The Ca^{2+} -calmodulin-dependent kinase II is activated in papillary thyroid carcinoma (PTC) and mediates cell proliferation stimulated by RET/PTC

Maria Rosaria Rusciano¹, Marcella Salzano¹, Sara Monaco¹,
Maria Rosaria Sapi², Maddalena Illario¹, Valentina De Falco¹,
Massimo Santoro¹, Pietro Campiglia³, Lucio Pastore^{4,5},
Gianfranco Fenzl², Guido Rossi^{1,6} and Mario Vitale^{2,7}

¹Department of Biologia e Patologia Cellulare e Molecolare, Università Federico II, Naples, Italy

²Department of Endocrinologia ed Oncologia Molecolare e Clinica, Università Federico II, Naples, Italy

³Department of Scienze Farmaceutiche, Università di Salerno, Salerno, Italy

⁴Department of Biochimica e Biotecnologie Mediche, Università Federico II, Naples, Italy

⁵CEINGE-Biotecnologie Avanzate, Naples, Italy

⁶CISI, Center for Basic and Clinical Immunological Sciences, Università Federico II, Naples, Italy

⁷Facoltà di Medicina, Università di Salerno, Salerno, Italy

(Correspondence should be addressed to M Vitale at Department of Endocrinologia ed Oncologia Molecolare e Clinica, Università Federico II, Via S. Panini, 5, Naples 80131, Italy; Email: mavitale@unina.it)

Abstract

RET/papillary thyroid carcinoma (PTC), TRK-T, or activating mutations of Ras and BRAf are frequent genetic alterations in PTC, all leading to the activation of the extracellular-regulated kinase (Erk) cascade. The aim of this study was to investigate the role of calmodulin-dependent kinase II (CaMKII) in the signal transduction leading to Erk activation in PTC cells. In normal thyroid cells, CaMKII and Erk were in the inactive form in the absence of stimulation. In primary PTC cultures and in PTC cell lines harboring the oncogenes RET/PTC-1 or BRAf^{V600E}, CaMKII was active also in the absence of any stimulation. Inhibition of calmodulin or phospholipase C (PLC) attenuated the level of CaMKII activation. Expression of recombinant RET/PTC-3, BRAf^{V600E}, or Ras^{V12} induced CaMKII activation. Inhibition of CaMKII attenuated Erk activation and DNA synthesis in thyroid papillary carcinoma (TPC-1), a cell line harboring RET/PTC-1, suggesting that CaMKII is a component of the Erk signal cascade in this cell line. In conclusion, PTCs contain an active PLC/ Ca^{2+} /calmodulin-dependent signal inducing constitutive activation of CaMKII. This kinase is activated by BRAf^{V600E}, oncogenic Ras, and by RET/PTC. CaMKII participates to the activation of the Erk pathway by oncogenic Ras and RET/PTC and contributes to their signal output, thus modulating tumor cell proliferation.

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Introduction

Papillary thyroid carcinoma (PTC) is the most common thyroid cancer, accounting for more than 80% of thyroid malignancies (Sherman 2003). It frequently carries genetic alterations, all resulting in the inappropriate activation of extracellular-regulated kinase (Erk) component of the mitogen-activated protein kinase (MAPK) signaling pathway, which plays a key role in the regulation of cell growth, differentiation, and

survival. RET/PTC and TRK-T rearrangements, and Ras and BRAf mutations are the most frequent genetic alterations in PTC, and all are responsible for thyroid tumorigenesis through the MAPK pathway (Greco *et al.* 1992, Santoro *et al.* 1996, Esapa *et al.* 1999, Nikiforov 2002, Kimura *et al.* 2003).

Most growth factor receptors generate multiple signals that regulate cell growth, proliferation, and survival, whose imbalance has dramatic effects on cell

fate (Demidenko & Blagosklonny 2008). We previously demonstrated in normal thyroid cells that integrin activation by fibronectin (FN) generates different converging signals leading to Erk activation and cell proliferation (Illario *et al.* 2003, 2005). In the same studies, we also provided evidence that calmodulin-dependent kinase II (CaMKII) is necessary to Raf-1 activation by Ras in the integrin → Ras → Raf-1 → MEK → ERK pathway. More recent studies suggest that the role of CaMKII in the Erk cascade is not restricted to integrins but it is rather a general phenomenon (Illario *et al.* 2009). CaMKII is a ubiquitous serine/threonine protein kinase involved in multiple signals and biological functions. It is a multimeric enzyme composed of 10–12 catalytic subunits, whose activation requires the binding to Ca^{2+} /calmodulin (Means 2000).

We extended our study to PTC to determine whether CaMKII plays a role in this neoplasia. Our results provide evidence that in PTC, CaMKII is constitutively activated by an upstream signaling. CaMKII is activated by RET/PTC and Braf^{V600E} and mediates the oncogenic signal leading to Erk activation and cell proliferation.

Materials and methods

Cell cultures, FN stimulation, vectors, and transfections

Tissue specimens were obtained at surgery from PTCs ($n=8$) and their contralateral lobes of subjects undergoing thyroidectomy. Informed consent for cell culture preparation was obtained from patients. Cell cultures were prepared as previously described (Vitale *et al.* 1997). Briefly, tissues were chopped by scalpels in small pieces and digested by type IV collagenase (Sigma Chemical Co.), 1.25 mg/ml in Ham's F-12 medium (F-12), and 0.5% BSA overnight at 4 °C under rotation. Cells were pelleted by centrifugation at 150 *g* for 5 min, washed twice in BSA-F-12, seeded in Petri dishes, and cultured in 5% CO₂ atmosphere at 37 °C in F-12 supplemented with 10% FCS. Medium was changed every 3–4 days, and cells were harvested by treatment with 0.5 mM EDTA in calcium- and magnesium-free PBS containing 0.05% trypsin. For each experiment, single individual cultures were used. The TAD-2 cell line, obtained by Simian virus 40 infection of human fetal thyroid cells, was generously donated by Dr T F Davies (Mount Sinai, New York, NY, USA). Thyroid papillary carcinoma cell lines TPC-1 (carrying RET/PTC-1) and BCPAP (carrying Braf^{V600E}) were cultured in F-12 supplemented with

10% FCS. Coated plates were prepared as follows: the plates were filled with PBS, 1% heat-denatured BSA (Sigma), or 100 µg/ml human FN (Collaborative Research, Bedford, MA, USA). After overnight incubation at 4 °C, the plates were washed thrice with PBS and used. Rat CaMKII α kinase-deficient mutant CaMKII α K42M was a generous gift of Dr A R Means. Ras^{V12}, RET/PTC-3, RET/PTC-3^{Y1015F}, and RET/PTC-3^{Y1062F} were subcloned into pcDNA3, myc-tagged expression vectors. Cells were transfected using Lipofectamine 2000 (Invitrogen).

Western blot and immunoprecipitation

For western blot analysis, cells were lysed in Laemmli buffer (0.125 mol/l Tris (pH 6.8), 5% glycerol, 2% SDS, 1% β -mercaptoethanol, and 0.006% bromophenol blue). Proteins were resolved by 7–10% SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon P; Millipore Corp., Bedford, MA, USA). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine in PBS, washed, and incubated for 1 h at 4 °C with primary antibodies and then washed again and incubated for 1 h with a HRP-conjugated secondary antibody. Finally, protein bands were detected by an enhanced chemiluminescence system (Amersham Biosciences). Computer-acquired images were quantified using ImageJ 1.39u, National Institutes of Health, USA. For immunoprecipitation, cells were lysed in immunoprecipitation buffer (0.05 mol/l Tris-HCl (pH 8.0), 0.005 mol/l EDTA, 0.15 mol/l NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 0.01 mol/l NaF, 0.005 mol/l EGTA, 0.01 mol/l sodium pyrophosphate, and 0.001 mol/l phenylmethylsulfonylfluoride). Rabbit polyclonal antibody reactive to all CaMKII isoforms (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and protein G plus/protein A agarose beads (Oncogene Science, Boston, MA, USA) were used to immunoprecipitate CaMKII from 1 mg total lysate. Mouse monoclonal antibodies to total- and phospho-Erk-1/2 were from Santa Cruz Biotechnology. Polyclonal antiphospho-CaMKII antibody (pT286-CaMKII) was from Zymed (Invitrogen).

CaMKII activity assay, inhibitors, and viruses

Cells were lysed in 200 µl of RSB buffer with 10 mM CHAPS, and 20 µl of the extracts were assayed in 50 µl of reaction mixture consisting of 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 0.5 mM dithiothreitol, 2 µM CaM, 100 nM microcystin, 50 µM ATP (1500 cpm/pmol [γ -³²P]ATP), and 0.1 mM substrate

peptide Autocamtide II. Total CaMK activity was determined by including 1 mM CaCl_2 in the mixture, whereas autonomous activity was measured in the presence of 2.5 mM EGTA. Ionomycin (Sigma) at a concentration of 500 ng/ml was used as a positive control for CaMKII activation. The reaction was carried out for 2 min at 30 °C, and 20- μl aliquots of the reaction mixture were spotted onto p81 phosphocellulose filters (Upstate Biotechnology, Charlottesville, VA, USA). Purified CaM and Autocamtide II were a kind gift from Dr A R Means (Durham, NC, USA). The CaMK inhibitor KN93 and the CaM inhibitors trifluoperazine (TFP) and *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W7) were purchased from Sigma. The CaMKII-specific inhibitor antCaNtide is derived from the endogenous CaMKII inhibitor protein CaMKIIN (Chang *et al.* 1998) and was made cell-permeable by the N-terminal addition of an Antennapedia-derived sequence (antCaNtide: RQIKIWFQNRRMKWKKRPPKLG QIGRSKRVIEDDRIDDLVK). ZD6474 was kindly provided by AstraZeneca. Rat CaMKII α kinase-deficient mutant CaMKII α K42M and truncated 1–290 CaMKII constitutively activated were subcloned into pAdTrack-CMV. The adenoviruses were generated using the AdEasy system available from Qbiogene and were a generous gift provided by Dr A R Means.

Detection of BRAF^{V600E} and RET/PTC rearrangements

Searching for BRAF mutations was performed on genomic DNA by mutant allele-specific PCR amplification as previously described (Sapio *et al.* 2006). Detection of RET/PTC rearrangements was performed by southern blot on reverse transcription-PCR products for the tyrosine kinase and extracellular domains. RNA from TPC-1 and NIH-3T3 cells was used as positive and negative controls respectively. The expression of the tyrosine kinase domain alone indicated the presence of a RET/PTC rearrangement.

[³H]thymidine incorporation

To determine DNA synthesis, cells were plated in a 24-well plate and serum-starved for 12 h in DMEM, 0.5% BSA. A total of 0.5 μCi [³H]thymidine was then added to the plates. After 24 h, the plates were gently washed with PBS and then with 10% trichloroacetic acid (TCA), and incubated 10 min with 20% TCA at 4 °C. The plates were washed with 5% TCA, and cells

were lysed with 0.2% SDS, 0.5 N NaOH for 15 min at 4 °C. The lysates were then resuspended in 5 ml scintillation fluid and counted in a β -counter (Becton Dickinson, Franklin Lakes, NJ, USA).

Figure 1 CaMKII and ERK phosphorylation in primary cultures of normal thyroid cells. Primary cultures of normal human thyroid cells were starved overnight from serum, resuspended by mild trypsinization and left in suspension for 30 min. (A) The cells were plated onto FN-coated plates for the indicated time or treated in suspension with 10% FCS for 30 min. (B) The cells were treated as before after a 30 min pre-treatment with 30 μM KN93, 1 μM TFP, or 15 μM W7. Erk and CaMKII phosphorylations were determined by western blot. Averages and s.d. of relative expressions of phosphorylated kinases were determined by scanning densitometry of three independent immunoblots. A value of 1 OD arbitrary unit was assigned to the unstimulated samples. In A and B, all experimental points were significant versus unstimulated points.

Figure 2 CaMKII is activated in PTC primary cultures. Primary cultures of PTC (six of eight analyzed are reported) were starved overnight from serum, resuspended, left in suspension, or plated onto FN-coated plates for 30 min and analyzed by western blot for total or phosphorylated CaMKII. Averages and s.d. of relative expressions of phosphorylated CaMKII were determined by scanning densitometry of independent immunoblots (PTC, $n=8$). A value of 1 OD arbitrary unit was assigned to the unstimulated samples.

Statistical analysis

Results are presented as the mean $\pm$ s.d. Statistical analysis was performed by using the *t*-test. The level of significance was set at $P < 0.01$.

Results

CaMKII is constitutively active in unstimulated PTC

Phosphorylation of Erk and CaMKII was determined by western blot in primary cultures of normal thyroids. Both kinases were not phosphorylated after serum starvation and 30 min suspension (Fig. 1A). Cell adhesion to FN or serum stimulation induced phosphorylation of both Erk and CaMKII. CaMKs inhibition by KN93 abrogated FN-induced Erk phosphorylation, and inhibition of calmodulin by W7 and TFP abrogated both CaMKII and Erk phosphorylations (Fig. 1B). We then analyzed CaMKII phosphorylation in primary cultures from eight PTCs. CaMKII was activated in starved cells left in suspension for 30 min, and FN stimulation did not induced any further phosphorylation (Fig. 2). This result indicates that in PTC, cells CaMKII is constitutively active and is no more modulated by integrins. Genetic analysis of six out of eight PTCs

demonstrated the presence of RET/PTC in one sample and BRAF^{V600E} in three. CaMKII phosphorylation in unstimulated and stimulated conditions was then analyzed in normal fetal immortalized thyroid cells (TAD-2) and in PTC cell lines harboring the oncogenes RET/PTC-1 (TPC-1) or BRAF^{V600E} (BCPAP; Fig. 3). While in TAD-2 cells, CaMKII was not phosphorylated in the absence of adhesion to FN, in PTC cell lines, this kinase was phosphorylated also upon serum starvation and denied adhesion, and FN did not induced any further kinase activation, as observed in PTC primary cultures.

CaMKII activation is mediated by a constitutive upstream Ca²⁺-dependent signaling

A PTC primary culture containing RET/PTC, TAD-2, and TPC-1 cells was starved from serum, resuspended by mild trypsinization and left in suspension for 30 min with or without the calmodulin inhibitor W7 or TFP. The cells were then lysed and CaMKII phosphorylation was determined by western blot (Fig. 4A). W7 strongly attenuated the phosphorylation of CaMKII in all cells. Also 1 μ M TFP, another calmodulin inhibitor, had the same effect (not shown). The inhibition of CaMKII activity by W7 in TPC-1 cells was confirmed in a dose-effect experiment, both by western blot and by *in vitro* kinase assay (Figs 4B and 4C). Calmodulin inhibition by W7 decreased the level of CaMKII phosphorylation in a dose-dependent manner, demonstrating the existence of a Ca²⁺-calmodulin-dependent signal responsible for the CaMKII constitutive activation.

Figure 3 CaMKII is activated in PTC cell lines. Primary cultures from a normal thyroid (NT) and cell lines TAD-2, TPC-1, and BCPAP were starved from serum, left in suspension or plated onto FN-coated plates for 30 min. CaMKII phosphorylation in cell lysates was determined by western blot. Averages and s.d. of relative expressions of phosphorylated CaMKII were determined by scanning densitometry. A value of 1 OD arbitrary unit was assigned to the unstimulated samples.

Figure 4 A Ca^{2+} -dependent signal mediates constitutive activation of CaMKII in PTC cells harboring RET/PTC. Primary culture from a PTC harboring RET/PTC (PTC), and cell lines TAD-2 and TPC-1 were starved from serum, left in suspension, plated onto FN-coated plates for 30 min, or treated for 30 min in suspension with the calmodulin inhibitor W7. CaMKII phosphorylation in cell lysates was determined by western blot. Averages and s.d. of relative expressions of phosphorylated CaMKII were determined by scanning densitometry. A value of 1 OD arbitrary unit was assigned to the samples treated with W7. In TPC-1 cells, the dose-dependent effect of W7 was determined on CaMKII phosphorylation (B) and enzymatic activity (C) by western blot or *in vitro* kinase assay. *Significant versus untreated.

CaMKII activation is PLC mediated

Phospholipase C (PLC) mobilizes calcium from the intracellular stores, thus it is an important modulator of the intracellular calcium concentration. To determine whether CaMKII activation was modulated by PLC, TPC-1 cells were treated with the PLC inhibitor U73122 at 10 μM for 15–120 min (Fig. 5A). The level of phosphorylation of PKC α was used to determine the inhibition of PLC. A modest attenuation of phosphorylation of both PKC α and CaMKII was visible at 60 min. PKC α was completely dephosphorylated after 120 min, while CaMKII and Erk phosphorylation was strongly reduced. The dose-dependent effect of U73122 on CaMKII phosphorylation was demonstrated by 2 h treatment (Fig. 5B).

RET/PTC and Ras^{V12} activate CaMKII

The oncogene RET/PTC generates multiple pathways, including Ras/Raf/Mek/Erk and PLC/PKC which in turn can activate the PLC–calcium pathway. We investigated whether this oncogene was responsible for the activation of CaMKII. TPC-1 cells were treated for 24 h with the RET kinase inhibitor ZD6474 at different concentrations (Fig. 6A). The observed inhibition of CaMKII phosphorylation suggested a role for RET/PTC. However, this inhibitor is not specific for RET as it also inhibits a few other tyrosine kinases. Therefore, we determined the level of CaMKII activation upon expression of RET/PTC-3 and its mutants. In TAD-2 cells, CaMKII activation was stimulated by RET/PTC-3. Also the mutants Y1015F and Y1062F (unable to activate the PLC and the Ras

Figure 5 PLC mediates CaMKII activation. (A) TPC-1 cells were treated with 10 μM U73122 for 15–120 min. Cell lysates were analyzed by western blot with antibodies to phosphorylated CaMKII and PKC α . (B) The cells were treated for 2 h with the indicated concentrations of U73122. Averages and s.d. of relative expressions of pCaMKII and pErk were determined by scanning densitometry of three independent immunoblots. A value of 1 OD arbitrary unit was assigned to the minor point. *Significant versus 0 point.

Figure 6 RET/PTC and Ras^{V12} activate CaMKII. (A) TPC-1 cells were treated for 24 h with ZD 6474 at the indicated concentrations, and pCaMKII was determined by western blot. TAD-2 (B) and COS7 (C) were transfected with 5 μg/plate of myc-tagged expression vectors for Ras^{V12}, RET/PTC-3, and RET/PTC-3^{Y1015F} (Y1015), or 2.5 μg/plate of RET/PTC-3^{Y1062F} (Y1062). The latter resulted too toxic for the cells at higher concentration. After 55 h, the cells were lysed, and pCaMKII and myc were determined by western blot. Averages and s.d. of relative expressions of phosphorylated CaMKII were determined by scanning densitometry of independent immunoblots. The values were normalized by myc values. A value of 1 OD arbitrary unit was assigned to the minor point. *Significant versus pcDNA3. **Significant versus RET/PTC-3.

pathways respectively) activated CaMKII, although less than the wild-type RET/PTC-3 (Fig. 6B). Similar results were obtained in COS cells (Fig. 6C). RET/PTC-3 and its mutant Y1015F appeared equally potent; however, the strong expression of the mutant could hidden a lower capability to stimulate

CaMKII activation. The treatment with lovastatin (here used as an inhibitor of Ras isoprenylation) for 6 h strongly attenuated the CaMKII activation by the mutant 1015. These results demonstrate that RET/PTC activates CaMKII through both the PLC and the Ras pathways.

CaMKII is required for Erk phosphorylation and cell proliferation

The effect of the inhibition of CaMKs was determined in TPC-1 cells. The two inhibitors, W7 and KN93, attenuated Erk phosphorylation in both cells, indicating that the CaMKs participate to signaling leading to Erk activation (Fig. 7A). Although CaMKII is the major CaMK affected by KN93, other more specific CaMKII inhibitors were also used to investigate the role of this kinase isoform in the TPC-1 cell line. The cells were starved from serum, treated with the specific CaMKII inhibitory peptide antCaNtide, and Erk phosphorylation was evaluated by western blot after 30 min of suspension (Fig. 7B). The inhibitor strongly reduced the level of Erk phosphorylation in a dose-dependent fashion. In a parallel experiment, TPC-1 cells were induced to express a dominant-negative CaMKII or an activated CaMKII mutant (Fig. 7C). The dominant-negative CaMKII and the activated mutants were expressed in the cells by infection with recombinant adenoviruses. Erk phosphorylation was reduced by the dominant-negative mutant, while the activated CaMKII mutant neither inhibited nor stimulated Erk phosphorylation. These results indicate that CaMKII activity is necessary to the endogenous signal leading to Erk phosphorylation in TPC-1 cells. The same effect was observed in PTC primary cultures harboring RET/PTC (not shown).

CaMKII is required for DNA synthesis in TPC-1 cells

To determine the role of CaMKII in DNA synthesis, the incorporation of [³H]thymidine was determined in TPC-1 cells stimulated with FCS or treated with different inhibitors (Fig. 7D). The cells were starved from serum and treated with FCS, the CaMKII inhibitors KN93 or antCaNtide, the Mek inhibitors PD98052 and U0126, or with the HMG-CoA inhibitor lovastatin that inhibits Ras prenylation and activity. Whereas FCS stimulated, all inhibitors reduced the [³H]thymidine incorporation to a similar low level, thus demonstrating that the DNA synthesis required active CaMKII, Mek, and Ras.

Figure 7 CaMKII is required for Erk phosphorylation. TPC-1 cells were starved from serum and treated for 30 min in suspension with the CaMKII-specific peptide inhibitors KN93 or W7 (A), or antCaNtide (B). Erk phosphorylation was evaluated by western blot. (C) The cells were infected with recombinant adenoviruses harboring an empty vector, a dominant-negative CaMKII mutant (CaMKIIIDN) or an activated CaMKII mutant (CaMKIIact). The expression of recombinant proteins was demonstrated by western blot with CaMKII antibody. Averages and s.d. of relative expressions of phosphorylated ERK1 and ERK2 were determined by scanning densitometry of independent immunoblots ($n=3$ in A, $n=2$ in B). A value of 1 OD arbitrary unit was assigned to the minor point. *Significant versus 0 point. (D) Two $\times 10^5$ TPC-1 cells were plated in 24-well plates, serum-starved for 12 h, and pre-treated for 30 min with FCS (10%), antCaNtide (5 μ M), KN93 (10 μ M), PD98052 (50 μ M), U0126 (30 μ M) or for 6 h with lovastatin (30 μ M). Then, in the same medium, [3 H]thymidine was added to the plates. After 24 h, the plates were washed and insoluble TCA radioactivity counted by scintillation fluid in a β -counter. In a parallel experiment, the cell cycle was analyzed by flow cytometry using propidium iodide. Data are reported as the mean $\pm$ s.d. of quadruplicates. All experimental points were significant versus unstimulated point.

BRaf^{V600E} activates CaMKII through a PLC/Ca²⁺/calmodulin-dependent mechanism

A PTC primary culture harboring BRaf^{V600E} and BCPAP cells were starved from serum, resuspended by mild trypsinization and left in suspension for 30 min with or without the calmodulin inhibitor W7. The cells were then lysed and CaMKII phosphorylation was determined by western blot (Fig. 8A). Like in cells carrying RET/PTC, W7 reduced the phosphorylation of CaMKII in both cells, demonstrating the existence of a Ca²⁺-calmodulin-dependent signal responsible for the CaMKII constitutive activation. Then, CaMKII activation was determined in BCPAP cells treated with the PLC inhibitor U73122 at 10 μ M for 60 or 120 min (Fig. 8B). Phosphorylation of CaMKII was strongly reduced after 120 min, demonstrating the role of PLC. To determine whether BRaf^{V600E} activates CaMKII, the expression of this oncogene was induced in TAD-2 cells (Fig. 8C). CaMKII

phosphorylation was increased by BRaf^{V600E}. The treatment of the cells with U73122 completely abrogated CaMKII phosphorylation, demonstrating that BRaf^{V600E} activates CaMKII through PLC. To determine the role of CaMKII on Erk activity in cells harboring BRaf^{V600E}, BCPAP cells were treated with KN93, W7, or antCaNtide. None of these inhibitors reduced significantly the phosphorylation of Erk, indicating that CaMKII is activated by BRaf^{V600E}, but does not mediate to the following Erk activation.

Discussion

CaMKII is a multimeric enzyme composed of 10–12 catalytic subunits. Activation of a single catalytic subunit requires its binding to Ca²⁺/calmodulin. This interaction allows the catalytic domain of one subunit to phosphorylate the Thr286 residue of the adjacent

Figure 8 BRaf^{V600E} activates CaMKII through a PLC/Ca²⁺-dependent signal. (A) Primary cultures from a PTC harboring BRaf^{V600E} (PTC) and BCPAP cells were left in suspension with or without W7 30 μ M for 30 min, and then CaMKII phosphorylation was determined by western blot. (B) BCPAP cells were treated for 60 or 120 min with U73122 30 μ M. The absence of PKC α phosphorylation indicates that PLC has been inhibited. (C) TAD-2 cells were transfected with 5 μ g/plate of myc-tagged expression vectors for RET/PTC-3 or 10 μ g/plate of BRaf^{V600E}. After 48 h, the cells were lysed and pCaMKII was determined by western blot. (D) BCPAP cells were treated in suspension with KN93, W7, or antCaNtide at the indicated micromolar concentrations for 30 min, and the Erk phosphorylation was determined by western blot. Averages and s.d. of relative expressions of phosphorylated CaMKII or Erk were determined by scanning densitometry of independent immunoblots. A value of 1 OD arbitrary unit was assigned to the minor point.

one. Once a subunit becomes phosphorylated, its kinase activity becomes independent of Ca²⁺/CaM binding. Thus, it follows that the level of CaMKII activation in a cell depends from the level of cytoplasmic Ca²⁺ concentration.

CaMKII was not activated in normal thyroid cells upon denied adhesion and serum starvation, while it was activated by serum and FN. In primary cultures from eight PTCs, CaMKII was activated also upon denied adhesion and serum starvation, and FN stimulation was unable to further increase its activity. The observation of this common molecular feature in all PTC analyzed is noteworthy, as PTC represents a group of tumors with quite heterogeneous genetic and molecular alterations. Even though the final activation of Erk is one of the most common molecular events of thyroid cell tumorigenesis, different genetic alterations have been described in PTC. Indeed, RET/PTC and TRK-T rearrangements, and Ras and BRaf mutations all lead to thyroid transformation through the MAPK pathway, a kinase cascade transducing mitogenic signals that regulates cell proliferation and differentiation.

Inhibition of CaMKII activation by calmodulin inhibitors demonstrates that this kinase maintains the physiologic Ca²⁺-dependent regulatory mechanisms in these tumors. To date, activating mutations of this kinase have not been described, and only truncated CaMKII generates a constitutive activated kinase no more dependent by the Ca²⁺/CaM binding (Means 2000).

Ca²⁺ impacts nearly every aspect of cell life, and multiple factors determine its final intracellular concentration (Clapham 2007). The cytoplasmic Ca²⁺ level is maintained lower than the extracellular compartment by continuous extrusion via the plasma membrane Ca²⁺ ATPase and the smooth endoplasmic reticular Ca²⁺ ATPase transporters, and by the Na/Ca exchangers. A mechanism for Ca²⁺ signaling is the release from the endoplasmic reticulum in response to receptor activation. Receptor tyrosine kinases can modulate the cytoplasmic Ca²⁺ level by activating PLC that in turn generates inositol (1,4,5) trisphosphate (IP3) from phosphatidylinositol 4, 5 bisphosphate. IP3 is a ligand for the IP3 receptor-mediated cationic channels spanning the membrane of the endoplasmic

reticulum. Their activation induces release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm. Thus, PLC through IP3 is an important factor concurring to the modulation of Ca^{2+} /CaMKII signaling. Our results indicate that the constitutive active Ca^{2+} /CaMKII signal in PTC cell lines is mediated by PLC.

As in PTC primary cultures, also in TPC-1 cell line CaMKII was activated in the absence of serum or FN stimulation. We chose TPC-1 as it represents a verified model of PTC cell line harboring RET/PTC-1 (Schweppe *et al.* 2008).

The RET/PTC recombinants are a group of cytoplasmic tyrosine kinases that activate multiple signaling pathways (Kimura *et al.* 2003, Knauf *et al.* 2003, Melillo *et al.* 2005, Ciampi & Nikiforov 2007). The RET tyrosine kinase domain contains several tyrosine residues that can be phosphorylated, participating in the biological effects of this oncogene. Among these phosphotyrosines, tyrosines 1015 and 1062 play an important role in RET/PTC signaling. Phosphorylated tyrosines 1015 and 1062 are docking sites for PLC γ and Shc respectively (Borrello *et al.* 1996, Arighi *et al.* 1997, Salvatore *et al.* 2000). It follows that RET/PTC could modulate the intracellular Ca^{2+} concentration via Y1015/PLC γ and thus in turn the enzymatic activity of CaMKII. Also Y1062 could participate in Ca^{2+} /CaMKII signaling being a docking site for Shc, which couples RET/PTC to Ras. Activated Ha-Ras binds and stimulates PLC ϵ , the last identified member of the PLC family (Kelley *et al.* 2001). Thus, besides RET/PTC, also oncogenic Ras represents a possible initiator of the PLC/ Ca^{2+} /calmodulin/CaMKII signaling. As expected, expression of recombinant RET/PTC-3 in two different cell lines was able to strongly stimulate CaMKII phosphorylation (Fig. 6). Both the Y1062/Ras and Y1015/PLC pathways appear to contribute to CaMKII activation, demonstrating that RET/PTC activates CaMKII through both pathways.

The analysis of eight PTCs, including some expressing BRAF^{V600E}, demonstrated that the constitutive CaMKII activation in PTC is not restricted to those harboring RET/PTC, and that other activating pathways must exist. RET/PTC, BRAF^{V600E}, and oncogenic Ras activate a common transcriptional program in thyroid cells that includes upregulation of the CXCL1 and CXCL10 chemokines and the CXCR4 chemokine receptor (Castellone *et al.* 2004, Melillo *et al.* 2005). Cellular responses to these chemokines are initiated by specific cell surface receptors of the G protein-coupled receptor family (Richardson *et al.* 1998, Zlotnik & Yoshie 2000). G protein-coupled

receptors (primarily Gq/11 subtypes), generate IP3 by activating PLC β and, like the receptor tyrosine kinases, modulate the cytoplasmic Ca^{2+} concentration. Thus, RET/PTC, BRAF^{V600E}, and oncogenic Ras can modulate the Ca^{2+} signaling through multiple mechanisms that can explain the basal CaMKII activity observed in every primary PTC analyzed, including those that do not harbor RET/PTC.

Inhibition of CaMKII obtained by antCaNtide and dominant-negative enzyme expression demonstrated that this kinase participates in the basal Erk activation in TPC-1. We previously showed that CaMKII is necessary for Raf-1 activation by integrins in human thyroid cells and by insulin in human fibroblasts (Illario *et al.* 2003, 2009, Monaco *et al.* 2009). It follows that Raf-1 is one possible component of the Erk pathway requiring active CaMKII in TPC-1 cells. To date, the role of CaMKII in the modulation of BRAF activity has not been investigated.

In BCPAP cells, inhibition of CaMKII did not produce any significant effect on Erk activation. This observation can be explained by the presence in this cell line of BRAF^{V600E}, which is constitutively activated. However, this does not exclude a role for CaMKII in tumors harboring BRAF^{V600E}, because it is involved in important cellular functions such as cell motility, a cellular function promoted also by BRAF^{V600E}, through chemokines and PLC, and noncanonical Wnt signaling which modulates cell fate during differentiation and transformation (Kremenevskaja *et al.* 2005, Melillo *et al.* 2005, Takada *et al.* 2007).

In conclusion, we have shown that CaMKII is activated in PTC by RET/PTC, oncogenic Ras, and BRAF^{V600E}. Our findings suggest that the PLC/CaMKII pathway could therefore provide appropriate targets for therapeutic intervention of tumors harboring RET/PTC. They also suggest that the role of CaMKII in medullary thyroid carcinomas induced by RET mutations should be investigated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Arighi E, Alberti L, Torriti F, Ghizzoni S, Rizzetti MG, Pelicci G, Pasini B, Bongarzone I, Piutti C, Pierotti MA *et al.* 1997 Identification of Shc docking site on Ret tyrosine kinase. *Oncogene* **14** 773–782.
- Borrello MG, Alberti L, Arighi E, Bongarzone I, Battistini C, Bardelli A, Pasini B, Piutti C, Rizzetti MG, Mondellini P *et al.* 1996 The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase Cgamma. *Molecular and Cellular Biology* **16** 2151–2163.
- Castellone MD, Guarino V, De Falco V, Carlomagno F, Basolo F, Faviana P, Kruhoffer M, Orntoft T, Russell JP, Rothstein JL *et al.* 2004 Functional expression of the CXCR4 chemokine receptor is induced by RET/PTC oncogenes and is a common event in human papillary thyroid carcinomas. *Oncogene* **23** 5958–5967.
- Chang BH, Mukherji S & Soderling TR 1998 Characterization of a calmodulin kinase II inhibitor protein in brain. *PNAS* **95** 10890–10895.
- Ciampi R & Nikiforov YE 2007 RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis. *Endocrinology* **148** 936–941.
- Clapham DE 2007 Calcium signaling. *Cell* **131** 1047–1058.
- Demidenko ZN & Blagosklonny MV 2008 Growth stimulation leads to cellular senescence when the cell cycle is blocked. *Cell Cycle* **7** 3355–3361.
- Esapa CT, Johnson SJ, Kendall-Taylor P, Lennard TW & Harris PE 1999 Prevalence of Ras mutations in thyroid neoplasia. *Clinical Endocrinology* **50** 529–535.
- Greco A, Pierotti MA, Bongarzone I, Pagliardini S, Lanzi C & Della Porta G 1992 TRK-T1 is a novel oncogene formed by the fusion of TPR and TRK genes in human papillary thyroid carcinomas. *Oncogene* **7** 237–242.
- Illario M, Cavallo AL, Bayer KU, Di Matola T, Fenzi G, Rossi G & Vitale M 2003 Calcium/calmodulin-dependent protein kinase II binds to Raf-1 and modulates integrin-stimulated ERK activation. *Journal of Biological Chemistry* **278** 45101–45108.
- Illario M, Cavallo AL, Monaco S, Di Vito E, Mueller F, Marzano LA, Troncone G, Fenzi G, Rossi G & Vitale M 2005 Fibronectin-induced proliferation in thyroid cells is mediated by $\alpha_3\beta_1$ integrin through Ras/Raf-1/MEK/ERK and calcium/CaMKII signals. *Journal of Clinical Endocrinology and Metabolism* **90** 2865–2873.
- Illario M, Monaco S, Cavallo AL, Esposito I, Formisano P, D'Andrea L, Cipolletta E, Trimarco B, Fenzi G, Rossi G *et al.* 2009 Calcium–calmodulin-dependent kinase II (CaMKII) mediates insulin-stimulated proliferation and glucose uptake. *Cellular Signalling* **21** 786–792.
- Kelley GG, Reks SE, Ondrako JM & Smrcka AV 2001 Phospholipase C(epsilon): a novel Ras effector. *EMBO Journal* **20** 743–754.
- Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE & Fagin JA 2003 High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC–RAS–BRAF signaling pathway in papillary thyroid carcinoma. *Cancer Research* **63** 1454–1457.
- Knauf JA, Kuroda H, Basu S & Fagin JA 2003 RET/PTC-induced dedifferentiation of thyroid cells is mediated through Y1062 signaling through SHC-RAS-MAP kinase. *Oncogene* **22** 4406–4412.
- Kremenevskaja N, von Wasielewski R, Rao AS, Schofl C, Andersson T & Brabant G 2005 Wnt-5a has tumor suppressor activity in thyroid carcinoma. *Oncogene* **24** 2144–2154.
- Means AR 2000 Regulatory cascades involving calmodulin-dependent protein kinases. *Molecular Endocrinology* **14** 4–13.
- Melillo RM, Castellone MD, Guarino V, De Falco V, Cirafici AM, Salvatore G, Caiazzo F, Basolo F, Giannini R, Kruhoffer M *et al.* 2005 The RET/PTC–RAS–BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. *Journal of Clinical Investigation* **115** 1068–1081.
- Monaco S, Illario M, Rusciano MR, Gragnaniello G, Di Spigna G, Leggiero E, Pastore L, Fenzi G, Rossi G & Vitale M 2009 Insulin stimulates fibroblast proliferation through calcium–calmodulin-dependent kinase II. *Cell Cycle* **8** 2024–2030.
- Nikiforov YE 2002 RET/PTC rearrangement in thyroid tumors. *Endocrine Pathology* **13** 3–16.
- Richardson RM, Ali H, Pridgen BC, Haribabu B & Snyderman R 1998 Multiple signaling pathways of human interleukin-8 receptor A. Independent regulation by phosphorylation. *Journal of Biological Chemistry* **273** 10690–10695.
- Salvatore D, Barone MV, Salvatore G, Melillo RM, Chiappetta G, Mineo A, Fenzi G, Vecchio G, Fusco A & Santoro M 2000 Tyrosines 1015 and 1062 are *in vivo* autophosphorylation sites in ret and ret-derived oncoproteins. *Journal of Clinical Endocrinology and Metabolism* **85** 3898–3907.
- Santoro M, Chiappetta G, Cerrato A, Salvatore D, Zhang L, Manzo G, Picone A, Portella G, Santelli G, Vecchio G *et al.* 1996 Development of thyroid papillary carcinomas secondary to tissue-specific expression of the RET/PTC1 oncogene in transgenic mice. *Oncogene* **12** 1821–1826.
- Sapio MR, Posca D, Troncone G, Pettinato G, Palombini L, Rossi G, Fenzi G & Vitale M 2006 Detection of BRAF mutation in thyroid papillary carcinomas by mutant allele-specific PCR amplification (MASA). *European Journal of Endocrinology* **154** 341–348.

- Schweppe RE, Klopper JP, Korch C, Pugazhenth U, Benezra M, Knauf JA, Fagin JA, Marlow LA, Copland JA, Smallridge RC *et al.* 2008 Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. *Journal of Clinical Endocrinology and Metabolism* **93** 4331–4341.
- Sherman SI 2003 Thyroid carcinoma. *Lancet* **361** 501–511.
- Takada I, Mihara M, Suzawa M, Ohtake F, Kobayashi S, Igarashi M, Youn MY, Takeyama K, Nakamura T, Mezaki Y *et al.* 2007 A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nature Cell Biology* **9** 1273–1285.
- Vitale M, Illario M, Di Matola T, Casamassima A, Fenzi G & Rossi G 1997 Integrin binding to immobilized collagen and fibronectin stimulates the proliferation of human thyroid cells in culture. *Endocrinology* **138** 1642–1648.
- Zlotnik A & Yoshie O 2000 Chemokines: a new classification system and their role in immunity. *Immunity* **12** 121–127.