Carboxy-terminal modulator protein induces Akt phosphorylation and activation, thereby enhancing antiapoptotic, glycogen synthetic, and glucose uptake pathways

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Submitted 9 November 2006; accepted in final form 3 July 2007

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Akt is then recruited to the membrane through the binding of its PH domain and these 3-phosphoinositides (6, 19). When Akt reaches the membrane, two phosphorylation sites (Thr308 and Ser473) of Akt are phosphorylated by phosphoinositide-dependent kinase (PDK)-1 (45, 46) and by PDK-2 (18, 28, 38, 39, 50), respectively. Recent reports have indicated that PDK-2 is very likely to be the mammalian target of rapamycin (mTOR)-Rictor complex (24, 43). Once phosphorylated at both sites, Akt is fully activated.

Recently, several proteins that modify the Akt activation state via direct binding to Akt (2, 4, 14, 34, 40) have been identified. Among them, carboxy-terminal modulator protein (CTMP) (23, 34) was reported to bind to the carboxy-terminal regulatory domain of Akt and to inhibit its activation. In addition, it was shown that stable CTMP overexpression in AKT8 cells inhibits tumor growth in nude mice. However, Akt is related not only to cell proliferation but also to antiapoptosis (12, 29, 35) and glucose metabolic processes such as glycogen synthesis, gluconeogenesis, glycolysis, and glucose uptake (16, 21, 25, 32, 47, 48, 51). The effects of CTMP on insulin signaling and on glucose metabolism have not previously been examined. Therefore, the initial aim of this study was to determine whether CTMP is a molecule involved in the insulin sensitivity of glucose metabolic processes, via its effect on Akt activity.

However, in our study, overexpression of CTMP was demonstrated to obviously enhance Akt phosphorylation regardless of whether a transient or a stable expression system was used. To our surprise, this is quite the opposite of previously reported results. When endogenous CTMP was suppressed by small interfering RNA (siRNA), Akt phosphorylations were reduced. Moreover, several cellular functions downstream from Akt, such as antiapoptotic and glucose metabolic processes, were shown to be enhanced by CTMP. We thus conclude that CTMP is a positive regulator of Akt.

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MATERIALS AND METHODS

Antibodies and immunoblotting. Anti-CTMP antibody was raised by immunizing rabbits with the keyhole limpet hemocyanin-conjugated 17 carboxy-terminal amino acids of CTMP and affinity purified on Affi-Gel-10 (Bio-Rad, Hercules, CA) columns to which the corresponding peptide had been coupled. The animal protocol was approved by the University of Tokyo Ethics Committee for Animal Experiments and strictly adhered to the guidelines for animal experiments of the University of Tokyo. Anti-Akt antibody was described previously (37). Anti-phospho-Thr308 Akt, anti-phospho-Ser473 Akt, anti-phospho-Ser256 Foxo1, and anti-phospho-Ser9 GSK-3β were purchased from Cell Signaling Technology (Beverly, MA). Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Immunoblotting was carried out with enhanced chemiluminescence (ECL Detection Kit, Amersham), and representative blots were obtained by exposing the films. The bands were quantitatively analyzed with Molecular Imager FX (Bio-Rad) without exposure of the films.

DNA constructs, expression vectors, and adenoviruses. Complete cDNA of human CTMP was amplified by PCR from a cDNA library of HeLa cells with a primer set based on the reported sequence (34). Amino-terminal FLAG tag was added, also by PCR. All the sequences were confirmed with a CEQ-2000XL DNA sequencer (Beckman Coulter, Tokyo, Japan). To prepare the plasmid expression vector, cDNA was subcloned into pcDNA3.1(-) (Invitrogen, Carlsbad, CA). To prepare the adenovirus for CTMP expression, the DNA construct was subcloned into a pAdexCAwt cosmid cassette and transfected with the parental viral genome into HEK293 cells as described previously (27). Adenovirus vectors for carboxy-terminally myc-tagged wild-type (WT) Akt, carboxy-terminally myc-tagged myr-Akt, and Escherichia coli β-galactosidase (LacZ) were described previously (27, 52). Adenoviruses were concentrated and purified by ultracentrifugation in a CsCl gradient as described previously (33).

Cell culture, adenoviral infection, and serum starvation. COS-1, HepG2, HeLa, and NIH3T3 cells were cultured in DMEM containing 4.5 g/l glucose, penicillin-streptomycin (Pen/Strep), and 10% fetal calf serum (FCS). HepG2, HeLa, and NIH3T3 cells were cultured in DMEM containing 4.5 g/l glucose, penicillin-streptomycin (Pen/Strep), and 10% fetal calf serum (FCS).

Fig. 1. Effects of transiently overexpressed carboxy-terminal modulator protein (CTMP) on Akt phosphorylation and activation. A: COS-1 cells were infected with various titers [multiplicity of infection (MOI) = 0.1–10] of LacZ or CTMP adenovirus. Phosphorylations of endogenous Akt at Thr308 or Ser473 without or with 50 nmol/l EGF stimulation were assayed by Western blotting with phospho-specific antibodies. Representative bands from duplicate experiments are shown. The multiplicity of CTMP expression at its mRNA level or protein level was assayed, and is shown at bottom. The protein level is indicated by inequality, because our antibody could not detect endogenous CTMP [not detected (ND)]. LacZ did not influence Akt phosphorylations, regardless of its MOI or EGF stimulation. CTMP enhanced these phosphorylations in a dose-dependent manner in the nonstimulated (basal) state. CTMP did not suppress Akt phosphorylations even when the cells were stimulated with EGF. B: HepG2 cells were infected with CTMP or LacZ (LZ) with adenoviral vectors. Phosphorylations of endogenous Akt at Thr308 (left) or Ser473 (right) in HepG2 cells without (no stim) or with 100 nmol/l insulin stimulation were assayed as in A and quantified in experiments performed in triplicate. Values are mean ± SE fold increase over LacZ without insulin. The enhancing effect of CTMP on Akt phosphorylation was also observed in HepG2 cells. C: Akt phosphorylation and kinase activity were assayed in HEK293 cells. HEK293 cells were transfected with the expression vector containing CTMP cDNA and were coexpressed with wild-type Akt with adenoviral vectors. Left: overexpression of CTMP and CTMP-induced increases in phosphorylations of Akt and GSK-3β. Right: CTMP-induced increase in Akt kinase activity with or without vanadate stimulation. Values are mean ± SE fold increase over vehicle without stimulation. ANOVA confirmed the significance of the enhancing effect of CTMP on Akt activity. D: Akt phosphorylation and kinase activity were assayed in COS-1 cells transfected with the expression vector containing CTMP cDNA. Right: overexpression of CTMP and CTMP-induced increases in phosphorylations of Akt. Left: CTMP-induced increase in Akt kinase activity with or without vanadate stimulation. Values are mean ± SE fold increase over vehicle without stimulation. ANOVA confirmed the significance of the enhancing effect of CTMP on Akt activity. CTMP or vehicle was coexpressed with wild-type Akt with adenoviral vectors. The effect of CTMP on Akt phosphorylations was enhanced. IP, immunoprecipitation.
calf serum (FCS) under a 5% CO2 atmosphere at 37°C. Adenoviral infection and serum starvation were carried out simultaneously for these cell types 24 h before the following experiments, i.e., the cells were incubated with adenovirus for 1 h, washed once with serum-free DMEM containing 0.2% BSA, and then incubated with that serum-free medium for 24 h. NIH3T3 cells stably transfected with CTMP constructs (pcDNA) were selected and maintained in DMEM containing 500 mg/l Geneticin. 3T3-L1 adipocytes were prepared from 3T3-L1 fibroblasts as described previously (41). Four days after the constructs (pcDNA) were selected and maintained in DMEM containing 0.2% BSA, and then incubated with that serum-free medium, these cell types 24 h before the following experiments, i.e., the cells were incubated with adenovirus for 1 h, washed once with serum-free DMEM containing 0.2% BSA, and then incubated with that serum-free medium for 24 h. NIH3T3 cells stably transfected with CTMP constructs (pcDNA) were selected and maintained in DMEM containing 0.2% BSA, and then incubated with that serum-free medium.

Plasmid transfection and Akt kinase assay. COS-1 cells or HEK293 cells were maintained in DMEM supplemented with 10% FCS (Life Technologies) and 50 U/ml Pen/Strep (GIBCO). Transfections were performed with the calcium phosphate technique (34). To obtain a cell line stably overexpressing CTMP, selection was carried out with G418 (Gibco BRL) and transient transfection was carried out with Lipofectamine.

After cells were first serum starved for 12 h in serum-free DMEM, they were stimulated with 100 μM pervanadate for 10 min at 37°C. Next, the cells were lysed in lysis buffer [mM: 50 Tris·HCl (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 sodium orthovanadate, 1 β-glycerophosphate, and 0.2 PMSF, with 1% Triton X-100] and centrifuged. The supernatants were then immunoprecipitated with anti-myc antibodies and protein G Sepharose beads. The immunoprecipitates were washed three times with lysis buffer and twice with kinase assay buffer [mM: 50 Tris·HCl (pH 7.5), 10 MgCl2, 1 EGTA, and 1 dithiothreitol]. Beads were resuspended in 45 μl of kinase assay buffer, and reactions were initiated by the addition of 5 μl of an ATP mixture containing 5 μM nonradioactive ATP, 2 μCi of [γ-32P]ATP (4,000 Ci/mmol), and 5 μM Cross tide and then incubated for 30 min at 30°C. The kinase reaction mixture was spotted onto a P81 filter (Whatman), the filters were washed three times with 0.5% (v/v) orthophosphoric acid, and the radioactivity remaining on the filters was measured with a BASStation2000 (Fujiﬁlm).

Adenoviral gene transfer and Akt kinase assay. COS-1 cells in 10-cm dishes, 24 h after serum starvation and viral infection [multiplicity of infection (MOI) 3], were stimulated without or with 50 ng/ml EGF for 10 min, washed once with ice-cold PBS, and lysed with 1 ml/dish of lysis buffer from an Akt kinase assay kit (Cell Signaling Technology). Insoluble materials were eliminated by centrifugation, and 200 μl of the supernatants was immunoprecipitated with 10 μl of immobilized Akt monoclonal antibody. Subsequent steps were carried out according to the manufacturer’s instructions.

RNA silencing of CTMP in HeLa cells. HeLa cells were seeded at 1 × 104 cells/well onto 24-well plates. At 24 h after seeding, siRNAs were transfected, i.e., 0.25 μg of siRNA of CTMP (target sequence: AACGATGGAAATATATACAT) or lamin (control siRNA, target sequence: AACTGGACTTCGAAAGAACA), 1.5 μl of RNAiFect transfection reagent (Qiagen, Tokyo, Japan), and DMEM containing 10% FCS (total 100 μl/well) were mixed, incubated for 10 min, and dropped into each well. At 36 h after transfection, cells were serum starved. After 12 h of starvation, the cells were stimulated without or with 1 μM insulin for 15 min and lysed with the lysis buffer from an RNAqueous kit (Ambion, Austin, TX) or Laemmli buffer. Total RNA was purified with the RNAqueous kit according to the manufacturer’s instructions. RNA was then reverse transcribed with Superscript III (Invitrogen). cDNA levels of CTMP and GAPDH (internal standard) were quantiﬁed with LightCycler and DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan). The primer sequences used were TCTGAGAAGCTTCTTACGTCGAA, CTAACACTCTGACATT for CTMP and GAAGGTGAAGGTCGGAGTC and GAAGATGTTAGGGATGTTCC for GAPDH.

Subcellular fractionation. In a 15-mm dish, COS-1 cells (24 h after virus infection at MOI 3) were washed with PBS and scraped off into 2 ml of HES (mM: 20 HEPES pH 7.4, 1 EDTA, 250 sucrose) containing protease/phosphatase inhibitors (mM: 1 PMSF, 1 orthovanadate, 40 β-glycerophosphate, 50 NaF). The cell suspension was homogenized with 10 strokes of a Teflon homogenizer and centrifuged at 600 g for 15 min to eliminate the nuclear fraction. The supernatant was ultracentrifuged at 250,000 g for 90 min, and the pellet was rinsed once with HES and lysed with 100 μl of lysis buffer (mM: 50 Tris pH 7.4, 100 NaCl, 10 EDTA, with 10% glycerol and 1% thovanadate, 40 μCi of [γ-32P]ATP, 5 μM Coomassie blue, and 5 μM Cross tide) and then incubated for 30 min at 30°C. The kinase reaction mixture was spotted onto a P81 filter (Whatman), the filters were washed three times with 0.5% (v/v) orthophosphoric acid, and the radioactivity remaining on the filters was measured with a BASStation2000 (Fujiﬁlm).

Adenoviral gene transfer and Akt kinase assay. COS-1 cells in 10-cm dishes, 24 h after serum starvation and viral infection [multiplicity of infection (MOI) 3], were stimulated without or with 50 ng/ml EGF for 10 min, washed once with ice-cold PBS, and lysed with 1 ml/dish of lysis buffer from an Akt kinase assay kit (Cell Signaling Technology). Insoluble materials were eliminated by centrifugation, and 200 μl of the supernatants was immunoprecipitated with 10 μl of immobilized Akt monoclonal antibody. Subsequent steps were carried out according to the manufacturer’s instructions.

Fig. 2. Positive effect of CTMP on Foxo1 and GSK-3β phosphorylations in HeLa cells. HeLa cells were infected with adenovirus to overexpress LacZ (LZ) or CTMP (CT) with wild-type Akt and stimulated without or with 100 nM insulin for 15 min. A: overexpressed Akt was conﬁrmed with Western blotting using anti-myc antibody (top). Phosphorylation of overexpressed Akt at Thr308 was assayed by Western blotting (middle), and phosphorylation levels were quantiﬁed (bottom). B and C: phosphorylation of Ser256 of Foxo1 and Ser9 of GSK-3β were assayed by Western blotting with the respective phospho-speciﬁc antibodies (B and C, top, respectively), and phosphorylation levels were quantiﬁed (B and C, bottom, respectively).
without or with 50 ng/ml EGF or 100 nM sodium orthovanadate for 10 min, washed once with ice-cold PBS, and lysed with lysis buffer (PBS containing 1% Nonidet P-40, 0.35 mg/ml PMSF, and 100 mM sodium orthovanadate). Insoluble materials were eliminated by centrifugation, and the supernatants were immunoprecipitated with anti-phosphotyrosine antibodies and protein G Sepharose. The PI3-kinase activity in the immunoprecipitants was measured as described previously (27).

UV-B irradiation and MTT assay of HeLa cells. HeLa cells were plated onto a 96-well culture plate at a density of $1 \times 10^4$ cells/well. At 24 h after seeding, the cells were infected with adenoviruses at an MOI of 3 for 1 h. After infection, the cells were washed once with serum-free DMEM containing 0.2% BSA and incubated in the same medium for 12 h. The medium was then replaced with PBS, and the plate was irradiated with UV-B (wavelength = 312 nm, energy = 8 mW/cm²; DT-20MCP UV illuminator, ATTO, Tokyo, Japan) for 6 min from the bottom face of the plate. The PBS was then replaced with 100 µl of serum-free DMEM containing 0.2% BSA, and the cells were incubated at 37°C. At 8 h after the irradiation, 10 µl of 12 mM 4,5-dimethylthiazol-2-yI-2,5-diphenyltetrazolium bromide (MTT) solution in PBS was added to each well, and the 37°C incubation was continued for 4 h. One hundred microliters of 10 mM HCl containing 10% SDS was added to each well. After vigorous mixing of each well by pipetting, the plate was incubated at 37°C for 2 h, each well was pipetted again, and absorbance at 570 nm was measured with a microplate reader. Each treatment combination (virus and UV irradiation) was examined four times ($n = 4$).

2-Deoxyglucose uptake assay. 3T3-L1 adipocytes in 24-well culture plates were infected with adenovirus at an MOI of 100. At 45 h after infection, the cells were washed once with DMEM containing 0.2% BSA and incubated in that medium for 3 h for serum starvation.
Next, glucose-free incubation was performed for 45 min in Krebs-Ringer phosphate buffer (8). Cells were then incubated with 0, 1, 10, or 100 nM insulin for 15 min, and 2-deoxy-o-[3H]glucose uptake during the subsequent 4 min was measured as described previously (3). Each treatment combination (virus and insulin) was examined twice.

Glycogen synthesis assay. 3T3-L1 adipocytes in 24-well culture plates were infected with adenovirus at an MOI of 100 in DMEM containing 4.5 g/l glucose and 10% FCS. At 40 h after infection, the cells were washed once with serum-free DMEM containing 1 g/l glucose and 0.2% BSA and then incubated with the same medium for 5 h. The cells were then incubated with 200 μl of the same medium containing 1.5 μCi/ml of o-[14C]glucose (230–370 μCi/mmole) and stimulated with 0, 0.1, 1, or 100 nM insulin for 3 h. After insulin stimulation, the cells were washed twice with ice-cold PBS and incubated with 200 μl of 10 N KOH at 4°C for 3 h. The cells were then scraped off, collected, and boiled with 2 μg of glycogen for 30 min. The lysate was mixed with 800 μl of ethanol and incubated at −20°C overnight. Tubes were centrifuged at 15,000 rpm for 20 min, and the supernatant was discarded. The glycogen pellets were rinsed once with 80% ethanol, dissolved in 200 μl of water, mixed with 800 μl of ethanol, and incubated again at −20°C overnight. The tubes were centrifuged, the pellets were dissolved in 200 μl of 0.1 N HCl and mixed with ACS II (Amersham Biosciences, Piscataway, NJ), and the incorporated 14C was quantified with a liquid scintillator. Each treatment combination (virus and insulin) was examined twice.

Statistical analysis. Figures 1–8 show means ± SE. To analyze the results of the experiments, Student’s unpaired t-test or two-way ANOVA with replication was used to demonstrate significant differences. With two-way ANOVA, mainly viral and growth hormone stimulation factors were assessed.

RESULTS

Transient CTMP overexpression enhanced Akt phosphorylation and activation in COS-1, HepG2, HEK293, and HeLa cells under both unstimulated and stimulated conditions. We created an expression vector as well as an adenovirus to express amino-terminally FLAG-tagged CTMP in cultured cells. In our subsequent experiments, COS-1, HeLa, and HepG2 cells and 3T3-L1 adipocytes were infected with this virus. We confirmed an ~22-kDa single band in the samples from these infected cells, using either anti-FLAG antibody or anti-CTMP antibody (Fig. 1A, top).

To investigate whether CTMP influences the phosphorylation state of Akt, we infected COS-1 cells with various titers of CTMP or LacZ (control) virus at an MOI of 3 and evaluated phosphorylations of Akt at Thr308 and Ser473 by immunoblotting with phospho-specific antibodies. As shown in Fig. 1A, CTMP enhanced endogenous Akt phosphorylations at both sites, in a viral dose-dependent manner, in the basal state, while control LacZ virus had no effect. The maximal level of Akt phosphorylation by CTMP overexpression was comparable with that induced by EGF stimulation. EGF stimulation had a small additional effect on Akt phosphorylation in CTMP-overexpressing cells (Fig. 1A, right 2 lanes), suggesting that high CTMP expression could induce nearly maximal Akt phosphorylation.

Subsequently, to confirm this phenomenon, we investigated the effects of CTMP overexpression on Akt phosphorylation by expressing CTMP in other types of cultured cells such as HepG2 and HeLa cells and 3T3-L1 adipocytes. In HepG2 cells, which are insulin-sensitive cells, CTMP produced a similar enhancement of Akt phosphorylation (Fig. 1B). Infection of HeLa cells produced a similar result (data not shown).

To rule out the possibility that the difference in overexpression systems between plasmid transfection and adenoviral gene transfer was responsible for the different results, CTMP was transiently overexpressed in HEK293 and COS-1 cells (Fig. 1, C and D, respectively) with an expression plasmid containing CTMP cDNA and the calcium phosphate method. In HEK293 cells, CTMP overexpression increased Akt phosphorylation as shown by immunoblotting with phospho-specific antibodies under both basal and vanadate-treated conditions (Fig. 1C, left). The phosphorylation of GSK-3β was also markedly increased by CTMP overexpression. Indeed, Akt kinase activity was increased by CTMP overexpression under both basal and vanadate-treated conditions (Fig. 1C, right). Very similar effects were also observed in COS-1 cells transfected with the CTMP expression plasmid (Fig. 1D). These results strongly suggest that CTMP overexpression increases Akt phosphory-
CTMP expression induced phosphorylations of Foxo1 and GSK-3β in HeLa cells. In the following experiments, we investigated the effects of CTMP on signals downstream from Akt. First, we examined phosphorylations of well-known Akt substrates, Foxo1(5, 31) and GSK-3β (11, 49), using their respective phospho-specific antibodies. Insulin induced both Foxo1 and GSK-3β phosphorylation in HeLa cells overexpressing Akt (Fig. 2, lane 1). Co-overexpression of CTMP enhanced the phosphorylation of both Foxo1 and GSK-3β in the basal state, compared with that of LacZ (Fig. 2, lane 2), although no significant difference was observed in the presence of insulin stimulation.

Stable overexpression of CTMP enhances phosphorylations of Akt and GSK-3β in NIH3T3 cells. To exclude the possibility that the difference between stable and transient overexpressions is responsible for the different results, CTMP was stably overexpressed in NIH3T3 cells by expression plasmid transfection followed by G-418 selection. As shown in Fig. 3A, two cell lines overexpressing CTMP (CTMP1 showing higher expression level than CTMP2) were prepared, and the expression level of Akt was confirmed to be unchanged. While basal Akt phosphorylation was not significantly altered in either CTMP-overexpressing cell line (Fig. 3B, left 3 lanes), Akt phosphorylation in response to vanadate stimulation was markedly enhanced in the CTMP-overexpressing cells (Fig. 3B, right 3 lanes). The phosphorylation of GSK-3β was also demonstrated to be significantly increased by stable CTMP overexpression under both unstimulated and vanadate-stimulated conditions (Fig. 3C). These results indicate that stable overexpression of CTMP increases Akt activity under vanadate-stimulated conditions.

siRNA of CTMP inhibited Akt phosphorylation. Next, we suppressed endogenous CTMP expression with siRNA to examine its physiological functions. The mRNA level of CTMP was determined by real-time PCR and standardized with that of GAPDH. siRNA decreased CTMP transcription to approximately one-fifth of that in HeLa cells transfected with lamin siRNA [lamin 1.00 ± 0.27, CTMP 0.21 ± 0.01 (arbitrary units); P = 0.002], although inhibition of CTMP protein expression could not be examined because the CTMP antibody was not sufficiently sensitive to detect endogenous CTMP protein in HeLa cells. Under these conditions, phosphorylations of Akt were partially but significantly suppressed at both Thr380 and Ser473 sites by CTMP siRNA to a greater extent than by lamin siRNA, as shown in Fig. 4A, top. Akt kinase activity was also slightly but significantly suppressed by treatment with CTMP siRNA (Fig. 4B).

CTMP enhanced membrane localization of Akt without affecting PI3-kinase activity. To elucidate the mechanism underlying the positive effect of CTMP on Akt activation, we prepared the membrane fraction of COS-1 cells overexpressing CTMP or control LacZ by adenoviral gene transfer at an MOI of 3. As shown in Fig. 5A, left, immunoblotting revealed that overexpression of CTMP markedly increased the amount of Akt in the membrane fraction in the basal state and also slightly but significantly increased that in the EGF-stimulated state.

Fig. 5. Mechanisms of CTMP effect on Akt. A: COS-1 membrane fraction was purified with ultracentrifugation and then electrophoresed and immunoblotted (IB) with anti-Akt antibody. CTMP markedly increased membrane-localized Akt, especially in the basal state. The calculated ratio of membrane Akt to total Akt is shown at right. The experiment was done in triplicate. The graph shows means ± SE, and 1 band representative of the 3 is presented. B: COS-1 cells were stimulated without or with 50 ng/ml EGF or 100 nM orthovanadate, and anti-phosphotyrosine-immunoprecipitated phosphatidylinositol 3-kinase (PI3-kinase) activity in the cell lysate was assayed. The graph shows the fold increase ± SE over LacZ without stimulation on a logarithmic scale. CT, CTMP. Each experiment was done 3 times, and 1 spot representative of the 3 is shown at top. PI3-kinase activity was unchanged by CTMP expression, regardless of stimulation. C: COS-1 expressing LacZ or CTMP (CT) were stimulated without or with EGF for 10 min and then incubated without or with 20 nM LY-294002 (LY) for 2 min. Akt phosphorylations at Thr380 were quantified in experiments conducted in triplicate, and representative bands are shown at top. The graph shows mean ± SE fold increases over LacZ without insulin. The enhancing effect of CTMP on Akt phosphorylations was reversed within 2 min of LY-294002 incubation.
The ratio of membrane Akt to total Akt was calculated and is shown in Fig. 5A, right.

To exclude the possibility that the effects of CTMP on Akt are mediated by PI3-kinase activation that is upstream from Akt, we assayed the PI3-kinase activity of anti-phosphotyrosine immunoprecipitants from LacZ- or CTMP-expressing COS-1 cells. As shown in Fig. 5B, PI3-kinase activity did not differ between samples from LacZ-expressing cells and those expressing CTMP.

However, it was shown that treatment with LY-294002 obviously attenuated the Akt phosphorylation induced by either CTMP overexpression or EGF stimulation within 2 min (Fig. 5C). Thus it is likely that CTMP does enhance Akt phosphorylation, but at least basal level PI3-kinase activity is necessary.

CTMP expression with Akt rescued HeLa cells from UV-B irradiation-induced apoptosis. One of the well-known functions of Akt is antiapoptosis. Therefore, we investigated whether CTMP overexpression produces an antiapoptotic effect on cultured cells. HeLa cells, 12 h after adenoviral infection, were irradiated with UV-B. Cellular viability after irradiation was assayed with the MTT assay. As shown in Fig. 6, expression of CTMP alone tended to increase cellular viability compared with LacZ, but the difference was not significant. When Akt was coexpressed, CTMP showed a marked and significant antiapoptotic effect. myr-Akt, a well-known constitutively active type of Akt, also showed an apparent antiapoptotic effect.

CTMP expression in 3T3-L1 adipocytes modestly enhanced glucose uptake. Akt activation is known to induce glucose uptake of adipocytes via translocation of GLUT4 to the plasma membrane. We investigated the effect of CTMP overexpression on the glucose transport activity of 3T3-L1 adipocytes (Fig. 7). The CTMP overexpression level in 3T3-L1 adipocytes is much lower, even with the adenoviral transfer system, than in other cell lines such as COS-1 or HepG2. Thus slightly increased Akt phosphorylation was observed only in the WT Akt-overexpressing cells in the basal state. Similarly, slightly increased GSK-3β phosphorylation was observed only in LacZ cells under basal conditions. While myr-Akt induced a fivefold increase in glucose uptake in the absence of insulin stimulation (30), CTMP overexpression induced a relatively mild (1.2-fold over that of LacZ) but significant increase in uptake. Insulin

Fig. 6. 4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay of UV-B-irradiated HeLa cells. HeLa cells in 96-well culture plates were infected with adenovirus in 6 combinations: LacZ (LZ), CTMP, wild-type (WT) Akt, both WT Akt and CTMP, myr-Akt, and both myr-Akt and CTMP. The MOI was 10 for each virus. At 12 h after infection cells were irradiated with UV-B, and 8 h after irradiation MTT was added. MTT uptake was assayed by absorption at 570 nm. Some of the t-test results are shown in the table (bottom).

Fig. 7. 2-Deoxyglucose uptake assay in 3T3-L1 adipocytes. 3T3-L1 adipocytes were infected with adenovirus in 6 combinations, as described in Fig. 5, at an MOI of 100 for each virus. The phosphorylations of Akt and GSK-3β were investigated for each of these combinations and are shown at top. The blot is representative of 4 independent experiments, and the CTMP-induced increase in Akt phosphorylation was observed when WT-Akt was compared with WT-Akt + CTMP. A CTMP-induced increase in GSK-3β phosphorylation was observed in the comparison between LacZ and CTMP. No such significant differences were seen under the insulin-stimulated conditions (data not shown). 2-deoxy-D-[3H]glucose uptakes with 0, 1, 10, and 100 nM insulin stimulation for 15 min were assayed twice each and are presented graphically at bottom. Repeated-measures 2-way ANOVA was conducted to detect the significance of differences in glucose uptake between pairs of viral conditions, and several of the results are shown in the table (middle).
dose-dependently increased glucose uptake. Comparison of both CTMP and WT Akt overexpression with Akt overexpression alone, in response to stimulation with each of the indicated concentrations of insulin, showed the additional augmenting effect of CTMP on glucose uptake to be significant, although also of a modest degree (20% increase). This observation suggests that the effects of CTMP and Akt on glucose uptake are additive and more significant than that of the LacZ control.

**CTMP expression in 3T3-L1 adipocytes enhanced glycogen synthesis.** Since Akt is also known to enhance glycogenesis via inhibition of GSK-3β, the effect of CTMP on glycogenesis in 3T3-L1 adipocytes was investigated (Fig. 8). With insulin stimulation, glycogen synthesis increased in a dose-dependent manner in control LacZ-infected cells. Since the effect of CTMP plus Akt was observed to differ between low-dose and high-dose insulin conditions, statistical analyses were performed separately for the low- and high-dose groups. CTMP expression significantly enhanced glycogen synthesis. The magnitude of this enhancement was high when cells were stimulated with low concentrations of insulin (0.1 and 1 nM). However, the synergistic effect of CTMP and WT Akt overexpressions on glycogen synthesis was observed only in the nonstimulated state. On the other hand, constitutively active Akt (myr-Akt) markedly increased glycogen synthesis in the basal state. However, when stimulated with a high concentration of insulin (100 nM), myr-Akt expression suppressed glycogen synthesis. This finding suggests that when the Akt signaling pathway is highly stimulated for a long period, some negative feedback suppression(s) is exerted on the glycogen synthetic pathway (insulin desensitization). Comparing 3T3-L1 adipocytes expressing solely WT Akt and those coexpressing WT Akt and CTMP, glycogen synthesis was higher in the latter when the cells were unstimulated or stimulated with a low concentration of insulin (0 or 0.1 nM). In contrast, glycogen synthesis was higher in the former when cells were stimulated with higher concentrations of insulin (1 and 100 nM). This is attributable to the same desensitization mechanism.

**DISCUSSION**

Akt is activated by 3-phosphoinositides and PDKs, but there exist several proteins that bind to Akt and modulate its activation state (2, 4, 14, 34, 40). CTMP was reportedly shown to bind to the carboxy terminus of Akt and to inhibit its phosphorylation and activation. However, our repeated careful experiments demonstrated that CTMP enhanced the phosphorylation and activation of Akt and its downstream signal pathways, regardless of whether a transient or a stable expression system was used. Furthermore, siRNA-mediated suppression of CTMP inhibited Akt phosphorylation. However, this suppression was minimal, possibly suggesting that the presence of a substantial basal level of Akt is required for CTMP to be functional in HeLa cells. These findings are quite contrary to those of a previous report (34), but this discrepancy is not attributable to differences in the cell line or the method used for transfection, because we utilized various cell lines and obtained essentially the same results. Thus we cannot explain the different conclusions.

CTMP is reportedly located mainly on the plasma membrane. Indeed, we found that overexpression of CTMP markedly enhances membrane localization of Akt. From this finding, we speculate that the mechanism underlying the enhancing effect of CTMP on Akt phosphorylation involves translocation of Akt to the plasma membrane. It is well established that, with targeting to the membrane, Akt conformational change occurs such that Thr308 and Ser473 are presented to the outside of the Akt molecule and phosphorylated by PDKs (36).

In the aforementioned previous report, the authors suggested that CTMP on the plasma membrane binds to Akt in the basal state and that CTMP binding to Akt does not completely block Akt phosphorylation in the stimulated state, but rather makes it more difficult. After phosphorylation is achieved, Akt would presumably disassociate from CTMP. However, this theory is somewhat difficult to understand, because it is unclear how CTMP suppresses Akt activation in the stimulated state, despite dissociating from Akt in that state.

Our interpretations appear to be more reasonable and are easier to understand: Akt is located mainly in the cytosol in the basal state, and CTMP, which is always on the plasma membrane, recruits Akt from the cytosol to the plasma membrane, leading to the phosphorylation of Thr308 and Ser473 of Akt by PDKs. Indeed, CTMP-induced membrane translocation of Akt was observed in the presence of wortmannin. We also confirmed that CTMP does not influence PI3-kinase activity, suggesting that the effects of CTMP on Akt are direct. However, the PI3-kinase inhibitor LY-294002 dephosphorylates CTMP-induced Akt phosphorylation. This finding indicates that CTMP-induced Akt phosphorylation is maintained by basal PI3-kinase activity and/or baseline concentrations of 3-phos-

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**Fig. 8.** Glycogen assay in 3T3-L1 adipocytes. 3T3-L1 adipocytes were infected with adenovirus in 6 combinations, as described in Fig. 5, at an MOI of 100 for each virus. [U-14C]glucose incorporations into glycogen with 3h of 0, 0.1, 1, or 100 nM insulin stimulation were each assayed twice. Repeated-measured 2-way ANOVA was conducted to detect the significance of differences between pairs of viral conditions, and several of the results are shown in the table. The comparison between CTMP and WT-Akt + CTMP, and that between WT-Akt and WT-Akt + CTMP, showed the effects of interactions. Therefore, data obtained with the lower (0, 0.1 nM) and higher (1, 100 nM) doses of insulin were analyzed separately for these 2 cases.

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<tr>
<th>comparison</th>
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<td>LZ</td>
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<td>LZ</td>
<td>WT-Akt</td>
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<td>WT-Akt</td>
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<td>myr-Akt</td>
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phosphoinositides. In addition, as shown in Fig. 5, the total amount of Akt may be slightly increased. We speculate that CTMP induces translocation of Akt to the membrane, and that membrane-bound Akt thereby becomes susceptible to phosphorylation by upstream kinases such as PDK-1, which requires PI3-kinase activation, and phosphorylation of Thr308 and Ser473 by PDKs. It is also likely that CTMP increases the stability of Akt, possibly because of the increased amount of Akt.

To evaluate whether CTMP influences the antiapoptotic function of Akt, we combined UV-B irradiation (20) and MTT assay (15) in HeLa cells. The result showed clearly that CTMP enhances antiapoptosis, especially with Akt coexpression. In the previous report, the authors showed stable expression of CTMP in AKT8 tumor cells to inhibit tumor growth. Their experiment was designed to observe tumor growth, which is a more integrated cellular process than a specific antiapoptotic function. Moreover, AKT8 cells highly express constitutively active Akt, and the apoptotic signal in these cells may differ from that in physiological cells. On the other hand, our experiment induced relatively short-term CTMP expression in HeLa cells, in which Akt signaling would be nearer to physiological conditions. Therefore, we believe that our results reflect the physiological function of CTMP, at least as regards the antiapoptotic effect.

Akt reportedly plays critical roles in insulin-induced glucose metabolism, i.e., glycogen synthesis and glucose uptake. As for glycogen synthesis, Akt has been established as directly phosphorylating and inactivating GSK-3β, which results in activation of glycogen synthase. As for glucose uptake, constitutively active Akt reportedly induces GLUT4 translocation, thereby increasing glucose uptake. Our experiments revealed that CTMP enhances both of these pathways, indicating that CTMP may function as an insulin-sensitizing molecule for glucose metabolism, possibly in relation to insulin sensitivity. In the glycogen synthesis assay, expression of myr-Akt, or coexpression of CTMP and Akt, induced insulin desensitization. This phenomenon may be attributable to glycogen synthesis not being regulated solely by the insulin/Akt/GSK-3β/glycogen synthase pathway but also by the insulin/protein phosphatase-1 pathway (10, 42), which may be suppressed by chronic Akt activation. At a minimum, the desensitization occurred in response to both myr-Akt expression and Akt-CTMP coexpression, which is consistent with our finding that CTMP strongly activates coexpressed Akt. On the other hand, the effects of CTMP on insulin-induced glucose uptake were modest, although statistically significant. From this finding, we speculate that CTMP leads to Akt activation mainly on the plasma membrane, while for efficient GLUT4 translocation activation of Akt in other intracellular compartments may be critical.

Recently, we (2) and another group (17) have identified a novel 200-kDa protein that binds to the carboxy terminus of Akt and markedly enhances Akt phosphorylation. This protein was termed Akt phosphorylation enhancer (APE), or Girdin. We have the impression that APE/Girdin and CTMP bind to the carboxy terminus of Akt, the mechanisms underlying the increases in Akt phosphorylation may be similar. We speculate that their binding to the carboxy terminus of Akt would induce conformational changes in Akt, thereby possibly making Akt more easily accessible to PDK-1 and PDK-2. Interestingly, APE/Girdin binds to actin, and CTMP is located at the plasma membrane. Thus both APE/Girdin and CTMP enhance Akt activity by modifying the conformation of the Akt carboxy terminus, but the former may function by interacting with the actin network and the latter at the plasma membrane. Further work is necessary to elucidate the similarities and differences in these proteins.

In summary, our experimental findings on CTMP overexpression and suppression in various cell systems allow us to draw the conclusion that CTMP enhances Akt phosphorylation and activation. The mechanism appears to involve membrane-localized CTMP recruiting Akt from the cytosol to the plasma membrane. CTMP-induced Akt activation results in phosphorylation of Akt substrates. It also activates multiple downstream Akt pathways, including antiapoptotic, glycogen synthetic, and glucose uptake processes. Therefore, CTMP may be involved in cellular antiapoptotic mechanisms and insulin sensitivity.

REFERENCES


