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BRAF Mediates RET/PTC-Induced Mitogen-Activated Protein Kinase Activation in Thyroid Cells: Functional Support for Requirement of the RET/PTC-RAS-BRAF Pathway in Papillary Thyroid Carcinogenesis

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In human papillary thyroid cancers (PTCs), mutations of *RET/PTC*, *NTRK*, *RAS*, or *BRAF* are found in about two thirds of cases with practically no overlap, providing genetic evidence that constitutive signaling along RET-RAS-BRAF-MAPK is key to their development. The requirement for BRAF in RET/PTC-mediated MAPK activation and gene expression has not been tested functionally. There are three RAF isoforms: ARAF, BRAF, and CRAF. Compared with the others, ARAF is a much weaker stimulator of MAPK. To determine the key RAF isoform mediating RET/PTC-induced ERK phosphorylation, we stably transfected doxycycline-inducible RET/PTC3-expressing thyroid PCCL3 cells with small interfering RNA vectors to induce selective knockdown of BRAF or CRAF. Conditional RET/PTC3 expression induced comparable ERK phosphorylation in CRAF knockdown and control cells but negligible ERK phosphorylation in BRAF knockdown cells. Selective knockdown of BRAF prevented RET/PTC-dependent down-regulation of the sodium iodide symporter, a gene that confers key biological effects of RET/PTC in PTCs. Moreover, microarray analysis revealed numerous RET/PTC-regulated genes showing requirement of BRAF for appropriate expression. These data indicate that BRAF is required for RET/PTC-induced MAPK activation in thyroid cells and support the notion that BRAF inactivation may be an attractive target for PTCs. (*Endocrinology* 147: 1014–1019, 2006)

RET REARRANGEMENTS (*RET/PTC*) resulting in constitutively active chimeric forms of this tyrosine kinase receptor are found in 5–30% of papillary thyroid carcinomas (PTCs) (1). This activation occurs through chromosomal recombination linking the N-terminal domain of unrelated partner gene and its promoter region to the C-terminal kinase domain of RET. RET is not normally expressed to a significant degree in thyroid follicular cells, but the promoter of the partner gene drives constitutive expression of the chimeric gene. There are at least 10 different types of *RET/PTC* chimeric genes that differ according to the 5′ partner gene involved in the rearrangement. *RET/PTC1* and *RET/PTC3* are the most common types, accounting for more than 90% of all of the rearrangements. The N-terminal domain of the RET/PTC1 and three oncoproteins is donated by the partner gene and has been shown to promote dimerization of the fusion protein and autophosphorylation of key tyrosine residues in a ligand-independent manner, resulting in constitutive activation of the kinase function of RET (2, 3). Several signaling pathways activated by RET have been identified. Among these, Tyr-1015 and Tyr-1062 are thought to be important for the oncogenic function of RET/PTC. Phosphorylation of Tyr-1015 and Tyr-1062 forms docking sites for phospholipase Cγ (PLCγ) and Shc, respectively (4–6). The Shc-RAS-MAPK pathway seems to be significant for RET-induced tumorigenesis (7).

Recently, a somatic point mutation of the *BRAF* gene leading to a V600E substitution, previously designated as V599E, has been identified as the most common genetic change in PTCs (36–69%) (reviewed in Ref. 8). This mutation is believed to disrupt a hydrophobic interaction between the P-loop and activation domains and converts BRAF into a catalytically active conformation (9). Interestingly, there is little or no overlap between PTCs with *RET/PTC*, *NTRK*, *RAS*, and *BRAF* mutations (10–12). This observation provides strong genetic evidence that constitutive signaling along the RET-RAS-BRAF-MAPK pathway is key to development of PTCs. However, this has not been examined experimentally in thyroid cells.

There are three RAF isoforms in mammalian cells: ARAF, BRAF, and CRAF (or RAF-1) (13). Dominant-negative mutations of *CRAF* and *CRAF* antisense constructs blocked proliferation and transformation induced by activated K-RAS and H-RAS in NIH3T3 cells (14). CRAF is expressed ubiquitously, whereas ARAF and BRAF are expressed in a tissue-specific manner. Although all RAF isoforms activate MAPK kinase (MEK), their activities are differentially regulated by upstream stimuli or other interacting molecules (15). ARAF and CRAF are more strongly activated by the oncogenic tyrosine kinase Src than by RAS, and both of these synergize to give maximal

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Abbreviations: MEK, MAPK kinase; NIS, sodium iodide symporter; PKA, protein kinase A; PKA-RIα, cAMP-dependent protein kinase regulatory subunit I α; PLCγ, phospholipase Cγ; PTC, papillary thyroid cancer; RNAi, RNA interference; siRNA, small interfering RNA.

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activation (16). In contrast, BRAF is strongly activated by oncogenic RAS alone. ARAF binds to RAS more weakly and is also a much weaker activator of MEK compared with BRAF and CRAF. In turn, BRAF seems to be a stronger stimulus for MEK than CRAF. The mechanisms that regulate MEK and ERK are quite well understood, whereas regulation of RAF is affected by other signaling pathways through phosphorylation, dephosphorylation, and protein-protein interactions (13, 15).

Besides the putative effects of RET mediated by Shc-RAS-RAF-MEK, the tyrosine kinase domain of RET also activates other signaling intermediates that can modulate RAF kinase activity, such as PLCγ-protein kinase C and Shc-phosphatidylinositol 3-kinase-Akt (17). Activation of protein kinase C has been shown to stimulate CRAF kinase activity. In contrast, Akt phosphorylates Ser-259 on CRAF and Ser-364 on BRAF and inhibits their function. Moreover, in thyroid cells, TSH binding of the TSH receptor results in cAMP generation and protein kinase A (PKA) activation, as well as cAMP induction of Rap1 through a PKA-independent pathway (18). Ser-43 on CRAF is directly phosphorylated by PKA and mediates PKA-induced inhibition in certain cell types and serum conditions (15). This site is not conserved in BRAF, despite which forskolin-induced PKA also inhibits BRAF activity. By contrast, Rap1 activates BRAF but inhibits CRAF (15).

Because of these multiple points of regulation, the effects of RET on RAF isoforms is of considerable interest in view of the critical role of these oncoproteins in thyroid cancer pathogenesis. Here we report that BRAF (but not CRAF) mediates RET-induced ERK phosphorylation and regulates expression of a number of genes in thyroid cells. This suggests that BRAF may be an attractive therapeutic target for the majority of PTCs.

Materials and Methods

Cell lines and transfections

PCCL3 cells, a clonal rat thyroid cell line requiring TSH for growth, were maintained in H4 medium consisting of Coon's medium/F-12 high zinc supplemented with 5% fetal bovine serum, 0.3 mg/ml L-glutamine, 1 mIU/ml TSH, 10 μg/ml insulin, 5 μg/ml apo-transferrin, 10 nM hydrocortisone, and penicillin/streptomycin. PTC3-5 cells were derived from PCCL3 cells to obtain doxycycline-inducible expression of RET/PTC3 as described previously (19). PTC3-5 cells were stably transfected with pcPUR+U6i-Cont (PTC3-Cont^{RNAi}-1 cells), -BRAF3 (PTC3-BRAF^{RNAi}3–6 cells), -BRAF4 (PTC3-BRAF^{RNAi}4–8 cells), -CRAF2 (PTC3-CRAF^{RNAi}2–16 cells), or -CRAF3 (PTC3-CRAF^{RNAi}3–3 cells) (Table 1) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Western blotting

Cells were lysed in a cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 50 mM NaF, 10 mM Na pyrophosphate, 2 mM phenylmethylsulfonylfluoride, 1 mM Na orthovanadate, and protease inhibitor cocktail (Sigma, St. Louis, MO). After measurement of protein concentration using Micro BCA Protein Assay Reagent (Pierce, Rockford, IL), 25 μg of each sample was separated by SDS-PAGE and blotted onto polyvinylidene difluoride or nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The following primary antibodies were used: anti-phospho-ERK E10 (Cell Signaling Technology, Beverly, MA), anti-ERK1 K-23 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-BRAF F-7 (Santa Cruz Biotechnology), anti-CRAF C-12 (Santa Cruz Biotechnology), anti-total Akt (Santa Cruz Biotechnology), anti-phospho-Akt (Cell Signaling Technology) anti-sodium iodide symporter (NIS) (a gift from Nancy Carrasco, Albert Einstein College of Medicine, New York, NY), and anti-RET (provided by Yuri Nikiforov, University of Cincinnati, Cincinnati, OH). The antigen-antibody complexes were visualized using horseradish peroxidase-conjugated antimouse or rabbit IgG antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence system (Amersham Biosciences). The membrane was stripped using a Restore Western Blot Stripping Buffer (Pierce) and reprobed several times. For quantitation, gel images were captured using the Kodak Image Station and band density was determined using the 1D Image Analysis software (Eastman Kodak, Rochester, NY).

Microarray analysis

The microarray analysis was performed essentially as described previously (20). Details can be found at http://microarray.uc.edu. Briefly, a rat 70-mer oligonucleotide library representing 27,342 genes (Qiagen-Operon, Alameda, CA) was spotted onto slides. The microarray slides were cohybridized with fluorescence (cyanine 3 or 5)-labeled cDNAs from PTC3-5 (or PTC3-BRAF^{RNAi}4–8) cells treated with or without 1 μg/ml doxycycline for 4 d. Three independent hybridizations by three separate RNA extractions with exchange of fluorescence dye color were performed to increase statistical power. Fold changes (Table 2) between cells treated with and without doxycycline in each cell line were calculated based on signal intensities on the spot. Statistical significance of the differential expression between treatment and control, was assessed by calculating *P* values for corresponding linear contrasts. Multiple hypothesis testing adjustment was performed by calculating false discovery rates (20).

Real-time RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Five micrograms of total RNA was reverse transcribed using a SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) with random hexamers. The following PCR amplifications were performed using QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) in a LightCycle instrument (Cepheid, Sunnyvale, CA). The cycle threshold value, which was determined using the second derivative, was used to calculate the normalized expression of the indicated genes using Q-Gene Software (21). The following primer pairs and annealing temperature were used: β-actin, 5′-ctgaaccctaaggccaaccgtg-3′ and 5′-ggcatacagggacagcacagcc-3′, at 56 C; α-crystallin B, 5′-ctacagccacttccctgagc-3′ and 5′-gtggaactccctggagatga-3′ at 60 C; and cAMP-dependent protein kinase regulatory subunit I α (PKA-RIα), 5′-gtgggtgaccaacatcagtg-3′ and 5′-tcttgcgtttcctcagtgtg-3′, at 55 C.

Results and Discussion

The purpose of this study was to clarify the key RAF isoform mediating RET/PTC-induced MAPK activation. To

TABLE 1. Sequences of RNAi target sites and oligonucleotides designed to construct siRNA expression vectors

Target site	Oligo design
BRAF3	5′caccGGAGtTAtATGTTGAAGTAtTgtgtgctgtccAGTACTTCAACATGTAGCTCCttttt-3′
GA-GGAGCTACATGTTGAAGTACT	3′-CCTCaATaTACAACTTCATaAcacacgacaggTCATGAAGTTGTACATCGAGGaaaaatacg5′
BRAF4	5′caccATATtAAGtAAATGATTgAGTgtgtgctgtccACTTAATCATTTGCTTGATATttttt-3′
GA-ATATCAAGCAAATGATTAAGT	3′-TATAaTTCaTTTACTAAcTCAcacacgacaggTGAATTAGTAAACGAACTATAaaaaatacg5′
CRAF2	5′caccACGTTCtACTtACAAtTCACAgtgtgctgtccTGTGAGTTGTGAGTGGAACGTttttt-3′
TC-ACGTTCCACTCACAACTCACA	3′-TGCAAGaTGAaTGTTaAGTGTcacacgacaggACACTCAACACTCACCTTGCAaaaaatacg5′
CRAF3	5′caccAGtTCTTGtTGTTTCtAAATTgtgtgctgtccAATTTGGAAACAGCAAGAGCTttttt-3′
AC-AGCTCTTGCTGTTTCCAAATT	3′-TCaAGAACaACAAAGaTTTAAcacacgacaggTTAAACCTTTGTCGTTCTCGAaaaaatacg5′

TABLE 2. Microarray data comparison between inducible RET/PTC3 and RET/PTC3 with knockdown of BRAF in PCCL3 cells

Ensembl gene ID	Gene description	PTC3 fold change	PTC3-BRAF^{RNAi} fold change	Ratio
ENSRNOG00000007159	Small inducible cytokine A2 precursor (CCL2/MCP1)	31.80	7.73	4.11
ENSRNOG00000018735	H-2 class II histocompatibility antigen, γ chain	26.62	6.70	3.97
ENSRNOG00000023998	Human: cystatin A (stefin A)	4.78	1.31[a]	3.64
ENSRNOG00000007457	Human: plasma protease C1 inhibitor precursor (C1 INH)	10.49	3.19	3.29
ENSRNOG00000018567	Solute carrier family 20	3.59	1.15[a]	3.13
ENSRNOG00000014548	Human: enhancer of filamentation 1 (HEF1)	−4.03	−1.35[a]	2.99
ENSRNOG00000009196	Human: membrane-associated nucleic acid binding protein	3.43	1.24[a]	2.76
ENSRNOG00000010524	α-Crystallin B chain	−2.78	−1.09[a]	2.54
ENSRNOG00000022552	Human: novel SH2-containing protein 3	−3.07	−1.23[a]	2.50
ENSRNOG00000000768	Ubiquitin D	27.88	11.21	2.49
ENSRNOG00000006104	Thyroglobulin precursor	−6.97	−2.81	2.48
ENSRNOG00000002436	Human: monocyte to macrophage differentiation protein	4.78	1.94	2.47
ENSRNOG00000022839	Human: interferon-induced protein with tetratricopeptide repeats 4 (IFIT4)	16.14	6.77	2.38
ENSRNOG00000014656	Cytochrome oxidase polypeptide VIII-heart, mitochondrial precursor	2.64	−1.12[a]	2.37
ENSRNOG00000011832	Pulmonary surfactant-associated protein D precursor (PSP-D)	6.28	2.67[a]	2.36
ENSRNOG00000013290	Mouse: nuclear receptor interacting protein 3	−3.24	−1.39[a]	2.33
ENSRNOG00000011800	Tissue factor precursor (TF)	8.63	3.73	2.31
ENSRNOG00000003148	Metalloproteinase inhibitor 2 precursor (TIMP2)	−4.10	−1.79	2.29
ENSRNOG00000024243	Urokinase plasminogen activator surface receptor	16.14	7.11	2.27
ENSRNOG00000001306	Human: cAMP-dependent protein kinase type I-α regulatory chain (TSE1)	4.31	1.91[a]	2.26

[a] Change is not statistically different from cells not treated with doxycycline ($P > 0.05$).

achieve this, we stably transfected clonal doxycycline-inducible RET/PTC3-expressing rat thyroid PCCL3 cells (PTC3-5 cells) with small interfering RNA (siRNA) expression vectors to induce selective knockdown of each RAF isoform. Because ARAF expression in PCCL3 cells was much lower than in NIH3T3 cells (data not shown) and ARAF kinase activity is much lower than BRAF or CRAF (16), we focused on BRAF and CRAF. First, to identify efficient RNA interference (RNAi) target sites, we used our own algorithm to choose four target sites for each RAF isoform: BRAF1–BRAF4 and CRAF1–CRAF4 (the details of the algorithm will be published elsewhere). We prepared hairpin-type siRNA expression vectors containing a human U6 promoter, two *Bsp*MI sites, and a puromycin-resistant gene (22, 23). We next synthesized oligonucleotides with the target sequence, a hairpin sequence (5′-gtgtgctgtcc-3′), terminator sequence, and overhanging sequence. As shown in Table 1, three mutations in the sense strand from C to t or from A to g, which generated G:U base pairing in siRNA, were inserted to reduce the mutation rates in *Escherichia coli* and facilitate sequence analysis (22, 23). Then we annealed the fragments and inserted them into *Bsp*MI sites. The sequences of all constructs were confirmed. Four siRNA constructs that target different regions of the respective RAF cDNA were tested for each isoform in transient assays (data not shown). The two siRNAs with highest knockdown efficiencies for each isoform (BRAF3, BRAF4, CRAF2, and CRAF3) were used to establish stable clones (Table 1).

We isolated several stable clones and checked the knockdown efficiencies. The best clones for each RNAi target site were chosen for functional experiments (PTC3-BRAF^{RNAi}3–6, PTC3-BRAF^{RNAi}4–8, PTC3-CRAF^{RNAi}2–16, and PTC3-CRAF^{RNAi}3–3). We then used these clones to determine the impact of selective knockdown of each RAF isoform on ERK phosphorylation after conditional expression of RET/PTC. To rule out RNAi off-target effects (nonspecific silencing), we used two different target sites for each RAF isoform in this key experiment. Moreover, the two target sites for the other RAF isoform can be considered as controls (*e.g.* RNAi clone for CRAF can be a control of RNAi for BRAF). As shown in Fig. 1, RET/PTC expression in every cell line was induced in a dose-dependent manner by doxycycline. RET/PTC expression levels between the clones were comparable. In control cells, ERK phosphorylation was induced by RET/PTC in a concentration-dependent manner. CRAF knockdown (PTC3-CRAF^{RNAi}2–16 cells and PTC3-CRAF^{RNAi}3–3 cells) did not affect RET/PTC-induced ERK phosphorylation. In contrast, RET/PTC elicited only a negligible induction of ERK phosphorylation in PTC3-BRAF^{RNAi}4–8 cells. In PTC3-BRAF^{RNAi}3–6 cells, ERK phosphorylation was slightly induced, probably due to lower knockdown efficiency of BRAF in this line. These data indicate that BRAF is required for RET/PTC-induced MAPK activation in thyroid cells. Although we believe the most likely explanation for requirement of BRAF for RET/PTC-mediated ERK activation is through the RAS signaling pathway, we cannot exclude the possibility that loss of BRAF eliminates TSH-dependent MAPK activation via Rap1. This is unlikely because of the previously demonstrated inhibition by RET/PTC of TSH-mediated cAMP generation through events upstream of Rap1 (19). Indeed, a similar requirement for BRAF was observed in the absence of TSH.

Paradoxically, there may be a very subtle greater induction of phospho-ERK1/2 levels in the CRAF knockdown cell line CRAF^{RNAi}3–3 compared with control RNAi. However, it should be noted that, despite our attempts to carefully titrate the level of RET/PTC induction, this cannot be done with complete accuracy, which detracts from our ability to draw strong conclusions from subtle changes.

Marais *et al.* (16) reported that kinase activity of BRAF acti-

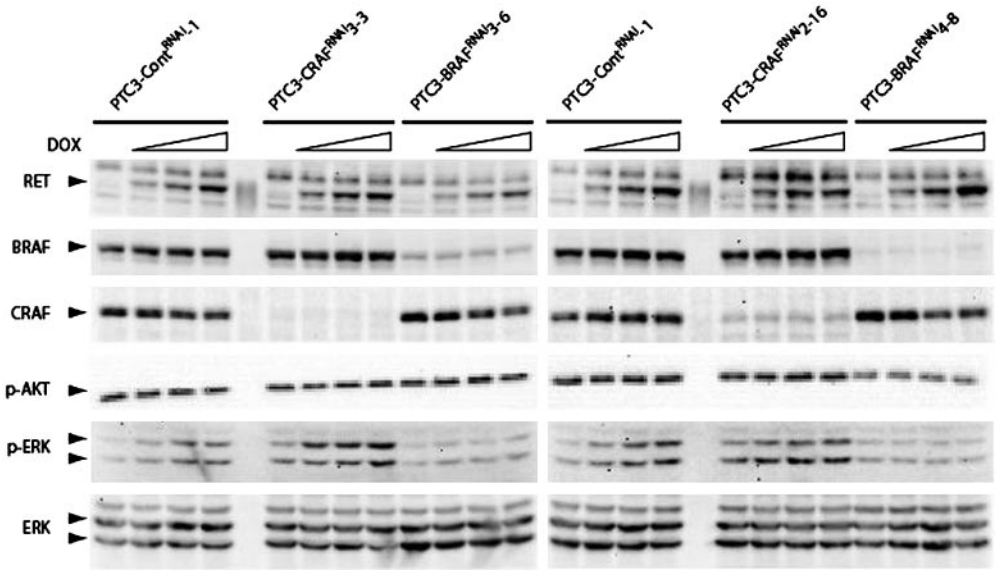

FIG. 1. BRAF regulates RET-induced ERK phosphorylation. The indicated cell lines were treated with different concentration of doxycycline (DOX; 0–500 ng/ml) for 48 h. A total of 25 μg of protein lysate was separated by 8% SDS-PAGE and blotted onto polyvinylidene difluoride membrane. The indicated primary antibodies were used. Similar results were obtained in three independent experiments.

vated by RAS is markedly greater than that of CRAF. However, after combined activation by RAS and Src, CRAF kinase activity approximates 60% of maximally activated BRAF (16). Because viral Src phosphorylates phosphatidylinositol 3-kinase, Shc, and PLCγ (24), we anticipated that RET/PTC might activate CRAF and that this isoform would at least play a partial role in activation of MEK. Of note, in the studies of Marais *et al.* (16), the RAF isoforms, oncogenic Src, or RAS were overexpressed in COS7 cells, whereas in our system, only the triggering stimulus (*i.e.* RET/PTC3) is overexpressed (16). Our data are consistent with studies in CRAF-deficient mouse embryonic fibroblasts, in which ERK activation by growth factors was not compromised, presumably due to compensation by BRAF (25, 26). Epidermal growth factor-induced ERK activation is also completely abrogated in BRAF-deficient mouse embryonic fibroblasts (27). In DT40 B-cells, CRAF was dispensable for B cell receptor-mediated ERK activation, whereas BRAF-deficient cells showed reduced ERK activation (28). Overall, these results are consistent with our finding that CRAF seems dispensable for RET/PTC-induced ERK activation (Fig. 1). However, because it is not possible to completely control for clonal variation and subtle differences in RET/PTC expression, we cannot exclude a minor role for CRAF in RET/PTC-induced ERK activation. It is possible that, in thyroid cells, TSH-induced cAMP signaling, presumably acting via Rap1, may suppress CRAF kinase activity, thus further enabling BRAF to function as a preferred downstream effector for RET/PTC.

The MAPK pathway plays an important role in growth and dedifferentiation of thyroid cells. Previous studies (19) indicate that acute expression of RET/PTC in PCCL3 cells inhibits TSH-dependent growth and slightly decreases cell viability. To examine whether these effects require BRAF or CRAF, growth curves were performed with the BRAF and CRAF knockdown cells lines. These showed that knockdown of neither BRAF nor CRAF prevented the inhibition of TSH-dependent growth by RET/PTC (data not shown). Similarly, there was no apparent interference by knockdown of either RAF isozyme on the morphological changes induced by RET/PTC3. Studies from several groups demonstrated that constitutive activation of RET-RAS-MAPK pathway impaired thyroid-specific gene expression, including NIS (29–31). Because of its physiological significance, we next investigated the effects of selective RAF isoform knockdown on NIS expression after conditional activation of RET/PTC. As shown in Fig. 2A, treatment with doxycycline for 3–6 d induced a decline of NIS expression in control and PTC3-CRAF^{RNAi}3–3 cells. However, in PTC3-BRAF^{RNAi}4–8 cells, NIS expression was not altered by RET/PTC. This indicates that BRAF mediates RET-induced inhibition of NIS expression and presumably of iodine transport, a function critical for hormone biosynthesis and for successful medical therapy of differentiated thyroid cancers.

To identify other genes regulated by RET/PTC via BRAF, we performed a microarray experiment using RNA isolated from PTC3-5 and PTC3-BRAF^{RNAi}4–8 cells. A comprehensive description of this microarray experiment will be reported elsewhere. Here we attempted to define a restricted RET/PTC-regulated gene set showing dependence on BRAF. We compared the fold change in gene expression between the two cell lines (RET/PTC with or without BRAF knockdown) 4 d after RET/PTC activation. The 20 genes showing the highest ratios are shown in Table 2. Using these experimental conditions, accuracy of the microarray is more than 90% for genes showing a 2-fold regulation (20). To further validate the microarray results and to explore the contribution of the presence or absence of both CRAF and BRAF to their regulation, we performed quantitative real-time RT-PCR for two biologically interesting genes. α-Crystallin B is a small heat shock protein that has been shown to inhibit apoptosis (32, 33). PKA-RIα is an inhibitory subunit of PKA (34). Inactivating mutations of the gene coding for this protein (*PRKAR1A*) are found in Carney complex, a familial multiple neoplasia syndrome associated

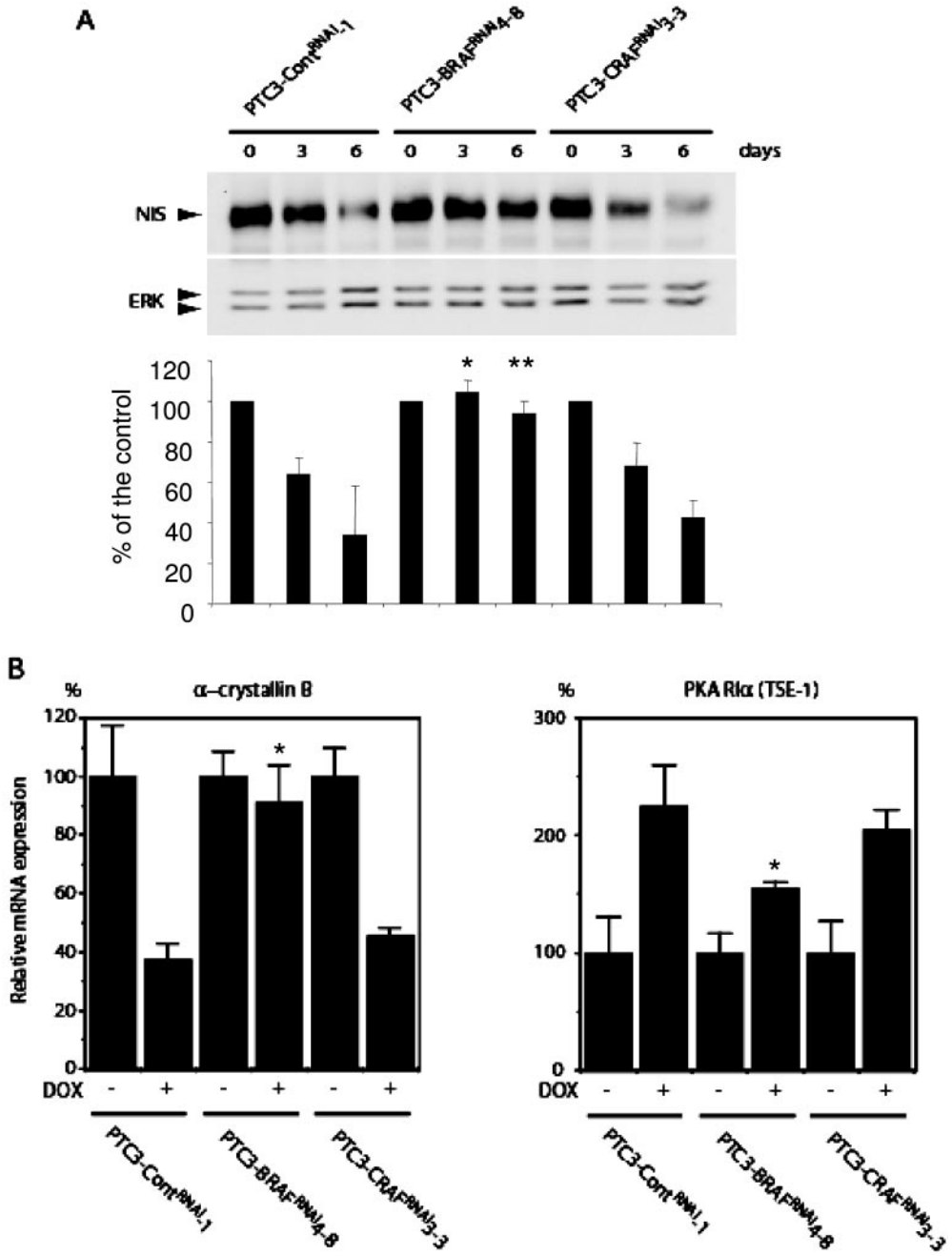

FIG. 2. A, Requirement of BRAF for RET/PTC3-regulated expression of NIS. The indicated cell lines were treated with 1 μg/ml doxycycline for the indicated time. A total of 25 μg of total cell lysates were separated by 9% SDS-PAGE and transferred to nitrocellulose membrane, and membranes were probed with the indicated antibodies. Quantitation of NIS protein levels after induction of RET/PTC3 is shown at the *bottom*. Data are the mean ± SEM of three independent experiments. NIS protein was normalized to total ERK levels and expressed as a percentage of NIS abundance in cells not exposed to doxycycline. *, $P = 0.00048$; and **, $P = 0.00230$ *vs.* NIS abundance at the corresponding time point in control cells. B, Requirement of BRAF and/or CRAF for RET/PTC3 regulation of expression of selected genes. The indicated cell lines were treated with or without 1 μg/ml doxycycline (DOX) for 6 d. Quantitative real-time RT-PCR was performed as described in *Materials and Methods*. Data are represented relative to expression of the indicated cells in the absence of doxycycline. Each bar represents the mean and SE of the data collected in triplicate. *, $P = 0.0025$; and **, $P = 0.027$ *vs.* expression at the corresponding time point in control cells.

with adrenocortical hyperplasia, abnormal mucosal, and skin pigmentation (34, 35).

As shown in Fig. 2B, α-crystallin B was down-regulated in control and PTC3-CRAF^{RNAi}3–3 cells but not in PTC3-BRAF^{RNAi}4–8 cells. Because conditional expression of RET/PTC or RAS in PCCL3 cells induces apoptosis (19, 36), BRAF may play a role in cell death through impairment of α-crystallin B expression.

PKA-RIα was induced in control and PTC3-CRAF^{RNAi}3–3 cells by about 2-fold, but the induction was modest in PTC3-BRAF^{RNAi}4–8 cells (Fig. 2B). There are two potential activator protein-1 binding sites in the 5′-flanking region of human PKA-

RIα gene (37), which could mediate this effect. Conditional expression of RET/PTC disrupts cAMP signaling (19), and these data raise the possibility that BRAF may mediate impairment of TSH-induced PKA activation through higher expression of PKA-RIα.

In conclusion, BRAF is required for RET/PTC-induced MAPK activation and regulation of a number of downstream target genes in thyroid cells, several of which may be of relevance to disease pathogenesis. This provides functional corroboration for the genetic evidence pointing to a central role of RET/PTC-RAS-BRAF-MAPK in thyroid cell transformation. Because mutations of *RET/PTC*, *RAS*, or *BRAF* account for about 70% of PTCs, these data predict that BRAF-specific antagonists may hold promise as a therapy for the majority of thyroid cancers.

Acknowledgments

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