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Journal of Cellular Biochemistry 113:1488–1500 (2012)

Journal of Cellular Biochemistry

Upregulation of mTORC2 Activation by the Selective Agonist of EPAC, 8-CPT-2Me-cAMP, in Prostate Cancer Cells: Assembly of a Multiprotein Signaling Complex

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ABSTRACT

Ligation of cell surface-associated GRP78 by activated α_2-macroglobulin triggers pro-proliferative cellular responses. In part, this results from activation of adenylyl cyclase leading to an increase in cAMP. We have previously employed the cAMP analog 8-CPT-2Me-cAMP to probe these responses. Here we show in 1-LN prostate cancer cells that 8-CPT-2Me-cAMP causes a dose-dependent increase in Epac1, p-Akt^{T308}, p-Akt^{S473}, but not p-CREB. By contrast, the PKA activator 6-Benz-cAMP caused a dose-dependent increase in p-CREB, but not Epac1. We measured mTORC2-dependent Akt phosphorylation at S473 in immunoprecipitates of mTOR or Rictor from 1-LN cells. 8-CPT-2Me-cAMP caused a two-threefold increase in p-Akt^{S473} and Akt^{S473} kinase activity in Rictor immunoprecipitates. By contrast, there was only a negligible effect on p-Akt^{T308} in Rictor immunoprecipitates. Silencing Rictor gene expression by RNAi significantly suppressed 8-CPT-2Me-cAMP-induced phosphorylation of Akt at Ser^{473}. These studies represent the first report that Epac1 mediates mTORC2-dependent phosphorylation of Akt^{S473}. Pretreatment of these cells with the PI 3-Kinase inhibitor LY294002 significantly suppressed 8-CPT-2Me-cAMP-dependent p-Akt^{S473} and p-Akt^{S473} kinase activities, and both effects were rapamycin insensitive. This treatment caused a two to threefold increase in S6 Kinase and 4EBP1 phosphorylation, indices of mTORC1 activation. Pretreatment of the cells with LY294002 and rapamycin significantly suppressed 8-CPT-2Me-cAMP-induced phosphorylation of S6 Kinase and 4EBP1. We further demonstrate that in 8-CPT-2Me-cAMP-treated cells, Epac1 co-immunoprecipitates with AKAP, Raptor, Rictor, PDE3B, and PDE4D suggesting thereby that during Epac1-induced activation of mTORC1 and mTORC2, Epac1 may have an additional function as a ''scaffold'' protein. J. Cell. Biochem. 113: 1488–1500, 2012.

KEY WORDS: RICTOR; mTORC2; S6 KINASE; 4EBP1; p-AKTS473; Epac1; PROSTATE CANCER CELLS; SIGNALOSOME

cAMP is a critical intracellular second messenger that mediates the action of extracellular signals. It regulates a wide variety of cellular processes including proliferation and apoptosis [Stork, 2003]. cAMP may either inhibit or stimulate cell proliferation in a PKA-dependent or PKA-independent manner [Stork, 2003; Bos, 2006]. In cells where cAMP stimulates cell proliferation in a PKA-independent manner, it activates Rap1 via Epac [Mei et al., 2002; Misra et al., 2002, 2008ab; Qiao et al., 2002; Misra and Pizzo, 2005; Bos, 2006]. Here the effects of cAMP are mediated by PI 3-Kinase/Akt signaling [Mei et al., 2002; Misra et al., 2002, 2008ab; Qiao et al., 2002; Misra and Pizzo, 2005; Bos, 2006]. As a consequence, enhanced DNA and protein synthesis, and cell proliferation occur [Misra and Pizzo, 2009]. Of significance, Rap1 is often elevated in highly metastatic prostate cancer cell lines [Bailey et al., 2009]. Epac, contains a COOH-terminal catalytic domain responsible for nucleotide exchange, an NH_2-terminal regulatory DEP domain responsible for membrane attachment, and either one cAMP binding domain in Epac1 or two in Epac2 [Bos, 2006]. 8-CPT-2Me-cAMP treatment of macrophages enhances Epac1 expression and its activation as well as Akt phosphorylation at T308 and S473 in a PI 3-Kinase dependent, but PKA-independent manner [Misra et al., 2008ab]. Silencing Epac1 expression by RNAi, or pretreatment with PI 3-Kinase or mTOR inhibitors suppresses 8-CPT-2Me-cAMP-induced upregulation of Epac1, Akt activation, and ultimately DNA and protein synthesis [Misra et al., 2008ab; Misra and Pizzo, 2009]. Prostate cancer cells treated with 8-CPT-2Me-cAMP demonstrate Epac1-induced activation of Rap1, activation of ERK1/2, and mTOR signaling pathways [Misra and Pizzo, 2009].

Akt, which exists as three isoforms, is activated in response to multiple extracellular signals acting through Tyr kinases or G-protein coupled receptors to regulate proliferation, and survival [Manning and Cantley, 2007]. It consists of an NH_2-terminal plekstrin homology domain (PH), a catalytic domain, and a COOH-terminal hydrophobic domain (HD) [Alessi et al., 1997]. Akt

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Received 11 August 2011; Accepted 29 November 2011 • DOI 10.1002/jcb.24018 • © 2011 Wiley Periodicals, Inc.
Published online 15 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

activation occurs through the binding of the PH domain to the phosphatidyl-3 kinase (PI 3-Kinase) products PtdIns 3,4,5-P3 (PIP3) and PtdIns 3,4-P2 (PIP2). For Akt1, this requires phosphorylation of T308 in the activation loop by PDK1 and S473 in the COOH terminal HD domain by mTORC2 [Alessi et al., 1997; Hanada et al., 2004; Guertin and Sabatini, 2007; Manning and Cantley, 2007; Alessi et al., 2009]. The tumor suppressor PTEN, a dual lipid/protein phosphatase, is frequently inactivated in human cancers, causing hyperactivation of Akt [Manning and Cantley, 2007; Guertin et al., 2009]. PTEN expression inversely correlate with Akt phosphorylation at S473, but not T308 [Wan and Helman, 2005; Kreisberg et al., 2004].

mTOR is a PI 3-Kinase-related Ser/Thr kinase that integrates signals from many external stimuli [Zoncu et al., 2011], and the mTOR pathways are frequently deregulated in human cancers [Martin and Hall, 2005; Zhou and Huang, 2010]. mTOR exists in two distinct complexes, mTORC1 and mTORC2. mTORC1 in addition to mTOR, contains the regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (MLST8/GβL), proline rich Akt substrate 40 kDa (PRAS40), and DEP-domain containing mTOR interacting protein (Deptor) [Martin and Hall, 2005; Grant, 2008]. Raptor regulates assembly of mTOR, recruitment of kinase substrates, and subcellular localization of mTORC1 [Martin and Hall, 2005; Grant, 2008; Laplante and Sabatini, 2009; Sengupta et al., 2010]. PRAS40 and Deptor in the dephosphorylated state repress mTORC1 activity which is relieved by Akt phosphorylation. Akt also activates mTORC1 by directly phosphorylating TSC1/TSC2 complex thus inhibiting its GAP activity and releasing GTP-bound Rheb to activate mTORC1 [Martin and Hall, 2005; Grant, 2008; Laplante and Sabatini, 2009; Sengupta et al., 2010]. The complex of rapamycin with its intracellular receptor FKBP12 binds directly to mTORC1 and inhibits mTORC1-mediated phosphorylation [Martin and Hall, 2005; Guertin and Sabatini, 2007; Grant, 2008; Alessi et al., 2009; Laplante and Sabatini, 2009; Sengupta et al., 2010]. When activated, mTORC1 phosphorylates two main regulators of mRNA translation and ribosome genesis p70 S6 Kinase (S6 Kinase) and 4EBP1 and thus stimulate protein synthesis. mTORC2 complex in addition to mTOR, contains Raptor-independent companion of mTOR (Rictor), MLST8 (GβL), Deptor, mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor 1 (protor/PRR5). Activated mTORC1 phosphorylates S6 Kinase which then phosphorylates Rictor inhibiting Akt signaling [Martin and Hall, 2005; Guertin and Sabatini, 2007; Manning and Cantley, 2007; Grant, 2008; Laplante and Sabatini, 2009; Sengupta et al., 2010].

In an earlier report we showed that exposure of prostate cancer and melanoma cells to 8-CPT-2Me-cAMP, by activating Epac1. Activation of the mTORC1 signaling cascade follows and this promotes proliferation in a PI 3-Kinase-dependent and rapamycin-sensitive manner [Misra and Pizzo, 2009]. We now have studied the role of Epac1 on Akt^{S473} activation by measuring mTORC2 kinase activities in Rictor immunoprecipitates of these cancer cells. In this report we show that exposure of prostate cancer cell to 8-CPT-2Me-cAMP activates mTORC2 kinase causing an upregulated phosphorylation of Akt1 at S473 in a PI 3-Kinase-sensitive but rapamycin-insensitive manner. Knockdown of Epac1 and Rictor expression by RNAi suppressed the activation of mTORC2. We further show that mTORC1 complexes in Raptor immunoprecipitates, phosphorylates S6 Kinase and 4EBP1, and Epac1 induced activation of mTORC1 and mTORC2 involves the assembly of a multiprotein signaling complex.

EXPERIMENTAL PROCEDURES

MATERIALS

Culture media were purchased from Invitrogen. 8-CPT-2Me-cAMP and N6-Benzoyladenosine-3′5′ cyclic mono-phosphate (6-Benz-cAMP) were purchased from Axxora LLC (San Diego, CA). Antibodies against mTOR, p-Akt^{T308}, p-Akt^{S473}, Akt1, S6 Kinase, p-S6 Kinase, 4EBP1, p-4EBP1, GβL, p-PRAS40, PRAS40, p-$CREB^{S133}$, and CREB were from Cell Signaling Technology (Beverly, MA). Antibodies against Epac1, Raptor, Rictor protor AKAP, PDE3B, and PDE4D were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mSINI antibody was purchased from Bethyl Laboratories, Inc. (Montgomery, TX). Anti-actin antibodies were from Sigma (St. Louis, MO). PHAS-1 was purchased from Stratagene. GST-S6 Kinase construct was generously provided by Prof. J. Blenis (Harvard Medical School, Boston, MA) and GST-S6 Kinase protein was expressed and purified according to the protocol provided by Prof. Blenis. [^{33}P]-γ-ATP (specific activity 3,000 Ci/mmol) was from Perkin-Elmer Life Sciences. Rapamycin and LY294002 were from Biomol (Plymouth, PA).

PROSTATE CANCER CELL LINES

In an earlier report we demonstrated that 8-CPT-2Me-cAMP upregulated mTOR signaling which were comparable in three prostate cancer cell lines, 1-LN, DU-145, and PC-3, and 1 melanoma cell line [Misra and Pizzo, 2009]. Therefore, we have used 1-LN, DU-145, and PC-3 prostate cancer cells to study the effect of 8-CPT-2Me-cAMP on mTOR2 activation. The highly metastatic 1-LN prostate cancer cell line is derived from less metastatic PC-3 cells and was a kind gift of Dr. Philip Walther (Duke University Medical Center, Durham, NC). DU-145 was purchased from ATCC. Cells were grown in 6- or 12-well plates in RPMI1640 medium supplemented with 10% FBS, 2 mM glutamine, 12.5 units/ml penicillin, 6.5 μg/ml streptomycin and 10 nM insulin (RPMI-S) in a humidified CO_2 incubator at 37°C. At about 90% confluency, the medium was aspirated and a fresh volume of RPMI-S medium added and desired cells used for experiments described below.

SPECIFICITY OF 8-CPT-2Me-cAMP AND 6-BENZ-cAMP ACTIVITY ON Epac1 AND p-CREB IN 1-LN PROSTATE CANCER CELLS

1-LN cells were grown as above in RPMI-S medium overnight in 6-well plates. The monolayers (3×10^6 cells/well/6-well plates) were washed with Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4, and 3.5 mM $NaHCO_3$ (HHBSS), and a volume of fresh RPMI-S medium added, cells incubated for 5 min for temperature equilibrium. The cells were exposed to varying concentration of either 8-CPT-2Me-cAMP or 6-Benz-cAMP generally for 30 min, unless otherwise stated. The reaction was terminated by aspirating the medium. A volume of lysis buffer containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 0.1% NP-40, 25 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.1 M sodium orthovanadate, 1 mM PMSF,

1 mM benzamidine and leupeptin (10 μg/ml) (lysis Buffer A) was added and cells lysed for 15 min over ice. Cell lysates were transferred to Eppendorf tubes and centrifuged at 1,000 rpm/5/min at 4°C to remove cell debris. The supernatants were transferred into new tubes and protein contents determined [Bradford, 1976]. To an equal amount of lysate protein a volume of 4× sample buffer was added, tubes boiled for 5 min, centrifuged, and the samples electrophoresed in 10% acrylamide gels. The protein bands on the gel were transferred to Hybond-P® membranes and the membranes immunoblotted with antibodies against either Epac1 or p-CREB. The detection and quantification of immunoblots was performed by ECF employing a Storm 850 Phosphorimager®. The specificity of antibodies used was determined by treating cells with non-immune antibodies and processed as above. Under these conditions no reactivity of the non-specific antibodies was observed. The respective membranes were reprobed for actin or CREB as the protein loading controls. In all subsequent studies similar control studies were performed.

MEASUREMENT OF p-Akt^{T308} AND p-Akt^{Ser473} BY WESTERN BLOTTING IN 1-LN PROSTATE CANCER CELLS TREATED WITH 8-CPT-2Me-cAMP

The effect of treatment with varying concentrations of 8-CPT-2Me-cAMP on p-Akt^{T308} and p-Akt^{S473} levels in 1-LN prostate cancer cells (3×10^6 cells/well/6-well plates) were determined by Western blotting as described above except that the respective blots were immunoblotted with antibodies specific for p-Akt^{T308} and p-Akt^{S473}, respectively. The respective membranes were reprobed for unphosphorylated Akt1 as a protein loading control.

CHARACTERIZATION OF THE COMPONENTS OF mTORC1 AND mTORC2 COMPLEXES IN 1-LN CANCER CELLS TREATED WITH 8-CPT-2Me-cAMP

1-LN cells (3×10^6 cell/well in 6-well plates) incubated overnight in RPMI-S medium were washed twice with cold HHBSS and a volume of RPMI-S medium added to each well. After temperature equilibration, cells in the wells were exposed to either buffer or 8-CPT-2Me-cAMP (100 μM/30 min) and incubated as above. Reactions were stopped by aspirating the medium and a volume of CHAPS lysis buffer (buffer B) containing 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 0.3% CHAPS and a Roche Protease inhibitor cocktail tablet (1 tablet/10 ml) was added and cells lysed over ice for 15 min. The lysates were transferred to separate Eppendorf tubes, centrifuged (1,000 rpm/5 min/4°C) and supernatants used for protein estimation [Bradford, 1976] and immunoprecipitation. Equal amounts of lysate protein (200–250 μg) were immunoprecipitated with Raptor antibodies (1:50, Santa Cruz, cat # sc81537) or, Rictor antibodies (1:50, Santa Cruz, cat # 81538) followed by the addition of 40 μl of Protein A agarose and contents incubated with rotation overnight at 4°C. Raptor and Rictor immunoprecipitates were recovered by centrifugation (2,000 rpm/5 min/4°C) and washed twice with cold lysis buffer B. To Raptor and Rictor immunoprecipitates a volume of 4× sample buffer was added, tubes boiled for 5 min, centrifuged, electrophoresed (4–20%, 12.5% or 10% acrylamide gels), transferred to Hybond-P® membranes and the membranes immunoblotted with antibodies specific for mTOR, Raptor, and Rictor, GβL, mS1N1, and Protor. The protein bands on the membranes were detected by ECF and quantitated in Storm860 Phosphorimager®.

ASSAY OF mTORC1 ACTIVATION OF S6 KINASE IN RAPTOR IMMUNOPRECIPITATE IN PROSTATE CANCER CELLS TREATED WITH 8-CPT-2Me-cAMP

mTORC1 activity in the Raptor immunoprecipitates from prostate cancer cells treated with 8-CPT-2Me-cAMP was assayed as described earlier [Misra and Pizzo, 2009]. Briefly, 1-LN, DU-145, and PC-3 prostate cancer cells (3×10^6 cells/well in 6-well plates) incubated overnight in RPMI-S medium were washed twice with cold HHBSS and a volume of RPMI-S medium to each well. After temperature equilibration, cells respective wells were exposed to either buffer or 8-CPT-2Me-cAMP (100 μM/30 min) and incubated as above. Reactions were stopped by aspirating the medium and a volume of CHAPS lysis buffer (buffer B) and cells lysed over ice for 15 min. The lysates were transferred to separate Eppendorf tubes, centrifuged (1,000 rpm/5 min/4°C) and supernatants used for protein estimation [Bradford, 1976] and immunoprecipitation. Equal amounts of lysate protein (200–250 μg) were immunoprecipitated with Raptor antibodies (1:50) as above followed by the addition of 40 μl of Protein A-agarose and contents incubated with rotation overnight at 4°C. In experiments where Raptor immunoprecipitates were used for assaying S6 Kinase phosphorylation, the immunoprecipitates was recovered by centrifugation (2,500 rpm/5 min/4°C) and first washed once with lysis buffer containing 10 mM Tris–HCl pH 7.2, 0.5% sodium deoxycholate, 0.1% NP-40, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 2 mM DTT, 10 μg/ml leupeptin, and 5 μg pepstatin followed by washing with above lysis buffer without NP-40 and containing 1 M NaCl and then with buffer containing 50 mM Tris–HCl pH 7.2, 5 mM Tris base and 100 mM NaCl [Misra and Pizzo, 2009]. Immunoprecipitates after each wash were recovered by centrifugation at 2,500 rpm for 5 min at 4°C. To the respective immunoprecipitates, 40 μl of reaction buffer containing 20 mM HEPES pH 7.2, 10 mM $MgCl_2$, 50 μM ATP, and 3 μg substrate peptide added. The reaction was started by adding 5 μCi of [^{33}P]γ-ATP. The contents were incubated for 15 min, centrifuged, electrophoresed in 12.5% gel, transferred to membrane and membrane autoradiographed. Phosphorylated S6 Kinase peptide bands were visualized and quantified as above. To examine the sensitivity of mTORC1 kinase towards rapamycin and the PI 3-Kinase inhibitor LY294002 cells were pretreated with rapamycin (100 nM/20 min) or LY294002 (20 μM/20 min) before adding 8-CPT-2Me-cAMP (100 μM/30 min) and cell lysates processed as above. Other details for autoradiographic visualization of S6 Kinase were identical to those described above [Misra and Pizzo, 2009].

ASSAY OF mTORC1-DEPENDENT 4EBP1 PHOSPHORYLATION IN RAPTOR IMMUNOPRECIPITATES

1-LN, DU145, and PC-3 prostate cancer cells were grown and processed as above. The lysates (200–250 μg protein) were immunoprecipitated with anti-Raptor antibodies (1:50) by the addition of 40 μl of protein G agarose slurry and the contents incubated with rotation overnight at 4°C. The supernatant was

aspirated and immunoprecipitates washed twice with lysis buffer B at 2,500 rpm for 5 min at 4°C and immunoprecipitates resuspended in 40 μl of kinase buffer containing 10 mM HEPES pH 7.4, 50 mM NaCl, 50 mM β-glycerophosphate, 0.1 mM EDTA, 1 mM DTT, 20 mM $MnCl_2$, 200 μM ATP, and 4 μg PHAS. The reaction was initiated by adding 5 μCi of [^{33}P]-γ-ATP, tubes incubated in a shaking water buffer, tubes boiled for 5 min, centrifuged and supernatant electrophoresed on 12.5% acrylamide gels. The proteins were transferred onto PDVF membranes which were autoradiographed. Phosphorylated peptide bands were visualized and quantified as above. To examine the sensitivity of mTORC1 kinase towards rapamycin and the PI 3-Kinase inhibitor LY294002 the cells were pretreated with either rapamycin (100 nM/20 min) or LY294002 (20 μM/20 min) before adding 8-CPT-2Me-cAMP (10 μM/30 min) and cell lysates processed as above. Other details for autoradiographic visualization of 4EBP1 were identical to those described above [Misra and Pizzo, 2009].

Akt^{S473} PHOSPHORYLATION IN RICTOR IMMUNOPRECIPITATES OF PROSTATE CANCER CELLS TREATED WITH 8-CPT-2Me-cAMP

1-LN, DU-145, and PC-3 prostate cancer cells were grown as above. Equal amount of lysate proteins (200–250 μg) were immunoprecipitated with anti-Rictor antibodies (1:50) by the addition of 40 μl of protein A agarose slurry and contents incubated with rotation overnight at 4°C. Rictor immunoprecipitates were washed with: (1) lysis buffer B supplemented with 0.5 M NaCl, (2) lysis buffer B, and (3) Tris–HCl (pH 7.4) supplemented with 1 mM DTT, 1 mM PMSF, and 1 mM benzamidine by centrifugation at 2,500 rpm for 5 min at 4°C. To each immunoprecipitate, 40 μl of kinase buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 20 μg/ml leupeptin was added followed by the addition of 30 μM of AKT^{S473} kinase substrate (NH_2-RRPHFPQFSYSA-COOH) to the respective tubes. The peptide (NH_2-GGEEEEYFELVKKKKCOOH (Zak3tide)) served as the control. The reaction was initiated by adding 50 μM ATP and 5 μCi of [^{33}P]-γ-ATP in each tube and the tubes were incubated for 30 min at 30° in a shaking water bath. The reaction was stopped by the addition of 5 μl of 0.5 M EDTA to each tube, the tubes centrifuged at 3,000 rpm for 3 min, 40 μl of each supernatant applied on p81 phosphocellulose paper (Whatman, NJ), allowed to dry and papers washed four times each time by immersing them in a liter of 1 N phosphoric acid for 3 min. The papers were rinsed with acetone and their radioactivity was counted in a liquid scintillation counter [Misra and Pizzo, 2005; Misra et al., 2006, 2008ab]. In preliminary experiments, the kinase activities of Akt^{T308} and Akt^{S473} (pmol [^{33}P]-γ-ATP incorporated into substrate/mg protein), towards the control peptide were always 50–60% of buffer control. Hence, control peptide activities are not being shown [Misra and Pizzo, 2005; Misra et al., 2006, 2008a].

MEASUREMENT OF Akt^{T308} PHOSPHORYLATION IN RICTOR IMMUNOPRECIPITATES OF CELLS TREATED WITH 8-CPT-2Me-cAMP

The details of measuring Akt^{T308} phosphorylation in Rictor immunoprecipitates of 1-LN cells treated with 8-CPT-2Me-cAMP were identical to those described above for Akt^{S473} phosphorylation except that the Akt^{T308} kinase substrate peptide (NH_2-KTFCGTPEY-LAPEVRR−COOH) was employed [Misra and Pizzo, 2005; Misra et al., 2006, 2008a].

THE EFFECTS OF SILENCING Epac1 GENE EXPRESSION ON PHOSPHORYLATION OF Akt^{S473} AND Akt^{T308} IN RICTOR IMMUNOPRECIPITATES OF CANCER CELLS STIMULATED WITH 8-CPT-2Me-cAMP

To understand the role of Epac1 in activation of mTORC2 in prostate cancer cells treated with 8-CPT-2Me-cAMP, the expression of Epac1 gene silencing by RNAi was performed [Misra and Pizzo, 2005, 2009; Misra et al., 2008ab]. The chemical synthesis of dsRNA homologous in sequence to the target Epac1 peptide sequence 204VAHLSN209 mRNA sequence 5′-TGT GGC CCA CCT CTC CAA CTC-3′ (Swiss Prot Epac1 primary accession number 095398) was performed by Ambion (Austin, TX). dsRNAs were prepared by annealing the sense 5′-UGG CCC ACC UCU CCA ACU CU-3′ and antisense 5′-GAG UUG GAG AGG UGG GCA ACA-3′ and annealed dsRNAs purified by Ambion. Silencing Epac1 gene expression prior to stimulation with 8-CPT-2Me-cAMP was done by transfection of cancer cells with 100 nM (48 h) of Epac1 dsRNA as described previously [Misra and Pizzo, 2009]. Control cells were transfected with equimolar concentration of scrambled RNA (Ambion Catalogue number 4610) as above. The magnitude of silencing Epac1 gene expression in transfected cells, as measured by Epac1 mRNA and Epac1 protein levels under our experimental conditions ranged between 60% and 65% [Misra and Pizzo, 2005, 2009; Misra et al., 2008ab]. The respective cells in 6-well plates were treated as: (i) lipofectamine + buffer, (ii) lipofectamine + 8-CPT-2Me-cAMP (100 μM/30 min), (iii) Epac1 dsRNA (100 nM/48 h) + 8-CPT-2Me-cAMP (100 μM/30 min), and (iv) scrambled dsRNA (100 nM/48 h) + 8-CPT-2Me-cAMP (100 μM/30 min) and incubated as described in the preceding sections. The reactions were terminated by aspirating the medium, a volume of Chaps lysis buffer B added, cells lysed over ice for 15 min, cell lysates transferred to Eppendorfs tubes, centrifuged (1,000 rpm/5 min/4°C), supernatants transferred to new tubes and their protein determined. Equal amounts of lysate proteins were used for immunoprecipitation with Rictor antibodies as described in the preceding section. The Rictor immunoprecipitates were washed and used for measuring their activity towards Akt^{S473} phosphorylation and Akt^{T308} as described in the preceding section. Remaining portions of cell lysates were used for determining the protein levels of p-Akt^{T308}, and p-Akt^{S473}, by Western blotting.

Akt^{S473} PHOSPHORYLATION IN RICTOR IMMUNOPRECIPITATES FROM 1-LN CELLS TRANSFECTED WITH RICTOR dsRNA

To confirm the role of mTORC2 in 8-CPT-2Me-cAMP-induced phosphorylation of Akt at S473, we silenced Rictor gene expression by RNAi. This treatment would disrupt the assembly of mTORC2 complexes and thus suppressed mTORC2 activation. The siRNA probe was purchased from Ambion (small-interfering RNA ID S226002) of the sense sequence (5′ → 3′) GGG UUA GUU UAC AAU CAG Ctt and antisense (5′ → 3′) GCU GAU UGU AAA CUA ACCtt. Cells were transfected with 100 nM of annealed Rictor dsRNA and

control cells were transfected with lipofectamine as described previously [Misra et al., 2008a]. Forty-eight hours after transfection, the control cells were stimulated with either buffer or 8-CPT-2Me-cAMP (150 μM/30 min/37°C). Cells for the negative controls were transfected with scrambled dsRNA (100 nM; 48 h, Ambion) and then stimulated with either buffer or 8-CPT-2Me-cAMP as above. The reactions were terminated by aspirating the medium and cells were lysed in a volume of CHAPS lysis buffer as described above. Protein contents of the respective lysates were determined and equal amounts of lysate proteins employed for immunoprecipitating Rictor and Rictor antibodies as described above. Rictor immunopreciptates were used for measuring phosphorylation of Akt at S473 as described above. Equal amounts of lysate proteins were also employed for determining levels of Rictor and p-Akt^{S473} by Western blotting as described above.

Akt^{S473} PHOSPHORYLATION IN mTOR IMMUNOPRECIPITATES FROM 1-LN

1-LN prostate cancer cells were grown as above. Equal amounts of lysate protein (200–250 μg) were immunoprecipitated with anti-mTOR antibodies (1:50) followed by the addition of 40 μl of protein A-agarose slurry and the contents incubated with rotation overnight at 4°C. The respective immunoprecipitates were washed and used for assay of Akt^{T308} and Akt^{S473} phosphorylation as described above.

MODULATION OF PROTEIN COMPONENTS OF mTORC1 AND mTORC2 COMPLEXES IN PROSTATE CANCER CELLS TREATED WITH 8-CPT-2Me-cAMP

mTOR nucleates two distinct multiprotein complexes mTORC1 and mTORC2 which are rapamycin-sensitive and rapamycin-insensitive, respectively. These two complexes participate in different pathways and recognize distinct substrates the specificity of which is determined by mTOR interacting proteins [Guertin et al., 2006]. In view of the importance of the presence of components for optimal mTORC1 and mTORC2 activation, in the next series of experiments we measured levels of Raptor, Rictor, PRAS40, and GβL in Raptor and Rictor immunoprecipitates by Western blotting as described above.

CO-IMMUNOPRECIPITATION OF Epac1, PDE3B, PDE4D, RAPTOR, AND RICTOR IN CELL LYSATES OF 8-CPT-2Me-cAMP TREATED CELLS

Next we evaluated whether 8-CPT-2Me-cAMP-induced activation of mTORC2 signaling involves the formation of a multiprotein complex in 1-LN prostate cancer cells by co-immunoprecipitation experiments [Gloerich et al., 2010; Rampersad et al., 2005; Hochbaum et al., 2011; Wilson et al., 2011]. 1-LN prostate cancer cells were grown and treated as above. Equal amounts of lysate proteins (200 μg) were used in separate experiments for immunoprecipitation in presence of protein agarose with AKAP (1:50) Epac1 (1:50), PDE3B (1:50), PDE4D (1:50), Raptor (1:50) and Rictor (1:50) antibodies, respectively, as described in the preceding sections. The immunoprecipitates, after incubating overnight were washed thrice with chaps lysis buffer (2,000 rpm/5 min/4°C). A volume of 4× sample buffer added, immunoprecipitates boiled for 5 min in a water bath, centrifuged, supernatants electrophoresed, and transferred to membranes. Immunoprecipitated proteins on each membrane were immunoblotted for AKAP, Epac1, PDE3B, PDE4D, Raptor, and Rictor and proteins on membranes visualized in a Storm Phosphorimager®.

RESULTS

SPECIFICITY OF 8-CPT-2Me-cAMP AND 6-BENZ-cAMP ACTIVITY ON THE EXPRESSION OF EPAC1 AND P-CREB IN 1-LN CANCER CELLS

In preliminary experiments we examined the effect of treating 1-LN cells with 8-CPT-2Me-cAMP and 6-Benz cAMP. 8-CPT-2Me-cAMP-treated cells expressed Epac1 protein without showing any effect on p-CREB^{S133}, the effector of PKA (Fig. 1A). In contrast, 6-Benz-cAMP treatment upregulated p-CREB^{S133} without affecting expression of Epac1 (Fig. 1A). These observations are consistent with the reported specificities of these cAMP analogs. Like 1-LN cells, treatment of DU-145 and PC-3 prostate cancer cells with 8-CPT-2Me-cAMP also upregulated the expression of Epac1 (Fig. 1B,C).

8-CPT-2Me-cAMP UPREGULATES THE EXPRESSION OF p-Akt^{T308} AND p-Akt^{S437} IN 1-LN CANCER CELLS

Treatment of 1-LN cancer cells with varying concentrations of 8-CPT-2Me-cAMP elevated p-Akt^{T308} and p-Akt^{S473} in a concentration-dependent manner (Fig. 1D), consistent with our previous observations [Misra and Pizzo, 2005, 2009; Misra et al., 2008a]. The maximal stimulation of p-Akt^{T308} and p-AKT^{S473} occurred at 100–150 μM of 8-CPT-2Me-cAMP; therefore, in the experiment described below this concentration was used. Incubation of cells with 150 μM of 8-CPT-2Me-cAMP for varying periods of time showed a maximal increase in phosphoprotein of Akt^{T308} and Akt^{S473} at about 20–30 min of incubation (Fig. 1D). Treatment of 1-LN cells with 6-Benz-cAMP showed no such effect on the upregulation of p-Akt^{T308}, or p-Akt^{Ser473} (Fig. 1D). Treatment of Du-145 and PC-3 cancer cells with (150 μM/30 min) also elevated levels and p-Akt^{308} and p-Akt^{473} compared to buffer-treated cells (Fig. 1E).

CHARACTERIZATION OF COMPONENTS OF mTORC1 AND mTORC2 COMPLEXES IN 1-LN CANCER CELLS STIMULATED WITH 8-CPT-2Me-cAMP

Before studying the kinase activities of mTORC1 and mTORC2 complexes towards S6 Kinase, 4EBP1, and Akt^{T473} in cancer cells, we first identified the components of these two complexes immunoprecipitated by anti-Raptor or Rictor antibodies, respectively (Fig. 2). Raptor immunoprecipitates contained mTOR, Raptor, GβL, and PRAS40 but no Rictor, mS1N1, or protor (Fig. 2). The Rictor immunoprecipitates contained mTOR, Rictor, GβL, mS1N1, and protor but no Raptor (Fig. 2). The same components have been reported by others in Raptor and Rictor immunopreciptates of various cell lines [Alessi et al., 1997; Martin and Hall, 2005; Laplante and Sabatini, 2009; Sengupta et al., 2010; Zoncu et al., 2011]. We have employed these Raptor and Rictor immunoprecipitates in the experiments described in the following sections.

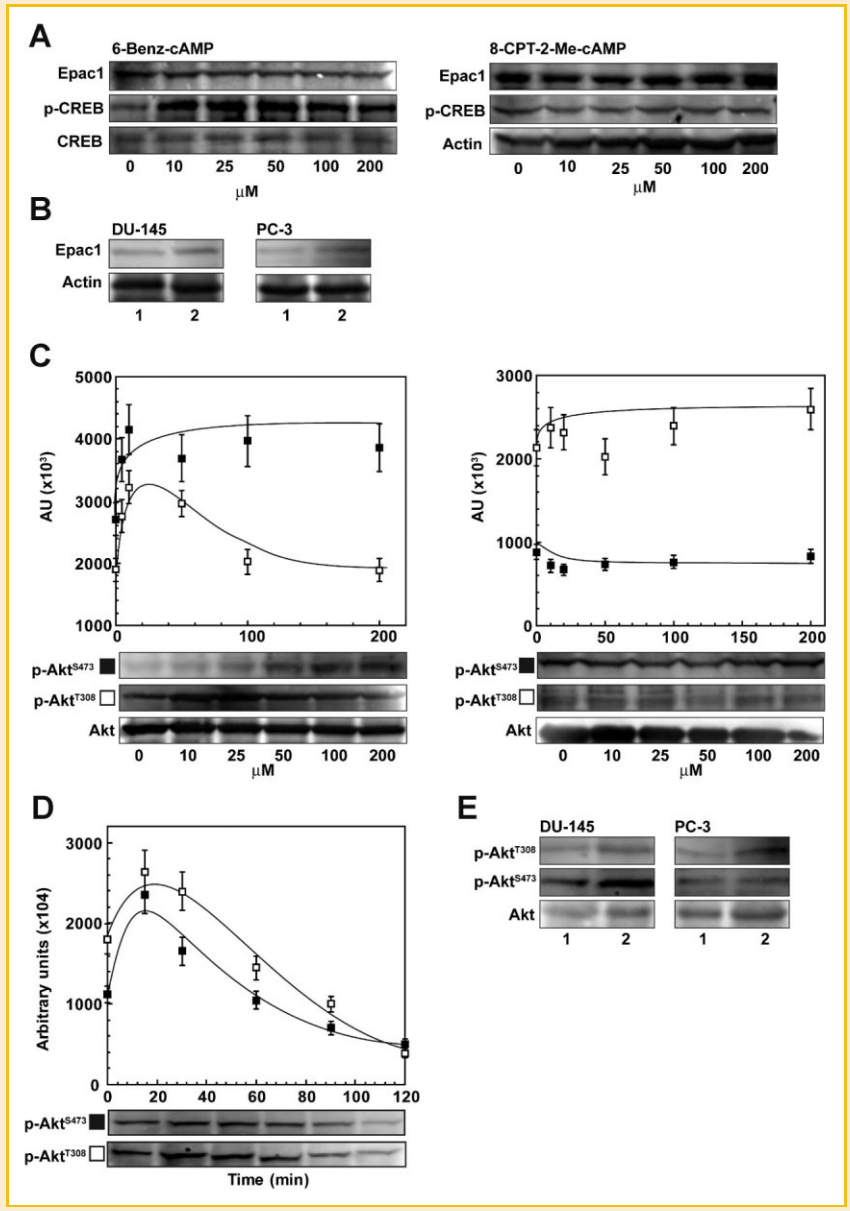

Fig. 1. Specificity of 8-CPT-2Me-cAMP and 6-Benz-cAMP with respect to Epac1 expression and p-CREB. Panel A: Effect of varying concentration of 6-Benz-cAMP and 8-CPT-2Me-cAMP on expression of p-CREB^{S133} and Epac1 in 1-LN cells. Panel B: Effect of 8-CPT-2Me-cAMP (100 μM/30 min) on expression of Epac1 in DU-145 and PC-3 cancer cells. Panel C: Effect of varying concentrations of 8-CPT-2Me-cAMP and 6-Benz-cAMP on p-Akt^{T308} (□) and p-Akt^{S473} (■) in 1-LN cancer cells. Panel D: Effect of time of incubation on 8-CPT-2Me-cAMP-induced increase in p-Akt^{T308} (□) and p-Akt^{Ser473} (■) in 1-LN cancer cells. Panel E: Effect of 8-CPT-2Me-cAMP (100 μM/30 min) on p-Akt^{T308} and p-Akt^{S473} in DU-145 and PC-3 cancer cells. Immunoblots shown along with their respective controls are representative of at least three independent experiments.

8-CPT-2Me-cAMP UPREGULATES Akt1 PHOSPHORYLATION AT S473 BY mTOR AND RICTOR IMMUNOPRECIPITATES IN PROSTATE CANCER CELLS

We then studied the role of mTORC2 kinase in the Akt^{S473} phosphorylation in Rictor immunoprecipitates of 1-LN prostate cancer cells treated with 8-CPT-2Me-cAMP (Fig. 3A,B). This treatment of 1-LN prostate cancer cells elevated Akt^{S473} phosphorylation by about 2.5-fold (Fig. 3A). This 8-CPT-2Me-cAMP-induced Akt phosphorylation by mTORC2 kinase was sensitive to the PI 3-Kinase inhibitor LY294002, but insensitive to rapamycin (Fig. 3A). Like 1-LN cells, DU145 and PC-3 cells showed a marked increase in phosphorylation of Akt^{S473} in Rictor immunoprecipitates (Fig. 3C). To further assess the role of Epac1 in Akt^{S473} phosphorylation, we silenced Epac1 gene expression by RNAi which resulted in significant inhibition of Akt phosphorylation at S473 (Fig. 3B). Similar responses of 1-LN cells treated with 8-CPT-2Me-cAMP or LY294002 and rapamycin prior to 8-CPT-2Me-cAMP or transfection with Epac1 dsRNA prior to 8-CPT-2Me-cAMP exposure were observed using Western blotting analysis (Fig. 3A,B). Consistent with previous studies in other cell types, Rictor immunoprecipitates

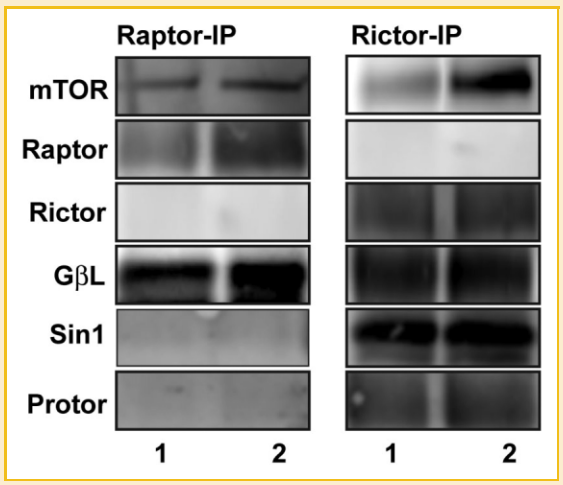

Fig. 2. Immunoblots showing protein components of Raptor and Rictor immunoprecipitates of 1-LN cells treated with 8-CPT-2Me-cAMP (100 μM/30 min). The lanes are: (1) buffer or (2) 8-CPT-2Me-cAMP-treated. A representative immunoblot from three to four experiments, in each case, is shown.

showed no significant phosphorylation of Akt^{Thr308} [Alessi et al., 1997, 2009; Manning and Cantley, 2007] (Fig. 3A,B). In contrast to Rictor immunoprecipitates, mTOR immunoprecipitates phosphorylate Akt at both T308 and S473 in 8-CPT-2Me-cAMP-treated 1-LN prostate cancer cells (Fig. 4). mTOR-immunoprecipitate-mediated phosphorylation of Akt at T308 was sensitive to both the PI 3-Kinase inhibitor LY294002 and rapamycin whereas Akt phosphorylation at S473 was sensitive to LY294002, but not to rapamycin (Fig. 4A). Transfection of cells with Epac1 dsRNA significantly inhibited mTOR immunoprecipitate-mediated phosphorylation of Akt at both T308 and S473 (Fig. 4B). Similar observations were made by Western blotting analysis of Akt phosphorylation at T308 and S473 by mTOR immunoprecipitates of 1-LN cells treated with 8-CPT-2Me-cAMP (Fig. 4C,D). By silencing Rictor expression by RNAi, we

Fig. 3. Phosphorylation of Akt^{S473} by Rictor immunoprecipitates of 1-LN cells treated with 8-CPT-2Me-cAMP. Panel A: Bar diagram showing the phosphorylation of Akt at S473 (■) and Akt at T308 (□) by Rictor immunoprecipitates and its modulation by LY294002 and rapamycin. The bars are: (1) buffer-treated; (2) 8-CPT-2Me-cAMP (100 μM/30 min); (3) LY294002 (25 μM/20 min) then 8-CPT-2Me-cAMP; and (4) rapamycin (100 μM/20 min) then 8-CPT-2Me-cAMP. The phosphorylation is expressed as fmol [^{33}P]-γ-ATP incorporated/mg protein and is mean ± SE from four individual experiments. The * indicates values significantly different from 8-CPT-2Me-cAMP treated cells at the 5%. Representative immunoblots of three experiments of p-Akt^{S473} in Rictor immunoprecipitates of cells treated as above are shown below the bar diagram. Panel B: Bar diagram showing the phosphorylation of Akt^{S473} (■) and Akt^{T308} (□) by Rictor immunoprecipitates of 1-LN cells. The bars are: (1) lipofectamine + buffer; (2) lipofectamine + 8-CPT-2Me-cAMP (100 μM/30 min); (3) Epac1 dsRNA (100 μM/48 h) then 8-CPT-2Me-cAMP; and (4) scrambled RNA (100 μM/48 h) then 8-CPT-2Me-cAMP. The phosphorylation is expressed as fmol [^{33}P]-γ-ATP incorporated/mg protein and is mean ± SE from three independent experiments. Values significantly different from 8-CPT-2Me-cAMP and scrambled RNA + 8-CPT-2Me-cAMP treated cells at 5% levels are marked as *. A representative immunoblot of three independent experiments showing protein levels of p-Akt^{S473} in cells treated as above are shown below the bar diagram. Panel C: The bar diagram showing phosphorylation of Akt at S473 by Rictor immunoprecipitates of DU-145 (■) and PC-3 (□) cells treated with 8-CPT-2Me-cAMP. The bars in both cells are: (1) buffer and (2) 8-CPT-2Me-cAMP (100 μM/30 min). The phosphorylation is expressed as fmol [^{33}P]-γ-ATP incorporated/mg protein and is mean ± SE from three individual experiments.

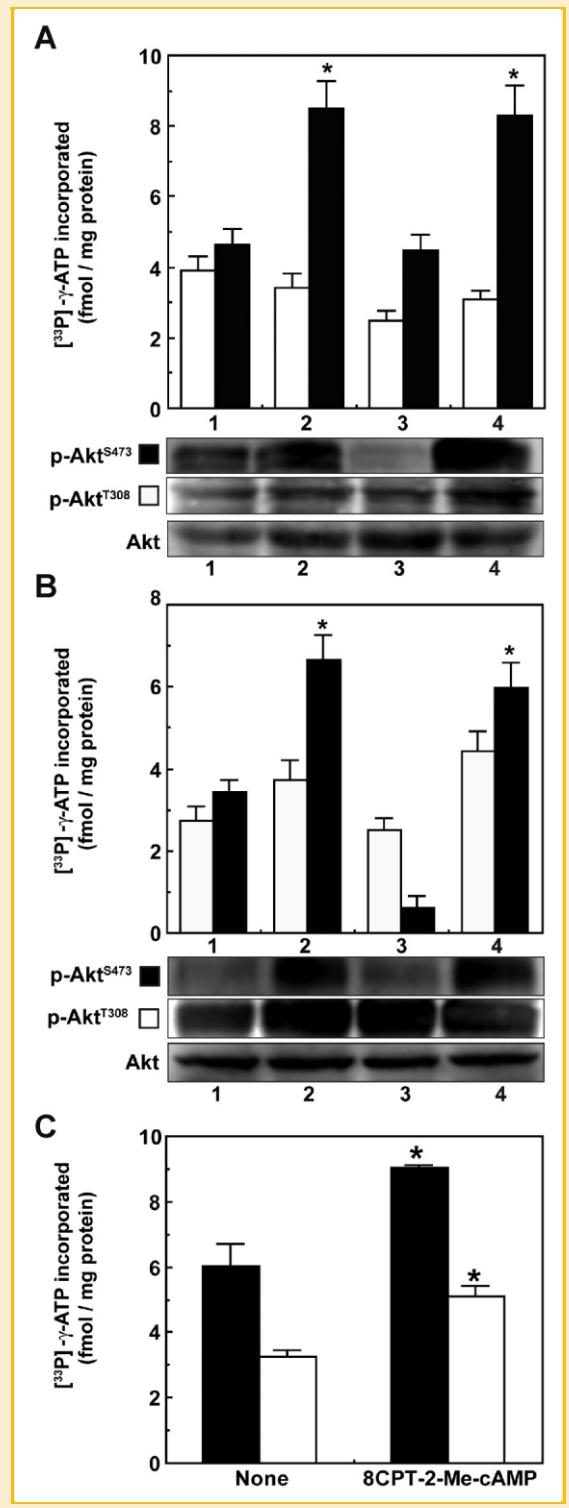

Fig. 3.

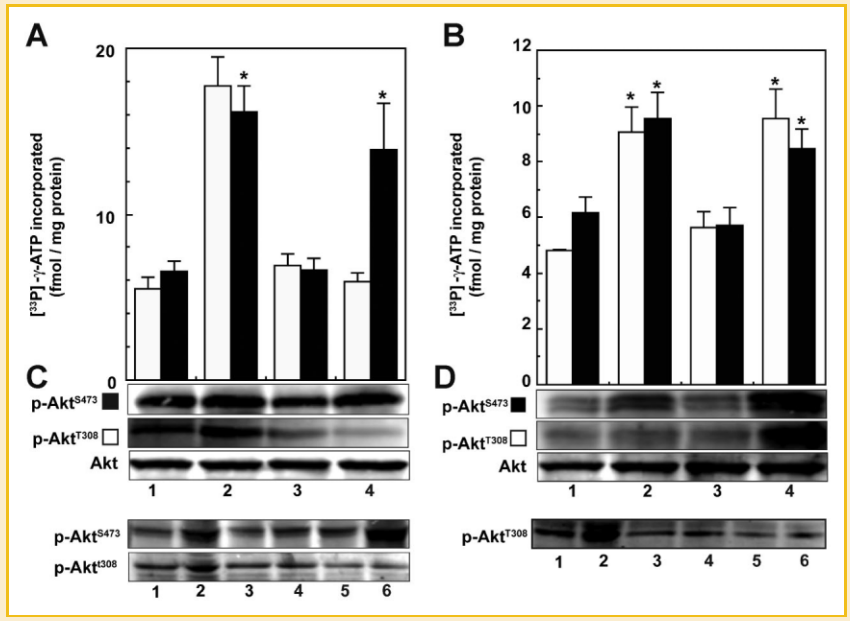

Fig. 4. Phosphorylation of Akt at T308 (□) and S473 (■) residues in mTOR immunoprecipitates of 1-LN prostate cancer cells treated with 8-CPT-2Me-cAMP. Panel A: The bars are: (1) buffer; (2) 8-CPT-2Me-cAMP (100 μM/30 min); (3) LY294002 (20 μM/20 min), then 8-CPT-2Me-cAMP (100 μM/30 min); (4) Rapamycin (100 μM/20 min) then 8-CPT-2Me-cAMP (100 μM/20 min). Panel B: Effect of silencing Epac1 gene expression by RNAi on phosphorylation of Akt at T308 and Akt at S473 in 1-LN cells treated with 8-CPT-2Me-cAMP (100 μM/30 min). The bars are: (1) lipofectamine + buffer; (2) lipofectamine + 8-CPT-2Me-cAMP (100 μM/30 min); (3) siEpac1 RNA (75 nM/48 h) then 8-CPT-2Me-cAMP (100 μM/30 min); and (4) scrambled RNA (75 μM/48 h) then 8-CPT-2Me-cAMP (100 μM/30 min). In Panel C and Panel D are shown the immunoblots of p-Akt^{T308} and p-Akt^{S473} in 1-LN cells treated as in Panel A and Panel B, respectively. Below Panel C is also shown the immunoblots of p-Akt^{T308} and p-Akt^{S473} from 1-LN cells treated with: (1) buffer; (2) 8-CPT-2Me-cAMP (150 μM/30 min/37°C); (3) LY294002 (20 μM/20 min/37°C); (4) Ly + 8-CPT-2Me-cAMP; (5) rapamycin (100 nM/20 min) and (6) rapamycin + 8-CPT-2Me-cAMP. Below the Akt^{T308} and Akt^{S473} immunoblot in Panel D is shown the immunoblot p-Akt^{Ser473} from 1-LN cells treated with: (1) buffer + lipofectamine; (2) lipofectamine + 8-CPT-2Me-cAMP (150 μM/30 min/37°C); (3) siEpac1 RNA (100 nM/48 h/37°C); (4) siEpac1 RNA + 8-CPT-2Me-cAMP; (5) scrambled RNA (100 nM/48 h/37°C); and (6) scrambled RNA + 8-CPT-2Me-cAMP. The phosphorylation of Akt at T308 and S473, the residues respectively is expressed as fmol [^{33}P] incorporated per mg protein and is mean ± SE from three to four individual experiments. The * indicates values significantly different at the 5% level from 8-CPT-2Me-cAMP-treated cells in Panel A and from 8-CPT-2Me-cAMP and scrambled RNA treated cells in Panel B. Immunoblots shown are representative of three individual experiments.

have further confirmed that 8-CPT-2Me-cAMP-induced upregulation of Akt^{S473} phosphorylation is brought about by mTORC2 (Fig. 5A). Suppression of the cellular Rictor pool (Fig. 6B) significantly inhibited phosphorylation of Akt^{S473}. These results are similar to those reported by determining the Akt^{S473} kinase activity [Guertin et al., 2006, 2009; Zoncu et al., 2011]. Full activation of Akt1 requires phosphorylated at both T308 and S473. A number of phosphatases and interacting partners modulate Akt1 subcellular localization, phosphorylation, and its kinase activity [Du and Tsichlis, 2005]; therefore, it is likely that modulations in the availability of Epac1 will affect downstream signaling and cellular responses.

8-CPT-2Me-cAMP TREATMENT OF PROSTATE CANCER CELLS UPREGULATES PHOSPHORYLATION OF S6 KINASE AND 4EBP1 IN RAPTOR IMMUNOPRECIPITATES

Activated mTORC1 kinase phosphorylates two downstream effectors S6 Kinase and 4EBP [Martin and Hall, 2005; Laplante and Sabatini, 2009; Sengupta et al., 2010; Zoncu et al., 2011]. In the absence of growth factors or nutrients, S6 Kinase is associated with eIF3. Upon addition of growth factors or nutrients, the mTOR/Raptor complex is recruited to eIF3 to phosphorylate S6 Kinase and 4EBP1. Phosphorylation of S6 Kinase leads to its dissociation from eIF3 and phosphorylated S6 Kinase binds to PDK1 which phosphorylates S6 Kinase [Hanrahan and Blenis, 2006; Houslay, 2010]. We have shown that mTOR immunoprecipitates of 1-LN, DU-145, and PC-3 cells treated with 8-CPT-2Me-cAMP elevate LY294002—and rapamycin-sensitive phosphorylation of S6 Kinase and 4EBP1 [Misra and Pizzo, 2009]. Downregulation of Epac1 also inhibits 8-CPT-2Me-cAMP induced phosphorylation of S6K and 4EBP1 in 1-LN cells [Misra and Pizzo, 2009]. We have now employed Raptor-immunoprecipitates to study the role of mTORC1 complex in phosphorylation of S6 Kinase and 4EBP1 in 1-LN, DU-145, and PC-3 cells stimulated with 8-CPT-2Me-cAMP (Fig. 5A–D). Like phosphorylation of S6 Kinase and 4EBP1 observed with mTOR-immunoprecipitates [Alessi et al., 1997], Raptor-immunoprecipitates also phosphorylates S6 Kinase and 4EBP1 (Fig. 5A–D). Pretreatment of 1-LN cells with rapamycin and LY294002 causes a marked suppression in 8-CPT-2Me-cAMP-induced phosphorylation of S6 Kinase and 4EBP1 (Fig. 6A,B).

MODULATING PROTEINS OF mTORC1 AND mTORC2 COMPLEXES IN PROSTATE CANCER CELLS TREATED WITH 8-CPT-2Me-cAMP

The mTORC2 complex in addition to mTORC contains Rictor, GβL, Deptor, mammalian stress-activated protein kinase interacting protein (mS1N1), and protein observed with Rictor (protor/PRR5). Rictor is obligatory for mTORC2 catalytic activity and recruitment of

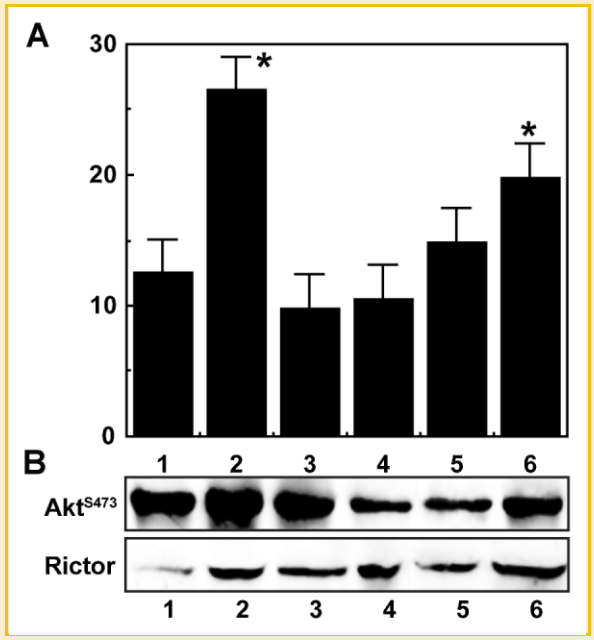

Fig. 5. Panel A: Phosphorylation of Akt^{S473} by Rictor immunoprecipitates from 1-LN cells treated with: (1) buffer + lipofectamine; (2) lipofectamine + 8-CPT-2Me-cAMP (150 μM/30 min/37°C); (3) siRictor RNA (100 nM/48 h/37°C); (4) siRictor RNA + 8-CPT-2Me-cAMP; (5) scrambled RNA (100 nM/48 h/37°C); and (6) scrambled RNA + 8-CPT-2Me-cAMP. Phosphorylation of Akt^{S473} is expressed as fmol [^{33}P] incorporated per mg protein and is the mean ± SE from four individual experiments. The * indicates values significantly different at 5% level from buffer and siRictor RNA-treated cells. Panel B: Representative immunoblots of three experiments of p-Akt^{S473} and Rictor of cell lysates of 1-LN cells treated as in Panel A. Protein loading control actin in Rictor immunoblot is shown below it.

substrates. In contrast to mTORC1, MLST8 is required for mTORC2 activity [Thediecke et al., 2007]. The activity of mTORC2 is controlled by PI 3-Kinase and is insensitive to energy status. Prolonged exposure to rapamycin inhibits mTORC2 activity by disrupting the assembly of new mTORC2 complexes [Sabatini, 2006]. mTORC1 activation-induced phosphorylation of S6 Kinase, phosphorylates Rictor at T1135 and inhibits Akt signaling [Dibble et al., 2009; Julian et al., 2010]. In a variety of cancer cells increased levels of Rictor correlating with Akt^{S473} phosphorylation have been observed [Masri et al., 2007; Sparks and Guertin, 2009]. In view of the importance of the presence of various components for optimal mTORC1 and mTORC2 activation, in the next series of experiments we measured the levels of Raptor, Rictor, PRAS40, and GβL under the experimental conditions by Western blotting (Fig. 7). Treatment of prostate cancer cells with 8-CPT-2Me-cAMP substantially elevated Raptor and Rictor levels (Fig. 7). This treatment also elevated p-PRAS40 and GβL levels (Fig. 7). The levels of these interacting proteins of mTORC1 and mTORC2 complexes were also affected by inhibitors pretreatment and by down-regulation of Epac1 expression by RNAi (Fig. 7). 8-CPT-2Me-cAMP-induced alterations in these interacting proteins would influence the activities mTORC1 and mTORC2 and the consequent cellular responses.

Epac1 FORMS A MULTIPROTEIN SIGNALING COMPLEX (SIGNALOSOME) WITH AKAP, RAPTOR, RICTOR, PDE3, AND PDE4D IN 1-LN PROSTATE CANCER CELLS STIMULATED WITH 8-CPT-2Me-cAMP

Scaffold proteins colocalize the molecules involved in the same signaling pathway and function as catalysts thereby activating different components in the signaling pathways. Scaffold proteins are instrumental in ensuring specificity and efficiency in regulating positive and negative feedback loops [Engstrom et al., 2010]. Activation of AKT via mTORC signaling may be scaffold-dependent [Tzatsos, 2009]. AKAP forms a signaling complex at the nuclear envelop of striated myocytes and neurons consisting of PKA, Epac1, PDE3B, PDE4D3, ERK5, MAPK, ryanodine receptor, calcineurin and phosphatase 2A [Dodge-Kafka et al., 2005; Tzatsos, 2009; Wilson et al., 2011]. AKAP anchors ERK1, PDK1, and p90RSK and regulates their activity [Dodge-Kafka et al., 2005]. AKAP9 interacts with Epac1 and promotes Epac1 stimulated microtubule growth [Sehrawat et al., 2011]. We have, therefore, investigated whether 8-CPT-2Me-cAMP-induced Epac1 signaling involves the assembly of a multiprotein complex (signalosome) in activating mTORC1 and mTORC2. We performed co-immunoprecipitation studies where we probed for Epac1, AKAP, Raptor, Rictor, PDE3B, and PDE4D immunoprecipitates, respectively, for AKAP, Epac1, Raptor, Rictor, PDE3B, and PDE4D in each case (Fig. 8). In Epac1 immunoprecipitates, Raptor, Rictor, AKAP, PDE3B, PDE4D co-immunoprecipitated with Epac1. In AKAP immunoprecipitates, Epac1, Raptor, Rictor, PDE3B, and PDE4D co-immunoprecipitated with AKAP. In Raptor immunoprecipitates, Epac1, AKAP, PDE3B, and PDE4D co-immunoprecipitated with Raptor. In Rictor immunoprecipitates, Epac1, AKAP, PDE3B, and PDE4D co-immunoprecipitated with Rictor. In PDE3B immunoprecipitates, Epac1, AKAP, Raptor, and Rictor co-immunoprecipitated with PDE3B. In PDE4D immunoprecipitates, Epac1, Raptor, Rictor, and AKAP co-immunoprecipitated with PDE4D (Fig. 8) In these signaling complexes, we propose that Raptor, Rictor, PDE3B, and PDE4D function to provide platforms for Epac1 binding and facilitate the recruitment of Epac1 via its DEP domain to the plasma membrane where it causes activation of mTORC1 and mTORC2.

DISCUSSION

Among the major signaling networks implicated in the proliferation and survival of prostate cancer cells in vitro and in vivo are the MAPK and PI 3-Kinase/Akt/mTOR pathways [Bellacosa et al., 2005; Gloerich and Bos, 2010]. These signaling pathways converge at the mTOR signaling cascade to promote cell survival and inhibit apoptosis [Gloerich and Bos, 2010]. Consequent to PI 3-Kinase activation, Akt is recruited to the plasma membrane where it is phosphorylated at T308 and S473 by PDK1 and mTORC2, respectively. The PH, catalytic, and COOH-terminal domains of Akt may interact with several Akt regulatory proteins including TCL1, JNK activating proteins, HSP90, COOH modulator protein, Epac1 [Misra et al., 2008a]. In an earlier report, we showed that treatment of prostate cancer cells with 8-CPT-2Me-cAMP upregulates DNA and protein synthesis which are impaired by pretreatment

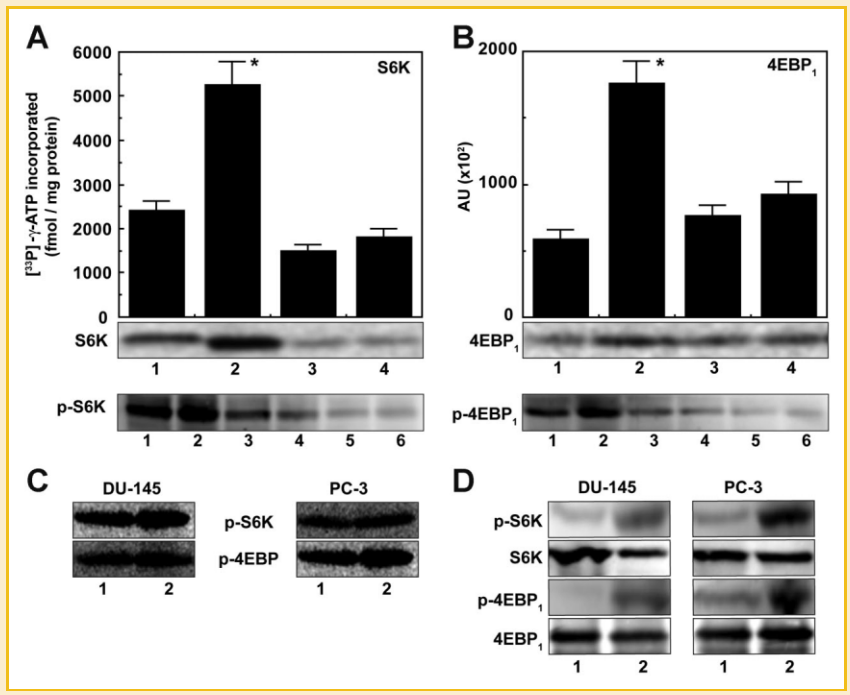

Fig. 6. 8-CPT-2Me-cAMP-induced activation of mTORC1 kinase in Raptor immunoprecipitates of 1-LN prostate cancer cells. Panels A and B: Autoradiograph showing the phosphorylation of S6 Kinase and 4EBP substrate peptides by Raptor immunoprecipitates in 1-LN cells. Lanes in both autoradiographs are: (1) buffer (2) 8-CPT-2Me-cAMP (100 μM/30 min), (3) LY294004 (20 μM/20 min) then 8-CPT-2Me-cAMP (100 μM/30 min), and (4) rapamycin (100 μM/20 min) then 8-CPT-2Me-cAMP (100 μM/30 min). Panel C: Autoradiographs showing phosphorylation of S6 Kinase and 4EBP in DU-145 and PC-3 cancer cells treated with 8-CPT-2Me-cAMP (100 μM/30 min). Panel D: Immunoblot showing the levels of p-S6 Kinase and p-4EBP in DU-145 and PC-3 prostate cancer cells treated with 8-CPT-2Me-cAMP as described in Panel C. Autoradiographs and immunoblots shown are representative of three individual experiments.

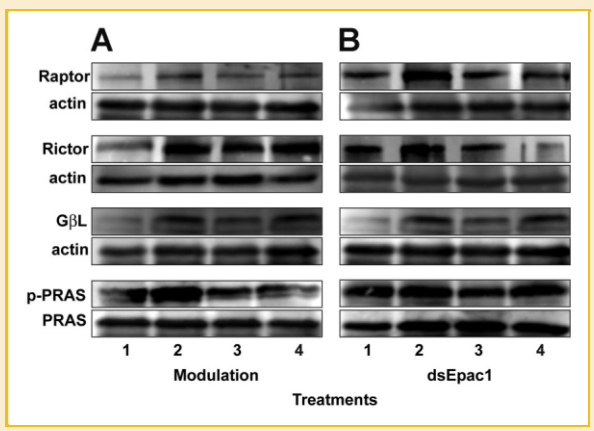

Fig. 7. Panel A: A representative immunoblot of three experiments showing the effect of PI3 Kinase and mTOR inhibitor on protein levels of Raptor and Rictor, GβL, and p-PRA40 in 1-LN cells treated with: (1) buffer; (2) 8-CPT-2Me-cAMP (100 μM/30 min); (3) LY294002 (25 μM/20 min) then 8-CPT-2Me-cAMP; and (4) rapamycin (100 μM/20 min) then 8-CPT-2Me-cAMP. Panel B: A representative immunoblot of transfection of 1-LN cells with siEpac1 RNA on protein levels Raptor, Rictor, GβL, and p-PRAs 40 min. 1-LN cells treated with: (1) lipofectamine + buffer; (2) lipofectamine + 8-CPT-2Me-cAMP (100 μM/30 min); (3) siEpac RNA (100 μM/48 h) then 8-CPT; and (4) scrambled RNA (100 μM/48 h) then 8-CPT-2Me-cAMP. Protein loading controls are shown below respective immunoblots. Representative immunoblots from three to four experiments are shown. The * indicates values significantly different at the 5% level from all other conditions.

with LY294002, a PI 3-Kinase inhibitor and rapamycin an mTORC1 inhibitor [Misra and Pizzo, 2009]. 8-CPT-2Me-cAMP-induced activation of mTORC1 as evaluated by S6 Kinase and 4EBP1 phosphorylation in mTOR immunoprecipitates was attenuated by pretreatment of cancer cells with PI 3-Kinase and mTOR inhibitors as well as by downregulation of Epac1 expression by RNAi [Misra and Pizzo, 2009].

Here we show: (1) treatment of 1-LN, prostate cancer cells with 8-CPT-2Me-cAMP, causes a dose-dependent upregulation of Epac1, p-Akt^{T308}, and p-Akt^{S473} without showing any effect on p-CREB^{Ser133}; (2) in contrast 6 Benz-cAMP, a selective agonist of PKA, shows no effect on Epac1 expression but upregulates the expression of p-CREB^{Ser133} and shows negligible effects on the expression of p-Akt^{T308} and p-Akt^{S473}; (3) 8-CPT-2Me-cAMP treatment of the prostate cancer cells, as compared to vehicle-treated cells, causes a two- to threefold increase in mTORC2 activation as evaluated by Akt^{S473} kinase activity in Rictor and mTOR immunoprecipitates; (4) Akt^{S473} kinase activity in Rictor and mTOR immunoprecipitates is sensitive to LY294002 but insensitive to rapamycin. Silencing Epac1 and Rictor expression by RNAi significantly inhibits Akt^{S473} kinase activity in Rictor and mTOR immunoprecipitates which otherwise is increased by two- to threefold compared to vehicle-treated cells. 8-CPT-2Me-cAMP-induced S6 Kinase and 4EBP1 phosphorylation by Raptor immunoprecipitates is sensitive to LY294002 and rapamycin; and (5) Epac1-induced activation of mTORC1 and mTORC2 is "scaffold"-dependent and involves the formation of a multiprotein

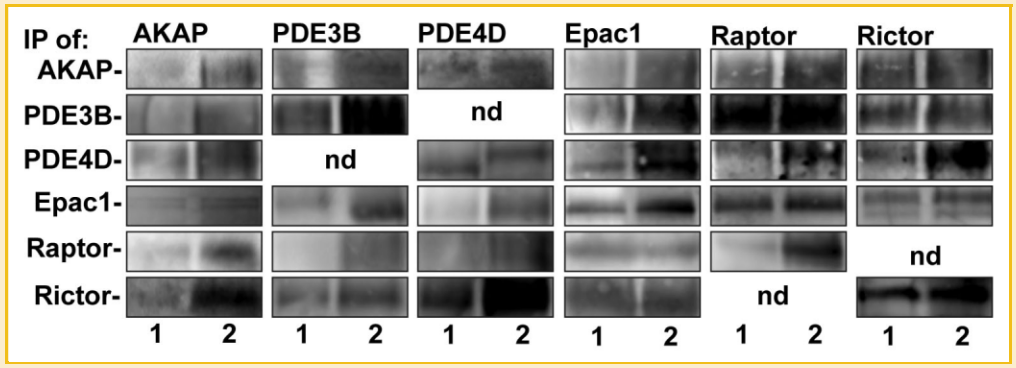

Fig. 8. Immunoblots of APAK, PDE3B, PDE4D, Epac1, Raptor, and Rictor immunoprecipitates (IP) showing co-immunoprecipitation of APAK, PDE3B, PDE4D, Epac1, Raptor, and Rictor in 1-LN cells treated with: (1) buffer; and (2) 8-CPT-2Me-cAMP (100 μM/30 min). Immunoblots shown are representative of three to four independent experiments. n.d. = not done.

signaling complex comprised of AKAP, Epac1, PDE3B, PDE4D, Raptor, and Rictor.

Protein–protein interactions aid in spatially organizing signal transduction and regulation of cellular functions [Dodge-Kafka et al., 2005; Tzatsos, 2009; Engstrom et al., 2010; Rampersad et al., 2010; Sehrawat et al., 2011; Wilson et al., 2011]. Spatial organization and recruitment of mediators of specific pathways are essential for ensuring signaling specificity and amplification. Global activation of Epac1 promotes accumulation of Rap1/GTP/Ras-GTP, which signal via activation of several effectors including PI 3-Kinase, phospholipase D, phospholipase Cε, Raf1, p38 MAPK [Rampersad et al., 2005; Wilson et al., 2011]. Integration of cAMP-Epac1 and PI 3-Kinase signals occurs via the assembly of signalosomes which contains mediators of specific pathways [Dodge-Kafka et al., 2005; Tzatsos, 2009; Engstrom et al., 2010; Sehrawat et al., 2011]. Adenylyl cyclase-induced increase in intracellular cAMP becomes compartmentalized due to local degradation by spatially restricted phosphodiesterases and confined targets of cAMP effector proteins. cAMP-Epac1 signaling is spatially regulated by diverse anchoring mechanisms reflecting many different functions assigned to Epac.

A kinase anchoring proteins (AKAP) are multivalent scaffold proteins targeting PKA to discrete cellular localization in combination with other signaling molecules [Dodge-Kafka et al., 2005; Thediecke et al., 2007]. AKAP anchors PKA and directs cellular localization of the complex while PDE4D3 functions as an adaptor protein for Epac1 and ERK5. Phosphorylation of PDE4D3 decreases its enzyme activity favoring local increase in cAMP and subsequent accumulation of PKA and Epac1 [Dodge-Kafka et al., 2005]. PDE3B binds to p84 subunit of PI 3-Kinase-γ and this interaction allows recruitment of PI 3-Kinase to PDE3B-Epac1 complexes. PDE3B-tethered Epac1 regulates PI 3-Kinase activity and thus regulates human endothelial cells adhesion, spreading and tubule formation [Wilson et al., 2011]. Assembly of a signalosome containing Epac1, PI 3-Kinase, and the cyclic nucleotide phosphodiesterase PDE3B has been reported. In this signalosome PDE3B-tethered Epac1 is regulated by PDE3B hydrolysis of cAMP and activated Epac1 promotes PI 3-Kinase mediated phosphorylation of Akt. Using immunoprecipitation studies, we show that in addition to colocalization of Epac1 with PDE3B, and PDE4D, it also colocalizes with Raptor and Rictor, the scaffolds for recruiting components of the mTORC1 and mTORC2 complexes. PDE4D enzyme is over expressed in the prostatic epithelial cells of patients with prostatic adenocarcinomas and plays a permissive role in promoting proliferation [Rahrmann et al., 2009]. PDE4D is also a binding partner of Rheb and inhibits mTORC1 activation by Rheb. Elevated levels of cAMP disrupt PDE4D and Rheb interaction and promote Rheb and mTORC1 interaction and mTORC1 activation [Rahrmann et al., 2009]. PDE4D interaction with Epac1 foster their interaction into vascular endothelial-cadherin based complexes which regulates Epac1 based stabilization of vascular endothelial-cadherin-based adhesion. PDE4D regulates both the activity and subcellular localization of Epac1 [Rampersad et al., 2005]. In PC-3 prostate cancer cells mTORC2-induced activation of Akt^{S473} is associated with plasma membrane localization of Rictor [Guertin et al., 2009]. Similar observations have also been reported in murine peritoneal macrophages treated with 8-CPT-2Me-cAMP [Misra et al., 2002, 2008b; Manning and Cantley, 2007; Huston et al., 2008; Bailey et al., 2009; Misra and Pizzo, 2009; Liu et al., 2010]. 8-CPT-2Me-cAMP-induced activation of Akt appears to be preceded by their physical association either before and/or after membrane recruitment. The DEP domain of Epac1 may play a role in membrane recruitment of Epac1 and/or multiprotein complexes of Epac1. Deletion of the DEP domain inhibits recruitment of Akt1 to the membrane and it remains mostly cytosolic [Bos, 2006]. In resting cells, Epac1 is localized in the cytosolic and perinuclear region and in stimulated cells, Epac1 also localizes in plasma membranes and the perinuclear region [Bos, 2006].

In summary, we demonstrate that 8-CPT-2Me-cAMP, an Epac1 specific analog of c-AMP, promotes mTORC1 and mTORC2 activation in prostate cancer cell lines. In addition to activation of Rap/Ras like proteins, Epac1 induces activation of mTORC1 and mTORC2 by the assembly of a multiprotein signaling complex comprising of AKAP–Epac1–PDE3B–PDE4D–Raptor–Rictor and thus appears to be scaffold-dependent. Based upon these observations, we propose a plausible mechanism of Epac1-induced

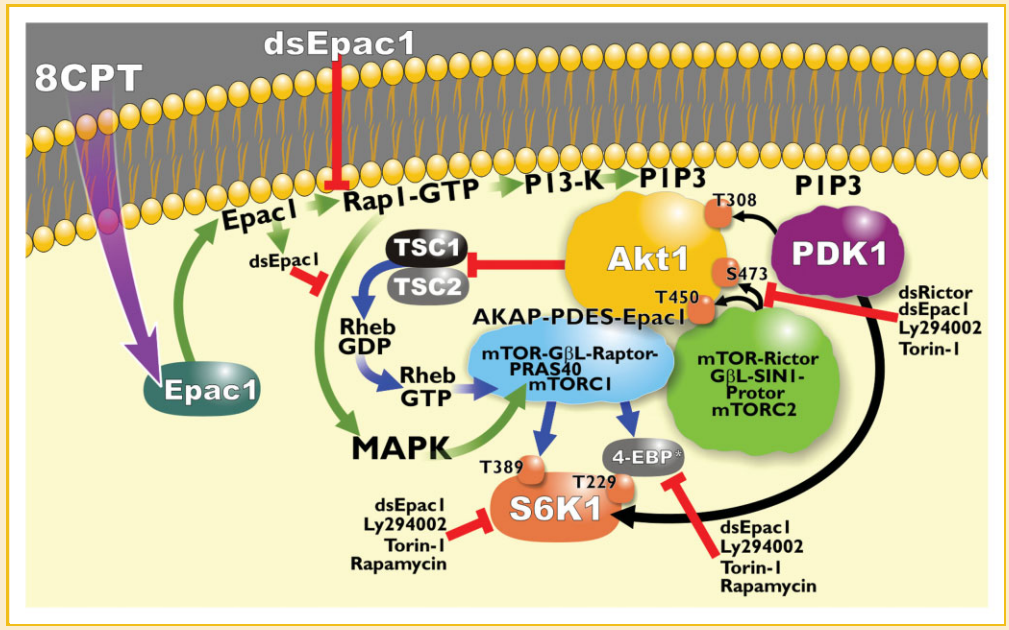

Fig. 9. A schematic representation of events leading to the assembly of multiprotein signaling complexes involved in mTORC1 and mTORC2 activation in 1-LN prostate cancer cells treated with 8-CPT-2Me-cAMP.

activation of mTORC1 and mTORC2. This involves the assembly of a signalosome consisting of AKAP, Epac1, PDE4D, PDE3B, Raptor, and Rictor and the translocation of this signalosome to the plasma membrane and nuclear envelop via DEP domain by Epac1. Protein–protein interactions facilitate the spatial organization of signal transduction events [Dodge-Kafka et al., 2005; Rampersad et al., 2004; Boulbes et al., 2011; Sehrawat et al., 2011; Wilson et al., 2011]. Previous studies have demonstrated that HEAT repeats are involved in the formation of rod-like structures which display binding sites for assembly of multiprotein complexes [Andrade and Bork, 1995]. Such repeats are known to occur in MTOR [Dennis et al., 1999]. This offers a plausible mechanism for our observations. The requirement for two mTOR complexes for full activation of Akt is dependent on the involvement of assembly of multiprotein signaling complexes. The critical step in the activation of Akt is its translocation to the plasma membrane and its phosphorylation at T308 and S473 followed by translocation to nuclei. Subcellular distribution of components of mTORC and mTORC2 complexes has been elusive. Recent studies have shown strong colocalization of Rictor and mTOR with the ER, but weaker colocalization with plasma membranes. mTORC2-dependent phosphorylation of Akt at S473 takes place on the surface of the ER [Boulbes et al., 2011 and references therein]. These observations strongly support the involvement of assembly of multiprotein signaling complexes in Epac1 induced activation of mTORC2. However, further studies are needed to understand the kinetics and mechanics of the assembly of this signaling complex. The nucleation of these complexes is facilitated by the continuous proximity of intracellular organelles within the cytoplasmic milieu. A schematic representation of mTORC2 activation involving multiprotein signaling complexes comprising of Epac1, PDE3, Raptor, Rictor, and AKAP is shown in Figure 9.

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