Published in final edited form as:

Cell Signal. 2008 January ; 20(1): 130–138.

Interaction between TCL1 and Epac1 in the activation of Akt kinases in plasma membranes and nuclei of 8-CPT-2-O-Me-cAMP-stimulated macrophages*

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Abstract

Epac1 is a cAMP-stimulated guanine exchange factor that activates Rap1. The protein product of the *T cell leukemia 1* (TCL1) proto-oncogene binds to Akt enhancing its kinase activity. TCL1 and Epac promote cellular proliferation because of their activating effects on Akt. Employing macrophages, we have studied the mechanisms whereby these proteins function in the regulation of Akt kinase activity. Cells were treated with 8-CPT-2-O-Me-cAMP, a cAMP analog which acts selectively and specifically via Epac1. Epac1 co-immunoprecipitated with TCL1 in plasma membrane and nuclear fractions of 8-CPT-2-O-Me-cAMP-stimulated macrophages. Interaction of TCL1 and Epac1 was also observed in a [¹²⁵I]GST-Epac1 pulldown assay. A two-threefold increase in Akt^{Thr-308} and Akt^{Ser-473} protein kinase activities and their phosphoprotein levels was observed in TCL1 immunoprecipitates of plasma membranes and nuclei of the treated cells. Elevated Akt^{Thr-308} protein kinase activity and its phosphoprotein levels were significantly reduced in TCL1 immunoprecipitates of plasma membranes of 8-CPT-2-O-Me-cAMP treated cells where *Epac1* gene expression was silenced. In contrast, Akt^{Ser-473} protein kinase activity and its phosphoprotein levels were reduced only in plasma membranes. Our studies suggest that a ternary complex of TCL1, Epac1, and Akt forms in activated macrophages both promoting Akt activation and regulating intracellular distribution of Akt.

Keywords

Cyclic AMP generation in macrophages; 8-CPT-2-O-Me-cAMP and cyclic AMP-dependent regulation in macrophages; Akt protein kinase activation; Epac-1 and TCL1 interaction; protein kinases Akt in nuclei and plasma membranes

INTRODUCTION

The *T cell leukemia* (TCL1) proto-oncogene is expressed at specific stages of human lymphocyte maturation. During fetal development, it is also expressed in organs such as liver, kidney, thymus, and gonads [1–4]. In later life, however, TCL1 is overexpressed in various B and T cell lymphomas, including EB virus-infected B cell and AIDS-related lymphomas [5, 6]. Increased expression also occurs in non-lymphoid tumors such as gonadal seminomas and dysgerminomas [1–4]. The product of the *TCL1* gene is a 14 kDa protein that is found both in

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nuclei and associated with plasma membranes [1–4]. Restricted physiological expression of TCL1 suggests that *TCL1* gene expression is tightly regulated. The 5' promoter region of the gene contains a TATA box with cis-regulatory elements for several promoters including Nur77 and NFκB [7,8]. Nur77 is involved in T cell apoptosis *in vivo* and controls mitochondrial-dependent cell death [9]. Nur77 is a direct target of TCL1 via its Akt-induced phosphorylation [3,7]. Overexpression of TCL1 in human embryonic cells promotes cell survival and proliferation and prevents TNF-induced apoptosis [1,2,4]. Transient expression of TCL1 in cells causes a tenfold increase in serum-induced [³H]thymidine uptake [10]. The Akt-Nur77-TCL1 regulatory loop plays a significant role in enhancement of Akt kinase activity, thus maintaining cellular survival and early development of cells *in vivo*. A potential mechanism by which TCL1 promotes cell survival and oncogenic transformation is by binding to Akt thereby increasing its enzymatic activity [1–4,10,11]. Akt is a central component of the PI 3-kinase signaling pathway which has emerged as a pivotal regulator of many cellular processes including apoptosis, proliferation, and differentiation [12]. Dysregulation of members of the Akt family is associated with cancers. Akt activation in response to growth factors and other extracellular stimuli involves membrane recruitment of Akt triggered by inositol phospholipids binding to its NH₂-terminal plekstrin homology (PH) domain [12]. At the membrane, Akt is activated by a process involving lipid-mediated protein dimerization and phosphorylation of two critical residues which are a Thr (308, 309, or 305) in the kinase domain and a Ser (473, 474, or 472) in the COOH-terminal hydrophobic motif of Akt1, Akt2, or Akt3, respectively [12]. The binding of TCL1 to Akt was shown by co-immunoprecipitation and a yeast two hybrid screen [10,11]. TCL1 binds to the PH domain of all Akt family members and increases the ability of Akt to phosphorylate its substrates both *in vivo* and *in vitro* [1–4]. In addition to augmenting the activation of Akt, TCL1 enhances the translocation of Akt into the nucleus where several of its substrates are located [10,11]. Akt and TCL1 lack nuclear localization signals, but TCL1-Akt complexes may recruit NLS-containing proteins to transport these proteins into the nuclei [1,3,4].

The binding of many hormones and growth factors to cellular receptors triggers activation of adenylyl cyclase thus producing cAMP from ATP [14]. cAMP regulates a number of processes through its downstream effectors which include PKA and guanine nucleotide exchange factors (GEFs) involved in the regulation of Ras-related proteins [15,16]. The effect of cAMP is idiosyncratic depending on cell type. Thus cAMP generation may either inhibit or stimulate cell proliferation in a PKA-dependent or independent manner [15,16]. Stimulation of cell proliferative effects by cAMP in a PKA-independent manner often requires activation of Rap1 via Epac [15,16]. Here the cell proliferative effects of cAMP are mediated by the activation of the PI 3-kinase/Akt signaling pathway as demonstrated by the lack of an effect by PKA inhibitors, in contrast to an inhibitory effect observed with PI 3-kinase inhibitors [19–22]. An increase in intracellular cAMP activates Akt1 by phosphorylating it on both Thr³⁰⁸ and Ser⁴⁷³ [25,24]. By serving as a cAMP binding protein with intrinsic GEF activity, Epac couples cAMP production to the activation of Rap1 [23,24]. Epac is a multidomain protein which exists in two forms, Epac1 and Epac2, which are GEFs for Rap1 and Rap2, respectively [23–24]. Both proteins contain a COOH-terminal catalytic region responsible for nucleotide exchange, and an NH₂-terminal inhibitory regulatory region which consists of a DEP (Disheveled, Egl, plekstrin) domain responsible for its membrane attachment and one cAMP domain [23–26]. Epac2 contains an additional cAMP binding site [23–26]. In a gene expression profile analysis, Epac was identified as a transcript expressed at higher levels in chronic lymphocytic leukemia of B cell origin compared to the normal B cell population. Activation of Epac in these leukemias is associated with decreased apoptotic activity.

In a recent report we showed that forskolin-induced cell growth and proliferation of murine peritoneal macrophages requires Epac1-Rap1 signaling [22]. Treatment of macrophages with forskolin or the cAMP analog 8-CPT-2-O-Me-cAMP causes a significant increase in plasma

membrane and nuclear protein kinase Akt^{Thr-308} and Akt^{Ser-473} activities [22]. This increase is PKA-independent, but PI 3-kinase dependent. The activated protein kinase Akt is associated with Epac1 [22]. Since both Epac1 and TCL1 have similar intracellular distribution, promote cellular proliferation, and activate the protein kinase Akt in a PI 3-kinase dependent manner, one could hypothesize that Epac1 and TCL1 may interact synergistically to promote Akt activation and cellular proliferation. We have tested this possibility by examining their interaction in 8-CPT-2-O-Me-cAMP-stimulated macrophages by their co-immunoprecipitation, a GST pulldown assay, silencing the *Epac1* gene expression by RNA interference, and analysis of Akt1 kinase activities in TCL1 immunoprecipitates of plasma membrane and nuclei of macrophages stimulated with 8-CPT-2-O-Me-cAMP.

EXPERIMENTAL/MATERIALS AND METHODS

Materials

Culture media were purchased from Invitrogen (Carlsbad, CA). 8-CPT-2-O-Me-cAMP was purchased from AXXORA, LLC (San Diego, CA). Antibodies against Akt1, phosphorylated Akt1 at Thr³⁰⁸ or Ser⁴⁷³, and TCL1 were purchased from Cell Signaling Technology (Beverly, MA). [³³P]γ-ATP (specific activity 3000 Ci/nmol) and ¹²⁵I-Bolton Hunter reagent (specific activity 2200 Ci/nmol) were from Perkin Elmer Life Sciences (Waltham, MA). GST-Epac1 protein was purchased from ProteinXLab (San Diego, CA). TCL1 protein was purchased from Genway (San Diego, CA). Alexa Fluor[®] 488 goat anti-rabbit IgG and Alexa Fluor[®] 568 goat anti-mouse IgG, were purchased from Molecular Probes (Invitrogen). Mab 414 antibodies were purchased from Covance (Berkeley, CA). Formalin (10% neutral buffered) was purchased from Sigma-Aldrich (St. Louis, MO). Goat serum was purchased from Biomedica (Foster City, CA). Peptide substrates for Akt1^{Ser-473} kinase, NH₂-RRPHFPQFSYSA-COOH, and for Akt1^{Thr-308} kinase, NH₂-KTFCGTPEYLAPEVRR-COOH, were synthesized by Genemed (San Francisco, CA). The control substrate peptide Zak3tide was purchased from Upstate (Temecula, CA). Anti-Epac1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents of highest grade available were procured locally.

Cell culture to obtain intact macrophages

The use of mice for these studies was approved by the Institutional Animal Use Committee in accordance with relevant NIH regulations. Thioglycollate-elicited peritoneal macrophages were obtained by lavage with Hanks' balanced salt solution containing 10 mM HEPES (pH 7.4) and 3.5 mM NaHCO₃ (HHBSS) from pathogen-free 6 week old C57BL/6 mice, obtained from Charles River (Raleigh, NC). The cells were washed with HHBSS and suspended in RPMI 1640 medium containing 2 mM glutamine, penicillin (12.5 units/ml), streptomycin (6 µg/ml) and 5% fetal bovine serum, placed in 6-well plates (3×10⁶ cells/well) and incubated for 2 h at 37°C in a humidified CO₂ (5%) incubator. The monolayers were washed with HHBSS three times to remove non-adherent cells [22], which were then incubated overnight in the above RPMI medium before study.

Isolation of murine peritoneal macrophages stimulated with 8-CPT-2-O-Me-cAMP

Peritoneal macrophages, which had adhered for 2 h (4×10⁶ cells/well in 6 well plates) in quadruplicate wells, were incubated overnight in RPMI 1640 medium. The monolayers were washed twice with HHBSS, a volume of RPMI medium added, and cells incubated for 5 min at 37°C for temperature equilibrium. In separate wells, cells were stimulated with buffer alone, or 8-CPT-2-O-Me-cAMP (200 µM/30 min). The reactions were terminated by aspirating the medium. At this stage, the stimulated cells were divided into two equal aliquots one of which was used for the isolation of nuclei and the other for plasma membranes.

Isolation of nuclei

The nuclear fraction from one aliquot of the stimulated cells was isolated as described previously [27–30]. Briefly, a volume of chilled homogenizing buffer containing 10 mM Tris•HCl (pH 7.5), 10 mM NaCl, 1 mM PMSF, 10 μ M benzamidine, and leupeptin (20 μ g/ml) was added to the cells on ice. After 10 min, the cells were scraped into clean tubes, and homogenized in a Dounce homogenizer with 15 up-down strokes over ice. The homogenates were transferred into clean tubes and centrifuged at $800 \times g$ for 10 min at 4°C. The pellet was suspended in HHBSS and layered over a 200 μ l cushion of 50% sucrose in HHBSS and centrifuged at 14 000 rpm in an Eppendorf microcentrifuge for 3 min at 4°C. The nuclear pellet was collected and lysed in a volume of lysis buffer containing 50 mM Tris•HCl (pH 7.5), 120 mM NaCl, 1% NP40, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and 10 μ g/ml leupeptin. The protein content of nuclear lysates was determined by the Bradford method [33]. Purity of the isolated nuclear fractions was evaluated by electron microscopy and enzyme assays as previously described [27–30] and showed that nuclear preparations demonstrated no more than 3–7% cross contamination with other subcellular fractions.

Isolation of the plasma membrane fraction

The other aliquot of stimulated cells was used for the isolation of plasma membranes as described previously [27–30]. Briefly, a volume of the above homogenizing buffer was added to the monolayers and the plates left on ice for 15 min. The cells were then scraped into clean tubes and homogenized in a Dounce homogenizer with 30 up-down strokes over ice. The homogenates were transferred into clean tubes and centrifuged at $800 \times g$ for 5 min at 4°C. The supernatant was carefully removed and layered onto a sucrose step gradient of 50 and 30% (3 ml each), and centrifuged at $200\,000 \times g$ for 75 min in a Beckman Coulter ultracentrifuge (model Optima LE-80K) at 4°C. The supernatant was carefully removed into a clean tube and saved for cytosol experiments. The enriched plasma membrane fraction at the interphase between the sucrose layers was removed and suspended in a volume of incubation buffer containing 25 mM HEPES (pH 7.4), 10 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 2 μ M leupeptin, 1 mM PMSF and 1 mM Ca²⁺. The purity of plasma membrane-enriched fraction as assessed by electron microscopy and marker enzyme assays [27–30] showed no more than 5–7% cross contamination with other subcellular fractions. The plasma membrane pellet was solubilized in a volume of lysis buffer containing 50 mM Tris•HCl (pH 7.5), 120 mM NaCl, 1% NP40, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and 10 μ g/ml leupeptin. The protein content of plasma membrane and cytosol lysates was determined by the Bradford method [31].

Assay for Akt^{Thr-308} and Akt^{Ser-473} protein kinase activities in the TCL1 immunoprecipitates of the plasma membrane and nuclear fractions of cells stimulated with 8-CPT-2-O-Me-cAMP

The Akt^{Thr-308} and Akt^{Ser-473} protein kinase activities in plasma membrane and nuclear fraction lysates were determined as described earlier [22,32]. Briefly, equal amounts of plasma membrane and nuclear fractions lysate protein (60 μ g each) from each group was immunoprecipitated with TCL1 antibodies (1:50) at 4°C overnight with gentle rotation. TCL1 immunoprecipitates were washed first with: lysis buffer supplemented with 0.5 M NaCl, then with lysis buffer, 50 mM Tris•HCl (pH 7.4), and finally with 50 mM Tris•HCl supplemented with 1 mM dithiothreitol, 1 mM PMSF, and 1 mM benzamidine by centrifugation at $800 \times g$ for 5 min at 4°C. To each immunoprecipitate, 40 μ l of cold kinase buffer containing 50 mM Tris•HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, and 20 μ g/ml leupeptin was added followed by the addition of 30 μ M Akt^{Thr-308} kinase substrate peptide (NH₂-KTFCGTPEYLAPEVRR-COOH or Akt^{Ser-473} kinase substrate peptide (NH₂-RRPHFPQFSYSA-COOH) in the respective tubes. The reaction was initiated by adding 50

μM of ATP and 2 μCi of $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ in each tube, and incubated for 30 min at 30°C with shaking. The reaction was stopped by adding 5 μl of 0.5M EDTA to each tube, centrifuged at 3 000 rpm for 3 min, 40 μl of each supernatant applied on p81 phosphocellulose paper (Whatman, NJ), allowed to dry, and the papers were washed four times, each time immersing them in a liter of 1N phosphoric acid for 3 min. The papers were rinsed with acetone and their radioactivity was counted in a liquid scintillation counter. In preliminary experiments the activity of these Akt kinases was also determined on a control peptide, (Zak3peptide) $\text{NH}_2\text{-GGEEEEYFELVKKKK-COOH}$ under identical conditions. The protein kinase activity of $\text{Akt}^{\text{Thr-308}}$ and $\text{Akt}^{\text{Ser-473}}$ (pmol $[\text{P}]$ $\gamma\text{-ATP}$ incorporated into substrate/mg protein) towards the control peptide were always about 60% of the buffer control. Hence, in later experiments the activities of Akt kinases towards Zak3peptide were not determined and are not being shown.

Determination of Epac1 and TCL1 association and activation of Akt in plasma membrane and nuclear fractions of cells stimulated with 8-CPT-2-O-Me-cAMP by co-immunoprecipitation and Western blotting

In separate experiments, equal amounts of plasma membrane and nuclear fractions lysate protein from macrophages stimulated with buffer or 8-CPT-2-O-Me-cAMP were immunoprecipitated with either anti-Epac1 or anti-TCL1 antibodies (1:50) at 4°C overnight with gentle rotation. The respective immunoprecipitates were washed by centrifugation (thrice at $2\,200 \times g$ for 5 min) with lysis buffer at 4°C . A volume of 4 x sample buffer was added to the respective immunoprecipitates and samples boiled for 5 min and centrifuged at 3 000 rpm for 3 min. Equal amounts of sample proteins were resolved by SDS-PAGE, protein bands transferred to Hybond-P[®] membrane, and immunoblotted with antibodies against Epac1, TCL1, phosphorylated Akt as p-Akt^{Thr-308} or p-Akt^{Ser-473}, respectively. The protein bands on membranes were visualized by ECF (Amersham Biosciences) on a Storm 800 Phosphorimager (Amersham Biosciences) and quantified using ImageQuant 5.2 software.

Silencing Epac1 gene expression in murine macrophages by RNAi

The chemical synthesis of dsRNA homologous in sequence to the target Epac1 peptide sequence ⁶⁴⁷EHLRDVT⁶⁵³ mRNA sequence 5'-AGG AGC ACC TGC GGG ATG TCA - 3' (Swiss PROT EPAC1 primary sequence accession number (Q8VVC8) was performed by Ambion (Austin, TX). For making dsRNA of the sense 5'-AGG AGC ACC UGC GGG AUG UCA - 3' and antisense 5'-UGA CAU CCC GCA GGU GCU CCU - 3' strands, oligonucleotides were annealed according to the manufacturer's instructions. Throughout the studies, handling of reagents was performed in an RNase-free environment. Briefly, equal amount of sense and antisense oligonucleotides were mixed in an annealing buffer and heated at 90°C for 1 min then for 1 h at 37°C in an incubator. The dsRNA preparation was stored at -20°C before use. Macrophages (1.5×10^6 cells/well in 6 well plates) incubated overnight in hexuplicate were washed twice with HHBSS, 2 ml of DMEM containing 10% FBS was added, and monolayers incubated for 16 h and transfected with Epac1 dsRNA by a previously reported protocol [27]. At the end of the incubation, the cells were stimulated either with buffer or 8-CPT-2-O-Me-cAMP (200 μM) and cells incubated as above for 30 min. The reaction was stopped by aspirating the medium. To demonstrate that the transfection of macrophages with dsRNA homologous in sequence to the target Epac1 gene does not produce any non-specific effects on target gene expression, the cells were transfected with equimolar concentrations of scrambled small interfering RNA (Silencer negative control, catalog number 4 610, Ambion) under conditions identical to those described above. These cells were used for the following experiments: (1) measurement of TCL1 protein levels by Western blotting, (2) assay of $\text{Akt}^{\text{Thr-308}}$ and $\text{Akt}^{\text{Ser-473}}$ protein kinase activities associated with immunoprecipitates from plasma membrane and nuclear fractions, and (3) assessment of p-Akt^{Thr-308} and p-Akt^{Ser-473} levels in TCL1 and Epac1 immunoprecipitates by Western blotting.

[¹²⁵I]GST-Epac1 pulldown determination of Epac1 and TCL1 association in cells stimulated with 8-CPT-2-O-Me-cAMP

GST-Epac1 protein (50 µg) was iodinated with [¹²⁵I]NaI by the Bolton-Hunter method [33]. [¹²⁵I]GST-Epac1 was mixed with washed glutathione-Sepharose-4B beads and incubated at 4°C overnight with gentle rotation. The beads were washed with chilled PBS. Aliquots of [¹²⁵I]GST-Epac1 beads were incubated with cell lysates of macrophages stimulated with buffer or 8-CPT-2-O-Me-cAMP (200 µM/30 min) or with purified TCL1 (15 µg each) overnight at 4°C with gentle rotation. The incubations were terminated by washing the beads with chilled PBS four times by centrifugation at 4°C. A volume of 4 x sample buffer was added to the beads, the beads boiled for 5 min, and then centrifuged. The supernatants were processed for gel electrophoresis and transfer of protein bands from gels to PVDF membranes as described above. The respective membranes were autoradiographed for Epac1 and Western blotted for Epac1 and TCL1 proteins as described in the preceding sections.

Determinations of subcellular localization of Epac1 and TCL1 in macrophages stimulated with 8-CPT-2-O-Me-cAMP by confocal microscopy

Thioglycollate-elicited peritoneal macrophages ($2-3 \times 10^5$) in RPMI 1640 medium containing additions as above were pipetted onto glass coverslips in 35 mm dishes and allowed to adhere for 2 h in a humidified CO₂ (5%) incubator at 37°C. Non-adherent cells were aspirated and monolayers washed with cold HHBSS twice, a volume of above RPMI medium was added to cells incubated overnight as above. Macrophages incubated overnight were washed once with the above medium, fresh medium added to cells, and the respective monolayers incubated with either buffer or 8-CPT-2-O-Me-cAMP (200 µM/30 min) as above. The reaction was terminated by aspirating the medium and monolayers washed thrice with chilled PBS. The cells were fixed with formaldehyde (4%) in PBS containing 0.1% Triton X-100 (PBST) for 25 min at 37°C. The fixed cells on coverslips were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature and washed with chilled PBST thrice. The permeabilized cells were incubated with 0.1% BSA in PBST for blocking non-specific binding for 2 h at room temperature with rotation. The incubations were terminated by aspirating the medium and incubating the cells with rabbit polyclonal Epac1 antibodies (1:100) and mouse Mab 414 antibodies (1:100) either singly or together in 0.1% goat serum overnight at 4°C with rotation. The incubations were terminated by washing the cells with chilled PBST thrice and incubated with Alexa Fluor 488 goat anti-rabbit IgG (green) (H+L) (1:100) and Alexa Fluor 568 goat anti-mouse IgG (red) (H+L) (1:100) either singly or together for 2 h at room temperature. The cells were washed thrice with chilled PBST (10 min each time) with rotation and coverslips mounted on glass slides and sealed with nail polish for confocal microscopy. In identical experiments 8-CPT-2-O-Me-cAMP treated, fixed, and permeabilized cells were incubated with rabbit polyclonal TCL1 antibodies (1:100) and mouse Mab 414 antibodies (1:100) and processed for confocal microscopy as described above. For the controls, buffer treated, fixed, and permeabilized cells were incubated with rabbit non-immune antibodies and mouse monoclonal Mab 414 as above and cells processed for confocal microscopy. Samples were analyzed on a Zeiss LSM 410 confocal microscope. Images were then processed with Adobe Photoshop CS2.

RESULTS

TCL1 co-immunoprecipitates with Epac1 in plasma membrane and nuclear fractions of 8-CPT-2-O-Me-cAMP-stimulated macrophages

To identify whether TCL1 and Epac1 form a complex in the intracellular milieu, we studied plasma membrane and nuclear fractions of 8-CPT-2-O-Me-cAMP-stimulated macrophages by co-immunoprecipitation experiments. Immunoblot analysis indicates a significant presence of both Epac1 and TCL1 in TCL1 immunoprecipitates of the plasma membrane and nuclear

fractions of 8-CPT-2-O-Me-cAMP-stimulated cells (Fig. 1A). Likewise, a significant presence of TCL1 is observed in Epac1 immunoprecipitates of plasma membrane and nuclear fraction of these cells¹. Treatment of the cells with the cyclic AMP analog also caused an increase in the total cellular levels of TCL1 and Epac1 (Figure 1B,C). Next we examined Epac1 and TCL1 interaction in cells stimulated with 8-CPT-2-O-Me-cAMP by a [¹²⁵I]GST-Epac1 pulldown assay (Figure 2). These studies employing [¹²⁵I]GST-Epac1-glutathione-Sepharose B also demonstrate complex formation of Epac and TCL1. Further demonstrating complex formation, purified TCL1 binds to the [¹²⁵I]GST-Epac1-glutathione-Sepharose 4B beads (Figure 2).

Activation of Akt protein kinases in TCL1 immunoprecipitates of plasma membrane and nuclear lysates of 8-CPT-2-O-Me-cAMP-stimulated macrophages

Binding of intracellular cAMP to Epac1 in stimulated cells triggers activation of Epac1-Rap1 signaling, one component of which is the activation of the protein kinase Akt which is sensitive to inhibition of PI 3-kinase [17–22]. Likewise, TCL1 facilitates activation of the protein kinase Akt consequent to its binding to the PH domain of Akt [1,3,4]. TCL1 also promotes nuclear translocation of activated kinases where a large number of its substrates are located [10,11]. Next we determined protein kinase Akt activities in TCL1 immunoprecipitates of plasma membrane and nuclear lysates from 8-CPT-2-O-Me-cAMP-stimulated cells, to identify whether TCL1-Epac1 interaction promotes activation of the protein kinase Akt (Figure 3). The Akt^{Thr-308} and Akt^{Ser-473} protein kinase activities in TCL1 immunoprecipitates of plasma membrane and nuclear fractions of macrophages treated with 8-CPT-2-O-Me-cAMP were about two-threefold higher than buffer-treated cells (Figure 3). Similar activation of Akt kinases in these subcellular fractions in Epac1 immunoprecipitates of 8-CPT-2-O-Me-cAMP-stimulated macrophages has been observed¹. These observations suggest that in cells treated with 8-CPT-2-O-Me-cAMP, there is a multimeric complex of Epac1-TCL1-Akt which promotes Akt kinase activation.

Transfection of macrophages with Epac1 dsRNA suppresses Akt kinase activities in TCL1 and Epac1 immunoprecipitates of plasma membrane and nuclear fraction lysates of cells treated with 8-CPT-2-O-Me-cAMP

If the formation of a complex of Epac1-TCL1-Akt is required for activation of Akt kinases, then reduced expression of these components would adversely affect the activation of Akt. Indeed this is found to be correct (Table 1). Macrophages were treated with dsEpac1 RNA directed against Epac1 RNA prior to stimulation with 8-CPT-2-O-Me-cAMP. This significantly reduced Akt^{Thr-308} kinase activity in TCL1 immunoprecipitates of both plasma membrane and nuclei compared to cells treated with lipofectamine and 8-CPT-2-O-Me-cAMP or lipofectamine and scrambled dsRNA, respectively. Transfection of cells with dsEpac1 RNA prior to 8-CPT-2-O-Me-cAMP stimulation also caused parallel reductions in the phosphoprotein levels of Akt^{Thr-308} in TCL1 immunoprecipitates of plasma membrane and nuclei compared to cells treated with lipofectamine and 8-CPT-2-O-Me-cAMP or lipofectamine and scrambled dsRNA, respectively as determined by Western blotting (Figure 4). Akt^{Ser-473} kinase activity in the TCL1 immunoprecipitate of plasma membranes was greatly reduced in cells treated with dsEpac1 RNA prior to stimulation with 8-CPT-2-O-Me-cAMP compared to cells treated with lipofectamine and 8-CPT-2-O-Me-cAMP or lipofectamine and scrambled dsRNA, respectively. However, no effect on Akt^{Ser-473} kinase activity or phosphorylation of Akt^{Ser-473} was observed in TCL1 immunoprecipitates of nuclei (Figure 4 and Table 1). Likewise, silencing *Epac1* gene expression greatly attenuated 8-CPT-2-O-Me-

¹This manuscript has been submitted for publication. U.K. Misra, S.J. Kaczowka, and S.V. Pizzo. The cAMP-sensor Epac1 upregulates plasma membrane and nuclear Akt kinase activities in 8-CPT-2-O-Me-cAMP-stimulated macrophages: Effect of *Epac1* gene silencing on Akt protein kinase activation.

cAMP-induced increase in p-Akt^{Thr-308} in Epac1 immunoprecipitates of both plasma membranes and nuclei while p-Akt^{Ser-473} decreased only in plasma membranes (Figure 4B).

Nuclear localization of TCL1 in 8-CPT-2-O-Me-cAMP-stimulated macrophages

In cells where agonists stimulate increased intracellular cAMP and promote cellular proliferation, Epac1 is enriched in plasma membranes and the perinuclear region [14,15,17, 19]. TCL1 also localizes in these regions [1,3,4]. Our studies suggest that TCL1 and Epac1 associate with each other in plasma membranes and nuclei and promote Akt activation and translocation to nuclei in cells treated with 8-CPT-2-O-Me-cAMP (Figures 1,2,3). We next examined the distribution of TCL1 and Epac1 in 8-CPT-2-O-Me-cAMP-treated cells by confocal microscopy (Figure 5). Consistent with our studies, both Epac1 and TCL1 co-localize in the nuclear/perinuclear region of cells stimulated with 8-CPT-2-O-Me-cAMP (Figure 5).

DISCUSSION

TCL1 is a 14 kDa protein which when dysregulated by elevated or continuous expression is implicated in the development of B and T cell leukemia and lymphomas and solid tumors in humans and transgenic mice [1–4]. Binding of TCL1 to Akt causes increased Akt phosphorylation which results in enhanced Akt signaling that is linked to cell survival and cell proliferation [1–4]. TCL1 can promote cellular proliferation by negatively regulating Nur77 consequent to its phosphorylation by Akt [3]. We have observed that forskolin and 8-CPT-2-O-Me-cAMP treatment of macrophages upregulates Epac1 expression. Epac1 co-immunoprecipitates with p-Akt^{Thr-308} and p-Akt^{Ser-473} kinases and concomitantly there is a significant increase in their phosphoprotein levels in Epac1 immunoprecipitates of plasma membrane and nuclear fractions of 8-CPT-2-O-Me-cAMP treated cells (Figure 4B). The present study further shows that treatment of macrophages with 8-CPT-2-O-Me-cAMP also elevates TCL1 protein levels. Epac1 co-immunoprecipitates with TCL1 and enhances Akt^{Thr-308} and Akt^{Ser-473} protein kinase activities in TCL1 immunoprecipitates of plasma membrane and nuclear fractions. Since Epac1 and TCL1 associate with each other, and enhance Akt kinase activities, our data suggest that a TCL1-Akt-Epac1 complex is involved in the activation of Akt kinases as well as their translocation to nuclei in macrophages treated with 8-CPT-2-O-Me-cAMP.

In this communication we show an interaction between Epac1-Rap1 and TCL1 signaling pathways in murine peritoneal macrophages treated with 8-CPT-2-O-Me-cAMP which specifically and selectively function via Epac1. This crosstalk appears to be at the level of Akt kinase activation which is vital for cellular survival and proliferation in numerous physiological and patho-physiological situations. Since both TCL1 and Akt share an ability to transform T cells they may participate in a common tumorigenic pathway. TCL1 could interact with the Akt before, during, and/or after membrane recruitment from the cytoplasm. TCL1 interacts with the Akt PH domain in the face opposite the PtdIns-P-binding pocket augmenting Akt kinase activity. TCL1 may also bind and stabilize Akt at the membrane with TCL1 and PtdIns-P interacting simultaneously on opposite faces of the PH domain. Activation and translocation of Akt to nuclei by TCL1 requires their physical association via the PH domain. Likewise physical association of Epac1 and Akt, possibly via DEP domains of Epac1, is required for Akt activation. We currently do not understand the mechanism by which Epac1, TCL1, and Akt interact and how this interaction promotes Akt activation and its nuclear translocation. Akt, like TCL1 and Epac1, lacks a nuclear localization signal (NLS). However, TCL1:Akt:Epac1 complexes might be able to recruit NLS-containing proteins which would deliver this complex to nuclei.

Macrophages are present throughout the body, functioning in innate immune surveillance and host defense mechanisms directed against pathogens. In response to stimuli, macrophages

undergo a series of processes including chemotaxis, phagocytosis, intracellular microbial killing and release of inflammatory cytokines. The cAMP-Epac1-Rap1 pathway regulates reorganization of the actin cytoskeleton and the resulting morphological changes are important for many cellular processes including cell migration, cell spreading and adhesion. The results presented in the preceding sections suggest that the Epac1-TCL1-Akt signaling pathway is important for macrophage survival and cell proliferation.

CONCLUSION

In this study, we demonstrate several points. First, in 8-CPT-2-O-Me-cAMP-stimulated macrophages both TCL1 and Epac co-immunoprecipitate with each other in both plasma membrane and nuclear fractions. Second, employing a GST-Epac1 pulldown assay, TCL1 derived either from cells or by addition of purified TCL1, binds to Epac1. Third, in cells stimulated with this cAMP analog, there is a two- to threefold increase in activation of Akt1. Finally, by confocal microscopy, TCL1 and Epac co-localize in the nuclear/perinuclear region of cells stimulated with 8-CPT-2-O-Me-cAMP.

Acknowledgements

This work was supported by Grant No. HL-24066. We wish to thank Mrs. Marie Thomas for valuable assistance in the preparation of this manuscript.

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Figure 1.

Co-immunoprecipitation of Epac1 with TCL1 in TCL1 immunoprecipitates of plasma membrane and nuclear fractions of cells treated with: (1) buffer and (2) 8-CPT-2-O-Me-cAMP (200 μ M/30 min). **Panel A.** A representative immunoblot of three to four experiments of Epac1 and TCL1 in plasma membrane ($\square$) and nuclear fraction ($\blacksquare$) obtained by precipitation with an anti-TCL1 antibody. The bar diagrams above respective immunoblots show changes in protein levels of Epac1 and TCL1 in arbitrary units $\pm$ SE from three to four individual experiments.

Panel B. An immunoblot of Epac1 and TCL1 in cell lysates of cells treated with: (1) buffer; and (2) 8-CPT-2-O-Me-cAMP. **Panel C.** Specificity of Epac1 and TCL1 antibodies. Immunoblots of non-immune IgG immunoprecipitates of plasma membrane and nuclear fractions of cells treated with (1) buffer; and (2) 8-CPT-2-O-Me-cAMP probes with Epac1 and TCL1 antibodies. This study demonstrates the specificity of the method.

Figure 2.

$[^{125}\text{I}]\text{GST-Epac1}$ pulldown assay showing association of TCL1 with Epac1. $[^{125}\text{I}]\text{GST-Epac1}$ -glutathione-Sepharose 4B beads were incubated with: (1) lysates of buffer-treated cells; (2) lysates of 8-CPT-2-O-Me-cAMP-treated cells; and (3) purified TCL1 protein, respectively.

Panel A. Autoradiograph of $[^{125}\text{I}]\text{GST-Epac1}$ in Epac1 and TCL1 immunoblots, respectively. S in **Panel A** is the $[^{125}\text{I}]\text{GST-Epac1}$ protein standard. **Panel B.** Immunoblots of Epac1 and

TCL1, respectively of protein adsorbed on $[^{125}\text{I}]\text{GST-Epac1}$ -glutathione-Sepharose beads. S in **Panel B** is the TCL1 and $[^{125}\text{I}]\text{GST-Epac1}$ protein standards, respectively. **Panel C.** $[^{125}\text{I}]\text{GST-Epac1}$ in autoradiographs (**Panel A**) and TCL1 and Epac1 immunoblot (**Panel B**),

respectively expressed in arbitrary units ($\times 10^3$) as the mean $\pm$ SE from two experiments performed in duplicate are shown below the respective panel. In the left panel, TCL1, the bars represent: $[^{125}\text{I}]\text{GST-Epac1}$ by autoradiograph (□) and TCL1 protein by immunoblot (■). In the right Panel, the bars are: $[^{125}\text{I}]\text{GST-Epac1}$ by autoradiograph (□) and Epac1 protein by immunoblot (■).

Figure 3.

Upregulation of Akt^{Thr-308} and Akt^{Ser-473} protein kinase activities in the TCL1 immunoprecipitates of plasma membrane and nuclear fractions. **Panel A.** The bars are: (1) Akt^{Thr-308} kinase activity in plasma membranes (pm) of buffer (□) and 8-CPT-2-O-Me-cAMP-stimulated cells (■); (2) Akt^{Ser-473} kinase activities plasma membranes of buffer (□) and 8-CPT-8-O-Me-cAMP-treated cells (■); (3) Akt^{Thr-308} kinase activities in nuclei (nuc) of cells treated with buffer (□) or 8-CPT-2-O-Me-cAMP (■); and (4) Akt^{Ser-473} kinase activities in nuclei of cells treated with buffer (□) and 8-CPT-2-O-Me-cAMP (■). Akt1 kinase activities are expressed as [³³P] γATP incorporated (pmol/mg protein) and are mean ± SE from three experiments. Immunoblots representative of three experiments of p-Akt^{Thr-308} and p-

Akt^{Ser-473} of plasma membranes and nuclei are shown below the bar graph where (1) indicates buffer-treated and (2) 8-CPT-2-O-Me-cAMP-treated. **Panel B.** Specificity of antibodies employed in these studies. Immunoblots of non-immune IgG immunoprecipitates of plasma membrane and nuclear fractions of cells treated with: (1) buffer or (2) 8-CPT-2-O-Me-cAMP probed for p-Akt^{Thr-308} and p-Akt^{Ser-473}.

Figure 4.

Immunoblots showing the changes in TCL1, Epac1, p-Akt^{Thr-308} and p-Akt^{Ser-473} in TCL1 immunoprecipitates (**Panel A**) and Epac1 immunoprecipitates (**Panel B**) of plasma membranes (pm) and nuclei (nuc) of 8-CPT-2-O-Me-cAMP-treated cells after silencing *Epac1* gene expression. The lanes are (1) lipofectamine + buffer; (2) lipofectamine + 8-CPT-2-O-Me-cAMP (200 μ m/30 min); (3) Epac1 dsRNA + 8-CPT-2-O-Me-cAMP; and (4) scrambled dsRNA + 8-CPT-2-O-Me-cAMP. The protein loading control actin is also shown.

Immunoblots shown are representative of two experiments performed in duplicate. The changes in levels of Epac1, TCL1, p-Akt^{Thr-308} and p-Akt^{Ser-473} in TCL1 and Epac1 immunoprecipitates, respectively are shown above the respective immunoblots and are expressed in arbitrary units $\pm$ SE from two experiments performed in duplicate. The bars in the respective panels are: p-Akt^{Thr-308} (□) and p-Akt^{Ser-473} (■).

Figure 5.
Nuclear/perinuclear region localization of Epac1 and TCL1 in cells treated with 8-CPT-2-O-Me-cAMP, buffer, or anti-IgG by confocal microscopy.

Table 1

Effect of silencing the expression of *Epac* gene on Akt kinase activities in the plasma membrane and nuclei of macrophages treated with 8-CPT-2-O-Me-cAMP.

TCL1 immunoprecipitate		
Plasma membrane	p-Akt^{Thr-308}	p-Akt^{Ser-473}
None	6.50 ± 1.17	6.60 ± 1.00
8-CPT-2-O-Me-cAMP	14.35 ± 1.33	10.30 ± 1.36
dsEpac1 + 8-CPT-2-O-Me-cAMP	9.16 ± 0.58*	6.21 ± 0.67*
Scrambled dsRNA + 8-CPT-2-O-Me-cAMP	15.46 ± 1.94	16.58 ± 2.60
Nuclei	p-Akt^{Thr-308}	p-Akt^{Ser-473}
None	4.19 ± 0.81	5.29 ± 0.17
8-CPT-2-O-Me-cAMP	7.21 ± 0.86	9.97 ± 0.68
dsEpac1 + 8-CPT-2-O-Me-cAMP	3.44 ± 0.13*	8.33 ± 0.13
Scrambled dsRNA + 8-CPT-2-O-Me-cAMP	8.61 ± 0.95	10.56 ± 1.56

N=3

Akt kinase activity = [³³P] γ-ATP incorporated (pmol/mg protein)

* = significantly different from lipofectamine + 8-CPT-2-O-Me-cAMP treated cells at 5% level by Students' (t) test.