Trihydrophobin 1 Is a New Negative Regulator of A-Raf Kinase*

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Our previous work indicated that instead of binding to B-Raf or C-Raf, trihydrophobin 1 (TH1) specifically binds to A-Raf kinase both in vitro and in vivo. In this work, we investigated its function further. Using confocal microscopy, we found that TH1 colocalizes with A-Raf, which confirms our former results. The region of TH1 responsible for the interaction with A-Raf is mapped to amino acids 1-372. Coimmunoprecipitation experiments demonstrate that TH1 is associated with A-Raf in both quiescent and serum-stimulated cells. Wild type A-Raf binds increasingly to TH1 when it is activated by serum and/or upstream oncogenic Ras/Src compared with that of “kinase-dead” A-Raf. The latter can still bind to TH1 under the same experimental condition. The binding pattern of A-Raf implies that this interaction is mediated in part by the A-Raf kinase activity. As indicated by Raf protein kinase assays, TH1 inhibits A-Raf kinase, whereas neither B-Raf nor C-Raf kinase activity is influenced. Furthermore, we observed that TH1 inhibited cell cycle progression in TH1 stably transfected 7721 cells compared with mock cells, and flow cell cytometry analysis suggested that the TH1 stably transfected 7721 cells were G0/G1 phase-arrested. Taken together, our data provide a clue to understanding the cellular function of TH1 on Raf isoform-specific regulation.

The human trihydrophobin 1 (TH1) gene is the homolog of Drosophila TH1, which was originally identified during the positional cloning of mei-41 (1, 2). The TH1 gene lies adjacent to mei-41 and was characterized further by the D. T. Bonthron group in 2000 (3). According to their studies, the TH1 gene was highly conserved from Drosophila to human by sequence comparison. The human TH1 gene was located in chromosome 20q13, which had a transcript product of 2.4 kb. Multiple tissues detected by Northern blots showed that TH1 was widely expressed. The human TH1 protein has been predicted having a molecular mass of 65.8 kDa and displays high levels of expression in cardiac and skeletal muscle, kidney, adrenal, and thyroid. Although ubiquitously expressed, its function is not clear at present. From protein sequence data-base analysis, this protein does not seem to have a potential kinase domain. Our previous work has identified TH1 as a new interaction partner of A-Raf kinase, which belongs to a serine/threonine kinase family involved in MAPK signal transduction pathways (4).

The mammalian MAPK signaling pathways have been found to play key roles in a wide range of cellular responses such as proliferation, differentiation, and apoptosis corresponding to multiple extracellular stimuli (5-9). Among them, the Raf/MEK/ERK pathway is so far the best described pathway, which is responsive to mitogenic and differential stimuli. It was depicted as a conserved three-kinase cascade pathway that consists of MKKKs, MKks, and ERKs (6-9). As members of MKKKs, the serine/threonine Raf kinase family is poised in the core of the three-kinase module, which is important in relaying upstream signals to the nucleus (10-12). In mammalian cells, the Raf kinase family is composed of three important isoforms: A-Raf, B-Raf, and C-Raf (also known as Raf-1). These Raf isoforms share high similarities in their sequence and structure as reported previously (11-13).

Most studies have revealed that the Raf isoforms are under different regulation and have individual functions. In brief, to their activation, although all three Raf isoforms interact with their upstream activator Ras and are activated by translocating to the membrane ((14, 15), studies in COS-7 cells showed that their activations were quite different. Unlike B-Raf, whose activation is sufficient by oncogenic Ras alone, A-Raf behaves like C-Raf, which needs synergism of oncogenic Ras and other tyrosine kinases such as Src to achieve full activation (16, 17). Adding to the complexity, B-Raf kinase activity was proven to be determined by Ras and other members of the small G protein family such as Rap1 in PC12 cells (18) or TC21 in NIH3T3 cells (16, 19, 20). Moreover, Rap1 has been reported to inhibit C-Raf, whereas TC21 interacts with B-Raf and C-Raf but not A-Raf in these studies. Upon activation, the three Raf isoenzymes display differences in their catalytic activities. As measured by protein kinase assays, B-Raf has the highest activity to phosphorylate their common substrate, MEK1, whereas A-Raf has the lowest activity, and C-Raf catalytic activity is intermediate between them (21, 22). Studies with dominant negative mutants (23) and antisense oligodeoxynucleotides (24-27) on Raf isoforms suggested that Raf family activities are also regulated differentially by multiple pathways including phosphatidylinositol 3-kinase (23), protein kinase A (18), protein kinase C (27, 28), and Akt (protein kinase B) (25, 29) pathways according to different extracellular...
Suppression of A-Raf Kinase by TH1

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The human SMMC-7721 hepatocarcinoma cells (Institute of Cell Biology, Academic Sinica) were maintained in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C under 5% CO2 in humidified air. The human embryonic kidney 293 (HEK293) cells kindly provided by Dr. Kun L. Guan (University of Michigan). Plasmid pBOS-B-Raf(KD)-(HA)2 was kindly provided by Dr. Sutor (Mayo Foundation). GST-MEK1 (K52), and GST-ERK1 for prokaryotic expression were obtained from Dr. Ji H. Zhao (Cornell University) and Dr. Jian G. Gu (Osaka University). Ma deletion mutants constructed into plasmid pLexA were cotransformed into S. cerevisiae and expressed in vivo as described in Methods. The full-length TH1 was cut with EcoRI (an EcoRI site is underlined) and XhoI/EcoRI using the primers A-Raf sense (5′-GCACTCGAGCTCTACGTGGACAGGAAACGCACC-3′ (the recognition site of EcoRI is underlined)) and antisense, 5′-TTTGGGCGCCGTTAGCAGGTCGTATCCAT-C-3′ (the recognition site of NotI is underlined), TH1 F1 (amino acids 1–372; sense, 5′-TAAGAATTTGTTACTGACGGTAGGACGGAGGGGGA-3′ (EcoRI) and antisense, 5′-ATGGCCGGCCGTTATCTTGTATGCTGTC-3′ (NotI)), TH1 F2 (amino acids 1–192; sense, 5′-GTCAATTCTAGTACGGAGGTACCTACAG-3′ (EcoRI) and antisense, 5′-TGCCGTTGGAATAAGCTTACACG-3′ (NotI)), TH1 F3 (amino acids 1–170; sense, 5′-TAAGAATTTTCTGTCAGCAGGTTACGGG-3′ (EcoRI) and antisense, 5′-TGACGGCCGCGCTTCTGATCGAGATG-3′ (NotI)). The PCR products of each deletion were cut using corresponding enzymes and cloned into pLexA, pBOS-B-Raf(KD)-(HA)2, and pLexA-A-Raf(KD)-(HA)2 expression vectors. The resulting plasmids were used to transfect S. cerevisiae. 

For binding assay in the yeast two-hybrid system, the full length of TH1 (4) and deletion mutants was cloned in-frame with the DNA binding domain of LexA (Clontech). Plasmid pLexA-A-Raf(KD)-(HA)2 and the glutathione S-transferase (GST) vector pcDNA3-GST-TH1 and pcDNA3-A-Raf and the eukaryotic expression vector pcDNA3.1-myeloid TH1 were obtained as described previously (4). To generate the deletion mutants of TH1, we constructed plasmids with pLexA-TH1 as the template using the following primers: TH1 F1 (amino acids 1–452; sense, 5′-GTCAATTCTCTGAGCAGGGATCCAGGGGGGGA-3′ (EcoRI) and antisense, 5′-TAAGAATTTGTTACTGACGGTAGGACGGAGGGGGA-3′ (EcoRI) and antisense, 5′-TTTGGGCGCCGTTAGCAGGTCGTATCCAT-C-3′ (the recognition site of NotI is underlined)), TH1 F2 (amino acids 1–372; sense, 5′-TAAGAATTTGTTACTGACGGTAGGACGGAGGGGGA-3′ (EcoRI) and antisense, 5′-ATGGCCGGCCGTTATCTTGTATGCTGTC-3′ (NotI)), TH1 F3 (amino acids 1–192; sense, 5′-GTCAATTCTAGTACGGAGGTACCTACAG-3′ (EcoRI) and antisense, 5′-TGCCGTTGGAATAAGCTTACACG-3′ (NotI)), TH1 F4 (amino acids 1–170; sense, 5′-TAAGAATTTTCTGTCAGCAGGTTACGGG-3′ (EcoRI) and antisense, 5′-TGACGGCCGCGCTTCTGATCGAGATG-3′ (NotI)). The PCR products of each deletion were cut using corresponding enzymes and cloned into pLexA-A-Raf(KD)-(HA)2 expression vectors. For fluorescence detection, plasmids pEGFP-N3 and pDsRed-C1 were purchased from Clontech Co. By PCR amplification, we cloned A-Raf in-frame into pDsRed-C1 at the site of Xhol/EcoRI using the primers A-Raf sense (5′-GACCTGCGCTACTAGTGGACAGGAAACGCACC-3′ (the recognition site of EcoRI is underlined)) and antisense (5′-GTCGAATTCTTGACGGAGGTACCTACAG-3′ (EcoRI) and antisense, 5′-GTCGAATTCTTGACGGAGGTACCTACAG-3′ (NotI)). The PCR product was cut with HindIII/Sall and ligated into pcDNA3.1-myc-his-TH1. The full-length B-Raf was generated by PCR with pLexA-B-Raf as the template using the primers B-Raf sense (5′-GCCACTGCGCTACTAGTGGACAGGAAACGCACC-3′ (the recognition site of EcoRI is underlined)) and antisense (5′-GTCGAATTCTTGACGGAGGTACCTACAG-3′ (EcoRI) and antisense, 5′-GTCGAATTCTTGACGGAGGTACCTACAG-3′ (NotI)). The PCR product was cut with HindIII/Sall and ligated into pcDNA3.1-myc-his-TH1. The full-length C-Raf was generated by PCR amplification using the primers C-Raf sense (5′-CATCTGGAGGACCATACGAGGGC-3′ (the recognition site of EcoRI is underlined)) and antisense (5′-CAGAATCTTTGAGAAGACGAGGCG-3′ (EcoRI) and antisense, 5′-CAGAATCTTTGAGAAGACGAGGCG-3′ (NotI)). The full-length TH1 was cut with EcoRI/BamHI from pcDNA3.1-myc-TH1 and cloned in-frame into pEGFP-N3. The plasmids containing oncogenic Ras and Src were the generous gifts of Dr. J. H. Zhao (Cornell University) and Dr. J. G. Gu (Osaka University, Japan), respectively. The plasmids containing GST-MEK1, GST-MEK1(K52), and GST-ERK1 for prokaryotic expression were kindly provided by Dr. Kun L. Guan (University of Michigan). Plasmid pBOS-A-Raf(RD)(HA)2 was kindly provided by Dr. Sutor (Mayo Foundation). All generated sequences and plasmids were confirmed by sequencing.

Yeast Interaction Assays—For interaction in yeast, a series of TH1 deletion mutants constructed into plasmid pLexA were cotransformed with pB42AD-A-Raf and empty pB42AD plasmid to verify the specificity of the two-hybrid assay using EGY48 yeast strain (Mata trp1 ura3-52 leu2-3,112 his3-Δ200 ade2-1 can1-100 leu2-2 his3-12,112 trp1-1 ura3-1 his3-Δ200 leu2-3,112 can1-100). Yeast transformation was performed by the lithium acetate method provided by the manufacturer (CloneTech).

In Vitro Binding Assay—GST-TH1, its GST-fused deletion mutants, and A-Raf protein prepared as pcDNA3 constructs were used to generate GST fusion proteins individually with the TNT® Coupled Reticulocyte Lysate System (Promega). PMA treatment followed the manufacturer’s instructions. A GST pull-down assay was performed as described previously (4, 59, 60). In brief, the labeled proteins were addressed immediately by constant mixing with 25 μl of glutathione-Sepharose beads in the binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl) 10%
glycerol, 10 mM NaF, 1% Nonidet P-40, 1 mM NaVO₃, 10 μg/mL protease
inhibitor, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride for 3 h at
4 °C. Then the beads were washed three times with the binding buffer
and boiled in SDS sample buffer. The bound proteins were analyzed by
autoradiography after they were resolved by 12% SDS-PAGE.

In Vivo Binding Assay—The COS-1 and HEK293 cells in 60-mm
wells (Nunc) were transiently transfected with 3 μg of pcDNA3.1-
myc-th1 plasmid alone or with the combination of 0.5 μg of
cDNA3-Ras and 0.5 μg of pcDNA3-Src. Total DNA was kept constant
by pcDNA3.1-myc-his empty vector. 48 h after transfection, the cells
were starved in 0.1% fetal bovine serum medium for 18 h and then
stimulated by the readdition of serum. Cell lysates were prepared, and
immunoprecipitation was performed as described previously (4, 59, 60).
In brief, immunoprecipitation was performed as described previously (4, 59, 60).
For each Raf isoform kinase assay,

RESULTS

Confocal Microscopic Analysis of Th1 and Raf Family—In
previous work (4) we reported that TH1 can bind specifically to
A-Raf both in vitro and in vivo. To elucidate the subcellular
localization of TH1 with different Raf isoforms, we selected
COS-1 cells to transfected with pEGFP, pEGFP-TH1, and pD-
SRed-A-Raf, -B-Raf, and -C-Raf plasmids, respectively. 48 h
after transfection, the cells were fixed and analyzed under confocal microscopy. As shown in Fig. 1a, the EGFP is
distributed in whole cellular compartments, especially in the nucleus,
whereas red fluorescent protein-fused A-Raf is distributed exclu-
sively in the cytoplasm. There is no colocalization between
them (Fig. 1c). The EGFP-fused TH1 protein is also distributed
in both the cytoplasm and nucleus (Fig. 1, c and d). After
coexpressing EGFP-fused TH1 and red fluorescent protein
fused A-Raf proteins, we detected the cells with fluorescence
microscope. By merging the separate projection images green-
only and red-only emission detection, we observed that the
double-transfected cells contained yellow granules indicating
colocalization of TH1 and A-Raf (Fig. 1b). As a further support
to our former results, the other two members of Raf family,
B-Raf and C-Raf, did not colocalize with TH1 when checked by
confocal microscopy (Fig. 1, c and d). We also got the same
results using SMMC-7721 hepatocarcinoma cells. Taken to-
gether, they confirmed the specific interaction between TH1 and
A-Raf in vivo.

Mapping of A-Raf Binding Regions in TH1—To investigate
the region in TH1 responsible for binding A-Raf, we first con-
structed a series of TH1 deletion mutants into plasmid pLexA
and tested for their binding abilities with A-Raf in a yeast
system. These mutant constructs were cotransformed either
with the empty pB42AD plasmid or with pB42AD-A-Raf into
EGY48 yeast cells. Cotransformants were tested for growth in
the absence of leucine and production of β-galactosidase.

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top panel), TH1 can bind either with unstimulated A-Raf (lane 2) or with stimulated A-Raf (lane 3,4,5). This suggests that TH1 and A-Raf may exist in a protein complex in cells and that complex formation does not require growth factor stimulation. We also found that strengthened bindings between activated A-Raf and TH1 were observed. The increased binding of A-Raf kinase to TH1 was correlated with the extent of its enzymatic activities, whereas the levels of total A-Raf protein were not changed (Fig. 3A, middle panel). As a control, the levels of TH1 protein precipitates were determined by a monoclonal antibody against the myc epitope (Fig. 3A, bottom panel). A similar result was achieved when we used HEK293 cells, which expressed a high level of endogenous A-Raf protein. As shown in Fig. 3B, more evidently, with serum and/or oncogenic Ras/Src stimulation, TH1 binds increasingly with samples containing activated A-Raf (lanes 4–6) rather than unstimulated sample (lane 3). To verify further whether A-Raf kinase activity plays a pivotal role in its association with TH1, we used a eukaryotic expression plasmid pBOS-A-Raf(KD)-(HA)2 (23) to express kinase-dead A-Raf protein to test its interaction with TH1. As shown in Fig. 3C, kinase-dead A-Raf protein still could bind to TH1 but showed no quantity difference in binding with TH1 upon stimulation. Direct comparison of the bindings between wild type A-Raf and kinase-dead A-Raf with TH1 reveals that TH1 preferentially associates with wild type A-Raf (Fig. 3D). Given these, it might be concluded that A-Raf activation state is essential for its association with TH1 in both cell lines. The increasing ability of activated A-Raf to bind with TH1 suggests that TH1 may play some role in regulating A-Raf kinase.

Overexpression of TH1 Inhibits A-Raf Kinase Activity upon Serum Stimulation—A-Raf is well known as an upstream activator of MEK1, which regulates MEK1 kinase activity in the MAPK/ERK pathway (13, 53). To investigate the relevance between the interaction and Raf kinase activities, we performed Raf protein kinase assays using kinase-deficient GST-MEK1 as substrate. Before starting the experiments, we tested the cross-reactivity of the A-, B-, and C-Raf antibodies purchased, respectively. We found that certain Raf antibodies

![Confocal microscopic analysis of the TH1 association with A-Raf](image-url)
failed to immunoprecipitate the other two Raf proteins (data not shown). COS-1 cells were transfected with different amounts of plasmids pcDNA3.1-myc-TH1 and were serum starved for 18 h before restimulation by complete medium containing 10% serum. The whole cell lysates from transfected cells and mock cells were immunoprecipitated with an anti-A-Raf polyclonal antibody. The in vitro kinase assay of the A-Raf immunoprecipitates revealed that A-Raf kinase activity was decreased significantly in the presence of TH1 in a dose-dependent manner (Fig. 4A). As a control, the A-Raf binding-deficient TH1 fragment F5 was introduced and had no effect on A-Raf kinase activity as shown in Fig. 4A (lane 1). It reflects that the association between TH1 and A-Raf is important for TH1 to regulate A-Raf kinase activity. We also performed the same kinase assay using HEK293 cells and anti-p-MEK1 antibody instead of using [32P] incorporation. The data confirmed the result in COS-1 cells (Fig. 4A, right panels). Because in our previous work, TH1 could interact specifically with A-Raf but not B-/C-Raf, we checked the other two Raf isoforms kinase activities under the same experimental conditions. As shown in Fig. 4B, neither B-Raf nor C-Raf kinase activity was influenced by TH1 overexpression. Liquid scintillation detection of phosphorylation of myelin basic protein substrates depicted the different effects of TH1 overexpression on each Raf kinase (Fig. 4C). It can be inferred that through direct specific interaction with A-Raf, TH1 uniquely suppressed A-Raf kinase activity but not B-/C-Raf kinase activities.

Overexpression of TH1 Inhibits the Kinase Activity of A-Raf Activated by Oncogenic Ras/Src—Although Ras is the best characterized upstream activator of Raf isoforms, it still needs other components including tyrosine kinases to achieve full activation of Raf kinases (16). We investigated further the effect of TH1 overexpression on A-Raf kinase activity upon constitutively activated oncogenic Ras (Val12) and Src (Y527F) stimulation. As shown in Fig. 5, COS-1 cells were transiently transfected with Ras (lanes 3 and 4) or Src (lanes 5 and 6) individually or in combination (lanes 7 and 8). When transiently expressing TH1 as indicated in Fig. 5 (lanes 4, 6, and 8), endogenous A-Raf precipitated by anti-A-Raf antibody was tested for its ability to phosphorylate GST-MEK1 in an in vitro kinase assay. Our data suggested that TH1 suppressed A-Raf kinase activities stimulated by Ras and/or Src. Combined together, TH1 functions as a negative regulator of A-Raf in the Ras-mediated MAPK/ERK pathway.

TH1 Overexpression Influenced SMMC-7721 Cell Cycle Progression and Cell Proliferation—Raf proteins have been implicated in regulating cell cycle progression or cell cycle arrest depending on their effects on both ERK signal intensity and duration. Studies have also indicated that A-Raf played an important role in cell cycle progression and proliferation in
vascular smooth muscle cells (24) and in hemopoietic cells (23). Because TH1 could inhibit A-Raf kinase activity, we wished to check its effect on cell cycle progression. We established several cell strains that stably express TH1 (TH1/7721) and GST-TH1 (GST/7721) in SMMC-7721 hepatocarcinoma cells and COS-1 cells. With flow cell cytometry analysis, cells stably expressing TH1 were found to be $G_0/G_1$ phase-arrested and cells in $S$ phase and $G_2/M$ phase were greatly diminished compared with 7721 cells and pcDNA3/7721 cells (Fig. 6A). However, the cells that express A-Raf-binding-deficient TH1 mutant F5 could not affect the cell cycle progression. At least three independent experiments were performed, and statistical comparison of the changes of cell cycle was presented in Fig. 6B. From growth curves (Fig. 6C), we could see that cells stably expressing TH1 grew much more slowly than mock cells, whereas TH1 F5 lack this ability, which was consistent with the FACS results. Taken together, TH1 overexpression does affect the biological behavior of SMMC-7721 hepatocarcinoma cells and COS-1 cells.

**DISCUSSION**

In mammalian cells, A-Raf belongs to the Raf kinase family, which consists of other two members, B-Raf and C-Raf, acting as a key intermediate that relays signals from upstream Ras and tyrosine kinases to downstream serine/threonine kinases. Sequence comparisons of all three Raf proteins reveal that they share a high degree of similarity, consisting of three conserved regions, CR1, CR2, and CR3. Among them, CR1 and CR2 form the N-terminal regulatory domain, and CR3 comprises the catalytic domain. The N-terminal regulatory domain functions to suppress the catalytic activity of Raf, and removal of this domain will lead to the constitutively active forms of all three Raf kinases. The C-terminal catalytic domain is mainly responsible for phosphorylation of downstream substrates (13, 64–66). The alignment of the three Raf isoforms indicates that the C-terminal kinase domain is highly conserved, and the N-terminal regulatory domain is less conserved. The variety of the N-terminal regulatory domain may account for the specific regulation of each Raf isoform. Our previous work has identified TH1 as a specific A-Raf interaction protein that is uniquely bound to the N-terminal 1–162 amino acids of A-Raf. These amino acid sequences correspond to the A-Raf CR1 regulatory domain, which contains the Ras-binding domain and the cysteine-rich domain responsible for Ras binding and A-Raf activation. Besides Ras, other molecules such as 14-3-3 and a putative lipid ligand were reported to bind to the cysteine-rich domain regardless of different Raf isoforms (10). TH1 may be unique in regulating A-Raf rather than B-Raf or C-Raf through specific binding with this domain.

The biological reasons underlying Raf diversity are not fully understood but imply that Raf isoforms have overlapping and unique functions. The regulation of Raf isoforms was a complex process in which different Raf isoforms combine common and unique mechanisms regulate Raf kinase activity as reported previously. Studying the molecular mechanism regulating each specific reaction has only begun in recent years. A specific reaction may be regulated by a specific protein–protein interaction. Studies on Raf isoforms have revealed that each Raf protein has specific interaction partners other than common activators and effectors. For instance, PA28$\alpha$, a subunit of the 11 S regulator of proteosomes, was found to bind specifically with B-Raf but not A-Raf or C-Raf. Meanwhile, A-Raf also has its unique interaction proteins such as casein kinase 2$\beta$, pyruvate kinase type M2, hTOM, and hTIM. Our previous study on A-Raf also depicted TH1 as a new specific interaction protein of A-Raf. Hopefully, all of these interaction proteins may contribute to a better understanding of regulation of Raf isoforms and their selective role in cellular regulation.

Our data presented in this work suggest that TH1 binds preferentially with activated forms of A-Raf kinase as shown in Fig. 3. A-Raf can be activated by serum alone or in combination with upstream oncogenic Ras and Src stimulation. From its ability to phosphorylate MEK1 substrate as indicated in Fig. 5, the active forms of A-Raf show increased binding with TH1 (Fig. 3). In this situation, A-Raf enzymatic activity was inhib-
FIG. 4. Effects of TH1 overexpression on MEK1 phosphorylation by Raf isoforms when serum stimulated. A, overexpression of TH1 down-regulated A-Raf kinase activity by serum stimulation in COS-1 (left column) cells and HEK293 (right column) cells. B, neither B-Raf nor C-Raf kinase activity was apparently affected by TH1 overexpression. C, relative intensity of the phosphorylation abilities on myelin basic protein substrates of A-Raf and B-/C-Raf. COS-1 or HEK293 cells were transiently transfected with 1 or 3 μg of pcDNA3.1-myc-TH1 or pcDNA3-GST-TH1(F5) as indicated. Total DNA was kept at 4 μg/transfection with pcDNA3.1-myc empty vectors. After 48 h, cell lysates were precipitated by A-Raf, B-Raf, C-Raf antibodies plus protein G-agarose. Inactive MEK1 proteins were added to the different precipitates. Raf isoform kinase activities were measured by phosphorylation of MEK1 by autoradiography or by anti-p-MEK1 antibody. Western blotting of endogenous A-Raf and myc-tagged TH1 verified equivalent individual protein levels. Similar results were obtained from at least three independent experiments.
ited by overexpression of TH1 either upon serum stimulation or upon upstream oncogenic Ras/Src stimulation as shown in Figs. 4 and 5. Taken together, the enhanced binding of TH1 with activated forms of A-Raf contributes to its down-regulation on A-Raf enzymatic activity. Thus, TH1 functions as a negative regulator of A-Raf to avoid excessive A-Raf activation in cells. But whether this binding affects the conformation of A-Raf or just brings active A-Raf away from the ERK pathway is still not clear.

Although TH1 can bind directly with A-Raf, little is known about its function at present. Sequence analysis of TH1 protein revealed that multiple protein kinase phosphorylation sites were found. Such phosphorylation sites include several casein kinase 2β phosphorylation sites, a protein kinase C phosphorylation site, and two unique phosphorylation sites: tyrosine protein kinase and cAMP/cGMP-dependent protein kinase phosphorylation (indicated in Fig. 2A). In addition to these protein kinase phosphorylation sites, TH1 also contains several N-myristoylation sites, which might be involved in the recruitment of TH1 to membrane for certain interactions and several N-glycosylation sites for further procession. We mapped out the regions in TH1 which are required for A-Raf binding as N-terminal amino acids 1–372. Interestingly, we have observed in an in vitro substrate phosphorylation assay that TH1 could really be phosphorylated by A-Raf, but which protein kinase phosphorylation sites were involved was not verified (data not shown here). Whether this phenomenon correlates with the TH1 function on A-Raf needs to be investigated further. The regulation of TH1 by A-Raf kinase may play a role in other biological activities, which are not clear at present.

Previous investigations have shown that the Raf protein kinase family members display differences in their abilities to
control cell cycle G₁ depending on their effects on both ERK signal intensity and duration (67). Low levels of Raf activity can promote cell cycle progression, whereas high levels of Raf activity can induce cell cycle arrest. Other studies also indicated that A-Raf played an important role in cell cycle progression and proliferation in vascular smooth muscle cells (24) and in hematopoietic cells (25). Recent studies have shown that in murine fibroblast cells only moderate Raf activity is necessary for G₁ progression and cell proliferation such as C-Raf and A-Raf, whereas potent Raf activation such as B-Raf activation may lead to a p21G1/S-mediated cell cycle arrest. The activation of either βB-Raf or delta Raf-1:ER in quiescent 3T3 cells was insufficient to promote the entry of the cells into DNA synthesis. By contrast, the activation of ΔA-Raf:ER in quiescent 3T3 cells was sufficient to promote the entry of the cells into S phase after prolonged exposure to β-estradiol (21). Based on these studies, our data showed that TH1 specifically suppressed A-Raf enzymatic activity, and this may contribute to the cell cycle arrest observed. In summary, we have confirmed that TH1 serves as a new specific negative regulator of A-Raf kinase and is involved in Ras/Raf/MEK1 signal pathway. Its effects on A-Raf kinase provide us a clue to the understanding of Raf isoform-specific regulation in those cells and the unique function of A-Raf on cell cycle progression through ERK signal pathway, but the exact mechanism underlying its effects on A-Raf kinase needs to be elucidated further.

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REFERENCES
1. Banda, S. S., Yamamoto, A. H., Mason, J. M., and Boyd, J. B. (1995) Mol. Gen. Genet. 246, 148–155
2. Hari, K. L., Santerre, A., Sekelsky, J. J., McKim, K. S., Boyd, J. B., and Hawley, R. S. (1995) Cell 82, 815–821
3. Bonhoute, D. T., Hayward, B. E., Moran, V., and Strain, L. (2000) Hum. Genet. 107, 163–175
4. Yin, X. L., Chen, S., and Gu, J. X. (2002) Mol. Cell. Biochem. 231, 69–74
5. Su, B., and Karin, M. (1996) Curr. Opin. Immunol. 8, 402–411
6. Widmann, C., Gibson, S., Sarpe, M. B., and Johnson, G. L. (1999) Physiol. Rev. 79, 143–180
7. Herrskowitz, I. (1995) Cell 80, 187–197
8. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Adv. Cancer Res. 74, 49–139
9. Cobb, M. H. (1999) Prog. Biophys. Mol. Biol. 71, 479–500
10. Taam, G., Eisenmann-Tappe, I., Fries, H. W., Treppmair, J., and Rupp, U. R. (1994) Trends Biochem. Sci. 19, 474–477
11. Yuryev, A., and Wengelneg, L. P. (1998) Cell Res. 8, 91–98
12. Williams, N. G., and Roberts, T. M. (1994) Cell 79, 821–828
13. Hagemann, C., and Rapp, U. R. (1999) Oncogene 18, 2713–2722
14. Marais, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) EMBO J. 14, 3136–3145
15. Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1994) Science 264, 1463–1467
16. Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. (1997) J. Biol. Chem. 272, 4578–4583
17. Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J., and Marais, R. (1999) EMBO J. 18, 2137–2147
18. Vossler, M. S., Yao, H., York, R. D., Pan, M. G., Rim, C. S., and Stork, P. J. (1997) Cell 89, 73–82
19. Rosario, M., Paterson, H. F., and Marshall, C. J. (2001) Mol. Cell. Biol. 21, 3750–3762
20. Rosario, M., Paterson, H. F., and Marshall, C. J. (1999) EMBO J. 18, 1270–1279
21. Pritchard, C. A., Samuels, M. L., Bosch, E., and McMahon, M. (1995) Mol. Cell. Biol. 15, 6430–6442
22. McCueber, J. A., Steinman, L. S., Hoyle, P. E., Blakoc, W. L., Weinstein, Oppenheimer, C. Franklyn, R. A., Cherwinski, H., Bosch, E., and McMahon, M. (1998) Leukemia 12, 1903–1929
23. Sura, S. L., Yaman, B. T., Armstrong, E. A., Abraham, R. T., and Kurnitz, L. M. (1999) J. Biol. Chem. 274, 7062–7070
24. Ciofi, C. L., Garav, M., Johnston, J. F., McGraw, K., Boggs, R. T., Hrenik, D., and Moniz, B. P. (1997) Mol. Pharmacol. 51, 383–389
25. Guan, K. L., Figueroa, C., Birtz, T. R., Zhu, T., Taylor, J., Barber, T. D., and Vojtek, A. B. (2000) J. Biol. Chem. 275, 27354–27359
26. Bosch, E., Cherwinski, H., Peterson, D., and McMahon, M. (1997) Oncogene 15, 1021–1033
27. Carroll, M. P., and May, W. S. (1994) J. Biol. Chem. 269, 1249–1256
28. Kuroda, S., Ohtsuka, T., Yamamori, B., Fukui, K., Shimizu, K., and Takai, Y. (2000) J. Biol. Chem. 275, 1539–1547
29. Fantl, W. J., Muslin, A. J., Kikuchi, A., Martin, J. A., Mac Nicol, A. M., Gross, R. W., and Williams, L. T. (1994) Nature 371, 612–614
30. Avruch, J., Zhang, X. F., and Marais, R. (1999) Trends Biochem. Sci. 24, 279–283
31. Yam, S. L., Chen, S., Yan, J., Hu, Y., and Gu, J. X. (2002) Biochim. Biophys. Acta 1589, 71–76
32. Shinkai, M., Masuda, T., Kariya, K., Tamada, M., Shimizu, K., and Takai, Y. (1997) J. Biol. Chem. 272, 3265–3271
33. Nobukwasa, S., Noh, S. J., Zhou, G., Dixon, J. E., and Guan, K. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6213–6217
34. Tateishi, I., Kubo, T., and Nishino, N. (2000) J. Biol. Chem. 275, 34772–34777
35. Nishino, N., Amano, M., and Nishino, N. (2000) J. Biol. Chem. 275, 34778–34782
36. Nishino, N., Amano, M., and Nishino, N. (2000) J. Biol. Chem. 275, 34773–34777
37. Nishino, N., Amano, M., and Nishino, N. (2000) J. Biol. Chem. 275, 34774–34778
38. Nishino, N., Amano, M., and Nishino, N. (2000) J. Biol. Chem. 275, 34779–34783
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