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PRECLINICAL STUDIES

Modulator of the PI3K/Akt oncogenic pathway affects mTOR complex 2 in human adenocarcinoma cells

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Summary

Chaetoglobosin K (ChK) is a natural product that has been shown to promote F-actin capping, inhibit growth, arrest cell cycle G2 phase, and induce apoptosis. ChK also has been shown to downregulate two important kinases involved in oncogenic pathways, Akt and JNK. This report investigates how ChK is involved in the receptor tyrosine kinase pathway (RTK/PI3K/mTORC2/Akt) to the centrally located protein kinase, Akt. Studies have reported that ChK does not inhibit PI3K comparable to wortmannin and does not affect PDK1 activation. PDK1 is responsible for phosphorylation on Akt T308, while mTORC2 phosphorylates Akt S473. Yet, Akt's two activation sites, T308 and S473, are known to be affected by ChK treatment. It was our hypothesis that ChK acts on the mTORC2 complex to inhibit the phosphorylation seen at Akt S473. This inhibition at mTORC2 should decrease phosphorylation at both these proteins, Akt and mTORC2 complex, compared to a known mTOR specific inhibitor, Torin1. Human lung adenocarcinoma H1299 and H2009 cells were treated with IGF-1 or calyculin A to increase phosphorylation at complex mTORC2 and Akt. Pretreatment with ChK was able to significantly decrease phosphorylation at Akt S473 similarly to Torin1 with either IGF-1 or calyculin A treatment. Moreover, the autophosphorylation site on complex mTORC2, S2481, was also significantly reduced with ChK pretreatment, similar to Torin1. This is the first report to illustrate that ChK has a significant effect at mTORC2 S2481 and Akt S473 comparable to Torin1, indicating that it may be a mTOR inhibitor.

Keywords Akt · mTORC2 · Adenocarcinoma · Chaetoglobosin K · Torin1

Introduction

Targeted cancer therapies and personalized medicine are working in a concerted effort to make cancer treatments more efficacious. Many of the overstimulated pathways that charge tumor growth involve receptors that are classified as receptor tyrosine kinases (RTK). There have been advances such as development of tyrosine kinase inhibitors that are able to attenuate a genetically altered pathway and demote tumorigenic activity. For example, erlotinib is an EGFR inhibitor that is used in treatment of lung and pancreatic cancers. [1, 2] EGFR alterations in certain non-small cell lung cancer (NSCLC) lines can account for roughly 10% of total mutations that are activated to increase oncogenic actions. [3] Inhibitors of a protein that is involved with signal transduction downstream of the receptor, PI-3-kinase (PI3K), are currently approved by the FDA, which are idelalisib [4] and copanlisib. [5] Interestingly, many of the RTKs can dimerize and increase the overall activity of the intracellular signals, such as the case with HER2 and EGFR [6]. Furthermore, these pathways are entangled in activity and come together in the RTK/PI3K/mTORC2/Akt signaling cascade.

Akt is a protein kinase that is a key component of several pathway cascades, including integrin, G-protein coupled receptors, IL-2, and the aforementioned RTKs. [7] Akt is phosphorylated at three residues that function in stabilization and activation of the kinase. Akt is stabilized by phosphorylation at the T450 site by the kinase complex mTORC2. [8] Akt is then phosphorylated at the T308 site by PDK1 at the membrane, downstream from the receptor and PI3K. [7, 9] Finally, full activation of Akt is completed at S473 by the mTORC2 complex. [10] Akt activation by PDK1 at T308 is increased by 5-fold over basal activity, while the additional phosphorylation at S473 by mTORC2 increases Akt kinase activity up to 16-fold. [10]

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Several clinical trials have been conducted to test possible therapies that act as dual kinase inhibitors, which affect two different kinases involved in a pathway. [11, 12] A highly characterized example of this type is wortmannin that covalently inhibits PI3K and inhibits mammalian target of rapamycin complex 2 (mTORC2), both of which are involved in the RTK/PI3K/mTORC2/Akt signaling pathway, leading to Akt S473 dephosphorylation. [13, 14] Furthermore, VS-5584 [15] PF-04691502 [16] and gedatolisib [17] are dual kinase inhibitors that have been studied in Phase I and II clinical trials. The benefit from these small molecules is that they may be able to rescind the overactive signaling from reaching the centrally active protein, Akt.

Chaetoglobosin K (ChK) is a natural product indolylcytochalasin with anti-tumor properties that has been shown to have activity on two distinct oncogenic pathways, specifically the Akt and JNK pathways. [18] Furthermore, ChK was found to induce apoptosis, inhibit cytokinesis, [19] and arrest the cell cycle in the G2 phase via a p53-dependent pathway in cancer cell lines. [20, 21] Moreover, ovarian tumor lines with wild-type p53 and mutant p53 were tested with ChK and the treatment inhibited secretion of VEGF through Akt/mTOR signaling. [22] Prior to these latest investigations, it was discovered that ChK had the ability to affect both activation sites at Akt, T308 and S473. [19] It was also found that receptor mediated signaling through PI3K was not inhibited using ChK. [23] PI3K produces the phospholipid, PIP_3, that signals downstream to the proteins, PDK1 and mTORC2. [24, 25] PDK1 phosphorylates Akt T308 and no change in phosphorylation of PDK1 was found in WB-*ras*1 cells treated with ChK. [18] PTEN, the inhibitor for the phospholipid PIP_3, was also found to not be affected by ChK. [18] Since ChK affects Akt S473, which is an effector of mTORC2, we began investigations to find out whether ChK acts on mTOR. mTORC2 is directly upstream from Akt S473, while mTORC1 is downstream of Akt. [25] mTORC1 is a complex of several proteins including mTOR, RAPTOR, mLST8, DEPTOR, and PRAS40 [26] and mTORC2 is comprised of mTOR, RICTOR, mLST8, mSIN1, and DEPTOR. [27] There are two phosphorylation sites for mTOR that distinguish the two different complexes. The autophosphorylation site, S2481, monitors the complex mTORC2, while S2448 is probed for complex mTORC1. [28–30] Two major downstream effectors of mTORC1 are p70S6K and 4E-BP1. [26, 31] Briefly, p70S6K targets the S6 ribosomal protein to launch protein synthesis at the ribosome and 4E-BP1 prevents cap-dependent translation. [27] The inhibition of mTOR by rapamycin decreases phosphorylation of p70S6K, which causes the loss of a negative feedback loop and increases phosphorylation at Akt S473. [32, 33] Both mTORC1 effectors were altered by ChK treatment, which may be due to ChK's effect on Akt, or a direct effect on mTOR, in one or both mTOR complexes. [18] The purpose of this research was to monitor the effects of ChK in human adenocarcinoma cells to determine whether it acts on mTOR in the mTORC2 complex to alter Akt S473 phosphorylation.

Materials and methods

Materials

H1299 and H2009 human lung adenocarcinoma cells were from the American Type Culture Collection (ATCC, Manassas, VA). Chaetoglobosin K was purified from *Diplodia macrospora* at a purity of >97% and provided by H. Cutler. [34] Gibco RPMI-1640 medium, Gibco fetal bovine serum (FBS), L-glutamine, Ponceau S, bovine serum albumin (BSA), methanol, phenylmethylsulfonyl fluoride (PMSF), glacial acetic acid and 10X phosphate buffered saline (PBS), were from Fisher Scientific (Pittsburgh, PA). Sterile dimethyl sulfoxide (DMSO) and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). Akt (#4685), mTOR (#2972), ß-actin (#4970), phospho-Akt (T308, #13038), phospho-Akt (S473, #4060), phospho-Akt (T450, #9267), phospho-mTORC2 (S2481, #2974), anti-rabbit IgG alkaline phosphatase (AP)-link (#7054), anti-rabbit IgG horseradish peroxidase (HRP)-link (#7074), Torin1, calyculin A, and 10X cell lysis buffer were purchased from Cell Signaling Technology (Beverly, MA). IGF-1 was from Bachem Inc. (Torrance, CA) and the only item used to be dissolved in PBS. DMSO was the vehicle for all others. Blotting-grade nonfat dry milk blocker, 10% Tween-20, tris–HCl pH 7.5, nonfat dry milk, 25x alkaline phosphatase color development buffer, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT), protein molecular mass standards, Clarity™ western ECL substrates, all electrophoresis and Trans-Blot® Turbo equipment and consumables were from Bio-Rad (Hercules, CA). Chemicals, reagents, and solvents not specifically mentioned previously were of analytical grade.

Methods

Cell culture

H1299 and H2009 human lung adenocarcinoma cells were grown in RPMI-1640 media supplemented with 2 mM/L L-glutamine and 10% FBS. H1299 cells were used between passages 7–20 and H2009 cells were passaged from 35 to 39. Confluent cells were subcultured by trypsinization, counted via an Accuri C6 flow cytometer, then plated at 5–20% for experimentation or continuation. Cells were incubated in an atmosphere of 5% CO_2 at 37 °C.

Cell protein extraction

Human lung carcinoma cells were grown to 80–90% confluence in 25 cm^2 flasks. Treated cells were washed with 12 mL of PBS and lysed with 300 μL of chilled 1X cell lysis buffer supplemented with 1 mM PMSF for 5 min on ice. Lysed cells were scraped and collected into microcentrifuge tubes. The lysates were subjected to sonication for ten aspirations in a pipette tip. Samples were centrifuged for 10 min at 16000 xg at 4 °C, and the supernatant was aliquoted to microcentrifuge tubes and stored at −20 °C.

Protein concentration assay

Protein concentrations were determined using the Bio-Rad DC protein assay using triplicates for each sample. BSA was used as a standard protein and absorbances were read at 750 nm using a Tecan plate reader.

Western immunoblot assay

4x Laemmli sample buffer was added to equal amounts of protein/lane, followed by proteins separated on 7.5% or 12% polyacrylamide SDS gels, then semi-wet transferred to PVDF membranes by Trans-Blot® Turbo in 7–10 min. Membranes were washed with H_2O, stained with lab-made Ponceau for 2–3 min, washed with water, scanned, then blocked using 4% nonfat dry milk, 0.1% Tween-20, 40 mM Tris pH 7.5 for 1–2 h. Specified primary antibodies were incubated separately with blots in block buffer overnight at 4 °C. Immunopositive bands were detected using alkaline phosphatase or HRP-linked anti-rabbit secondary antibody and development with BCIP/NBT or Clarity ECL substrate mix. Selected blots were reprobed by a brief re-hydration in methanol and washed with water, followed by 1-h incubation in block buffer, then primary antibody incubation and development as described above. If membranes were stripped for reprobing, they were stripped while rocking for 30–40 min at 50 °C (25 mM glycine, 1% SDS, HCl, pH 2.0), and verified via reblocking and incubation with HRP-link antibody, then proceeded as normal for additional probing. For densitometric quantification, dried blots were scanned on a HP Scanjet 4400C scanner and band intensities measured using UN-SCAN-IT software (version 7.1) from Silk Scientific, Inc. (Orem, UT). Chemiluminescence was captured via a Gel Doc XR+ coupled with a CCD camera and the image was analyzed by Image Lab software from Bio-Rad (Hercules, CA).

Akt activity assay

The Cyclex© AKT/PKB kinase Assay/Inhibitor Screening Kit (CY-1168) was used in combination with recombinant human Akt1 (CY-E1168–1) as per the instructions to the kit. DMSO was used as the vehicle for all test compounds at 2.5 μL per well. Samples were read on a FLUOstar Omega plate reader at 450 nm.

Statistics and graphs

The data collected from each experiment were processed through Statistix v8.1. One-way ANOVA was used to compare three or more different groups, or the student t-test was used to compare only two groups. A p-value of less than 0.05 is designated (*), while a p-value less than 0.01 is designated (#). Graphpad 6.1 was used to illustrate the densitometric quantification of the Western blots and were plotted as the mean ± S.D.

Results

Chaetoglobosin K affects phosphorylation of Akt kinase at sites T308, T450, and S473 in H1299 cells, which is comparable to the known mTOR inhibitor, Torin1

ChK has been previously reported to affect Akt at the phosphorylation sites T308 and S473 in WB-*ras* and H1299 cells. [18, 19] However, the T450 site has not been investigated. Torin1 has been extensively characterized for its effects on Akt and has been shown to decrease phosphorylation at S473. [31, 35, 36] While most evidence suggests that Akt T308 is not affected by Torin1, in HeLa cells it was shown to affect T308 in short-term usage with recovery within 48 h. [36] A much higher concentration will begin to have an effect at T308 with other cells types. [31] Torin1 affects the S473 site much more specifically than other PIKK family proteins due to its affinity at mTOR. [12, 31] ChK and Torin1 were compared to each other in H1299 human lung adenocarcinoma cells. The cells were treated with ChK (10 μM) or Torin1 (100 nM) for 90 min at 37 °C to see their effect at all three major phosphorylation sites of Akt (Fig. 1b). A graphical representation of the densitometry quantification of the Western blots is shown (Fig. 1a). Torin1 was statistically significant at Akt T308 and S473 compared to control. ChK was also statistically significant at Akt S473. The dephosphorylation at Akt T308 was evident, although not significant with ChK. Akt T450 was not affected much compared to the other two sites, but Torin1 was statistically significant.

Chaetoglobosin K affects phosphorylation of Akt kinase in IGF-1 stimulated cells similarly to Torin1

To investigate the effects of ChK on Akt kinase further, H1299 cells were stimulated with IGF-1 (50 ng/mL) for 30 min and ChK was used to pretreat the cells to inhibit the

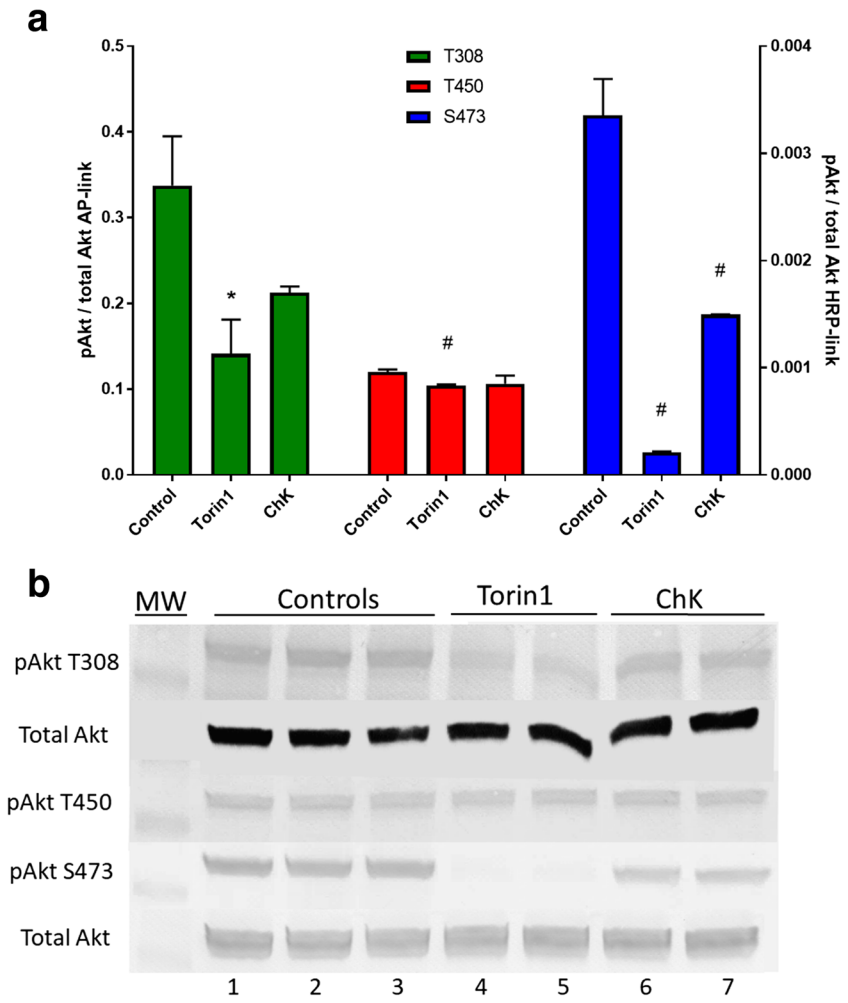

Fig. 1 Effects of ChK and Torin1 on Akt phosphorylation in H1299 cells. Densitometric quantification of replicate bands for each blot is represented in **a** and illustrate the mean ± S.D. (* = $p < 0.05$, # = $p < 0.01$, compared to control). **b** Cells were grown to 80–90% confluence in 25 cm^2 flasks, treated with vehicle (DMSO), Torin1 (100 nM), or ChK (10 μM) for 90 min, then extracted for Western Blot analysis. Akt T308 (top panel), total Akt (second-from-top panel), Akt T450 (middle panel), Akt S473 (second-from-bottom panel), total Akt (bottom panel) were probed as describe in Material and Methods. Treatment groups were DMSO (lanes 1–3), Torin1 (lanes 4–5), ChK (lanes 6–7). Molecular weight (MW) marker is set to the left. For the top two panels, the Akt T308 was stripped, verified no residual antibody remained, reprobed for total Akt using chemiluminescence. T308 was normalized to the chemiluminescent total Akt. Akt T450 and S473 were normalized to the colorimetric total Akt. The graphs and statistical results were virtually identical when Akt S473 was normalized to either total Akt, chemiluminescent or colorimetric (data not shown). Data are a representation of at least two independent experiments. Each individual treatment is at least a $n = 4$

increased phosphorylation at Akt S473. Fig. 2a and b show that ChK significantly inhibited IGF-1 stimulation of Akt S473 phosphorylation while not affecting total Akt. Densitometry quantification (Fig. 2a) showed that ChK+ IGF-1 was significantly lower compared to IGF-1, indicating that ChK modulates phosphorylation of Akt S473 and inhibits IGF-1 stimulation at that site. ChK was then compared to Torin1 in IGF-1 stimulated H1299 cells (Fig. 2c, d). While Torin1 was more potent than ChK at the concentration used, both were able to significantly inhibit IGF-1 phosphorylation at Akt S473 ($p = 0.0106$ IGF-1 vs ChK+IGF-1 and $p = 0.0019$ IGF-1 vs Torin+IGF-1). Further evaluation of Akt kinase at the other two major sites, T308 and T450, showed that both Torin1 and ChK significantly inhibited phosphorylation at Akt T308 (Fig. 2c). Akt T450 was not significantly affected by IGF-1, ChK, ChK+IGF-1, or Torin+IGF-1.

Chaetoglobosin K decreases phosphorylation of Akt kinase in Calyculin A stimulated H1299 cells comparable to Torin1

Calyculin A (12.5 nM) was used to treat H1299 cells in order to inhibit the phosphatases PP1 and PP2a, which increased phosphorylation at Akt T308 and S473 (Fig. 3a, b). Cells were pretreated with either ChK (10 μM) or Torin1 (100 nM) for 1 h then followed by calyculin A for 30 min. ChK was shown to behave in calyculin A treated cells (Fig. 3a) similarly to the

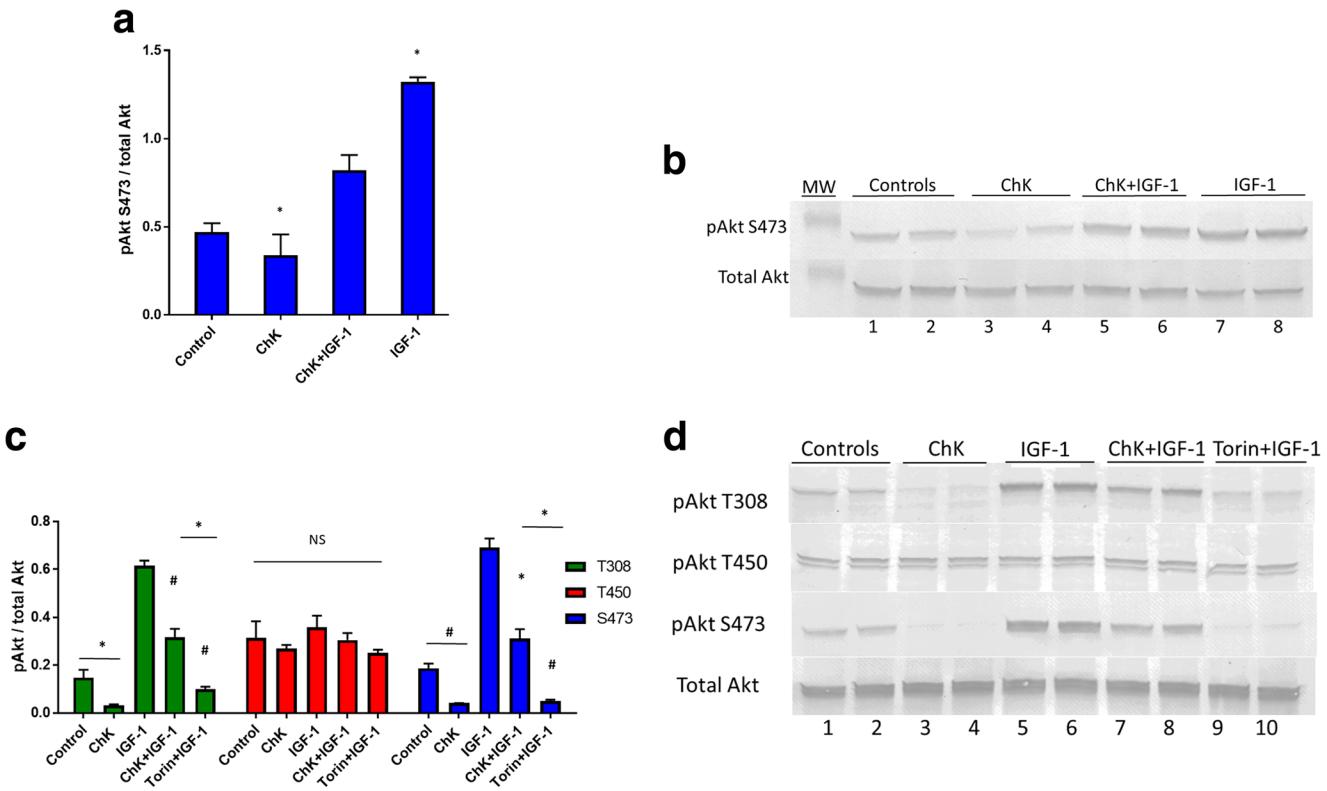

Fig. 2 ChK affects all three sites of Akt kinase similar to Torin1 in IGF-1 stimulated H1299 cells. (**a, b**) H1299 cells were grown to 80–90% confluence in 25 cm2 flasks, treated with vehicle (DMSO/PBS) or ChK (10 μM) for 90 min, or pretreated with ChK or DMSO and stimulated with IGF-1 (50 ng/mL) for 30 min. **b** Cells were extracted for Western blot analysis at Akt S473 and total Akt. Treatment groups are: vehicle (lanes 1–2), ChK (lanes 3–4), ChK+IGF-1 (lanes 5–6), and IGF-1 (lanes 7–8). Densitometric quantification of the bands for each blot is illustrated in **a** and represents the mean ± S.D. (* = $p < 0.05$ compared to ChK+IGF-1). (**c, d**) Cells were grown to 80–90% confluence in 25 cm2 flasks, treated with vehicle (DMSO/PBS) or ChK (10 μM) for 90 min, pretreated with DMSO, ChK, or Torin1 (100 nM) for 60 min and stimulated with IGF-1 (50 ng/mL) for 30 min, then extracted for Western Blot analysis at Akt T308 (top panel), Akt T450 (top-middle panel), Akt T450 (lower-middle panel), total Akt (bottom panel) as describe in Material and Methods. Treatment groups are: vehicle (lanes 1–2), ChK (lanes 3–4), IGF-1 (lanes 5–6), ChK+IGF-1 (lanes 7–8), Torin+IGF-1 (lanes 9–10). Densitometric quantification for each duplicate bands and blot are characterized in **c** and represent the mean ± S.D. (* = $p < 0.05$, * = $p < 0.01$ compared to IGF-1). Data are a representation of at least two independent experiments. Each individual treatment is at least a $n = 4$

IGF-1 treated cells (Fig. 2c). ChK alone decreased phosphorylation at Akt S473 ($p = 0.0087$, Fig. 3a). Calyculin A increased phosphorylation at Akt S473 by approximately 2.6 fold compared to control. Pretreatment with ChK in the calyculin A treated cells significantly abrogated phosphorylation at the S473 site ($p = 0.0307$). Torin1 pretreatment significantly inhibited calyculin A phosphorylation of Akt S473 ($p = 0.0141$), comparably to ChK.

mTORC2 phosphorylation at S2481 is inhibited by Chaetoglobosin K in IGF-1 stimulated cells

To further understand the changes seen on Akt kinase in H1299 human lung adenocarcinoma cells, upstream mTORC2 was analyzed at the S2481 site for changes in phosphorylation. The experiments analyzed for Akt kinase at the three sites, T308, T450, and S473, were also used for the mTORC2 S2481 analysis. As shown in Fig. 4a, IGF-1 increased phosphorylation at mTORC2 S2481 and both ChK and Torin1 significantly inhibited IGF-1 phosphorylation ($p = 0.0053$ IGF-1 vs. ChK+IGF-1 and $p = 0.0115$ IGF-1 vs. Torin+IGF-1). Additionally analyzed were ChK+IGF-1 versus Torin+IGF-1, which resulted in no significant difference ($p = 0.0709$). The inhibition of phosphorylation at mTORC2 S2481 by ChK mirrors the same response seen at Akt S473. This is a direct agonism of the IGF-1/PI3K/mTORC2 pathway by IGF-1 and the inhibition of mTOR by a potent and selective inhibitor, Torin1, has been matched by ChK.

mTORC2 is affected by treatment with calyculin A and both Chaetoglobosin K and Torin1 inhibit mTORC2 S2481 phosphorylation

Investigating further into the mechanism of ChK in H1299 cells, calyculin A stimulated cell experiments were analyzed for mTORC2 S2481 phosphorylation. Fig. 5a indicates that calyculin A increased phosphorylation at mTORC2 S2481

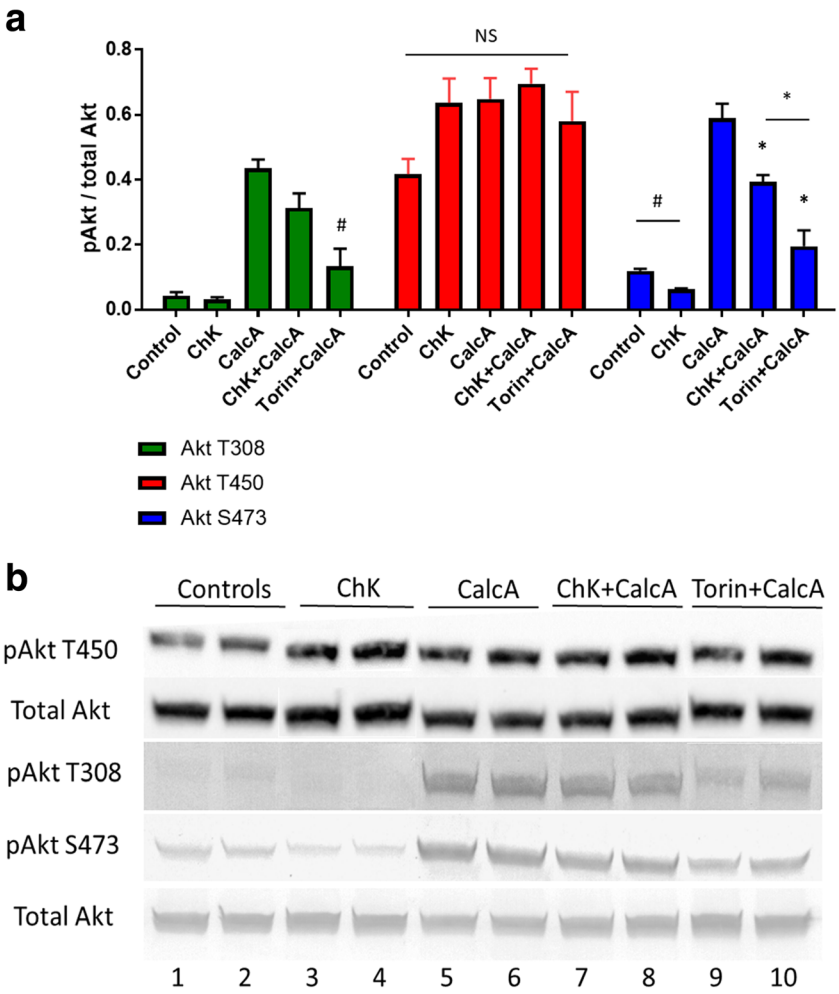

Fig. 3 Phosphatase inhibition increases Akt phosphorylation and is abrogated by ChK in H1299 cells. Graphical representation of the densitometry quantification in shown in **a**. (* = $p < 0.05$, # = $p < 0.01$ compared to CalcA) Cells were treated with either vehicle (DMSO, 90 min), ChK (10 μM, 90 min), or calyculin A (12.5 nM, 30 min) with a 60 min DMSO, ChK, or Torin1 (100 nM) pretreatment, then extracted as described in the Material and Methods section. **b** Proteins were electrophoresed through a 12% SDS/PAGE gel and probed for Akt T450 (top panel), total Akt (second-from-top panel), Akt T308 (middle panel), Akt S473 (second-from-bottom panel), total Akt (bottom panel). Akt T450, chemiluminescent total Akt, and Akt T308 were analyzed on the same blot. Akt T450 is normalized to the chemiluminescent total Akt. T308 and S473 were normalized to the colorimetric total Akt. Treatment groups were: vehicle (lanes 1–2), ChK (lanes 3–4), calyculin A (lanes 5–6), ChK+CalcA (lanes 7–8), Torin+CalcA (lane 9–10). Data are a representation of at least two independent experiments. Each individual treatment is at least a $n = 4$, except for Torin+CalcA $n = 3$

compared to control, and both ChK and Torin1 were able to impede calyculin A phosphorylation at that site. ChK pretreatment in the calyculin A treated lanes was statistically different compared to calyculin A lanes ($p = 0.0405$). Additionally, the Torin+CalcA treated cells were also different compared to calyculin A treated cells ($p = 0.0037$). Furthermore, ChK+CalcA and Torin+CalcA were also different ($p = 0.0082$). Calyculin A inhibits phosphatases PP2a and PP1 that have direct activity at Akt T308 and S473 [37, 38]. Calyculin A increases phosphorylation at mTORC2 S2481 [30] and Akt S473 [38]. These results are the first to suggest that ChK and Torin1 act similarly in H1299 cells to alter phosphorylation of the mTORC2 complex (Fig. 4a, 5a) and Akt (Fig. 2c, 3a) following PP1/PP2a inhibition or IGF-1R stimulation.

Chaetoglobosin K produces similar trends in H2009 human lung adenocarcinoma cells at mTOR and Akt kinases

H2009 cells were cultured and treated in the same manner as the H1299 cells. The treatment time was increased from 90 min to 210 min for H2009 cells based on results from a kinetic study to optimize effects on Akt S473 phosphorylation that ranged from one to 4 h (data not shown). Fig. 6a illustrates Akt S473 was significantly reduced in ChK+IGF-1 lanes compared to the IGF-1 lanes in Fig. 6b ($p = 0.0028$). Torin1 pretreatment also prevented IGF-1 stimulation ($p = 0.0001$). Akt T308 did not show a significant change with ChK treatment; however, Torin1 was able to prevent IGF-1 stimulation at Akt T308 ($p = 0.03$). mTORC2 S2481 was affect by ChK

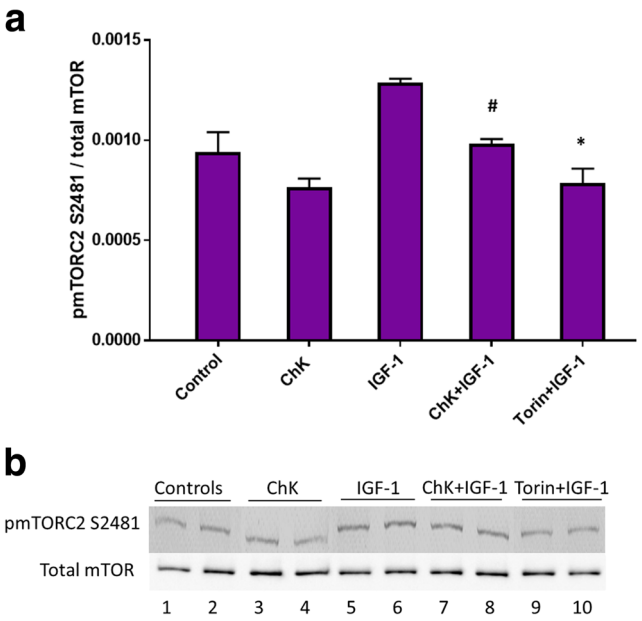

Fig. 4 IGF-1 stimulated H1299 cells are inhibited by ChK at mTORC2 S2481 autophosphorylation site. These blots were analyzed from the same experiments that were analyzed for Akt S473 in IGF-1 stimulated cells. Densitometric quantification for each replicate band and blot are characterized in **a** and represent the mean ± S.D. (* = $p < 0.05$, # = $p < 0.01$ compared to IGF-1) **b** Cells were treated with vehicle (DMSO/PBS) or ChK (10 μM) for 90 min, pretreated with DMSO, ChK, or Torin1 (100 nM) for 60 min and stimulated with IGF-1 (50 ng/mL) for 30 min, then extracted for Western Blot analysis. 7.5% SDS/PAGE gels were electrophoresed and blotted for mTORC2 S2481 or total mTOR. The same blot was used for both phospho- and total antibody without the use of a stripper. Treatment groups are: vehicle (lanes 1–2), ChK (lanes 3–4), IGF-1 (lanes 5–6), ChK+IGF-1 (lanes 7–8), Torin+IGF-1 (lanes 9–10). Data are a representation of at least two independent experiments. Each individual treatment is at least a $n = 4$

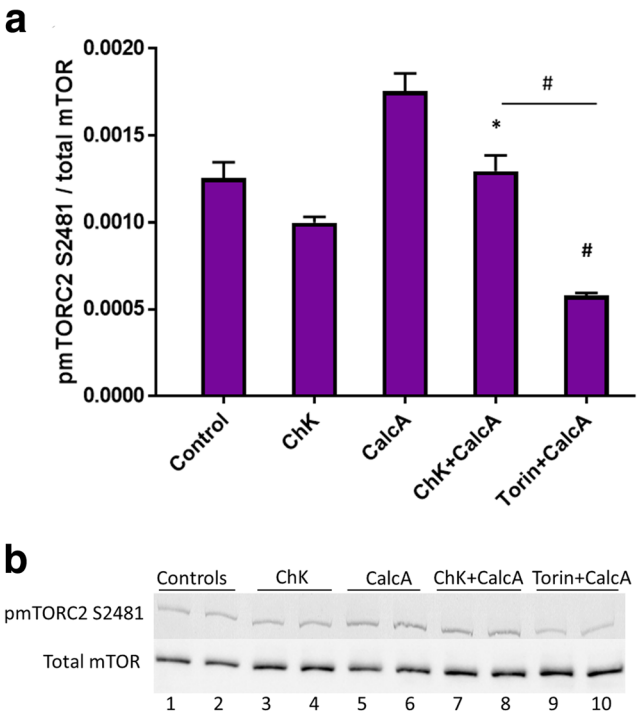

Fig. 5 mTORC2 S2481 autophosphorylation is hindered by ChK and Torin1 in calyculin A treated H1299 cells. Densitometric quantification for each replicate band and blot are characterized in **a** and represent the mean ± S.D. (* = $p < 0.05$, # = $p < 0.01$ compared to CalcA) **b** 80–90% confluent cells were treated with either vehicle (DMSO, 90 min), ChK (10 μM, 90 min), calyculin A (12.5 nM, 30 min) with a 60 min DMSO, ChK, or Torin1 (100 nM) pretreatment, then extracted as described in the Material and Methods section. PVDF membranes were probed for mTORC2 S2481 (colorimetric) or total mTOR (chemiluminescence). The same blot was used for each phospho- and total antibody without the use of a stripper. Treatment groups were: vehicle (lanes 1–2), ChK (lanes 3–4), calyculin A (lanes 5–6), ChK+CalcA (lanes 7–8), Torin+CalcA (lanes 9–10). Data are a representation of at least two independent experiments. Each individual treatment is at least a $n = 4$, except for Torin+CalcA $n = 3$

pretreatment with IGF-1 stimulation and this decreased the maximum dose response by IGF-1 ($p = 0.0693$), but these were just below significance. Torin1 was able to significantly decrease IGF-1 stimulation at mTORC2 S2481 ($p = 0.0351$).

Chaetoglobosin K is not an active site Akt inhibitor

To ensure that the cellular effects seen by ChK were not due to inhibition of Akt kinase, we monitored ChK for its ability to inhibit isolated Akt kinase in vitro. Results show that ChK had no effect on Akt activity, compared to the positive control, staurosporine, in Fig. 7.

Discussion

Cancer treatments are improving and changing as more research is finding new proteins that are involved in the signaling cascades from the RTKs to alter expression of oncogenic factors. One of those proteins of interest has been mTOR and its two complexes, mTORC1 and mTORC2. Since the discovery of the elusive PDK2 to be renamed mTORC2 in 2005 [10], hundreds of clinical trials have investigated the use of rapamycin and rapalogs, which are allosteric inhibitors of mTOR by inhibiting binding of FKBP12 to mTOR. [39] Direct catalytic site inhibitors, such as Torin1 and Torin2, have been designed to investigate the inhibition of both mTOR complexes. [12, 31, 40] Other dual kinase PI3K/mTOR or direct mTOR inhibitors have been developed that have endured Phase I/II clinical trials. [41] The use of rapamycin and the rapalogs, such as everolimus, acutely inhibit mTORC1 rather than mTORC2, which causes a feedback loop to propagate the signaling from the RTK down through mTORC2, which increases phosphorylation at Akt S473, thus promoting cell growth and survival rather than growth inhibition. [32, 33, 42] Torin1 inhibits both mTOR complexes and it was reported that mTORC2 complexation and inhibition reduced β-cell viability in cell lines and isolated human and rat islets, moreover than rapamycin. [43] A new third generation

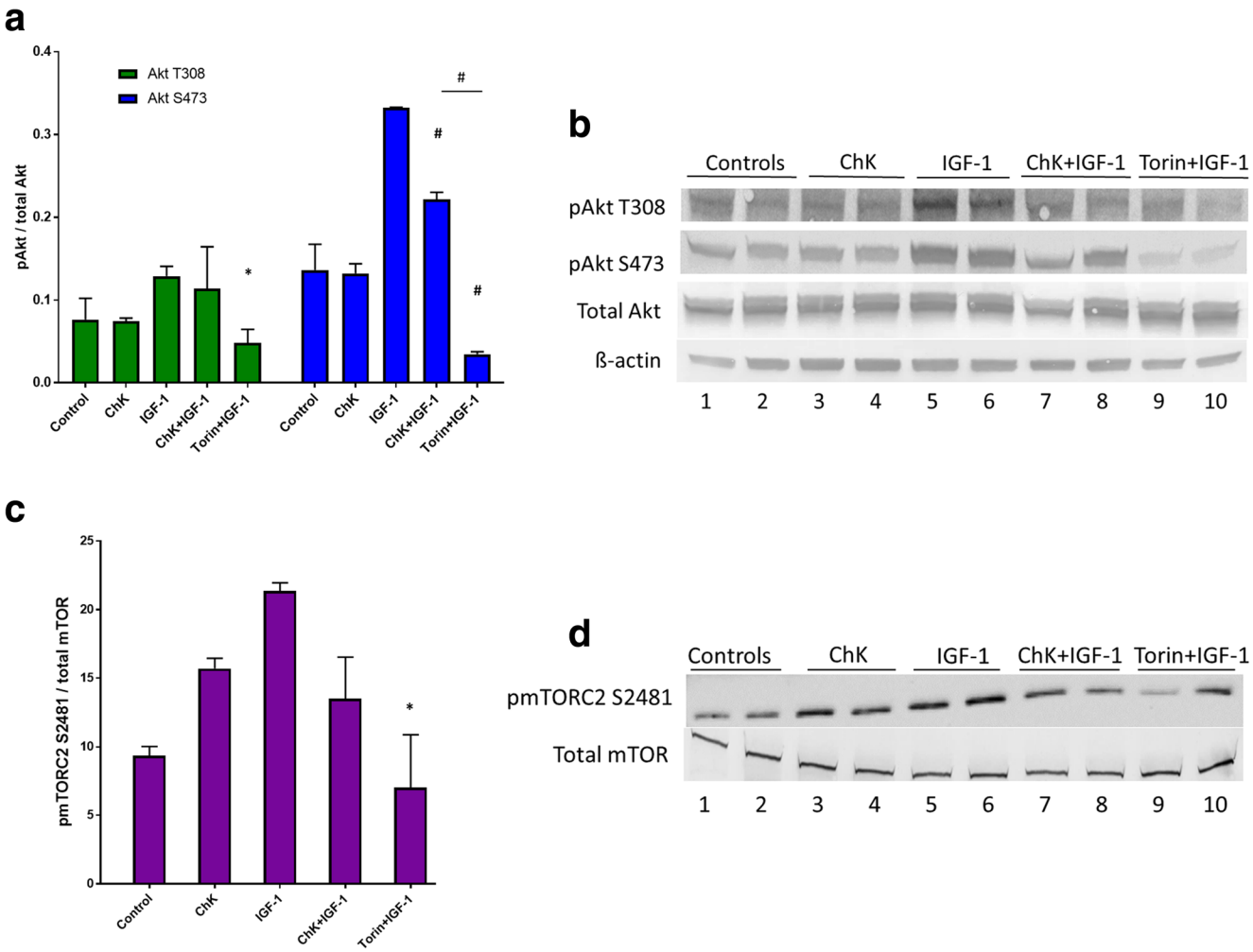

Fig. 6 H2009 cells have similar trends for effects with ChK treatment at Akt S473, T308 and mTORC2 S2481 with IGF-1 stimulation. Densitometric quantification for each replicate band and blot are characterized in **a** and **c**, and represent the mean ± S.D. (* = $p < 0.05$, # = $p < 0.01$ compared to IGF-1) **b** Cells were treated with vehicle (DMSO/PBS) or ChK (10 μM) for 210 min (lanes 1–4), pretreated with DMSO, ChK, or Torin1 (100 nM) for 180 min and stimulated with IGF-1 (50 ng/mL)(PBS) for 30 min (lanes 5–10), then extracted for Western Blot analysis. 12.5% SDS/PAGE gels **b** were used for all Akt and ß-actin antibodies, while 7.5% gels **d** were electrophoresed and blotted for mTORC2 S2481 or total mTOR. Two different blots using identical samples from the same experiment were used for the analysis in Fig. 6c and d. Treatment groups are: vehicle (lanes 1–2), ChK (lanes 3–4), IGF-1 (lanes 5–6), ChK+IGF-1 (lanes 7–8), Torin+IGF-1 (lanes 9–10). Data are a representation of two independent experiments

of mTOR inhibitors called RapaLinks have been designed to inhibit mTOR complexation and directly inhibit the ATP-catalytic site. [44] The results from these third-generation mTOR inhibitors remain to be elucidated.

ChK is a natural product that has been studied over the last few decades that may be a viable option to study in animals to assess its ability to suppress tumor growth, as well as adverse effects. The dose used in the current and previous studies [18, 19, 22] are in the low micromolar range, which presents ChK as a drug that may have a large therapeutic window. Compared to Torin1, ChK may be a practical option as it has been shown not to affect glucose uptake into cells [21] and it may not induce pancreatic ß-cell destruction, as reported with Torin1 treatment. [43] The effects on mTORC1 S2448 are needed to see the overall activity of ChK at the mTOR complexes. However, the indolylcytochalasian has several positions along the structure that could be altered to enhance efficacy and potency at Akt S473 and mTORC2 S2481. ChK has also been shown to inhibit cytokinesis [19], which is in agreement with its recent report to halt cells in the G2 phase [20]. It was able to morphologically alter the cells while under treatment, as expected due to its activity at capping the plus-end of actin filament formation. [21] Torin1 was not seen to morphologically alter the cell structure as ChK treatment did (data not shown). No study has concluded that ChK is a direct mTOR catalytic site inhibitor, like Torin1 has been described. [31]

This study characterizes and illustrates that ChK modulates activity at mTORC2 S2481 and Akt S473 similar to the mTOR catalytic site inhibitor, Torin1, in H1299 cells. A comparable effect of ChK on Akt S473 was observed in H2009

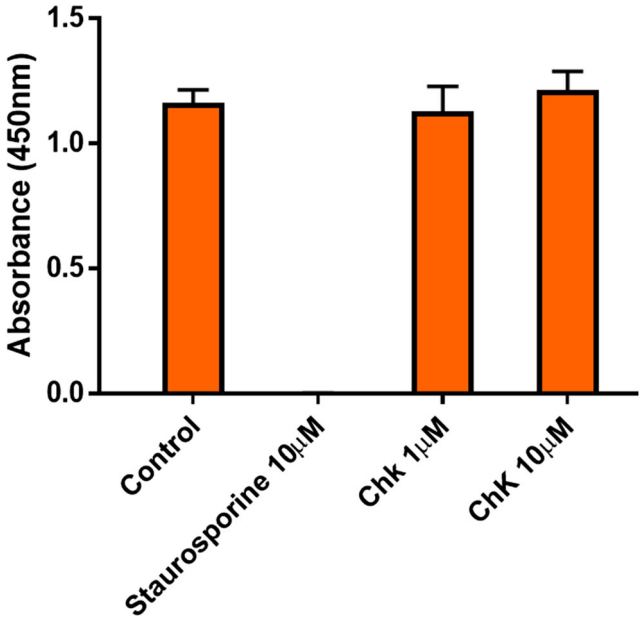

Fig. 7 ChK does not directly affect Akt kinase activity. Instructions for the assay were followed according to manufacturer. DMSO was used as control. The Akt kinase kit used a microtiter plate coated with a synthetic peptide substrate that was phosphorylated by Akt kinase. An antibody that specifically binds to the phosphorylated substrate was used, along with a HRP-linked secondary, to report a colorimetric value. Staurosporine is a known inhibitor of Akt kinase. Values are the mean ± S.D. ($n = 4$)

adenocarcinoma cells with a similar trend of effects on mTORC2 S2481. It is possible that a finer adjustment of the ChK treatment time using H2009 cells may have better optimized effects on mTORC2 S2481 phosphorylation. We also present evidence that ChK does not act on Akt kinase (Fig. 7). ChK prevented an increase in phosphorylation due to IGF-1 stimulation or calyculin A treatment at Akt S473 (Figs. 2c, 3a) and mTORC2 S2481 (Figs. 4a, 5a) at 90 min. ChK also prevented IGF-1 stimulation in H2009 cells for 210 min at Akt S473 (Fig. 6a). Previous studies treated WB-*ras*1, H2009, H1299, OVCAR-3, and A2780/CP70 cells with ChK for 24 h. [18–20, 22] A time course study at 4, 12, and 24 h in H2009 cells revealed that a four-hour treatment reduced phosphorylation at Akt S473 by more than 50% compared to 12 or 24 hours. [23] This study is the first to demonstrate that the kinetics of this potential anti-cancer molecule are rapid enough to see both a morphological change and phosphorylation changes at a target protein complex, mTORC2, and its effector protein, Akt kinase.

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Compliance with ethical standards

Conflict of interest Blair P Curless declares that he has no conflict of interest. Nne E Uko declares that she has no conflict of interest. Diane F Matesic declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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