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PCR differential display-based identification of regulator of G protein signaling 10 as the target gene in human colon cancer cells induced by black tea polyphenol theaflavin monogallate

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ABSTRACT

We have previously reported that black tea polyphenol theaflavin monogallate (TF-2) suppressed COX-2 and induced apoptosis in human colon cancer cells [Lu, J.B., Ho, C.-T., Ghai, G., Chen, K.Y., 2000. Differential effects of theaflavin monogallates on cell growth, apoptosis and Cox-2 gene expression in cancerous versus normal cells. Cancer Res. 60, 6465–6471.]. We now extended the study by using PCR-based differential display to search for genes that were selectively induced by TF-2. Here we report the identification of Regulator of G-binding protein signaling 10 (RGS10) as the target gene, which was induced as early as 4 h after the TF-2 treatment. We then examined the effect of TF-2 on several other RGS genes and found that, in addition to RGS10, TF-2 induced the expression of RGS14, but not RGS4. Other tea polyphenols, including theaflavin-3,3'-digallate (TF-3) and (−)epigallocatechin-3-gallate (EGCG), also induced the expression of RGS10 and RGS14, but not RGS4. However, theaflavin (TF-1), which does not contain the gallate moiety, was ineffective. These results showed for the first time that tea polyphenols can induce the expression of selective RGS genes and that the gallate moiety may be important in this induction. In view of the role of RGS in modulating G-protein mediated signal transduction pathways, our findings may be significant since dysregulation of G-signaling is prominently implicated in carcinogenesis.

1. Introduction

The major polyphenols in black tea are dimeric flavanols called theaflavins (TF-1, TF-2, and TF-3). Theaflavins share close structural similarity to EGCG, the green tea polyphenol (Fig. 1). Tissue culture and animal experiments indicate that these compounds possess biological activities that may be of clinical importance, including chemoprevention and anti-inflammation. Tea polyphenols have been shown to block carcinogenesis in animal models (Na and Surh, 2006; Yang et al., 2006). Clinical studies have demonstrated good efficacy of EGCG or green tea extracts containing EGCG in patients with cervical lesion or high-grade prostate intraepithelial neoplasia, albeit not complete response (Nagle et al., 2006). Tea polyphenols are known to affect gene expression and signal transduction pathways that are pathologically relevant (Hwang et al., 2007). For example, both TF-2 and EGCG have been shown to suppress COX-2 gene expression (Lu et al., 2000; Peng et al., 2006). To understand the molecular basis of the biological effects of tea polyphenols, one approach is to identify their target genes by using genome-wide screening method. In this regard, we have initiated a PCR-based cDNA differential display analysis to search for and to clone genes specifically up- or down-regulated by TF-2. One of the cDNA fragments that we have cloned turns out to be RGS10. In view of the crucial role of RGS family in controlling G-protein signaling (Ross and Wilkie, 2000), we have further examined the effects of TF-2 on other RGS genes and to determine whether the effects on RGS gene expression were limited only to TF-2. We found that tea polyphenols that contain gallate group, including TF-2, TF-3 and EGCG, are all effective in inducing RGS10 and RGS14 gene, albeit with slightly different kinetics and potency. Dysregulation of G-protein signaling, including G proteins and RGS proteins, play prominent roles in tumorigenesis (Kelly et al., 2006; Cao et al., 2006). Although the effects of tea polyphenols on G-protein signaling pathways have been reported (Na and Surh, 2006; Yang et al., 2006; Nagle et al., 2006; Wang and Mukhtar, 2002), the current study shows for the first time that tea polyphenols are able to induce the expression of some RGS genes.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco BRL (Gaithersburg, MD). Other chemicals were

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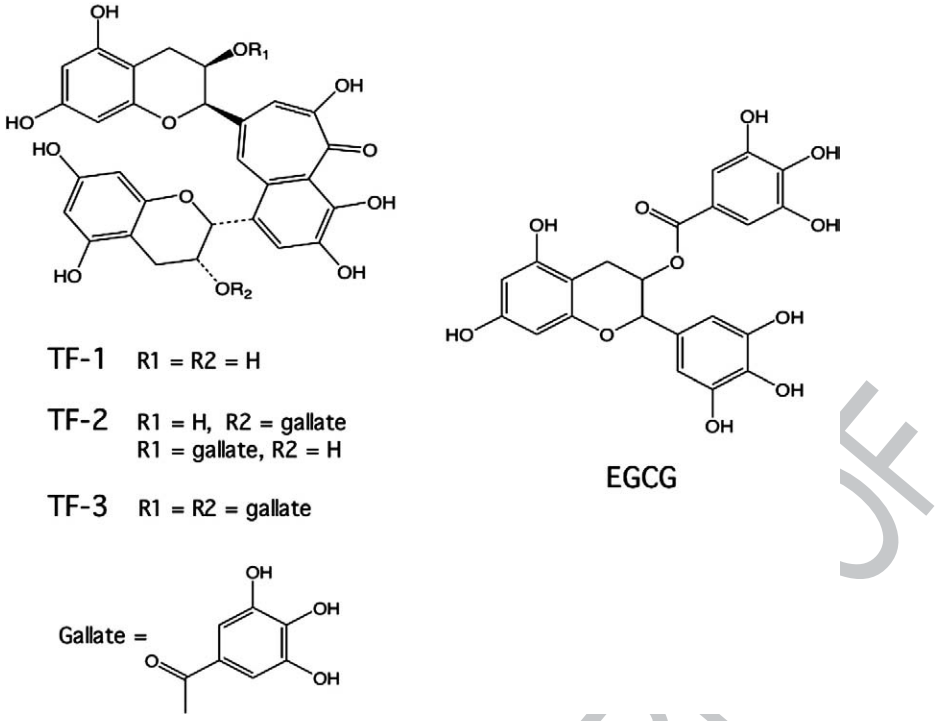

Fig. 1. Chemical structures of major black tea and green tea polyphenols. TF-1, theaflavin; TF-2, theaflavin-3-monogallate and theaflavin-3'-monogallate; TF-3, theaflavin 3,3'digallate; EGCG, (−) epigallocatechin-3-gallate. TF-1 is the only one without gallate group.

from Sigma (St. Louis, MO). Theaflavin polyphenols were purified from black tea powder (Lu et al., 2000). The TF-2 used in this study is a mixture of two theaflavin monogallate isomers as previously described (Lu et al., 2000). (−) Epigallocatechin-3-gallate (EGCG) was a kind gift of Dr. Chi-Tang Ho, Rutgers University. PCR primers H-T_{11}A, H-AP-1, H-AP-3, and H-AP-6 were purchased from GenHunter (Nashville, TN). Other oligo primers were synthesized by Integrated DNA Technologies, Inc. (Piscataway, NJ). [α-^{32}P] dATP and [α-^{32}P] dCTP (>3000 Ci/mmol) were obtained from ICN (Costa Mesa, CA). Hybond-NX nylon membrane was purchased from Amersham Life-Science Co. (Arlington Heights, IL).

2.2. Cell culture and RNA isolation

Human colon cancer Caco-2 cells (ATCC #HTB-37) were purchased from American Type Culture Collection, Rockville, MD. Human colon cancer cell HCT116 was a gift from Dr. Bert Vogelstein, Johns Hopkins medical School. Cells were maintained in Dulbecco's medium containing 10% fetal bovine serum at 37 °C, 5% CO_2. Cells at 80–90% confluence were treated with individual tea polyphenols and were harvested at indicated time. Total RNA was prepared using RNeasyTM Total RNA Kit (Qiagen Inc. Chatsworth, CA) and further treated with RNase-free DNase to remove DNA.

The effect of TF-2 on apoptosis was monitored by DNA laddering assay as described previously (Gosslau et al., 2008).

2.3. Differential display assay

Total RNA (0.2 μg) was reverse transcribed in a 20 μl final reaction volume containing 25 mM Tris–Cl (pH 8.3), 1.5 mM $MgCl_2$, 5 mM DTT, 37.5 mM KCl, 200 μM dNTP, 1 μM primer H-T_{11}A (5'-AAGCTT TTTTTTTTTA-3') and 1 μl SuperScript™ RNase H reverse transcriptase (Gibco BRL, Grand Island, NY) and incubated at 65 °C for 5 min, 37 °C for 60 min and 75 °C for 5 min. For PCR amplification, 2 μl of reverse-transcribed sample was mixed with 18 μl of buffer containing 10 mM Tris–Cl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 μM primers, 2 μM dNTP, 0.5 μl [α-^{32}P] dATP, 0.2 unit Taq DNA polymerase (Qiagen Inc.). The random primers used are H-AP1 (5'-AAGCTT GATTGCC-3'), H-AP3 (5'-AAGCTTTGGTCAG-3'), and H-AP6 (5'-AAGC TTTTACCGC-3'). The reaction was subjected to 30 cycles of amplification

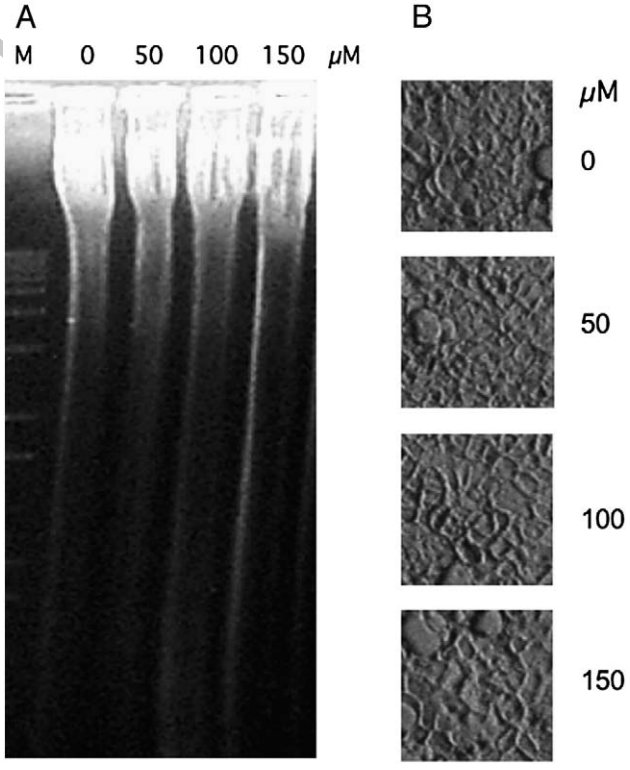

Fig. 2. Effect of TF-2 on DNA laddering and cell viability of confluent Caco-2 cells. Caco-2 cells grew to confluence were treated with TF-2 at 0, 50, 100, and 200 μM. (A) DNA laddering assay. Cells were harvested at 24 h and processed for DNA isolation and agarose gel electrophoresis. DNA was visualized by ethidium bromide staining. (B) Cell morphology at 24 h after treatment with TF-2 at different concentrations as indicated.

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at 94 °C for 30 s, 40 °C for 2 min and 72 °C for 30 s, and with final extension at 72 °C for 5 min. The PCR products (3.5 μl) were mixed with 2 μl of formamide loading buffer and heated at 80 °C for 2 min before loading on a 6% polyacrylamide sequencing gel. After electrophoresis, the gel was dried and exposed to Kodak XAR5 film.

2.4. Purification of PCR product, cloning and sequencing

The differentially expressed cDNA band was excised from the polyacrylamide gel. The cDNA fragment was eluted by boiling the gel slice in TE buffer (0.01 M Tris–Cl, pH7.5, 1 mM EDTA). After ethanol precipitation, the cDNA was re-amplified using the appropriate primer pair. The PCR product was electrophoresed on a 1.2% agarose gel and the band was extracted by the Gel Slice Kit (Bio-Rad Lab., Hercules, CA). The isolated cDNA fragment was subcloned and used for probe labeling and sequencing. The re-amplified cDNA was subcloned into TOPO-TA vectors (pCR 2.1, Invitrogen). The dideoxynucleotide chain-termination method for DNA sequencing using was performed by Integrated DNA Technologies, Inc. (Coralville, IA). BLAST analysis was performed to search for the identity of the cDNA fragments.

2.5. Reverse transcription and PCR amplification (RT–PCR)

Total RNA (1 μg) from cells was reverse transcribed into cDNA by incubating with SuperScriptTM RNase H reverse transcriptase (Gibco BRL, Grand Island, NY) using Oligo(dT)12-18 as primer. For PCR amplification, gene specific primers were designed. The sequences of the sense and antisense primers for various genes were:

HG3PDH (sense, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'; antisense, 5'-CATGTGGGCC ATGAGGTCCACCAC-3'), RGS10 (sense, 5'-AGCC TCAAGAGCACAGCCAAAT-3'; antisense, 5'-GCACGTCCTGAGAGGAAATT CCT-3'), RGS14 (sense, 5'-TCTGGAGAAGCACGGCTTGAG-3'; antisense, 5'-TGCAGCTGTCCTGGACTGTTG-3'), RGS4 (sense, 5'-AAGATGAATCGA GACT TGAGGAA-3'; antisense, 5'-ATAGTCTAGTCATTGTCTGAATCACTG-3'). PCR was performed under conditions where the yield of the amplified product was linear with respect to the amount of input RNA for each pair of gene specific primers. Housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR products were electrophoresed on 1% agarose gel containing 0.5 μg/ml ethidium bromide and photographed under UV light.

2.6. Probe labeling and Northern blot analysis

The cDNA probe was labeled with [α-^{32}P]dCTP using Ready-To-Go™ DNA Labeling Beads (Amersham Pharmacia Biotec, Inc., Piscataway, NJ). Total RNA (8 μg) of each sample was electrophoresed in formaldehyde-denatured 1% agarose gel and then transblotted onto nylon membrane for hybridization and detection.

3. Results

3.1. Differential display of DD-1 cDNA from TF-2 treated cells

Black tea theaflavin TF-2 is capable of suppressing COX-2 gene expression in confluent Caco-2 and WI38VA cells (Lu et al., 2000). To search for the early target genes of TF-2 in Caco-2 cells using differential display approach, we have used the dosage range of TF-2 (50 μM) and the time point (12–16 h) at which cells remained viable. Fig. 2 showed that confluent Caco-2 cells treated with TF-2 at a dosage less than 100 μM for up to 24 h remained viable without appreciable DNA fragmentation. The original differential display method relies on the use of an oligo-dT anchor primer and a random primer to amplify the cDNA pools obtained from the control and treated samples in order to allow easy identification and recovery of cDNAs that exhibit differential expression (Liang and

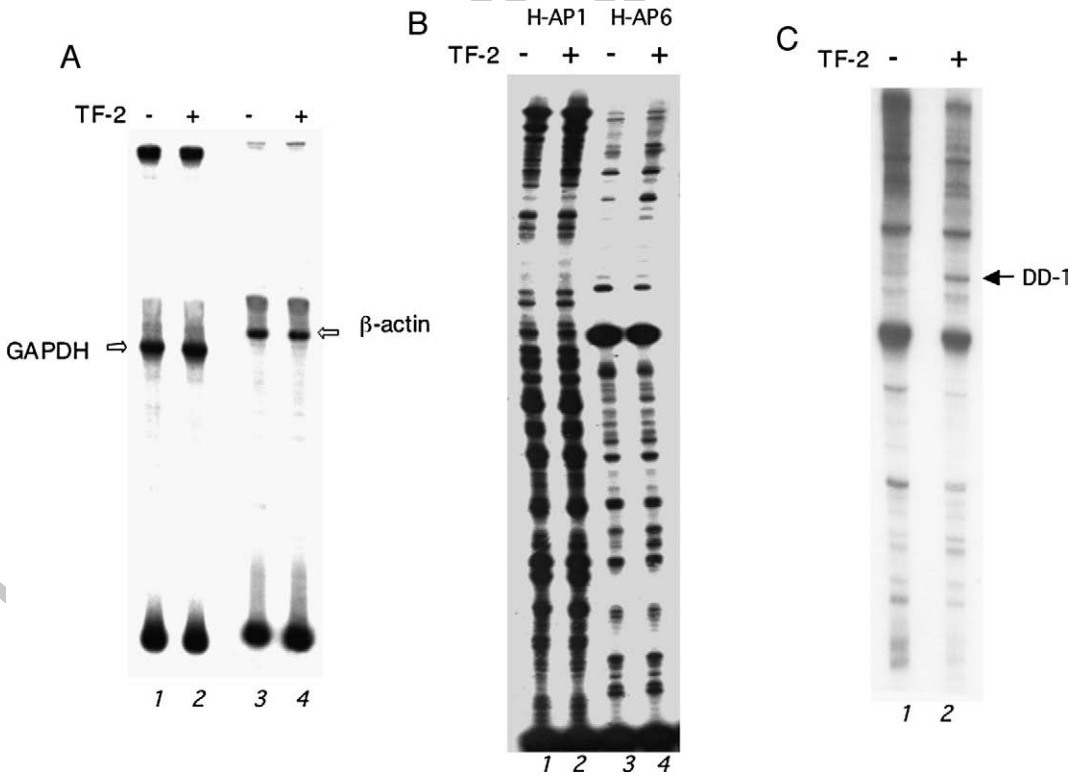

Fig. 3. The PCR differential display of cDNA expressed in Caco-2 cells. (A) Autoradiogram of internal control. TF-2 treatment did not affect the expression of two house-keeping genes, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and β-actin. (B) Autoradiogram of cDNA fragments derived from Caco-2 cells treated with (+) or without (−) TF-2 (50 μM) for 12 h by PCR differential display using primers H-T11A and H-AP1 (lanes 1 and 2) and H-AP6 (lanes 3 and 4). (C) Autoradiogram of cDNA fragments derived from Caco-2 cells with (+) or without (−) TF-2 treatment for 12 h by PCR amplification using primer H-AP3. Arrow indicates the fragment differentially expressed in two cell samples.

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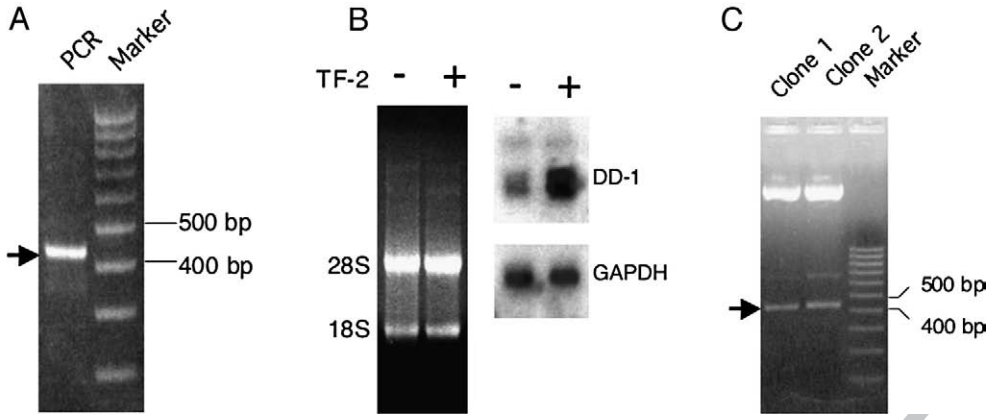

Fig. 4. Confirmation of cDNA fragment DD-1 and molecular cloning. (A) Re-amplification of the excised cDNA band using H-AP3. Arrow indicates the PCR product DD-1. (B) Northern blot analysis of DD-1 in Caco-2 treated for 12 h with (+) or without (−) TF-2 (50 μM). Left, quality of total RNA; right, Northern blot of DD-1 and house keeping gene GAPDH. (C) Restriction analysis of the cloned DD-1. Plasmids from transformants, clones 1 and 2, were digested with *EcoR*1. Arrow indicates the correct size of the DD-1 insert.

Pardee, 1992). Based on the observation that palindromic sequences occur more frequently within open reading frame than in untranslated regions, a modification of PCR differential display using a pair of palindromic primers has been reported (Bao et al., 1999). To search for the target genes, we have attempted to identify the cDNA fragments that exhibit differential expression using both approaches. To ensure the quality and quantity of the cDNA pools derived from the control and TF-2 treated cells, two house keeping genes, β-actin and GAPDH were first amplified and compared (Fig. 3A). The cDNA displays of the control and treated cells were then compared by using either a pair of anchor (H-T_{11}A) and random primer or a single palidromic primer. Fig. 3B shows the results using the pair of H-T_{11}A with H-AP1 or with H-AP6. In both cases, we did not observe clear difference in the cDNA display from control and treated samples. Nonetheless, the consistency of the profile confirms the robustness of the method. Fig. 3C shows the palindromic PCR-cDNA display using a single primer H-AP3. One band, marked as DD-1, exhibiting a clear up-regulation in the treated sample, was chosen for further analysis.

3.2. Cloning, sequencing and identification of DD-1

The DD-1 band on the gel was excised and re-amplified by PCR using the same palindromic primer. A single band at 440-bp was apparent, indicating that DD-1 represented a single molecular species (Fig. 4A). Northern blot analysis using the amplified PCR product as a probe showed that the expression of DD-1 was indeed significantly enhanced in Caco-2 cells 12 h after TF-2 treatment (Fig. 4B), suggesting that DD-1 was a putative target gene of TF-2. We therefore proceeded to clone DD-1 for sequencing and identification. Two clones were isolated and subjected to restriction analysis. Both clones contained an insert with correct size as expected for DD-1 (Fig. 4C). The cloned DD-1 fragment was then subjected to DNA sequencing and BLAST analysis. The sequencing and BLAST results as summarized in Fig. 5 indicated that the DD-1 fragment was derived from an ORF of the homo sapiens regulator of G protein signaling 10 (RGS10), a member of the RGS family genes critical in regulating G-signaling pathways (Ross and Wilkie, 2000).

3.3. Effect of TF-2 on RGS10, 14 and 4 genes

Once RGS10 was identified as the target gene of TF-2, it is of interest to examine whether any other RGS members may also serve as candidate targets of TF-2. For this purpose, we have tested two other RGS genes. Fig. 6 shows the time course of the effects of TF-2 on the expression of RGS10, RGS4 and RGS14. Both Northern blot analysis (Fig. 6A) and RT–PCR (Fig. 6B) revealed that TF-2 induced the expression of RGS10 within 4 h of the treatment. TF-2 is also effective in inducing RGS14 gene, with peak value occurred at about 16 h after

aagc**tttggctag**cnngtgaagattttaagnaaatgcaagatangacgcagatncaggaaaagncaanggagatcnncatgacctttctgtccagcaaggcctcatcannggtcaacgtgnaggcagtctcggctcaanganagatcctggaagtaccgcacnctctgatgttccagaactccaggnncagatctttaatctcatgaagtacgacagctagagccgctttcttaagtctgacttgnntttaaaacacaagcgaacggaggaannggaagaagatttgcctgatgctcaaactgcagntaaaagagcttccagaatttatnacacatgagccccccantaagccgggactggcagcttnaagaagcaaaggaanttcctctcaggacgtgccgngtttatcattgctttgttnnttgtaagnactgaaatgt

Sequences producing significant alignments:	Score (bits)	E Value
gb|AF045229|AF045229 Homo sapiens regulator of G protein si...	422	e-116
emb|X56657|ML5S23S M.leprae 5S rRNA and 23S rRNA genes and ...	42	0.13
gb|U00014|U00014 Mycobacterium leprae cosmid B1549	42	0.13
dbj|AB018302.1|AB018302 Homo sapiens mRNA for KIAA0759 prot...	38	2.0
ref|NM_002926.1|RGS12| Homo sapiens regulator of G-protein ...	38	2.0
emb|Z81360|MTCY4C12 Mycobacterium tuberculosis H37Rv comple...	38	2.0
gb|AF045022|AF045022 Bos taurus phosphatidic acid-preferrin...	38	2.0
gb|AF030112|AF030112 Homo sapiens regulator of G protein si...	38	2.0
gb|AF030109|AF030109 Homo sapiens regulator of G protein si...	38	2.0
gb|AF030111|AF030111 Homo sapiens regulator of G protein si...	38	2.0
gb|AF030110|AF030110 Homo sapiens regulator of G protein si...	38	2.0

gb|AF045229|AF045229 Homo sapiens regulator of G protein signaling 10 mRNA, complete cds Length = 753
Identities = 416/457 (91%), Gaps = 35/457 (7%)
Strand = Plus / Plus

Fig. 5. Sequencing of DD-1 and BLAST analysis. The *E* value shows that DD-1 sequence is almost identical to a portion of human RGS10 coding sequence.

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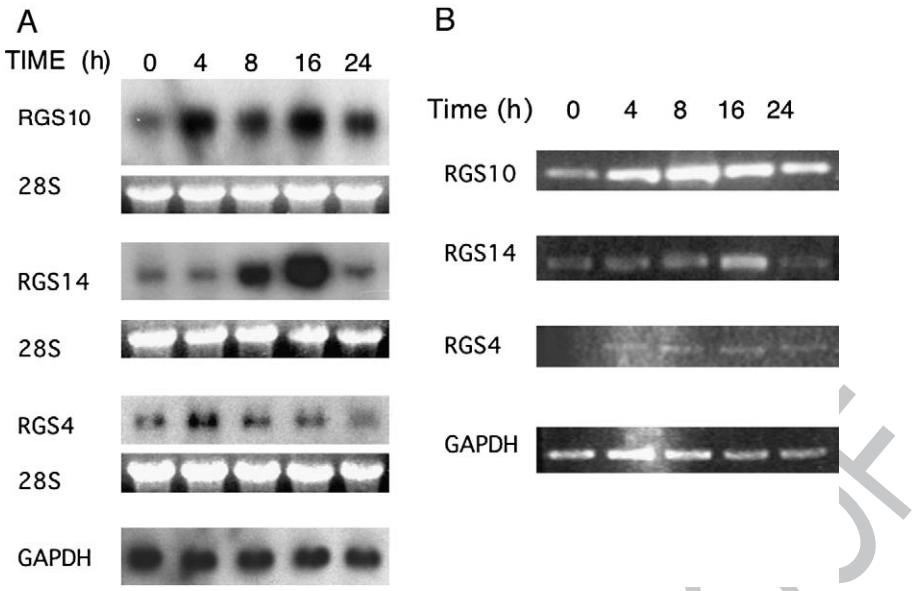

Fig. 6. Time course of the effects of TF-2 on the expression of three RGS genes. Caco-2 cells at 80% confluence were treated with TF-2 (50 μM) for various times as indicated. Cells were harvested at indicated time point and total RNA prepared for Northern blot analysis or RT–PCR by the procedures as described in Materials and methods. (A) Northern blot analysis of the effect of TF-2 on Caco-2 cells; (B) RT–PCR analysis of the effect of TF-2 on Caco-2 cells.

treatment. In contrast, TF-2 has little effect in inducing the expression of RGS4 gene.

We next examined the dose–response of the effect of TF-2 on the induction of RGS10 and RGS14 (Fig. 7). TF-2 at 10 μM induced the expression of RGS10 by more than 4-fold at 16 h. Maximal effect, up to 8-fold induction, appeared to occur at the TF-2 dosage between 50 to 100 μM. TF-2 at low concentrations (e.g. 10 μM) was less effective in inducing RGS14 than RGS10. However, at concentrations higher than 50 μM, TF-2 became equally effective in inducing both RGS10 and RGS14 genes. The finding that TF-2 exhibited different dose–response effect to the three RGS genes, RGS10, RGS14, and RGS4, is consistent with the notion that genes in the RGS family are differentially regulated (Ross and Wilkie, 2000).

3.4. Effects of other tea polyphenols on RGS gene expression

To determine whether other tea polyphenols are also capable of affecting RGS gene expressions, we have compared the effects of TF-1, TF-2, TF-3 and EGCG on the expression of the three RGS genes, RGS 4, RGS10 and RGS14. The results summarized in Fig. 8A and B indicated that RGS10 was prominently induced by TF-2, TF-3 and EGCG, but not by TF-1. TF-2 and TF-3 were also effective in inducing RGS14. All four

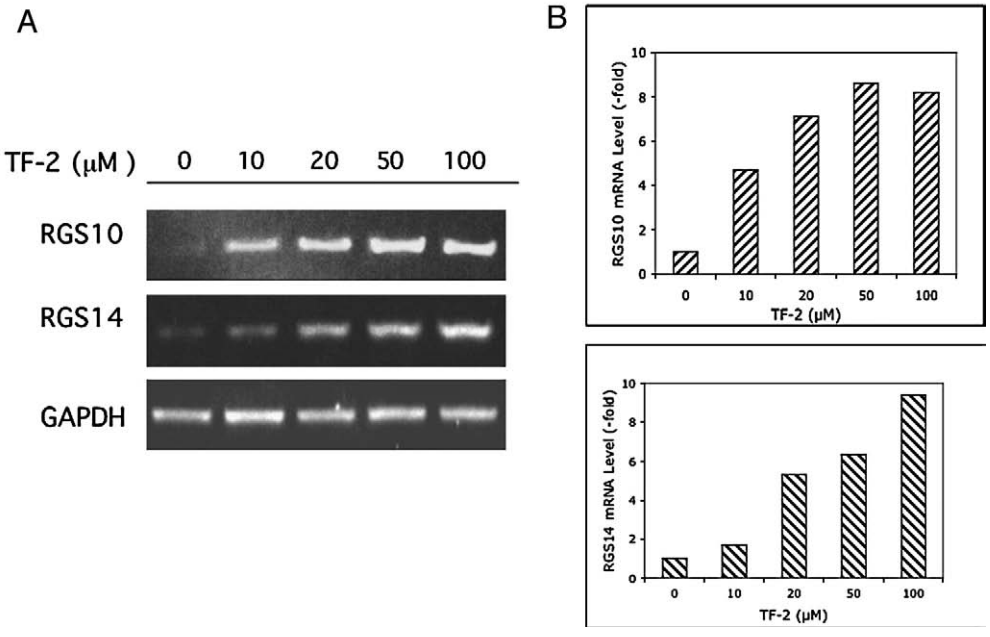

Fig. 7. Dose–response of the effects of TF-2 on RGS10 and RGS14 gene expressions. Caco-2 cells at 80% confluence were treated with TF-2 at various concentrations for 16 h. Cells were harvested and total RNA prepared for RT–PCR as described in Materials and methods. (A) The RT–PCR data of a representative experiment. (B) Densitometric tracing of the RT–PCR data. The relative levels of RGS10 and RGS14 mRNAs at each dosage were normalized against that of the GAPDH mRNA. The number represents an average of three measurements with standard errors <10%. For the control, the normalized RGS mRNA was set as 1.

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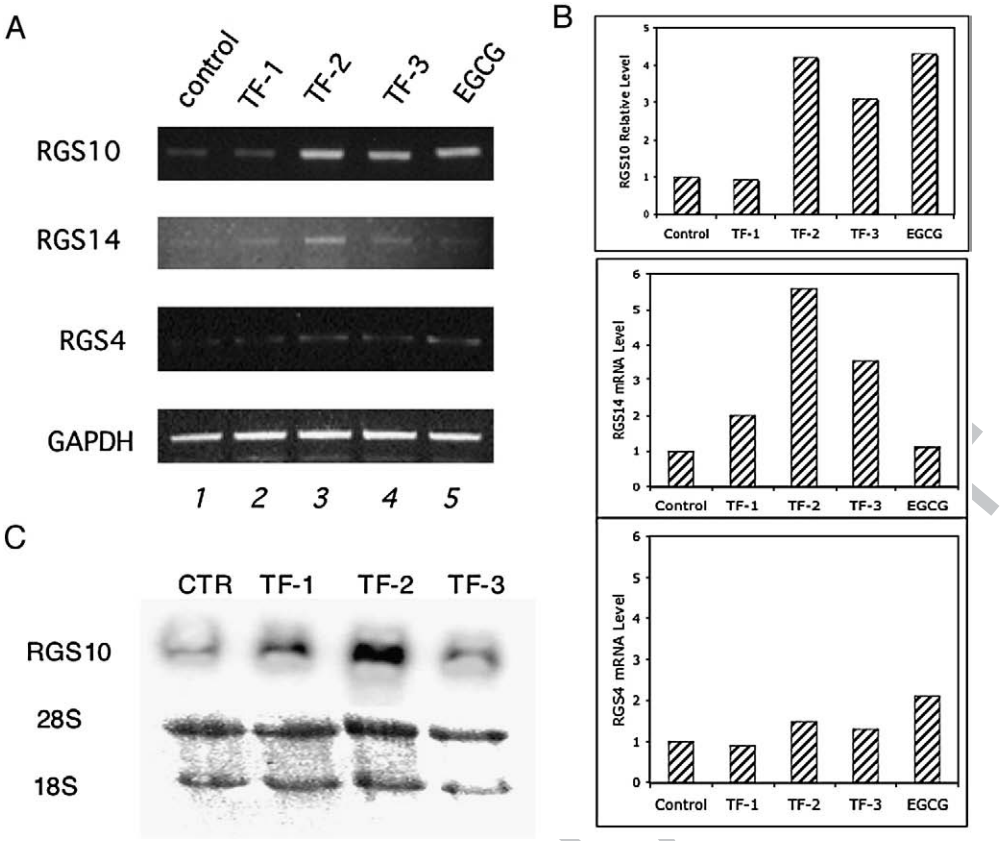

Fig. 8. Effects of tea polyphenols on the RGS expression. (A) Representative RT–PCR profile. Caco2 or HCT116 cells at 80% confluence were treated with solvent vehicle (control), TF-1 (50 μM), TF-2 (50 μM), TF-3 (50 μM), or EGCG (50 μM) for 16 h. Cells were harvested and total RNA prepared for RT–PCR. (B) Densitometric tracing of the RT–PCR data in (A). The relative mRNA levels of RGS4, RGS10 and RGS14 were all normalized against the GAPDH for each sample. The number represents an average of three measurements with standard errors < 10%. For the control, the normalized RGS mRNA level was set as 1. (C) Northern blot analysis of the effect of TF-1, TF-2, and TF-3 on the expression of RGS10 in HCT116 cells.

polyphenols tested had little or no effect on RGS4. As shown in Fig. 1, the most obvious common feature shared by TF-2, TF-3 and EGCG is that they all contain gallate moiety (Fig. 1). it is therefore tempting to speculate that this gallate moiety may provide the structural requirement for RGS gene up-regulation. We also tested the effect of theaflavins on RGS10 in another colon cancer cell line HCT116. Fig. 8C showed that TF-2 did induce RGS10, indicating that the effect is not limited to only one cell line.

4. Discussion

During the past decade extensive research has revealed many clinically relevant biological properties of tea polyphenols, ranging from anti-oxidative, anti-proliferative, anti-inflammatory to pro-apoptotic (Na and Surh, 2006; Yang et al., 2006; Nagle et al., 2006; Hwang et al., 2007). However, our understanding of the molecular basis and causal relationship of various biological events elicited by tea polyphenols is limited. In addition, the structure–activity relationship of these polyphenols and the mechanism by which they affect a diverse range of signaling pathways remained to be determined. With the advent of powerful gene profiling and other molecular techniques, progress aimed at bridging these gaps should accelerate. In this study, we describe the use of PCR-differential display, a gene profiling technique, that enables us to identify RGS10 as one of the target genes of TF-2 (Figs. 3–5). The effect of TF-2 on RGS10 is prominent; within 4 h we observed a more than 4-fold increase of the RGS10 transcript level (Fig. 6). This finding represents the first demonstration that tea polyphenols such as TF-2 can modulate RGS family genes. Since only the steady state level of RGS10 transcript was measured, the mechanism of RGS induction could be controlled at either transcriptional or posttranscriptional level.

The regulators of G protein signaling (RGS) were originally discovered as negative regulators of G-protein signaling in *Saccharomyces cerevisiae* since they facilitate the rate of GTP hydrolysis (Watson et al., 1996; Berman et al., 1996; Berman and Gilman, 1999). The G-protein signaling utilizes the heterotrimeric guanine-nucleotide-binding-proteins (G-proteins) to link receptors (signal recognition elements) and effectors (signal generators) to trigger diverse physiological responses. G-protein signaling represents the most prevalent signaling pathways in animals (Ross and Wilkie, 2000; Berman and Gilman, 1999). The step essential to G-protein signaling is the on-and-off switching mechanism via GTP hydrolysis and exchange (Ross and Wilkie, 2000; Watson et al., 1996; Berman et al., 1996; Berman and Gilman, 1999; Sowa et al., 2000). RGS proteins act as GTPase-activating proteins (GAPs), allowing rapid deactivation of G-protein signaling pathways *in vivo*, which is 10- to 100-fold faster than the rate of GTP hydrolysis of G subunits *in vitro* (Sowa et al., 2000). RGS proteins are not only capable of binding G-proteins, they can also interact with specific G-protein coupled receptors (GPCRs) and effectors such as adenylyl cyclase, GIRK channels, PLC-β_1 and Ca^{2+} channels, providing highly specific and selective regulation (Posner et al., 1998; Abramow-Newerly et al., 2006). Thus RGS proteins, by providing an additional layer of regulation of G signaling, play a pivotal and critical role in controlling the duration and specificity of physiological responses elicited by G-signalling pathways.

Using palindromic PCR-differential display we were able to show that TF-2 induces the expression of RGS-10 and RGS-14 (Figs. 6 and 7). In addition, we found that TF-3 and EGCG are also effective in inducing the expression of RGS-10 (Fig. 8). These findings are significant in light of the important role of RGS in modulating G-signaling pathways. Among the four tea polyphenols that we have tested, only TF-1 is ineffective in inducing either RGS-10 or RGS-14. It is certainly of

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interest to note that TF-1 is also the only polyphenol here that totally lacks gallate component (Fig. 1). If indeed gallate moiety is essential for tea polyphenols to affect RGS10 gene expression, the information may be useful for probing the protein target site of the gallate-containing polyphenols. The findings that TF-2 exhibit different dose–response and kinetics for different RGS genes (Figs. 6 and 7) and that different polyphenols have different effects on a particular RGS gene (Fig. 8) are also potentially significant. It is conceivable that through selective induction of RGS, individual tea polyphenols can selectively suppress a subset of G-protein signaling and thus affect only a certain cell physiology accordingly. In this regard, it is of interest to note that most of the genes known to be repressed by EGCG are associated with the G-protein signaling network (Wang and Mukhtar, 2002).

In summary, our findings allow us to propose that the diverse effects of tea polyphenols mediated via G-signaling pathways may be attributable to their selective effects on RGS genes. In addition, the identification of RGS10 and 14 as the target genes of tea polyphenols TF-2 and EGCG provide us with an upstream anchor to further explore the mechanistic basis for the actions of these small molecules.

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