Telomerase Is Regulated by c-Jun NH2-Terminal Kinase in Ovarian Surface Epithelial Cells

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Abstract

Telomerase activity is present in >90% of all tumors and appears to be regulated by the phosphatidylinositol 3-kinase signaling pathway. Here we demonstrate that Akt is not involved in the signaling cascade for telomerase regulation in ovarian surface epithelial cells. However, we showed that c-Jun NH2-kinase induces telomerase activity, that inhibition of JNK by JIP abrogates telomerase activity, and that JNK expression activates transcription of a reporter gene fused to the hTERT promoter sequence. Consequently, our data show that JNK is a key regulator of telomerase activity and, hence, may provide new perspectives on tumorigenesis that could be exploited for novel therapeutic strategies.

Introduction

Because telomerase is present in >90% of human tumors and is absent from most normal somatic cells (1), it is the most widely expressed and specific cancer marker presently known (2). Consequently, understanding the molecular mechanisms regulating telomerase is of significant clinical importance. Transcriptional control of telomerase includes alternate splicing of hTERT mRNA transcripts, hTERT promoter methylation (3), and binding of one or more transcription factors, including c-Myc, to the hTERT promoter (5). Protein kinase C (6), DMSO (7), calcium (8), and zinc (9) are also reported to affect telomerase activity, illustrating the complexity associated with telomerase regulation. We and others have previously shown that PI 3-kinase can up-regulate telomerase activity (10, 11). In the present study, we show that the primary target of PI 3-kinase, Akt, did not affect telomerase activity in human OSE cells. However, JNK, also a downstream target of PI 3-kinase, increased telomerase activity, induced hTERT transcription, and led to induced reporter activity by transcriptional activation of hTERT. Therefore, this is the first report to identify JNK as a key regulator of telomerase activity.

Methods and Materials

Cell Culture. Telomerase-positive ovarian carcinoma cell lines OV1063, OV2008, OVCA3, SW626, and CaOV3 were used. Four nonmutagenic SV40 large-T antigen-transfected OSE cell lines, FHOSE 118, FHOSE 1816–686, NFHOSE 80, and FHOSE 1816–575, derived from normal ovarian surface epithelium (12), were also used in this study. FHOSE 118, NFHOSE 80, and FHOSE 1816–575 cells were determined previously to be telomerase negative (13). Cells were maintained in Medium 199/H11022 (1:1; Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal bovine serum (HyClone, Logan, UT) and 10 μg/ml gentamicin (Life Technologies, Inc., Grand Island, NY) in a humidified 5% CO2/95% air atmosphere. Cell growth was determined by MTS assay as described previously (8).

Treatment with Anisomycin. To determine whether JNK signaling was involved in telomerase regulation, SW626 or FHOSE 118 cells were serum starved for 24 h, treated for 8 or 24 h with 20 or 40 μM of anisomycin (Sigma), a potent stimulator of JNK, and then assayed for telomerase activity. Parallel cultures of cells were collected for RT-PCR.

Transfections. To examine the role of JNK signaling in telomerase regulation, Flag-tagged JNK, Flag-tagged JIP, which is a specific inhibitor of JNK, HA-tagged AA, and GFP were used for transfection. Cells were serum starved for 24 h and then transfected with 3 μg of DNA using Lipofectamine reagent (Life Technologies, Inc., Grand Island, NY). Cells were collected at 24 h after transfection and assayed for telomerase activity. In all transfection experiments, parallel cultures transfected with GFP were used as a control for transfection efficiency.

Telomerase Assay. To quantitatively detect changes in telomerase levels, all cells were assayed for telomerase activity using the telomerase PCR-ELISA (Roche Molecular Biochemicals, Indianapolis, IN), as described previously (13) and according to the manufacturer’s instructions. After PCR-ELISA, telomerase activity was detected using a Dynex-MXR plate reader (Dynex Technologies, Chantilly, VA) and recorded as absorbance units. These values were expressed as a fold increase above control levels, with the control value used as the denominator for the determination of fold increase for the treated samples. For graphical representation of the effect of Akt, anisomycin, JNK, or JIP on telomerase activity, control values for the untreated samples of all cell lines were set at 1.0. Telomerase activity is shown ±SE.

Immunoprecipitation and Western Blot Analysis. Cells were lysed on ice using a modified RIPA buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.5% NP40, 5 mM NaF, 1 mM Na3VO4, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, 1 mM benzamidine, and 10 μg/ml trypsin inhibitor]. Lysates were then centrifuged at 12,000 × g at 4°C for 10 min, and the protein concentrations of the supernatants were determined as described previously (10). Protein extracts were solubilized in SDS gel loading buffer (60 mM Tris base, 2% SDS, 1% β-mercaptoethanol). Samples containing equal amounts of protein (25 μg) were separated on a 10% SDS-PAGE and electroblotted onto Hybond-ECL nitrocellulose membranes (Amersham Life Sciences, Piscataway, NJ) by wet transfer. Immunoblotting was performed using antibodies against JNK (1:1000), phospho-JNK (1:1000), phospho-c-Jun (1:1000), phospho-SEK1 (1:2000), Akt (1:1000), phospho-Akt (1:1000; Cell Signaling, Beverly, MA), β-actin (Sigma; 1:5000), HA (1:1000), and Flag (1:1000). Blots were visualized using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions.

RT-PCR. To examine the contribution of transcriptional control in telomerase regulation, RT-PCR was performed as described previously (10). To insure there was no DNA contamination, each sample for reverse transcription was prepared in duplicate, with the duplicate preparation lacking reverse transcriptase (14). The cDNA samples were amplified using the Perkin-Elmer GeneAmp kit. The hTERT primers used were hTERT-S (CGGAAGAGTTCTCCACGT CACACTTCATGA) for an internal control. The amplified products were then separated by electrophoresis on a 9% polyacrylamide gel, stained with 1× SyberGreen (FM BioProducts, Rockland, ME), and analyzed...
TELOMERASE IS REGULATED BY JNK IN OSE CELLS

Akt Is Not Involved in Telomerase Regulation in OSE Cells. To determine whether Akt, the primary target of PI 3-kinase, was the downstream target of PI 3-kinase in the regulation of telomerase, several telomerase-positive cancer cell lines and telomerase-negative normal cell lines were surveyed for telomerase activity (Fig. 1A).

Parallel cultures were assayed by immunoprecipitation and Western immunoblotting for phospho-Akt as well as Akt. Working within the linear range of AKT phosphorylation, we found an inverse correlation between telomerase activity and Akt activation. Telomerase-negative cell lines demonstrated higher levels of phosphorylated Akt than the telomerase-positive cell lines. Densitometric analysis confirmed this finding. When normalized to total Akt levels, endogenous phosphorylated Akt levels in telomerase-negative cells were 1.8-fold greater than in telomerase-positive cells, as determined by using ImageQuant software. In addition, transfection of activated AA into the telomerase-negative FHIOSE 118 cells did not induce telomerase activity (Fig. 1B). Western blot analysis using anti-HA confirmed successful transfection of AA.

Anisomycin Induces Telomerase Activity. Because JNK is also a downstream target of PI 3-kinase, SW626 and FHIOSE 118 cells were treated with anisomycin, a known stimulator of JNK, and then assayed for telomerase activity (Fig. 2). Treatment with anisomycin resulted in a 3- and 3.5-fold increase in telomerase activity in SW626 cells by 8 and 24 h, respectively (Fig. 2A). After anisomycin treatment, RT-PCR revealed de novo transcription of hTERT mRNA in FHIOSE 118 with the Kodak EDAS 120 Digital Analysis System. Net hTERT mRNA intensities from treated samples were normalized to their corresponding β-actin mRNA levels and were expressed as a percentage of the control samples that were similarly normalized to their corresponding β-actin mRNA levels.

Luciferase Assay. The hTERT promoter-luciferase construct called pGL3-1375 (15) was used to measure hTERT transcriptional activity in the FHIOSE 118 cell line. pGL3-1375 contains a 1375-bp promoter fragment of hTERT fused to the luciferase reporter gene in pBluescript (Stratagene, La Jolla, CA). Transient cotransfections were performed as mentioned above, FHIOSE 118 cells were transfected using Lipfectamine (Life Sciences Technologies) with the indicated amounts of JNK or JIP cDNA. The total amount of transfected DNA was kept constant in each experiment by adding vector-only plasmid. A plasmid expressing the bacterial β-galactosidase gene was also cotransfected in each experiment to serve as an internal control for transfection efficiency. Cells were collected at 48 h, and transcriptional activity was measured as a function of luciferase activity using the Luciferase Assay System (Promega Corp., Madison, WI) according to the manufacturer’s instructions and as described previously (16). At the time of collection, cells were observed microscopically to ensure that cells were viable and that there were no signs of apoptosis. Transcriptional activity was expressed as relative luciferase activity ± SE, after normalization with β-galactosidase activity. Each transfection was performed in triplicate.

Statistical Analysis. Samples for telomerase PCR-ELISA and luciferase assay were run in triplicate, and the data were subjected to the Student’s t test analysis for determination of statistical significance.

Results

Akt Is Not Involved in Telomerase Regulation in OSE Cells. To determine whether Akt, the primary target of PI 3-kinase, was the downstream target of PI 3-kinase in the regulation of telomerase, several telomerase-positive cancer cell lines and telomerase-negative normal cell lines were surveyed for telomerase activity (Fig. 1A).
and phospho-c-Jun. Using Western blot analysis, membranes were probed with phospho-SEK1, phospho-JNK, and phospho-e-Jun. β-actin was used as a loading control. Parallel cultures were examined for endogenous levels of phospho-JNK using Western blot analysis. JNK was used as a loading control. Several telomerase-negative and -positive cell lines were examined for endogenous levels of phospho-JNK using Western blot analysis. JNK served as a loading control.

Endogenous JNK Activity Is Increased in Telomerase-positive Cells. Telomerase-negative FHIOSE 118 and telomerase-positive SW626 cells were compared for endogenous levels of members of the JNK signaling pathway (Fig. 3A). After immunoprecipitation, cell lysates were probed with anti-phospho-SEK1, anti-phospho-JNK, and anti-phospho-c-Jun. Western blot analysis revealed high levels of phosphorylated proteins. In contrast, SW626 cells were found to have high levels of phosphorylated SEK1, JNK, and c-Jun. β-actin was used as a loading control. Several telomerase-positive ovarian cancer cells and telomerase-negative, nontumorigenic ovarian cell lines were also surveyed for endogenous levels of JNK, phosphorylated JNK, and telomerase activity. We found that telomerase-positive cell lines have elevated endogenous phospho-JNK levels when compared with telomerase-negative cell lines (Fig. 3B).

JNK Plays a Role in Telomerase Regulation. To confirm a role for JNK in telomerase regulation, the telomerase-negative FHIOSE 118, NFHIOSE 80, and FHIOSE 1816–575 cells were transfected with GFP or JNK, whereas the highly telomerase-positive CaOV3 ovarian cancer cells were transfected with GFP and JIP (Fig. 4). The FHIOSE 118 cells were also cotransfected with JNK and the JNK inhibitor protein, JIP. FHIOSE 118 cells transfected with JNK exhibited an 8-fold induction of de novo telomerase activity, whereas transfection with GFP or JNK + JIP did not induce detectable telomerase activity (Fig. 4A). Furthermore, JIP was able to suppress JNK-induced telomerase activity in the FHIOSE 118 cells (Fig. 4A). Successful transfections were confirmed by Western blot analysis (Fig. 4B, inset), and transfection efficiency in FHIOSE 118 cells was ~70% as visualized compared with GFP controls. To confirm the effect of JNK, two additional telomerase-negative cell lines, NFHIOSE 80 and FHIOSE 1816–575 cells, were also transfected with JNK (Fig. 4A). Twenty-four h after transfection with JNK, NFHIOSE 80 cells exhibited a 5.75-fold increase in telomerase activity, and the FHIOSE 1816–575 cells showed a 3-fold increase. For NFHIOSE 80 and FHIOSE 1816-
575 cells, transfection efficiencies were approximately 55 and 30%, respectively, as visualized compared with GFP controls. Transfection of JIP into CaOV3 cells resulted in a 10-fold decrease in telomerase activity (Fig. 4B), confirming the role of JNK in the regulation of telomerase. For CaOV3 cells, transfection efficiency was ~35% as visualized compared with GFP controls. GFP did not affect telomerase activity (data not shown). Successful transfections were confirmed by Western blot analysis (Fig. 4B, inset).

**JNK Is Capable of Activating the hTERT Promoter, and This Activation Can Be Inhibited by JIP.** To clearly demonstrate the role of JNK in telomerase regulation, it was necessary to determine whether JNK was capable of activating transcription of a reporter gene fused to the hTERT promoter sequence (Fig. 4C). Telomerase-negative FHIOSE 118 cells were transfected with either the hTERT promoter reporter construct ∆ JNK, ∆ JIP. Samples were collected 48 h after transfection, and luciferase activity was measured (Fig. 4C). The hTERT promoter reporter was activated by as little as 0.05 μg of JNK, resulting in a 3.5-fold increase in reporter gene activity. In addition, JIP was able to abolish reporter activity when cotransfected with JNK, but alone, JIP did not induce reporter activity.

**Discussion**

Our study is the first to identify JNK as a regulator of telomerase. This data are in agreement with previous studies that suggested a connection between the PI 3-kinase pathway and telomerase activity. Although hTERT may have a consensus sequence for the Akt kinase, our attempts to induce telomerase activity in the OSE cell system with activated Akt were unsuccessful. This would suggest that Akt, the primary downstream target of PI 3-kinase, does not contribute to telomerase regulation in OSE cells. Instead, we showed that JNK, also a target of PI 3-kinase, is involved in telomerase regulation.

Although the hTERT promoter has been shown to respond to c-myc, many binding sites have been identified for several different transcription factors within the promoter region (17). This would suggest a complex mechanism for telomerase regulation that is probably cell type or tissue specific. However, we revealed that JNK alone was capable of activating transcription of the luciferase gene fused to the hTERT promoter sequence in our OSE cell system. Although it is not known precisely how JNK activates the hTERT promoter, it is possible that JNK-mediated phosphorylation and activation of c-Jun can lead to c-Jun binding to any of several AP1 sites present throughout the hTERT promoter (18). In support of this idea, Satoru and Inoue have demonstrated that loss of the AP1 site in the hTERT promoter (18). In support of this idea, Satoru and Inoue have demonstrated that loss of the AP1 site in the hTERT promoter significantly reduces hTERT expression and consequently, telomerase activity.

In summary, our study is the first to indicate that JNK can induce telomerase activity by transcriptional activation of hTERT. The initial extracellular signal may stimulate PI 3-kinase and cascade through SEK1, then JNK. JNK, in turn, could activate c-Jun, causing transcription of hTERT and subsequent activation of telomerase. Certainly, additional studies are warranted to completely delineate the signaling pathway for JNK-mediated telomerase regulation. These data will have a significant impact on the development of chemotherapeutic agents to target telomerase.

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**References**

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