution and incubated with membranes for 1 h at RT. Membranes were washed twice in blocking solution (10 min each wash), then four times in TBST (10 min each wash). Finally, blots were incubated with enhanced chemiluminescence Western blotting detection reagent (Amersham) for 1 min, then wrapped and exposed to autoradiographic film for 1–5 min. The extent of antibody binding was standardized to α-tubulin (for Akt2) [28] or Akt2 (Akt-P and Erk-P) and quantitated by densitometry (UltraScan XL laser densitometer; Pharmacia LKB, Piscataway, NJ). Standardized protein levels were expressed as fold-difference (mean ± SEM) versus a designated reference tissue or treatment (arbitrarily set at one).

Preparation of Genomic DNA for Evaluation of Oligonucleosome Formation

Genomic DNA was prepared from all treated cells and corresponding controls as previously reported [4, 29], with the exception that the total amount of DNA electrophoresed was 0.5 to 1 μg per lane and was detected using SYBR Green (Molecular Probes, Inc., Eugene, OR).

Data Analysis

All experiments were replicated a minimum of three times unless otherwise stated. Replicate data within an ex-

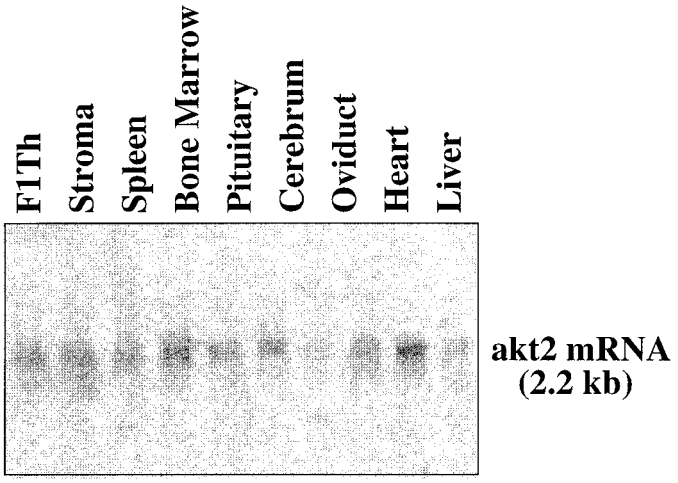

FIG. 1. Widespread expression of the 2.2-kb akt2 mRNA transcript in various tissues from the hen. This blot was replicated once with similar results. F1 Th, Theca tissue from the largest preovulatory follicle; stroma, ovarian stromal tissue.

periment were routinely expressed as fold-difference compared to some reference point (usually freshly collected [T_0] tissue). Such data were subsequently compared by one-way ANOVA (without including the standardized reference data), with significant interactions ($P < 0.05$) partitioned using the Fisher protected least-squares-difference multiple-range test (Statview; Abacus Concepts, Inc., Berkeley, CA). In some instances, the original (i.e., untransformed) data were further analyzed by the paired *t*-test (e.g., T_0 vs. $T_{6\ h}$ − FBS [Con] for all experiments, $T_{5\ min}$ vs. Con, see Fig. 5).

RESULTS

Chicken akt2 cDNA

A partial chicken akt2 cDNA (741 nucleotides in length) comprising the entire carboxyterminal catalytic and regulatory domains is available as Genbank accession no. AF181260. Nucleic acid identity to the human akt2 sequence within the region characterized is 84%. Identity of the deduced amino acid coding sequence (a total of 245 amino acids) to the human homologue is 92%, whereas the overall homology, including conserved substitutions, is 96%. By comparison, the amino acid identity and similarity to the related chicken Akt1 is calculated to be 85% and 92%, respectively. Both of the highly conserved Thr (corresponding to amino acid 309 from the human Akt2 sequence) and Ser (corresponding to amino acid 474) residues, which in mammalian species are phosphorylated by PDK, are present in the deduced chicken Akt2 amino acid sequence. Approximately 172 amino acids from the kinase domain are included within the partial cDNA, and amino acid identity within this region exceeds 97% compared to the human homologue.

Expression of akt2 mRNA

Northern Blot analysis of chicken akt2 mRNA detects a 2.2-kilobase (kb) transcript that is widely expressed within tissues from the hen (Fig. 1). Although the transcript size for chicken akt1 has not yet been reported, specificity of this signal for akt2 is assumed considering that the nucleic acid homology of the chicken akt2 cDNA probe compared to the corresponding chicken akt1 sequence (Genbank accession no. AF039943) was determined to be 72%. A second, slightly smaller transcript is also observed; however, its identity, whether it be an alternatively spliced variant, akt1, or akt3, remains to be established.

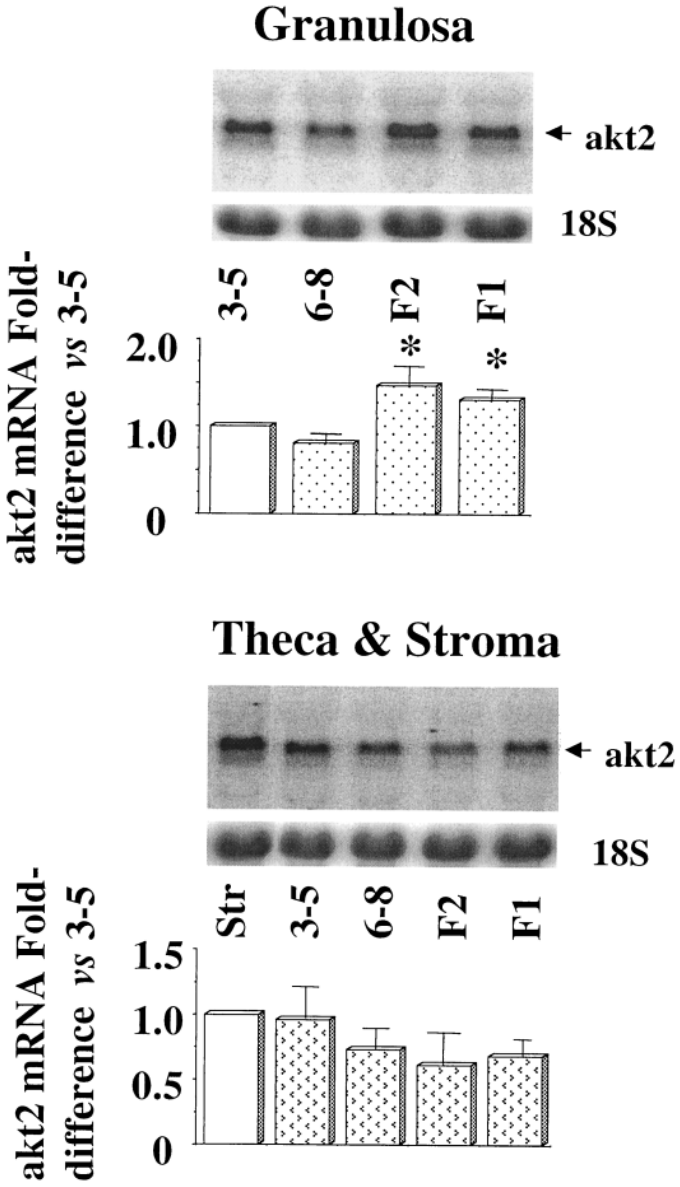

FIG. 2. Expression of akt2 mRNA in granulosa and theca tissue during follicular development. Data are expressed as fold-difference versus 3- to 5-mm follicles (granulosa) or Str (theca) and were analyzed without including the values standardized to one. F2 and F1, Second-largest and largest preovulatory follicle, respectively; Str, ovarian stromal tissue; 3–5 and 6–8, size of follicle (mm). $^*P < 0.05$ versus 6–8.

Levels of akt2 mRNA are elevated within the granulosa layer from preovulatory (F1 and F2) follicles compared to prehierarchal (6- to 8-mm) follicles ($P < 0.05$; Fig. 2). By comparison, no significant differences were found in levels of the akt2 transcript detected within the theca cell layer during follicular development.

Expression of Akt2 Protein in Hen Granulosa Cells

Protein levels for Akt2 are 2.5- to 3.4-fold higher within the granulosa cell layer from preovulatory (F3–F1) versus prehierarchal (6- to 8-mm) follicles ($P < 0.05$; Fig. 3). Interestingly, Akt2 protein levels were not elevated within

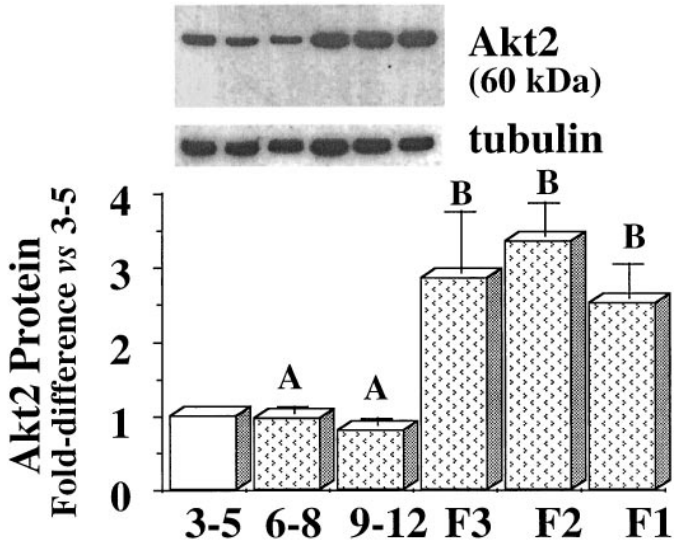

FIG. 3. Immunoreactive Akt2 protein levels in the granulosa cell layer during follicular development. Data are standardized to α-tubulin and expressed as fold-difference versus 3- to 5-mm follicles. $^{A,B}P < 0.05$.

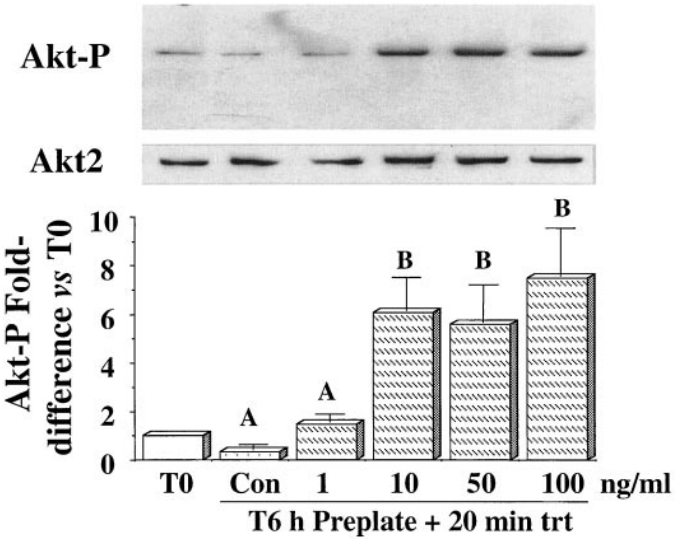

FIG. 4. Levels of Akt-P in granulosa cells precultured in the absence of serum for 6 h, followed by a 20-min treatment with 0, 1, 10, 50, or 100 ng IGF-I/ml. $^{A,B}P < 0.05$.

granulosa cells from follicles most recently selected into the preovulatory hierarchy (9–12 mm). Levels of total Akt2 protein do not change within either preovulatory or prehierarchal follicle granulosa cells following up to 20 h of culture either in the absence or the presence of IGF-I or LH (data not shown).

Akt Phosphorylation in Cultured Preovulatory Follicle Granulosa Cells

Analysis of all experiments in which preovulatory follicle granulosa cells were precultured for 6 h in the absence of FBS (Con) determined that levels of Akt-P are consistently decreased compared with those of freshly collected (T_0) cells ($P = 0.004$, n = 34, paired *t*-test). A 20-min IGF-I treatment at doses of 10, 50, and 100 ng/ml induced Akt-P in cultured preovulatory follicle granulosa cells ($P < 0.05$; Fig. 4). Given that no significant differences were found in the extent of phosphorylation among these doses, a 50-ng/ml dose of IGF-I was utilized in all subsequent experiments. Phosphorylation of Akt was significantly increased within 5 min after treatment with IGF-I ($P < 0.05$ compared to the 0-min control, paired *t*-test), and induced levels were maintained for up to 16 h (Fig. 5).

A comparison of various growth factors determined that IGF-I is the most potent inducer of Akt-P (13.9- ± 2.27-fold increase vs. T_6 Con, $P < 0.05$), whereas TGF-α (50 ng/ml) is somewhat less potent (8.37- ± 1.79-fold increase vs. T_6 Con). Fibroblast growth factor, PDGF, LH (Fig. 6), and 8-br-cAMP (1 mM; data not shown) failed to consistently initiate Akt-P after a 20-min treatment period. The effects of two medium supplements were also evaluated, and it was determined that a 20-min treatment with ITS, but not with 2.5% FBS, increases Akt-P (8.2- ± 2.1-fold vs. cells cultured in the absence of FBS, $P < 0.05$).

Effects of IGF-I on Oligonucleosome Formation

Granulosa cells from preovulatory follicles cultured for up to 44 h in the presence of 2.5% FBS remained viable, as evidenced by morphology and the virtual absence of detached cells (data not shown), and they showed little to no evidence of apoptotic cell death, as indicated by oligonucleosome formation (Fig. 7). By contrast, serum withdrawal induced oligonucleosome formation after 33 and 44 h of culture, whereas cotreatment with IGF-I (50 ng/ml) prevented this effect.

Effects of LY294006 and U0126 on Phosphorylation and Oligonucleosome Formation

Whereas treatment with the PI 3-kinase-inhibitor LY294006 effectively blocked IGF-I- and TGF-α-induced Akt-P in granulosa cells from preovulatory follicles (Fig. 8, top), the inhibitor had no effect on TGF-α-induced Erk-P (Fig. 8, bottom). In the absence of serum, IGF-I treatment induced only minimal Erk-P. Conversely, the MEK-inhibitors U0126 (Fig. 9) and PD98509 (data not shown) failed to inhibit levels of IGF-I- or TGF-α-induced Akt-P but effectively prevented growth factor-induced Erk-P. The single

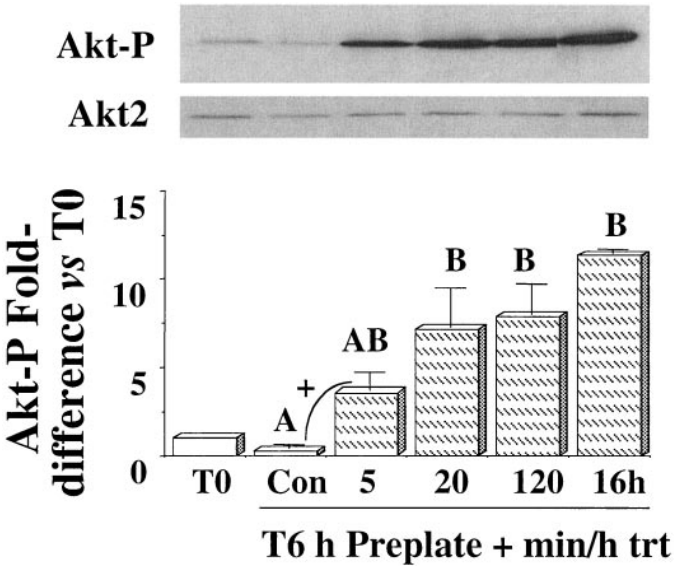

FIG. 5. Time-course for IGF-I (50 ng/ml)-induced Akt phosphorylation in preovulatory follicle granulosa cells precultured for 20 h, then treated for 0, 5, 20, 120 min, or 16 h. $^{A,B}P < 0.05$; $+P < 0.05$ versus 0-min control (Con) by paired Student *t*-test.

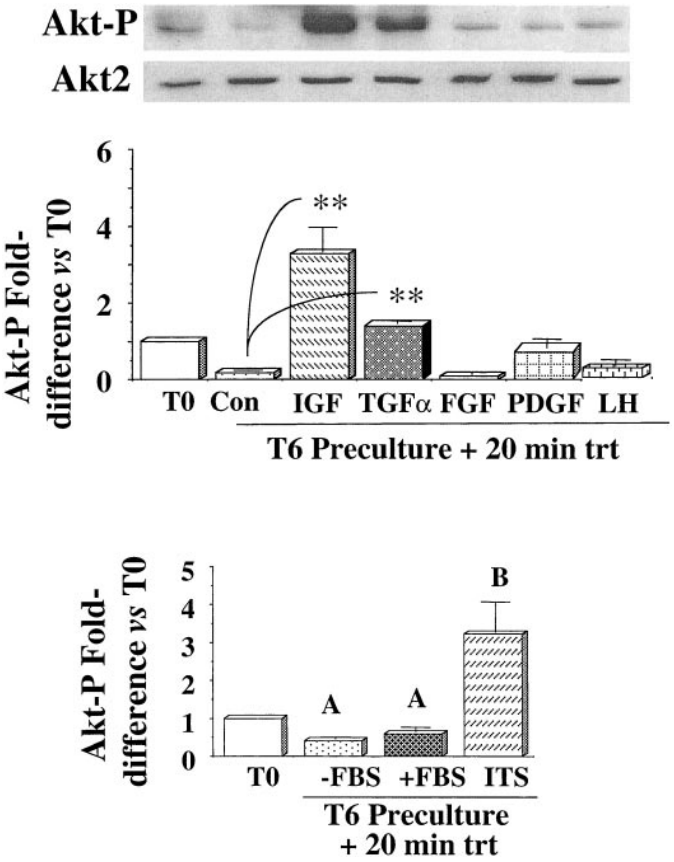

FIG. 6. Top) Extent of Akt-P in granulosa cells from preovulatory follicles in response to IGF-I (50 ng/ml), TGF-α (50 ng/ml), FGF (50 ng/ml), PDGF (50 ng/ml), or LH (100 ng/ml). $^{**}P < 0.05$ versus the absence of growth factor or LH (Con). Bottom) Levels of Akt-P following a 20-min treatment in the absence (−) or presence (+) of 2.5% FBS or ITS (1×). In both experiments, cells were precultured for 6 h in the absence of FBS, then treated with the appropriate agent for 20 min. Levels of Akt-P were standardized to total Akt2 protein and expressed as fold-difference versus T_0. $^{A,B}P < 0.05$.

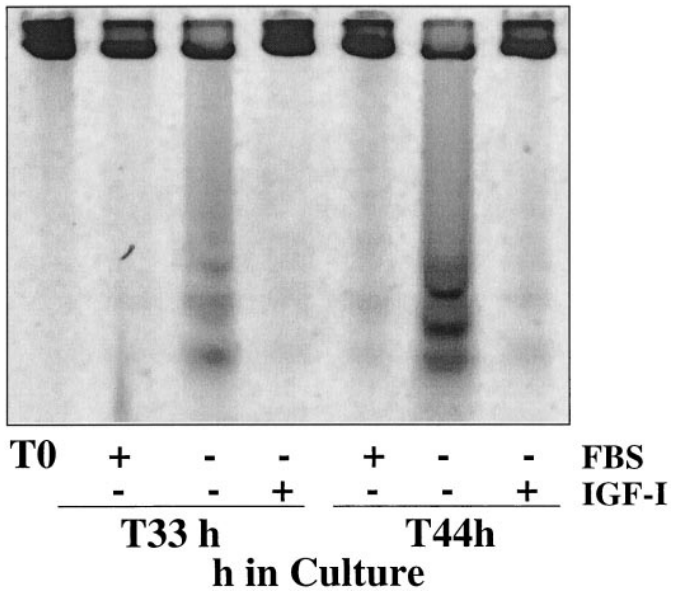

FIG. 7. Ability of IGF-I (50 ng/ml) treatment to prevent oligonucleosome formation in cultured preovulatory follicle granulosa cells following 33 or 44 h of serum withdrawal. This experiment was repeated twice with similar results. FBS, 2.5% fetal bovine serum.

immunoreactive band detected for Erk-P in all likelihood represents the total phosphorylated Erk1 and Erk2, as reported by previous authors [30].

Finally, inhibition of PI 3-kinase signaling with LY294006 induces oligonucleosome formation in preovulatory follicle granulosa cells following a 20-h treatment. The oligonucleosome formation-inducing effects of LY294006 cannot be attributed to general cell toxicity, because cotreatment with either 8-bromo-cAMP (1 mM) or LH (100 ng/ml) reduced this effect (Fig. 10). By comparison, inhibition of MEK signaling using U0126 (Fig. 10) or PD98509 (data not shown) for a comparable period of time failed to induce an increase in oligonucleosome formation compared to that in control cultured cells.

DISCUSSION

The recent identification of chicken Akt1 (Genbank accession no. AF039943) and Akt2 (present report), together with the deduced high degree of homology observed within functionally active domains compared to their respective mammalian counterparts, strongly suggest conservation of PI 3-kinase/Akt survival signaling between these vertebrate groups. The data reported herein pertaining to the activation and consequences resulting from inactivation of this signaling pathway in hen granulosa cells further support this

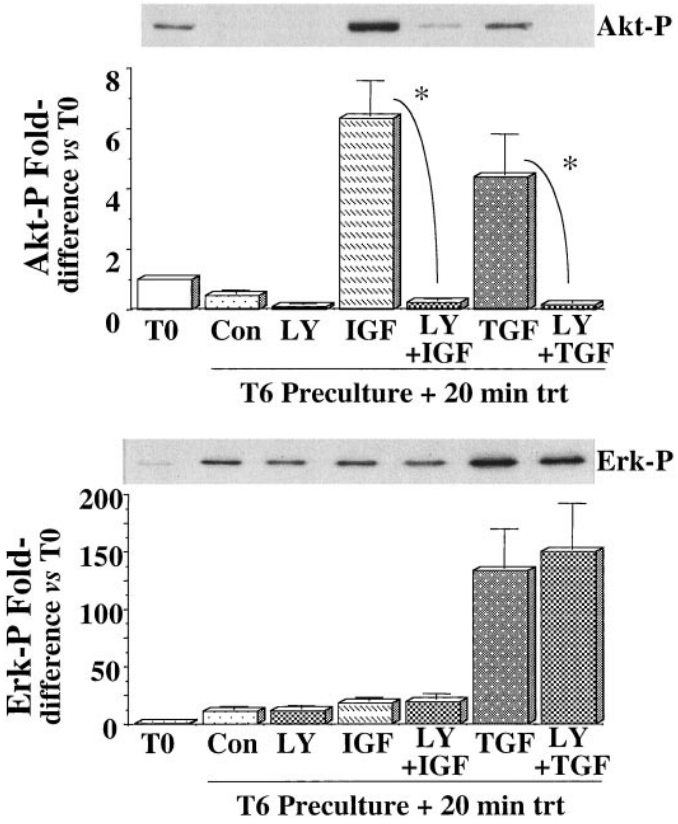

FIG. 8. Top) Inhibition of IGF-I (50 ng/ml)- and TGF-α (50 ng/ml)-induced Akt-P by the PI 3-kinase-inhibitor LY294002 (50 μM). Bottom) Lack of inhibitory effect by LY294002 on IGF-I- or TGF-α-mediated Erk-P. Preovulatory follicle granulosa cells were precultured in the absence of serum for 6 h, then pretreated for 1 h without or with LY294002, and finally treated for 20 min in the absence or presence of growth factor. $^{*}P < 0.01$.

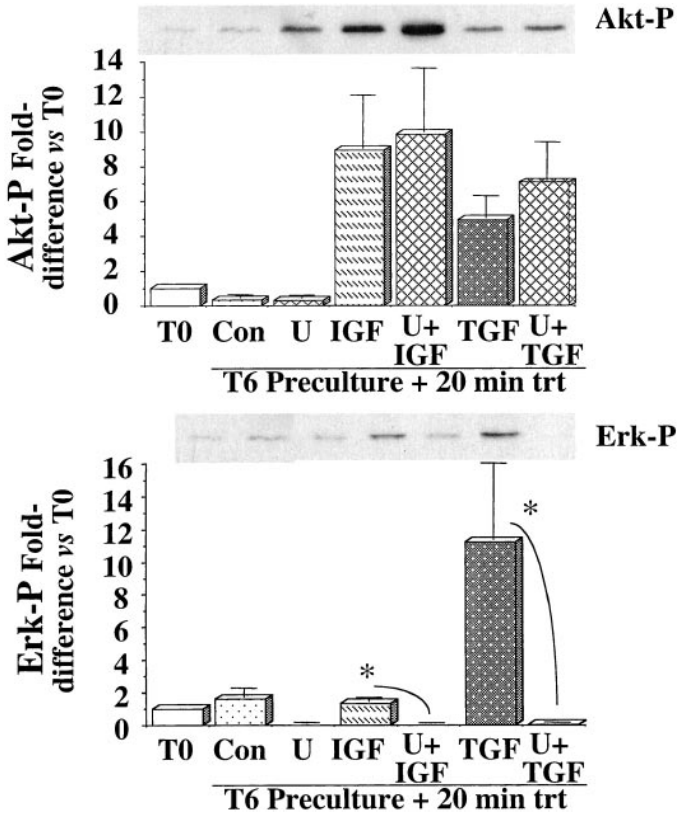

FIG. 9. Top) Lack of inhibitory effect by the MAP kinase-inhibitor U0126 (50 μM) on IGF-I (50 ng/ml)- or TGF-α (50 ng/ml)-mediated Akt-P. Bottom) Inhibition of IGF-I and TGF-α-induced Erk-P by U0126. Preovulatory follicle granulosa cells were precultured in the absence of serum for 6 h, then pretreated for 1 h without or with U0126, and finally treated for 20 min in the absence or presence of growth factor. $*P < 0.01$.

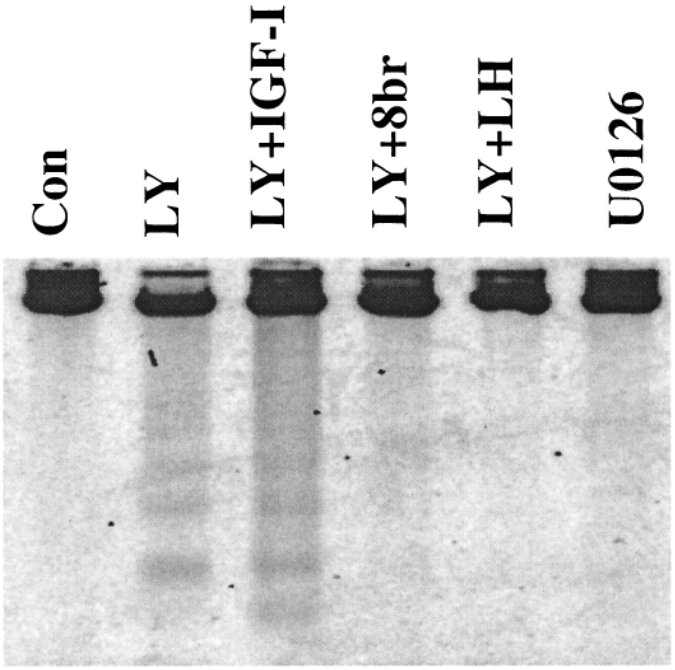

FIG. 10. The PI 3-kinase-inhibitor LY294002 (LY; 50 μM) induces oligonucleosome formation, both in the absence and presence of IGF-I (50 ng/ml), in preovulatory follicle granulosa cells following 20 h of culture, whereas cotreatment with LH (100 ng/ml) or 8-bromo-cAMP (cAMP; 1 mM) prevents this effect. By contrast, treatment of cells with U0126 (50 μM) fails to induce oligonucleosome formation. The treatments shown were repeated at least twice with similar results.

logical speculation. Furthermore, the widespread expression of the akt2 mRNA transcript among various tissues from the hen, including theca tissue, suggests a more ubiquitous role for this signaling pathway in cells of multiple origins.

The estimated 2.2-kb akt2 mRNA transcript is smaller than the 4.1-kb transcript reported for human akt2 [18], but the apparent size of the expressed protein as determined by Western blot analysis (60 kDa; Fig. 3) is similar [31]. To our knowledge, essentially no information has been reported to date regarding characteristics of the chicken Akt1 protein, but the mammalian Akt1 protein has been reported to migrate, with an apparent size of 59 kDa [32]. Thus, given the relatively small degree of cross-reactivity to Akt1 predicted from the primary sequence of the epitope used to generate the Akt2 antiserum (see *Materials and Methods*), the immunoreactivity depicted in Figure 3 may represent some small contribution from Akt1. Nevertheless, although there appear to be cell type-dependent differences in the relative expression and activation of Akt1 compared to Akt2, the consequences for downstream signaling for both isoforms are predicted to be similar [14, 33, 34].

The pattern of Akt2 protein expression within the granulosa cell layer during follicular development generally parallels that for mRNA (Fig. 3 vs. Fig. 2). The finding of elevated Akt2 in preovulatory versus prehierarchal follicles is consistent with the proposal that Akt signaling represents an important cell survival pathway in apoptosis-resistant cells. It is interesting to note, however, that levels of Akt2 are not increased within granulosa cells from the follicle most recently selected into the preovulatory hierarchy (9–12 mm), even though the cells from such follicles have, by this time, acquired the cell death resistance characteristic of larger (F3–F1) follicles [2]. This timing of increased Akt2 during follicular development is similar to that previously noted for Bcl-xLONG [35], and it indicates that increased levels of these putative cell survival proteins are the result, not the cause, of follicle selection.

Given stage-specific differences in Akt2 protein levels, it was interesting to evaluate potential factors responsible for regulation of Akt protein expression. However, levels of Akt2 remain unchanged after 20 h of culture in the absence or the presence of the putative cell survival factors, IGF-I and LH (data not shown). By contrast, results of previous studies have shown that levels of Bcl-xLONG are increased by 4.8-fold (in prehierarchal follicle granulosa) to 1.7-fold (in preovulatory granulosa) following a 20-h treatment with 8-bromo-cAMP [35]. Mechanisms responsible for the 2.5-fold or higher levels found in preovulatory follicle granulosa cells remain to be elucidated, but it is significant that upregulation of Akt expression in mammalian cells has generally been correlated with terminal differentiation [5]. Moreover, although Akt signaling has also been linked to enhancing cell proliferation [17], this possibility is considered less likely, because preovulatory follicle granulosa cells are relatively nonmitotic, except within the relatively limited germinal disk region [36].

The time frame to induction of Akt phosphorylation in response to IGF-I (Fig. 5) together with the effective doses (Fig. 4) are generally comparable to those recently reported in pig granulosa cells [25]. Whereas TGF-α was also found to stimulate significant levels of Akt phosphorylation in hen granulosa cells, neither FGF nor PDGF were found to be effective (Fig. 6). The absence of response to the latter mammalian growth factors cannot be attributed to biolog-

ical inactivity, because each factor has previously been demonstrated to induce plasminogen-activator activity and/or to promote mitosis in hen granulosa cells [37, 38]. Although PDGF has recently been reported to activate PI 3-kinase/Akt signaling in the porcine theca cell layer [39], the potential for its activity within the hen theca cell layer was not tested in the present studies.

Both IGF-I and the type-I IGF receptor mRNA expression have been reported to be expressed within the granulosa and theca cell layers of hen follicles throughout follicular development [40]. This suggests that the cell survival actions of IGF-I can be mediated in either an autocrine or a paracrine fashion. Moreover, immunoreactive EGF/TGF-α receptor and TGF-α have been localized to the hen granulosa cell layer during follicular development [41], and TGF-α has been implicated as an important cell survival factor in prehierarchal follicle granulosa cells [4]. These findings, together with reports that TGFα has 100- to 300-fold greater affinity for the chicken EGF/TGF-α receptor than EGF [42, 43], suggest a physiological role for TGF-α in mediating cell survival effects within the granulosa cell layer, at least in part, through Akt signaling.

The absence of serum for as little as 6 h results in a significant decline in Akt-P levels, and prolonged deprivation (33–44 h) eventually leads to cell death. The findings that IGF-I is capable of reversing the effects of serum starvation (Fig. 7) but that inhibition of PI 3-kinase activity induces pronounced oligonucleosome formation in cultured cells (Fig. 10) further support a functional role for Akt signaling in maintaining granulosa cell survival. On the other hand, conflicting reports have appeared with regard to the role of MEK/Erk signaling in cell survival, because this pathway has been proposed to prevent (human luteinized granulosa and rat pheochromocytoma cells) [44, 45] or have no effect on (Rat-1 fibroblasts) [8] the progression of trophic factor withdrawal-induced apoptotic cell death. Although the data provided herein fail to support a critical function for MEK/Erk signaling in the short-term maintenance of granulosa cell viability (Fig. 10), recent unpublished data strongly implicate this pathway in the regulation of hen granulosa cell differentiation (specifically LH receptor and steroid acute regulatory protein expression).

From a technical perspective, it was also interesting to evaluate the effects of the frequently used cell culture medium supplements FBS and ITS on the activation of Akt. The finding that ITS, but not 2.5% FBS, induced rapid Akt phosphorylation to a level not unlike that stimulated by IGF-I (Fig. 6) might explain the mechanism and can support the usefulness of this supplement in maintaining cell survival in a defined medium. The observation that this level of FBS is effective in maintaining cell cultures for prolonged periods of time (Fig. 7) suggests the activation of alternative mechanisms for promoting cell survival. In fact, IGF-I has recently been reported to mediate cell survival in both Akt-dependent and -independent pathways [46].

In addition to the presence of an active Akt signaling cell survival pathway within granulosa, the relative resistance of large preovulatory (compared to prehierarchal) follicle granulosa cells to apoptotic cell death has also been correlated with increased expression of the death suppressive genes, bcl-xLONG [4, 35], bcl-2 [28], and inhibitor of apoptosis protein 1 [27]. The existence of multiple, and potentially independent, mechanisms responsible for granulosa cell survival is not surprising given the relative importance of maintaining follicle survival from an evolutionary perspective. In support of the proposal for multiple, independent pathways is the finding that LH and the cAMP analogue 8-bromo-cAMP are each capable of maintaining cell viability in cultures treated with the PI 3-kinase-inhibitor LY294006 (Fig. 10). It has previously been determined that such treatments induce expression of both Bcl-xLONG [35] and Mcl-1 (a related bcl-2 gene family member; unpublished data). It is significant to note that although in the present studies neither LH nor 8-bromo-cAMP induced Akt-P in the short term, FSH induced a biphasic increase in Akt-P in estrogen-primed, immature rat granulosa cells, with the highest levels of phosphorylation occurring after 48 h of culture [47]. Thus, some degree of cross-talk among cell survival pathways is clearly a possibility, and this potential deserves further investigation.

In summary, the data presented here support the role for active PI 3-kinase/Akt signaling in maintenance of the preovulatory follicle granulosa layer. Evidence is presented for the ability of both IGF-I and TGF-α to serve as effective promoters of Akt signaling, but additional growth factors and/or cytokines may also play a physiological role. Yet to be adequately addressed within the granulosa cell model from any species are the downstream intracellular (e.g., at the level of the mitochondria) [48] and genomic (e.g., transcription factors) mechanisms activated by the PI 3-kinase/Akt signaling pathway. Moreover, an additional challenge for the immediate future will be to unravel the cross-talk occurring among signaling pathways, cellular survival proteins, and death-inducing proteins that impact on cell survival.

ACKNOWLEDGMENT

We thank Ms. Z. Yin for excellent technical assistance during the early stages of this study.

REFERENCES

1. Kaipia A, Hsueh AJ. Regulation of ovarian follicle atresia. Annu Rev Physiol 1997; 59:349–363.
2. Johnson AL. The avian ovarian hierarchy: a balance between follicle differentiation and atresia. Poult Avian Biol Rev 1996; 7:99–110.
3. Hsueh AJ, Eisenhauer K, Chun SY, Hsu SY, Billig H. Gonadal cell apoptosis. Recent Prog Horm Res 1996; 51:433–455.
4. Johnson, AL, Bridgham JT, Witty JP, Tilly JL. Susceptibility of avian ovarian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of *bcl-x*long gene expression. Endocrinology 1996; 137:2059–2066.
5. Coffer PJ, Jin J, Woodgett JR. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochemical J 1998; 335:1–13.
6. Alessi DR, Cohen P. Mechanism of action and function of protein kinase B. Curr Opin Genet Dev 1998; 8:55–62.
7. Downward J. Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 1998; 10:262–267.
8. Kulik G, Klippel A, Weber MJ. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol 1997; 17:1595–1606.
9. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997; 91:231–241.
10. Peterson RT, Schreiber SL. Kinase phosphorylation: keeping it all in the family. Curr Biol 1999; 9:R521–R524.
11. Belham C, Wu S, Avruch J. Intracellular signalling: PDK1—a kinase at the hub of things. Curr Biol 1999; 9:R93–R96.
12. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998; 282:1318–1321.
13. Kane LP, Shapiro VS, Stokoe D, Weiss A. Induction of NF-kappaB by the Akt/PKB kinase. Curr Biol 1999; 9:601–604.
14. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three alts. Genes Dev 1999; 13:2905–2927.

15. Du K, Montminy M. CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem 1998; 273:32377–32379.
16. Cheng JQ, Altomare DA, Klein MA, Lee WC, Kruh GD, Lissy NA, Testa JR. Transforming activity and mitosis-related expression of the AKT2 oncogene: evidence suggesting a link between cell cycle regulation and oncogenesis. Oncogene 1997; 14:2793–2801.
17. Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A 1999; 96:4240–4245.
18. Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci U S A 1992; 89:9267–9271.
19. Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V, Ferrandina G, Benedetti Panici S, Neri G, Testa JR. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer 1995; 64: 280–285.
20. Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK, Testa JR. Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proc Natl Acad Sci U S A 1996; 93:3636–3641.
21. Nakatani K, Thompson DA, Barthel A, Sakaue H, Liu W, Weigel RJ, Roth RA. Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. J Biol Chem 1999; 274:21528–s1532.
22. Liu AX, Testa JR, Hamilton TC, Jove R, Nicosia SV, Cheng JQ. AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. Cancer Res 1998; 58: 2973–2977.
23. Johnson AL. Apoptosis-susceptible versus -resistant granulosa cells from hen ovarian follicles. In: Tilly JL, Strauss III JF, Tenniswood M (eds.), Cell Death in Reproductive Physiology. New York: Springer-Verlag; 1996: 78–92.
24. Flaws JA, DeSanti A, Tilly KI, Jacid R, Johnson AL, Hirshfield AN, Tilly JL. Vasoactive intestinal peptide-mediated suppression of apoptosis in the ovary: mechanisms of action and evidence of a conserved anti-atretogenic role through evolution. Endocrinology 1995; 136: 4351–4359.
25. Westfall SD, Hendry IR, Obholz KL, Rueda BR, Davis JS. Putative role of the phosphatidylinositol 3-kinase-Akt signaling pathway in the survival of granulosa cells. Endocrine 2000; 12:315–321.
26. Tilly JL, Kowalski K, Johnson AL. Stage of follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod 1991; 44:305–314.
27. Johnson AL, Bridgham JT, Digby MR, Lowenthal JW. Expression of the inhibitor of T-cell apoptosis (ita) gene in hen ovarian follicles during development. Biol Reprod 1998; 58:414–420.
28. Johnson AL, Bridgham JT, Witty JP, Tilly JL. Expression of bcl-2 and nr-13 in hen ovarian follicles during development. Biol Reprod 1997; 57:1096–1103.
29. Tilly JL, Kowalski KI, Johnson AL, Hsueh AJ. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology 1991; 129:2799–801.
30. Soler RM, Dolcet X, Encinas M, Egea J, Bayascas JR, Comella JX. Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. J Neurosci 1999; 19:9160–9169.
31. Jones PF, Jakubowicz T, Hemmings BA. Molecular cloning of a second form of rac protein kinase. Cell Regul 1991 2:1001–1009.
32. Jones PF, Jakubowicz T, Pitossi FJ, Maurer F, Hemmings BA. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc Natl Acad Sci U S A 1991; 88: 4171–4175.
33. Kim YB, Peroni OD, Franke TF, Kahn BB. Divergent regulation of Akt1 and Akt2 isoforms in insulin target tissues of obese Zucker rats. Diabetes 2000; 49:847–856.
34. Okano JI, Gaslightwala I, Birnbaum MJ, Rustgi AK, Nakagawa H. Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation in esophageal cancer cells. J Biol Chem 2000; 275:30934–30942.
35. Johnson AL, Bridgham JT, Jensen T. Bcl-X(LONG) protein expression and phosphorylation in granulosa cells. Endocrinology 1999; 140: 4521–4529.
36. Tischkau SA, Bahr JM. Avian germinal disc region secretes factors that stimulate proliferation and inhibit progesterone production by granulosa cells. Biol Reprod 1996; 54:865–870.
37. Tilly JL, Johnson AL. Effect of several growth factors on plasminogen activator activity in granulosa and theca cells of the domestic hen. Poult Sci 1990; 69:292–299.
38. Roberts RD, Ellis RC. Mitogenic effects of fibroblast growth factors on chicken granulosa and theca cells in vitro. Biol Reprod 1999; 61: 1387–1392.
39. Taylor CC. Platelet-derived growth factor activates porcine thecal cell phosphatidylinositol-3-kinase-Akt/PKB and ras-extracellular signal-regulated kinase-1/2 kinase signaling pathways via the platelet-derived growth factor-beta receptor. Endocrinology 2000; 141:1545–1553.
40. Onagbesan OM, Vleugels B, Buys N, Bruggeman V, Safi M, Decuypere E. Insulin-like growth factors in the regulation of avian ovarian functions. Domest Anim Endocrinol 1999; 17:299–313.
41. Onagbesan OM, Gullick W, Woolveridge I, Peddie MJ. Immunohistochemical localization of epidermal growth factor receptors, epidermal-growth-factor-like and transforming-growth-factor-alpha-like peptides in chicken ovarian follicles. J Reprod Fertil 1994; 102:147–153.
42. Lax I, Johnson A, Howk R, Sap J, Bellot F, Winkler M, Ullrich A, Vennstrom B, Schlessinger J, Givol D. Chicken epidermal growth factor (EGF) receptor: cDNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor alpha. Mol Cell Biol 1988; 8:1970–1978.
43. Onagbesan OM, Woolveridge I, Peddie MJ. Characterization of transforming growth factor-alpha receptors in the avian ovary: alterations in ligand binding to granulosa cells during follicular maturation. J Endocrinol 1996; 149:171–179.
44. Oliver RH, Khan SM, Leung BS, Yeh J. Induction of apoptosis in luteinized granulosa cells by the MAP kinase kinase (MEK) inhibitor PD98059. Biochem Biophys Res Commun 1999; 263:143–148.
45. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995; 270:1326–1331.
46. Kulik G, Weber MJ. Akt-dependent and -independent survival signaling pathways utilized by insulin-like growth factor I. Mol Cell Biol 1998; 18:6711–6718.
47. Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS. Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB)/Akt and serum and glucocorticoid-induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 2000; 14: 1283–1300.
48. Kennedy SG, Kandel ES, Cross TK, Hay N. Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. Mol Cell Biol 1999; 19:5800–5810.