Akt and PKC are involved not only in upregulation of telomerase activity but also in cell differentiation-related function via mTORC2 in leukemia cells

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Abstract

We have shown previously that PI3K/Akt pathway is active after cell differentiation in HL60 cells. In the present study, we have investigated whether additional molecules, such as protein kinase C (PKC), are involved in the regulation, not only of telomerase, but also of leukemia cell differentiation. We show that PKC activates telomerase and is, itself, activated following VD3- or ATRA-induced differentiation of HL60 cells, as was observed for PI3K/Akt. To clarify the significance of PI3K/Akt and PKC pathway activation in leukemia cell differentiation, we examined the active proteins in either the downstream or upstream regulation of these pathways. In conjunction with the activation of Akt or PKC, mTOR and S6K were phosphorylated and the protein expression levels of Rictor were increased, compared to Raptor, following cell differentiation. Silencing by Rictor siRNA resulted in the attenuation of Akt phosphorylation on Ser473 and PKCα/βII phosphorylation, as well as the inhibition of Rictor itself, suggesting that Rictor is an upstream regulator of both Akt and PKC. In addition, in cells induced to differentiate by ATRA or VD3, Nitroblue-tetrazolium (NBT) reduction and esterase activity, were blocked either by LY294002, a PI3K inhibitor, or by BIM, a PKC inhibitor, without affecting cell surface markers such as CD11b or CD14. Intriguingly, the silencing of Rictor by its siRNA also suppressed the reducing ability of NBT following VD3-induced cell differentiation. Taken together, our results show that Rictor associated with mTOR (mTORC2) regulates the activity of both Akt and PKC that are involved in cell functions such as NBT reduction and esterase activity induced by leukemia cell differentiation.

Introduction

Telomerase is active in immature somatic cells and tumor cells and is suppressed in differentiated cells (Meyerson et al., 1997); however, the regulatory mechanism that controls telomerase activity during cell differentiation remains unclear. Recently, induced pluripotent stem (iPS) cells derived from differentiated somatic cells were shown to represent a new source of stem cells for medical applications. These cells express high telomerase activity during iPS cell generation(Takahashi et al., 2007). To identify which factors affect telomerase activity during the differentiation of iPS cells, it is important to obtain a better understanding of the mechanisms of telomerase regulation.

Studies of protein phosphorylation have indicated that the up-regulation of telomerase activity during megakaryocytic differentiation and T lymphocyte activation is dependent on protein kinase C (PKC) (Counter et al., 1998; Sheng et al., 2003; Weng et al., 1996). PI3K/Akt has also been proposed as a key signaling protein in telomerase regulation (Kawauchi et al., 2005). On the other hand, it is known that the activation of protein kinase C and the PI3K pathway also plays a critical role in signal transduction leading to the differentiation of leukemic cells (Aihara et al., 1991; Makowske et al., 1988; Zhang et al., 2006; Zhao et al., 2004). These findings suggest

that the mechanisms regulating telomerase activity are complex and that telomerase may be regulated by more than one mechanism during cell differentiation.

We have reported previously that the down-regulation of telomerase activity occurs during myeloid or erythroid differentiation in hematopoietic cells (Yamada et al., 1998), and mTOR which is known as one of the downstream signaling proteins for Akt that regulates cell proliferation and initiation of cap-dependent translation, was increased (Yamada et al., 2008). The mTOR protein exists as two distinct evolutionarily-conserved complexes: mTORC1 and mTORC2 (Sparks and Guertin, 2010). In this study, we have extended our previous research to investigate changes in, and differences between, signal transduction pathways concerning the mTOR pathway and the role of PKC in relation to telomerase activity during monocytic or granulocytic differentiation.

Materials and methods

Cell lines

HL60, a human acute myeloblastic leukemia cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and was maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 in air. For cell starvation, HL60 cells were cultured in RPMI-1640 medium containing 0.1% FBS.

Chemicals and antibodies

1α, 25-dihydroxyvitamin D3 (VD3) and all-*trans*-retinoic acid (ATRA) were purchased from Sigma (St. Louis, MO, USA). LY294002 and Bisindolylmaleimide (BIM) were purchased from Calbiochem (La Jolla, CA, USA) and reconstituted in dimethyl sulfoxide (DMSO). Propidium iodide staining solution was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Nitroblue tetrazolium (NBT) was purchased from Muto Chemical Co. (Tokyo, Japan). Antisera against rabbit Akt, phospho-Akt (Ser473), and phospho-PKCα/βII were purchased from Cell Signaling Technology (CST, Beverly, MA, USA). The mouse monoclonal antibody against p21 was purchased from BD Pharmingen (San Diego, CA, USA). Rabbit PKCα and

phospho-PKCα (Ser657) were purchased from Upstate Biotechnology (Lake Placid, NY, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. The rabbit antibody against mTOR was generously provided by Dr. Peter J. Houghton (St. Jude Children's Research Hospital, Memphis, TN, USA), and the rabbit antibody against phospho-mTOR (Ser2448) was purchased from Cell Signaling Technology. Polyclonal rabbit antibodies directed against hTERT were purchased from Calbiochem and Abcam (Cambridge, UK), and rabbit ß-actin antiserum was purchased from Sigma.

Cell culture and induction of differentiation

HL60 cells were induced to differentiate by exposure to 1 to 4×10^{-8} M ATRA or 1 to 10×10^{-9} M VD3 for one to five days. Differentiation was assessed by the detection of immunophenotypic changes in addition to the identification of morphological changes by light microscopy. The optimum concentration of each inducer was determined from the results of preliminary experiments. Cell viability was assessed by trypan blue dye exclusion.

NBT reduction assay and cytochemical staining

We performed an nitroblue-tetrazolium (NBT) reduction assay on extracts of PMA-stimulated cells as detailed in a previous report (Gianni et al., 1996). To determine the rate of NBT positivity, we counted the number of cells that reduced NBT

to formazan (which produces a blue cell color) and the number of cells without NBT-reducing activity (white cells). Cytochemical staining for Giemsa and α-naphthyl butyrate esterase was performed using previously published techniques (Li et al., 1973).

Immunophenotyping

We immunophenotyped samples using a panel of monoclonal antibodies and flow cytometry, as reported previously(Yamada et al., 2008). The monocyte fraction was determined as the percentage of $CD14^+$ cells, and differentiation to mature granulocytes was assessed by the shift from $CD11b^-CD14^-$ cells to $CD11b^+CD14^-$ cells.

Reverse transcription-polymerase chain reaction (RT-PCR)

We isolated total RNA using Isogen (Nippongene, Tokyo, Japan), and synthesized cDNA using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) as reported previously(Yamada et al., 1998). The resulting cDNA (25 ng) was subjected to PCR amplification, and the products were visualized by agarose gel electrophoresis and staining of the gels with ethidium bromide. The relative concentrations of the PCR products were determined by comparing the amount of the product in each lane to *β-actin*. The PCR primers used were: 5'-TGTGCTGGGCCTGGACGATA-3' and 5'-ACGGCTGGAGGTCTGTCAAGGTAG-3' for *hTERT,* and

5’-CTTCTACAATGAGCTGCGTG-3’ and 5’-TCATGAGGTAGTCAGTCAGG-3’ for *ß-actin*.

Telomerase assay and quantification of enzyme activity

Telomerase activity was measured using the telomere repeat amplification protocol (TRAP), as previously described (Kim et al., 1994). To investigate the effects of PKC on telomerase, lysates obtained from starved HL60 cells were incubated either with or without recombinant PKC (Upstate Biotechnology) in reaction buffer consisting of 20 mM HEPES/NaOH (pH 7.5), 0.03% Triton X-100, 100 mM $CaCl_2$, and a lipid activator (Upstate Biotechnology) for 10 min at 30°C. Aliquots of the pretreated mixture were used for the TRAP assay.

RNA interference

For our small interfering RNA (siRNA) transfection experiments, we harvested untreated HL60 cells in the exponential growth phase. Separate aliquots of 2 x 10^6 cells were transfected with a double-stranded siRNA, targeting Rictor mRNA, or a control nonsilencing siRNA (purchased from Dharmacon, Lafayette, CO, USA) using the Amaxa Nucleofector electroporation technique (Amaxa, Gaithersburg, MD, USA), according to the manufacturer's guidelines. The final concentration of each siRNA was 100 nm/l and the siRNA sequence for targeting Rictor was designed using

siRNA-design software (Dharmacon). Untransfected cells and control siRNA (siCont) transfected cells were used as negative controls. One hour after transfection with siRNA, 5 nM of VD3 or solvent alone was added to the cell cultures. After three days of culture, the cells were harvested for Western blot analysis of Rictor, p-Akt and P-PKC protein expression.

Immunoprecipitation and immunoblotting

Cells ($2x10^7$) were lysed in RIPA buffer and lysates precleared with protein G-sepharose were incubated with appropriate antibodies for 2 h or overnight and processed as reported before (Kawauchi et al., 2005). For Western blotting, protein samples containing equal amounts of sample buffer were subjected to electrophoresis on a polyacrylamide gel and then were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was incubated with primary antibodies, followed by reaction with horseradish peroxidase-conjugated anti-rabbit IgG. Immunoreactive bands were visualized using the ECL Plus detection reagent according to the manufacturer's protocol (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

Statistical Analysis

The data are expressed as the means ± SD of three or more independent experiments. Statistical analysis was performed using the two-tailed Student's *t* test for paired data. $P < 0.05$ was considered to be statistically significant.

Results

Increased phosphorylation of PKC after differentiation of HL60 cells

We investigated PKC phosphorylation, which is known to be associated with various functions in differentiated cells. As shown in Figure 1, the phosphorylation of PKCα/βII (Thr638/641) and PKCα (Ser657) was increased by exposure to either of the differentiation inducers and p21 expression was slightly increased in the late stage of differentiation.

Increased expression of Raptor and Rictor during differentiation of HL60 cells

Activation of mTOR and AKT which was observed in the late stage of differentiation from our previous experiment and that of PKC in this experiment, made us extend our investigation to mTOR-related signaling proteins. We found that Raptor and Rictor were overexpressed after both monocytic and granulocytic differentiation. Rictor showed a greater degree of overexpression and mTOR-associated Rictor protein showed an increase by day 3 after the induction of differentiation (Figure 2).

Effects of recombinant PKC, and it's inhibitor on telomerase activity

We have observed previously that PKC and AKT differentially regulate telomerase activity during differentiation of leukemic cells(Nakatake et al., 2007; Yamada et al., 2008). To investigate whether PKC activate telomerase in HL60 cells,

we performed a TRAP assay after incubating cell lysates with recombinant PKC. We obtained lysates from HL60 cells after four days of serum starvation. Pretreatment with 100 or 500 ng of recombinant PKC enhanced telomerase activity in the lysates by 1.2-fold and 1.3-fold, respectively (Figure 3A). In addition, cells were starved for 2 days without FBS and then incubated with either DMSO or BIM, a PKC inhibitor, for 5 min, after which the cell extracts were assayed for telomerase activity. Telomerase activity was suppressed after short-term exposure to BIM (Figure 3B). BIM suppressed the transcription of *hTERT* in cultured HL60 cells and also suppressed the phosphorylation of PKCα during the differentiation of HL60 cells (Figure 3C,D). These results suggest that activation of telomerase in HL60 cells involves transcriptional or post-translational regulation via a pathway that includes PKC in addition to PI3K/Akt.

Phosphorylation of Akt and PKC during differentiation is related to cell functions

To determine the significance of the activities of Akt and PKCα during cell differentiation, we examined the effects of the PI3K inhibitor, LY294002, and the PKC inhibitor, BIM, on cell maturation and function. Preincubation with LY294002 or BIM did not significantly affect the expression of surface differentiation antigens (Table 1), but it did decrease NBT reduction and esterase enzymatic activity, which are normally

observed in differentiated cells (Figure 4, 5).

Knockdown of Rictor by RNA interference

To elucidate the role of signaling proteins in cell differentiation, we analyzed the relationship between the activation of mTOR signaling and the phosphorylation of Akt or PKC. HL60 cells were treated with Rictor-siRNA or a nonsilencing siRNA control, and were then incubated with VD3 or ATRA for three days, followed by Western blot analysis. In these experiments, 3-day culture instead of 5 days was used to avoid unreliable data derived from increased number of dead cells at longer incubation times as a result of cell damage after Nucreofector transfection. Rictor protein levels were markedly down-regulated within 72 h of transfection with Rictor siRNA (Figure 6). In addition, we observed the down-regulation of p-Akt (Ser473) and p-PKCα/βII in the same samples and a decrease in the number of cells showing NBT-reducing activity. These data suggests that phosphorylation of Akt (Ser473) and PKCα is modulated by Rictor and related to enzymatic activity.

Discussion

In our previous report, when HL60 cells were cultured in the presence of the differentiation inducers, VD3 or ATRA, Akt phosphorylation, which is associated with activation of its kinase activity, gradually increased following a transient decline(Yamada et al., 2008). In this experiment, PKC phosphorylation also increased during the late stage of differentiation. Short-term incubation of HL60 cells with the PKC inhibitor, BIM, suppressed telomerase activity, while pretreatment of starved-cell lysates with recombinant PKC produced a dose-dependent enhancement in telomerase activity. These results suggest that in addition to transcriptional regulation, PKC may play important roles in the post-translational regulation of telomerase activity in HL60 cells. These findings are consistent with previous report that was shown in the case of Akt(Zhang et al., 2006). Based on our data, it is difficult to determine whether the effects of PKC are direct or indirect. However, taken together with reports showing that the catalytic subunits of telomerase have a specific amino acid sequence that is recognized by PKC(Sheng et al., 2003), our results strongly suggest that PKC act as post-translational modulators of telomerase activity in HL60 cells. Akt and PKC activate the transcription of a wide variety of genes, particularly those involved in cell proliferation, including *hTERT*, and genes involved in apoptosis and cell survival

(Blume-Jensen and Hunter, 2001; Nicholson and Anderson, 2002; Vivanco and Sawyers, 2002). Given that epigenetic control of telomerase transcription occurs before the late stage of increased phosphorylation of Akt or PKC in differentiating HL60 cells (Yamada et al., 2008), it is likely that phosphorylation of these signaling proteins affects proteins involved in differentiation but not those involved in telomerase activity.

The expression of mTOR, which is known to be a downstream signaling protein of Akt, was also increased during the late stage of differentiation(Yamada et al., 2008). The mTOR protein exists as two distinct evolutionarily-conserved complexes: mTORC1 and mTORC2(Sparks and Guertin, 2010). The mTORC1 complex contains mTOR associated with the adaptor protein, Raptor (regulatory associated protein of mTOR), and is the target of rapamycin, a potent immunosuppressive compound with anti-cancer properties. In contrast, mTORC2 is not inhibited by rapamycin by virtue of the fact that it forms a complex with the adaptor proteins Rictor (rapamycin-insensitive companion of mTOR) and Sin1 (Guertin and Sabatini, 2007). The activation of mTORC1 stimulates mRNA translation through its downstream substrates, such as S6Ks and 4E-BPs, and also regulates protein synthesis(Beretta et al., 1996; Gingras et al., 1998; Holz et al., 2005; Yamada et al., 2008). The finding of an unexpected increase in the level of phosphorylated S6K with respect to that of

phosphorylated mTOR, suggests the involvement of mTOR pathway in the regulation of HL60 cell differentiation(Yamada et al., 2008). In particular, relative over expression of Rictor than Raptor made us investigate the role of mTORC2 in leukemic cell differentiation.

The upstream regulators of mTORC2 are currently unknown, although mTOR/Rictor mediates the polarization of the actin cytoskeleton and cell spreading (Jacinto et al., 2004; Sarbassov et al., 2004), which suggests that mTORC2 may be crucial for cell function. Relative to the large body of research on mTORC1, our understanding of Rictor and the mTORC2 complex is only just emerging. Rictor has begun to attract attention due to its role in Akt (Ser473) phosphorylation (Sarbassov et al., 2005), while mTORC2 is known to regulate the phosphorylation of the hydrophobic motif in PKCs (Guertin et al., 2006; Jacinto et al., 2004). Using siRNAs and inhibitors of PI3K and PKCα, we have shown that the phosphorylation of Akt (Ser473) and PKCα is modulated by Rictor in differentiated cells. These results show that Rictor is necessary for cell maturation and enzyme activity such as NBT reduction and esterase during leukemic cell differentiation.

Although the exact mechanisms by which the two mTOR complexes are regulated in response to differentiation remain unknown (Huang and Manning, 2008),

our results provide an important first step towards understanding the regulation of mTORC2 and its related proteins that occur during myeloid cell differentiation.

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Conflict of interest: The authors declare no competing financial interest.

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Figure legends

Figure 1. Increased phosphorylation of PKC after differentiation of HL60 cells

HL60 cells were incubated with VD3 or ATRA for the periods of time indicated and expression of signaling proteins was determined by Western blotting with ß-actin as the loading control. Values represent means ± SD of three independent experiments and representative data are shown.

Figure 2. Increased expression of Raptor and Rictor during differentiation of HL60 cells

(A) HL60 cells were incubated with VD3 or ATRA for the periods of time indicated and the expression levels of Raptor, Rictor and mTOR were determined by Western blotting with ß-actin as the loading control. Raptor and Rictor were overexpressed after both monocytic and granulocytic differentiation. Rictor showed a greater degree of overexpression. (B) HL60 cells were incubated with VD3 for the indicated times and cell lysates were immunoprecipitated with antibody directed against mTOR and then were immunoblotted with the antibodies as indicated. mTOR-associated Rictor protein showed an increase by day 3 after the induction of differentiation as indicated by fold change in mTOR-associated Rictor when compared

with that for Raptor (only the data with VD3 is shown). These experiments were repeated with similar results, and representative data are shown.

Figure 3. Effects of recombinant PKC and the PKC inhibitor BIM on telomerase activity

(A) Cells were starved for 4 days and cell extracts were assayed for telomerase activity with or without the addition of recombinant PKC. A representative ladder indicating telomerase activity (upper) and relative telomerase activity (lower) are shown. A significant increase in telomerase activity was observed after the addition of recombinant PKC.

(B) Cells were starved for 2 days and then incubated with either DMSO or 10 μM BIM for 5 min, after which the cell extracts were assayed for telomerase activity. A representative telomerase ladder and relative telomerase activity are displayed.

(C) Cells were incubated with 10 μM BIM for the indicated periods of time, and then expression of *hTERT* and *β-actin* mRNA was measured. *β-actin* is the internal control and relative expression is shown.

(D) Cells were pre-incubated with BIM or DMSO and differentiation was induced with VD3 or ATRA for the indicated periods of time and then expression of signaling

proteins was measured by Western blotting with β -actin as the loading control.

Values represent the means ± SD of three independent experiments and representative data are shown. *, $P < 0.05$.

Figure 4. Effects of the PI3K inhibitor, LY294002, and the PKC inhibitor, BIM, on NBT reduction

HL60 cells were pre-incubated with LY294002, BIM or DMSO (negative control) and then differentiation was induced with VD3 or ATRA for the periods of time indicated. We then performed the NBT reduction assay. The rate of NBT positivity was determined either by measuring NBT reduction to formazan or by inspecting the number of cells visually for NBT-reducing activity (blue cells). Values represent the means ± SD of four separate experiments and representative data are shown. *, $P < 0.05$.

Figure 5. Effects of a PI3K inhibitor and a PKC inhibitor on enzyme activity

HL60 cells were pre-incubated with the concentrations of LY294002, BIM or DMSO indicated and then differentiation was induced with VD3. The number of differentiated monocytes exhibiting esterase enzymatic activity was reduced in the

presence of these inhibitors.

Figure 6. The mTORC2 complex regulates activation of key cellular proteins

To clarify the role of mTORC2 with respect to the regulation of proteins that are involved in differentiation, we measured the expression of VD3 and ATRA-modulated proteins, including p-Akt and p-PKCα, in HL60 cells after siRNA-mediated knockdown of Rictor. Cells were treated with VD3 for 3 days instead of 5 days in the presence or absence of Rictor-siRNA to avoid unreliable data.derived from increased number of dead cells at 5 days as a result of cell damage after Nucreofector transfection. (A) The inhibition of Rictor expression by Rictor siRNA resulted in the down-regulation of Rictor, pAkt and p-PKC protein expression, which suggests that Rictor is required for the activation of these proteins. (B) In accordance with this theory, we observed a decrease in the number of cells with NBT-reducing activity in cells treated with Rictor siRNA. Values represent the means ± SD of four independent experiments and representative data are shown. *, $P < 0.05$.

Only the results of differentiation with VD3 are shown.

Figure 7. Diagram of changes in key signaling proteins and telomerase during

differentiation of HL60 cells

Each differentiation agent caused a significant increase in several signaling proteins (including Akt, mTOR, p70S6K, PKC, Raptor, and Rictor) and a decrease in 4EBP1 three days after the induction of differentiation, as explained in the text. The telomerase protein disappeared before the activation of Akt or PKC-α, which might account for the suppression of telomerase activity in differentiated cells. The kinase activity of mTOR/Rictor is essential for the phosphorylation of both Akt and PKC in differentiated cells, and the presence of active forms of Akt or PKC-α suggests that these proteins have a role in enzyme activity after differentiation.