Phosphatidylinositol Triphosphate Kinase-dependent and c-Jun NH$_2$-Terminal Kinase-dependent Induction of Telomerase by Calcium Requires Pyk2

Michelle Y. Alfonso-De Matte and Patricia A. Kruk
Department of Pathology, University of South Florida and the H. Lee Moffitt Cancer Center, Tampa, Florida

Abstract
Calcium signaling has been linked to activation of Pyk2, a calcium-dependent, focal adhesion kinase-related, non-receptor tyrosine kinase. Signaling via Pyk2 can activate c-jun NH$_2$-terminal kinase (JNK). Calcium has also been shown to activate phosphatidylinositol triphosphate kinase and/or JNK. Here, we show that calcium signaling in ovarian surface epithelial cells not only induces telomerase activity via JNK but also activates Pyk2. Moreover, telomerase activation by Pyk2 requires JNK activation. In contrast, a kinase-deficient Pyk2 construct failed to activate either JNK or telomerase. Finally, we demonstrate that Pyk2 is capable of driving the human telomerase reverse transcriptase promoter, resulting in telomerase activation. These data suggest a novel role of Pyk2 for telomerase regulation.

Introduction
The proline-rich tyrosine kinase, Pyk2, is a non-receptor tyrosine kinase that can be activated by inflammatory cytokines, UV radiation, tumor necrosis factor-$\alpha$, and changes in calcium levels (1). Overexpression or altered activity of Pyk2 has been linked to changes in cell adhesion, as well as invasion and metastasis of various malignancies (2). Also, Pyk2 can activate phosphatidylinositol triphosphate kinase (PI 3-kinase; Ref. 3), which, in turn, can phosphorylate JNK (4) as well as the mitogen-activated protein kinase signaling pathway (5), demonstrating that Pyk2 is an important signaling protein. We have shown previously that external stimuli such as UV and DMSO regulate telomerase activity in a PI 3-kinase/JNK-dependent but Akt-independent manner (6, 7). In addition, we have shown that calcium, a key signaling molecule, can also induce telomerase activity in ovarian surface epithelium (OSE) cells, although the exact signaling pathway was not identified (8). In the present study, we examined whether calcium, similar to UV and DMSO, regulates telomerase activity in a PI 3-kinase/JNK-dependent manner, and we defined a novel role for Pyk2 propagation of calcium-mediated, PI 3-kinase/JNK-dependent telomerase induction.

Materials and Methods
Treatment with Calcium, Ly294002, Anisomycin, and EDTA. An SV40 large T-antigen-transfected cell line of overtly normal, telomerase-negative OSE, FHOISE 118 (6–9), was serum starved for 24 h and then treated with either 10 mM CaCl$_2$ ± 3 mM EDTA (Sigma-Aldrich, St. Louis, MO) or ± 10 $\mu$M LY294002 (LY) in ethanol (Sigma-Aldrich), the specific PI 3-kinase inhibitor (10), as described previously (6, 8). Cells were also treated with 20–40 $\mu$M anisomycin in ethanol (Sigma-Aldrich), a well-known JNK activator, as described previously (11). Control cells were treated with vehicle alone. Cells were collected at 24 h and assayed for telomerase activity.

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 (6) and according to the manufacturers’ instructions. Telomerase activity was recorded as absorbance units. These values were expressed as fold increases above control levels, with the control value used as the denominator for the determination of fold increase for the treated samples. For graphical representation, control values were set at 1.0. Telomerase activity is shown ± SE.

Transfection. The FHIOSE 118 ovarian epithelial cell line as well as the telomerase-positive SW626 and highly telomerase-positive CaOV3 ovarian cancer cells (6) were transfected with either vector alone, Flag-tagged JNK, Flag-tagged JIP (JNK inhibitor protein), Myc-tagged wild-type (WT) Pyk2, or Myc-tagged kinase-deficient (KD) Pyk2 using Lipofectamine reagent (Life Sciences Technologies, Grand Island, NY) in serum- and antibiotic-free media where serum was added back after 5 h of transfection. In addition, some samples were treated with 10 mM CaCl$_2$. Cells were collected at 24 h and assayed for telomerase activity. Green fluorescent protein was used to determine transfection efficiency. All transfections were confirmed by immunoblot (8).

Immunoprecipitation and Western Blot Analysis. Cells were lysed on ice using a modified RIPA buffer (7). Lysates were collected, and for Pyk2, 700 $\mu$g of protein were immunoprecipitated overnight at 4°C and then washed three times with PBS. Protein extracts were solubilized in SDS gel loading buffer (7), and 30 $\mu$g of protein were loaded directly onto the gel. Immunoblotting was performed using anti-JNK (1:1000), anti-Myc (1:1000) anti-Phospho-JNK (1:1000), anti-Phospho-c-Jun (1:1000), anti-HA (1:1000), anti-Flag (1:1000; Cell Signaling, Beverly, MA), anti-phosphorylated and unphosphorylated Pyk2 (1:1000; Upstate Biotech., Lake Placid, NY), or β-actin (1:5000; Sigma-Aldrich). Blots were visualized using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturers’ instructions.

Luciferase Assay. The human telomerase reverse transcriptase (hTERT) promoter-luciferase construct, pGL3–1375 (12), was used to measure hTERT transcriptional activity in the FHIOSE 118 cells. Transient cotransfections were performed as mentioned above, with the indicated amounts of Pyk2, reporter, and β-galactosidase CDNA. β-galactosidase was used to normalize transfection efficiencies, serving as an internal control. Expression of luciferase was measured 48 h after transfection using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturers’ instructions and as described previously (7). At the time of collection, cells were microscopically observed to ensure cell viability with no signs of apoptosis. Transcriptional activity was expressed as relative luciferase activity ± SE, after normalization with β-galactosidase activity.

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 and Discussion
To determine whether calcium, similar to UV and DMSO, regulates telomerase activity in a PI 3-kinase/JNK-dependent manner, serum-starved FHIOSE 118 cells were treated with 10 mM CaCl$_2$ ± 10 $\mu$M LY, a well-known PI 3-kinase inhibitor (10). We have confirmed previously the specificity of LY as an inhibitor of PI 3-kinase using in vitro kinase assays (6). Furthermore, we have also shown both LY and...
were transfected with either vector alone, JNK, or JIP/H11006 vector as well as JNK, activation, FHIOSE 118 cells were transfected with a control induction. Confirming a role for PI 3-kinase in calcium-mediated telomerase contrast, LY inhibited all calcium-mediated telomerase induction, resulted in a 2.2-fold induction of telomerase activity at 24 h. In A), Calcium treatment alone assayed for telomerase activity (Fig. 1 A). Calcium treatment alone treated with vehicle alone. Samples were collected at 24 h and wortmanin capable of inhibiting telomerase activity (6). Control cells were treated with vehicle alone. Samples were collected at 24 h and assayed for telomerase activity (Fig. 1A). Calcium treatment alone resulted in a 2.2-fold induction of telomerase activity at 24 h. In contrast, LY inhibited all calcium-mediated telomerase induction, maintaining telomerase levels comparable with that of the control, confirming a role for PI 3-kinase in calcium-mediated telomerase induction.

To establish a role for JNK in calcium-mediated telomerase activation, FHIOSE 118 cells were transfected with a control vector as well as JNK, ± the JIP, ± CaCl2 (Fig. 1B). Calcium treatment resulted in a 4.1-fold increase in telomerase levels, in agreement with our previous reports (8). In comparison to the Fig. 1A, the lower level of induced telomerase activity reported may have been attributable to the serum starvation requirement when using kinase inhibitors. Transfection with JNK resulted in a 4.5-fold increase in telomerase induction. When JNK-transfected cells were treated with calcium, telomerase levels increased 5-fold, but an additive effect was not seen. This may be attributable to a threshold capacity for telomerase induction or that calcium activates JNK, which is already overexpressed, stimulating the same signaling pathway, resulting in the same response. Cotransfection with JNK and JIP inhibited the telomerase-inducing effects of JNK, whereas JIP inhibited the stimulatory effects of calcium, maintaining telomerase levels similar to those of the control.

**Fig. 1.** Calcium induces telomerase activity in a PI 3-kinase- and JNK-dependent manner. A, FHIOSE 118 cells were treated with 10 mM CaCl2 ± 3 mM EDTA for 8 h. Western blot was performed using anti-Pyk2 or anti-phospho-tyrosine. B, FHIOSE 118 cells were either treated with 10 mM CaCl2 or transfected with Myc-tagged WT- or KD-Pyk2. After 24 h, immunoblotting was performed using anti-phospho-JNK antibody, anti-JNK, and actin as a loading control. Values shown represent densitometric analysis using ImagQuant software. C, FHIOSE 118 cells were transfected with either a vector control or WT-Pyk2 ± 10 mM LY for 24 h. Immunoblotting was performed using phospho-JNK and actin as well as anti-Myc to confirm transfection of WT-Pyk2. Values shown for phospho-JNK represent densitometric analysis. All transfections were confirmed by immunoblot (data published previously; Ref. 7).

To show that both PI 3-kinase and JNK were required for telomerase induction, we stimulated JNK activity with anisomycin, a potent and well-known JNK activator (11), ± LY and assayed for telomerase activity (Fig. 1C). Anisomycin alone at 20 and 40 μM was capable of inducing de novo telomerase activity 2.4- and 3.5-fold, respectively, LY inhibited anisomycin-induced telomerase activity. Therefore, although calcium induces telomerase activity in telomerase-negative cells (Fig. 1A), the induction occurs in a PI 3-kinase/JNK-dependent manner (Fig. 1, B and C).

To determine whether Pyk2 mediated calcium induction of telomerase, SW626 cells were treated 10 mM CaCl2 ± EDTA, a calcium chelator (Fig. 2A). Western blot analysis revealed a 3-fold increase in the level of phosphorylated Pyk2 after treatment with calcium. Furthermore, EDTA inhibited Pyk2 phosphorylation, maintaining phospho-Pyk2 levels similar to those seen in the control sample.

In addition, to show a requirement for JNK in Pyk2-mediated telomerase induction, FHIOSE 118 cells were either treated with 10 mM CaCl2 or transfected with WT- or KD-Pyk2. Cells were collected at 24 h, and Western blot analysis was performed for phospho-JNK (Fig. 2B). Although FHIOSE 118 cells demonstrated no endogenously activated JNK, treatment with 10 mM CaCl2 resulted in a 379%
increase in phosphorylation of JNK. Transfection with WT-Pyk2 resulted in a 258% increase in JNK activation, in contrast with the KD-Pyk2 that did not activate JNK (Fig. 2B).

To determine whether Pyk2-mediated JNK activation affected JNK protein levels and possibly required PI 3-kinase (Fig. 2C), FHIOSE 118 cells were transfected with WT-Pyk2 ± LY, and Western blot analysis was performed for JNK, P-JNK, actin, and Myc-epitope to confirm transfection. Although control samples demonstrated no activation of JNK, cells transfected with WT-Pyk2 showed a 277% increase in P-JNK, whereas LY treatment resulted in a 47% decrease in P-JNK. Hence, Pyk2 propagates calcium-mediated signaling through PI 3-kinase (Fig. 2C) and JNK (Fig. 2B).

Having recognized that Pyk2 propagates calcium signaling through PI 3-kinase and JNK, and that calcium-mediated telomerase induction was PI 3-kinase- and JNK-dependent, we then assessed whether PI 3-kinase-, JNK-, or calcium-dependent induction of telomerase requires Pyk2. FHIOSE 118 cells were transfected with WT-Pyk2 and assayed for telomerase activity (Fig. 3A). Transfection with WT-Pyk2 resulted in a 7.5-fold increase in de novo telomerase activity, showing that Pyk2 alone was capable of inducing telomerase activity.

To determine the effect of PI 3-kinase or JNK on WT-Pyk2-mediated telomerase induction, FHIOSE 118 cells were transfected with WT-Pyk2 and either cotransfected with JIP or treated with LY (Fig. 3A). When WT-Pyk2 was cotransfected with JIP or treated with LY, telomerase levels remained at that of the control.

To further examine the involvement of Pyk2 for calcium-mediated induction of telomerase, the FHIOSE 118 cells were transfected with KD-Pyk2 ± 10 mM CaCl₂, and then assayed for telomerase activity at 24 h (Fig. 3B). Although calcium treatment alone resulted in a 4-fold increase in telomerase activity, transfection with KD-Pyk2 had no effect on telomerase levels. As expected, transfection of KD-Pyk2, in addition to calcium treatment, abolished calcium’s ability to induce telomerase activity. We also transfected the highly telomerase-positive CaOV3 cancer cell line with KD-Pyk2 and assayed for telomerase activity at 24 h (Fig. 3C). After transfection, KD-Pyk2 was able to abrogate telomerase activity by ~90% when compared with the control samples.

When FHIOSE 118 cells were cotransfected with an hTERT-promoter luciferase reporter and WT-Pyk2, we found that WT-Pyk2 was sufficient to drive the hTERT promoter, resulting in a 14-fold increase in reporter activity (Fig. 3D). Additionally, JIP was able to partially inhibit WT-Pyk2’s ability to drive the promoter, indicating a role for JNK in the Pyk2-mediated transcription of hTERT. Taken together, these data implicate Pyk2 as a key regulator of calcium-mediated telomerase induction, suggesting a novel function for Pyk2, and further support the role of Pyk2 as an oncogene.

Our study is the first to identify Pyk2 as a potential regulator of...
telomerase in OSE cells. Our data also delineate a novel pathway for calcium signaling that involves Pyk2 as an early kinase that may propagate signaling through PI 3-kinase and JNK, ultimately leading to transcription of hTERT (Figs. 3D and 4). The clinical significance of calcium signaling in OSE is often not realized. Before ovulation, the peritoneal levels of calcium fluctuate in preparation for rupture of the follicle (13). Interestingly, it has been reported that as the ovum pushes outward and the follicle ruptures, extracellular calcium levels increase. Changes in calcium levels may stimulate damaged OSE to proliferate in an attempt to repair the ovulation-induced damage. In turn, as shown in the present study, the elevation in calcium might stimulate de novo induction of telomerase activity, contributing to the proliferative state of the post-ovulatory OSE. It is believed that repeated ovulatory damage and repair of the OSE contributes to ovarian cancers (14–16), resulting in >15,000 deaths annually (17). Therefore, it is important to understand the role of calcium-mediated activation of telomerase for diagnostic and potential therapeutic intervention. With an outlined pathway for manipulating telomerase, it may be possible to target any one or all of these signaling proteins for chemotherapeutic intervention.

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References

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