The TC21 oncoprotein interacts with the Ral guanosine nucleotide dissociation factor

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TC21 is a highly oncogenic member of he Ras superfamily of small GTP binding proteins. We have used the yeast two hybrid system to identify proteins that interact with an oncogenic form of the TC2I protein. cDNA clones encoding the carboxy-terminal region of the RalGDS protein were isolated from human B-cell and HeLa cDNA libraries. RalGDS is an exchange factor that stimulates GDP dissociation from Ral, another member of the Ras superfamily of proteins. The interaction between RalGDS to TC21 is direct and appears to be mediated by the effector domain of TC21 and the carboxy-terminal region of RalGDS. Moreover, RalGDS only binds to TC21 in its active, GTP-loaded configuration. These results suggest that RalGDS might be an effector molecule for TC21 and may participate in cross-talking between Ral and TC2I signalling path- ways.

Keywords: TC21; RalGDS; effector dornain

Introduction

To date, more than 50 members of the Ras superfamily of **GTP** binding proteins have been identified in mammalian cells. These small GTP-binding proteins have important! regulatory functions in a variety of cellular processes including proliferation, differentia- tion, transport, and cellular architecture (Bourne *et al.,* 1990, 1991). In spite of the structural and biochemical similarities among the members of this superfamily of proteins, the only ones known to have significant! levels of oncogenic activity are the th**ree H-, K-** and N-Ras proteins (Barbacid, 1987). Recently, it has been shown that TC21 (also known as R-Ras2), a molecule highly related to the three Ras proteins, displays comparable transforming properties upon undergoing the same missense mutations (Chan *et al.,* 1994; Graham *et al.,* 1994). These observations have raised the possibility that TC21 may play a similar role as Ras in mitogenic signaling (Graham *et al.,* 1994). Lower levels of transformation have also been observed in similar mutated alleles of R-Ras (Saez *et al.,* 1994), RhoA (Khosravi-Far *et al.,* 1994) and *Aplysia* Rho (Perona *et al.,* 1993).

TC21 displays a significant degree of homology (55%) with each of the three Ras proteins and with R- Ras (89% homology, 78% identity) (Drivas *et al.,* 1990). Regions of homology encompass all critical motifs characteristic of the Ras proteins including the effector domain (residues 32 to 40 in the Ras proteins) which is believed to play a critical role in their interactions with downstream signaling elements (Polakis and McCormick, 1993). Mutations in this domain abolish the interaction of Ras proteins with a number of putative effector molecules including the Ser/Thr kinase Raf (Vojtek *et al.,* 1993). The interactions between Ras and Raf is believed to allow the translocation of Raf to the plasma membrane, a step required for activation of its kinase activity (MacDonald *et al.,* 1993; Stokoe *et al.,* 1994; Lecvers *et al.,* 1994). The effector domain of Ras also plays a critical role in mediating its interaction with RasGAP, a putative Ras effector molecule that stimulates the intrinsic GTPase activity of Ras (Martín *e1 al.,* 1992; Polakis and McCornick., 1993). In addition to the above mentioned effectors, it has been reported that the catalytic subunit of PI3-kinasc (Rodriguez-Viciana *et al.,* 1994) and the MEK kinase interact with the Ras in a GTP dependent manner (Russell *et al.,* 1995).

Likc all the other members of the Ras superfamily of proteins (except for Ran/TC4), TC21 needs to be prenylated for proper biological function (J Carboni, personal communication). However, and unlike the related Ras proteins, TC21 can become farnesylatcd and geranyl-geranylated with similar efficiency (Car- boni *et al.,* 1995). These observations have led to the hypothesis that, at least in cells in culture, TC2l can replace Ras activity when these proteins are rendered inactive by treatment with inhibitors of farnesyl transferase, thus explaining the lack of toxic effccts observed with this class of compounds.

In an effort to elucidate the signa! transduction pathway(s) utilized by TC21, we have used the yeast two hybrid system to isolate putative interacting proteins (Fields and Song, 1989), an approach previously used to successfully identify a number of signaling elements within the Ras pathway (Votjek *et al.,* 1993; Van Aelst *et al.,* 1993). We report here that the active, GTP-bound form of TC21 specifically interacts with RalGDS, an exchange factor for Ral, a non-transforming member of the Ras superfamily of proteins (Albright *et al.,* 1993).

Results

Isolation of TC21 interacting proteins

We used the yeast two hybrid system of Fields and Song (1989) to identify proteins that may interact with TC21 and, therefore, participate in its signaling pathway(s). As a 'bait', we used pMLB24, a plasmid

generated by fusing the oncogenic Va122 mutant of TC21 with the Gal4 DNA-binding domain (GBD) in a vector (pM LB6, see Materials and methods) that directs low· levels of expression of the GBD fusion proteins. The CJBD-TC2lVal22 fusion protein lacks its carboxy-terminal prenylation signal in order to prevent its locali1ation in the plasma membrane. The oncogenic version of TC2l was chosen to perform the screening to favor finding molecules that may spccifically recognize TC2l in its activated conformation. As the tester strain we used a derivative of YPB2 which, in addition of the bait plasmid, contains two Gal4- inducible markers, *H IS3* and *lacZ.* This tester strain was re-transformed with either HcLa or human B-cell cDNA libraries constructed in pGAD-Gl-1 and pACT, respectively, two vectors that direct the synthesis of fusion proteins containing the Gal4 transcriptional activation domain (GAD).

A total of 4.8 x 10" (HeLa cDNA library) and 3 x 10" (B-cell **cDNA** library) transformants were placed in selective media containing 5 mM 3-(3-amino-l ,2,4-triazole) (see Materials and methods). Transformants that showed histidine prototrophy after two weeks of culture were subsequently screened for their ability to produce /J-galactosidase using a filler lift assay (Breeden and Nasmyth 1985). A total of ten His' β- gal' colonies (eight from the HeLa library and two from the B-cell library) were identified.

To test whether the phenotypes of these colonies were dependent upon the interaction of their resident GAD fusion protein(s) with the GBD-TC21Val22 fusion protein used as a bait, library-derived plasmids were selectively recovered by complementation of the */euB6* mutation in *E coli* Ml-14 cells and subsequently used to transform YPB2 cells expressing either the GBD alone (pMLB6), the GBD-TC21Val22 fusion protein (pMLB24) or an unrelated GDB fusion protein that contains the N-terminal region (amino acids 1-· 120) of the Vav protein (pMLB14). In a representative experiment, one of the two plasmids recovered from the YPB2 cells transformed with the human B-cell library, pMLB25, interacted with the GBD-TC21Val22 fusion protein in a specific manner as determined by the lack or activation of the control plasmids expressing · either the GDB alone or the GDB-Vav fusion protein (Figure 1). Similar results were obtained with three of the eight plasmids recovered from the HeLa cDNA library {data not shown). None of the other cDNA clones isolated from these screenings encoded known proteins. To date we have not obtained sufficient evidence to determine whether these proteins interact with TC21 under physiological conditions.

Clones interacting with TC21 encode the carboxy-terminal region of RalGDS

Sequence analysis of the positive clones identified above revealed that each of these cDNAs represented overlapping clones encoding the carboxy-terminal region of RalGDS, a guanosine nucleotidc dissocia- tion stimulator (GDS) specific for Ral;\ and Ralll (Albright *el al.,* 1993) (Figure 2). RalGDS is a membcr of the CDC25 family of GDP/GTP exchangc factors that possesses a catalytic domain in the middle of the molecule (rcsiducs 412-499), two regions containing PEST sequcnccs (residues !96-318 and 557-728) and six blocks of homology with the carboxy-tcrminal region of CDC25 (Albright el *al.,* 1993). Intcrestingly, the same region of RalGDS that binds to TC2l has been shown to interact with the highly rclatcd l-1- and K- and R-Ras proteins as wcll as with Rap (Kikuchi *el al.,* 1994; l-lofer *e1 al.,* 1994; Spaargaren and Bischoff 1994).

RalGDS binds TC21 in a GTP dependent manner

The binding of exchange factors and effector molecules to small GTPases is highly dependent of their nucleotide binding status (Lai el *al.,* 1993). To determine whether the binding of RalGDS to TC21 occurs in a nucleotide-dependent manner, the carboxy- terminal region of RalGDS was fused to bacteria! MBP (see Materials and methods), absorbed on an amylose resin and incubated with purified wild type GST-TC21Gly22 fusion protein in the presence of

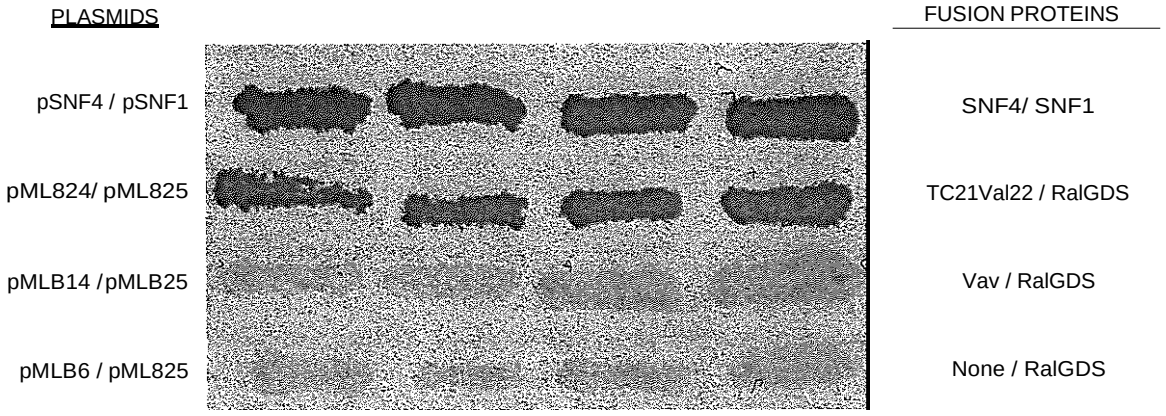

Figure 1 Specific interaction ofTC21Val22 with Ra\GDS in yeast two hybrid system. 1 *µg* ofpGBT derivative vectors containing either no insert (pMLB6), the oncogenic TC21Va122 isoform without the CAAX isoprenylation motif (pMLB24), or the amino terminal region of Vav (pMLBl4) were co-transfected in yeast ce!!s (YPB2 strain) with 1 *µg* of pMLB25, a pGAD-GH-derived vector containing sequences encoding residues 767 to 895 of Ra!GDS. Plasmids encoding the SNF4 and SNFI yeast proteins were used as positive controls. Four independent transfectants selected by Leu-Trp prototrophy werc tested far transactivation of the *LacZ* gene as previously described (Fields and Song, 1989)

either GDP or GTP-/-S, a non-hydrolizabic analoguc of GTP. A.ftcr incubation, compicxcs wcrc washcd, fr 1ctionatcd by SDS PAGE and submittcd to \Vcstern blot analysis using an anli-GST monoclonal antibody against the GST protein. To show that similar amounts of RaIGDS are prcscnt in cach linc, the filler was rcblottcd with anti-MBP polyclonal antibody (Figure 3B). As a negativc contrnL the MBP-RalGDS protcin \vas incubatcd with a GST fusion protcin containing sequcnccs derivcd from CDC42, a rclatcd mcmbcr of the Ras supcrfamily of GTP-binding protcins. As sl10wn in Figure 3A. MBP-RalGDS binds to TC21. but only whcn it has bccn incubatcd with GTP-;·-S. Th.: specificity or the intcraction betwecn RalGDS and TC2 I is further i!lustrated by the lack of binding_ of the MBP-RalGDS protcin tn GST-CDC42.

The effector domain of TC21 is required to interact with RalGDS

To idcntify the domain(s) of the TC2I protcin involved in the interaction with RalGDS, we cxamined the

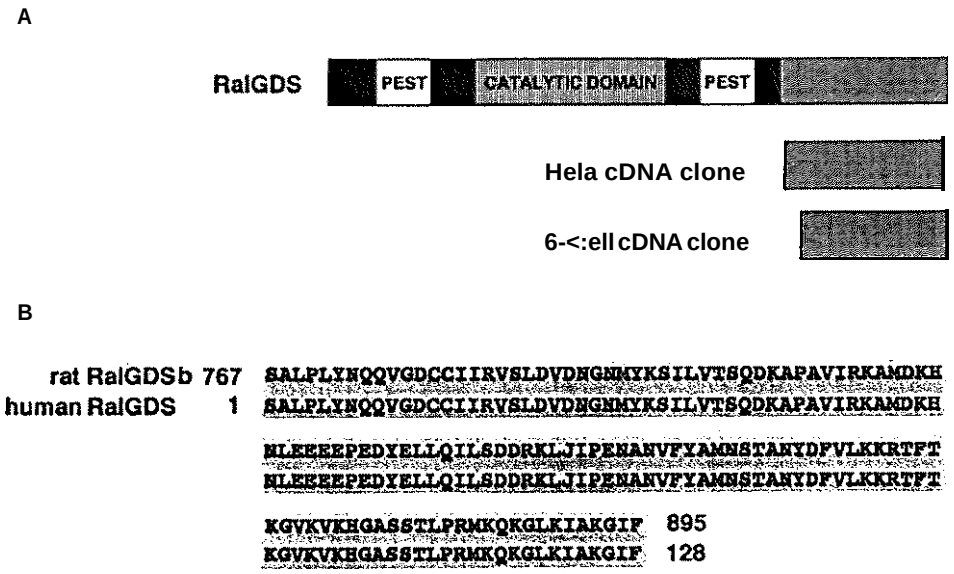

Figure 2 The carboxy-tenninal domain of Ra!GDS is responsible for TC21 binding. **(A)** Schemalic representation of the RalGDS full-lcngth proteín and the ovcr!apping carboxy-terminal regions isolated from the indicated cDNA libraries used in the ycast two hybrid system. **(B)** Alignment of the amino acid sequence of the carboxy-tenninal region of rat Ra!GDS (residues 767 to 895) with that oí the human cDNA clone isolated from the B-cell cDNA library

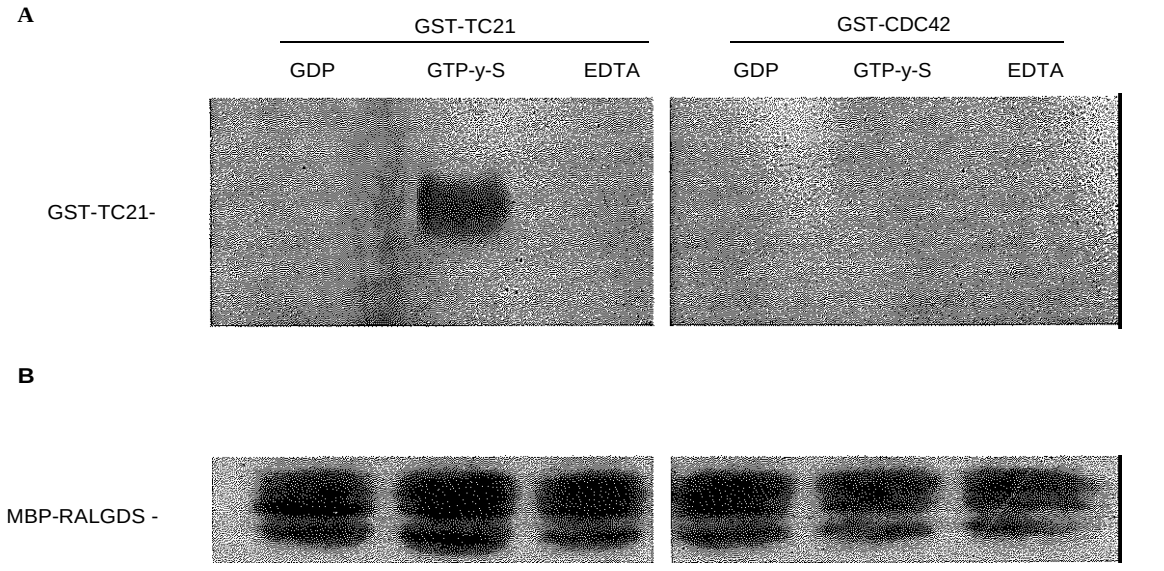

Figure 3 RalGDS binds specifically to the GTP-bound forro ofTC21. **(A)** GST-TC2l or GST-CDC42 fusion proteins were loaded with either GDP, GTP y-S or EDTA and incubated with amylose beads coated with a MBP fusion protein containing the carboxy- terminal region of RalGDS. Bound complexes were fractionated by SDS-PAGE and immunoblotted with anti GST antibodies as described in Materials and ffi('-thods. The migration of the bound GST-TC21 fusion protein is indicated by an arrow. **(B)** Nitrocellulose filter was then re-probed with anti-MBP antibody in order to show the equal amounts of MBP Ra!GDS present in each lane

ability of the MBP-RalGDS fusion protein to bind to a series of dcletion mutants of TC2 !. These mutants include a series of pGEX-5X derivativcs cncoding_ dcletions of the oncogenic TC2 I Va122 prolcin lacking either the 20 (pMLB28), 40 (pMLll29) or 49 (pM un 1) amino-terminal residucs (Figure 4A). 1n addition, we also gcncratcd a dcrivativc of pMLB29 which encodcs a truncatcd TC21 protein in which residue Thr45 has bcen replaced by Ala (pMBL30) (Figure 4A). As indicated above, TC21 must be in its active GTP-bound forn1 to recogmze RalGDS. Delction of amino terminal rcgions encompassing the GXXGXKX consensus GTP-binding motif may alter the ability of the mutants to bind GTP and subsequently to recognize RalGDS. Therefore, wc first examined whether the TC2 I mutant protcins described in Figure 4A retain their ability to bind GTP. To this end, each of the mutant proteins were incubated with [y-"P]GTP as indicated in Materials and methods. As illustrated in Figure 4B, each of the TC2l mutants display significant levels of GTP binding activity.

Next, we examined whether these TC2I mutants proteins could recognize RalGDS. Bacteria! lysates expressing MBP-RalGDS were incubated with each of the above protein mutants and the resulting complexes fractionated by SDS-PAGE and analysed by Western blot using anti-MBP antibodies. As illustrated in Figure 4B, deletíon of the 20 or 40 amino-terminal rcsiducs of TC2 I has no cffccl on its ahility to intc-ract with RalGDS. Ho\vcvcr, rcmoval of thosc amino acid rcsiducs (4 l to 49) corrcsponding to the dTector domain üf the Ras protcins, compk-tely aholishcd its hinding to RalGDS. Thesc rcsults are reminisccnt of those obtaincd ,vith Ras prntcins \Vhich rcquired an intact cffector domain to intcract with Ra!GDS. Interestingly, rcplacemcnt of a thrconinc rcsiduc (Thr45, which corresponds to Ras Thr35) hy alaninc had no effect on the ability of TC2I to intcract with RalGDS (Figure 4C), whereas a similar substitution in Ras complctely abolished its binding to RalGDS. These observalions suggcst that whereas TC21 and Ras utilizc similar domains to recognize RalGDS (Kikuchi *et al.*, 1994; Hofer *et al.*, l 994; Spaargarcn and Bischoff l 994), their interaction must involvc somewhat distinct residues, perhaps reflecting an overall distinct conformation of their respective effector domains.

In vivo binding of TC21 and RalGDS

To determine whether the association betwecn TC2 l and RalGDS occurs *in vivo,* we incubatcd cell lysatcs derived from B22-4! 1 cells, a NIH3T3-derived cell line tha t overexpresses the oncogenic TC2 l Val22 protein with antibodies raised against RalGDS. The resulting immunoprecipitates were fractionated by SDS-PAGE and probed with antibodies against TC2L As

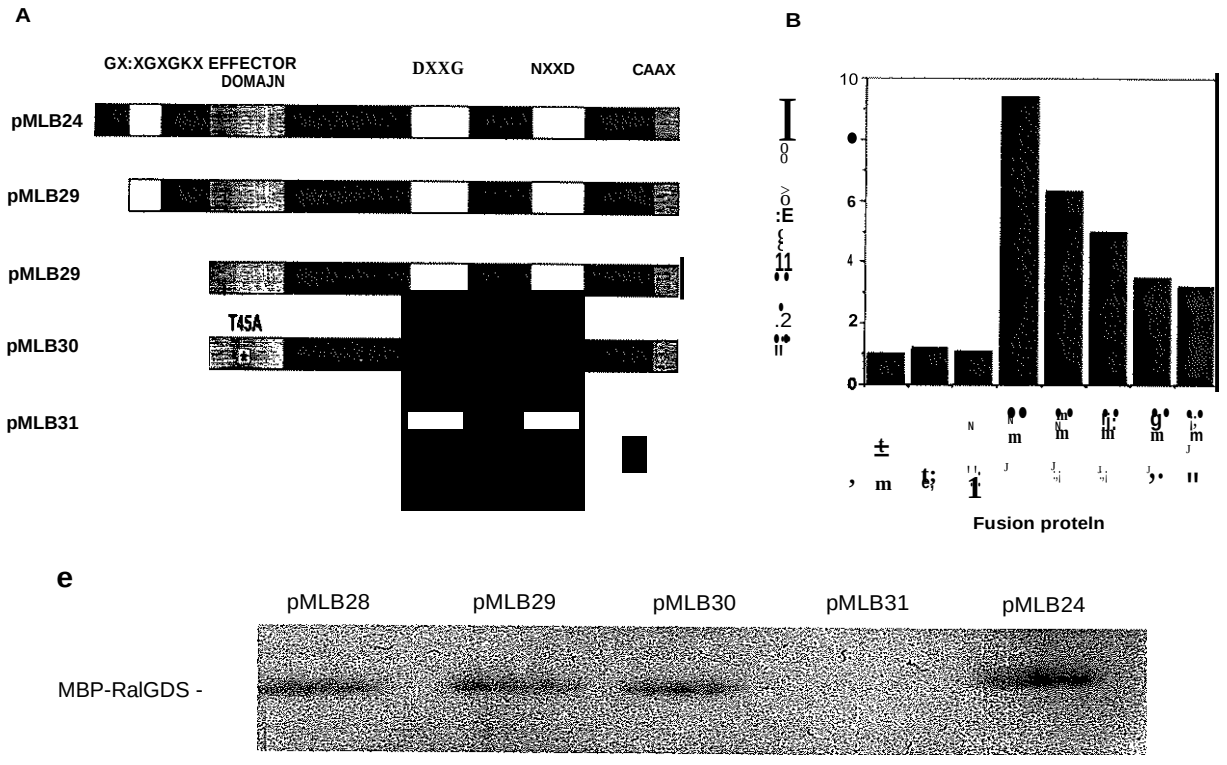

Figure 4 Ra!GDS binds to the effector domain of TC2l. **(A)** Schematic representation of the GST-TC21 fusion proteins used in this study. They include ful! length TC2l (pMLB24) and deletion mutants lacking the 20 (pMLB28), 40 (pMLB29) and 49 (pMLB3l) amino-terminal residues. pMLB30 encocles a GST-TC21 fusion protein carrying the same 40 amino acid de!etion as pMLB29 plus a miscoding mutation (indicated by an asterisk) in which the Thr45 residue has been replaced by Ala45. **(B)** AH the TC2l mutants were loaded with GTP in optima! conditions, GST alone anda non-related fussion protein (GST-MEKK) were used as negative controls. (C) Binding of the above GST-TC2l fusion proteins to Ra!GDS. Bacteria! lysates expressing MBP-RalGDS were incubated with the indicated GST-TC21 mutant proteins. After incubation, complexes were washed, fractionated by SDS- PAGE and immunoblotted with anti-MBP antibodies. The migration of the bound MBP-Ra!GSD fusion protein is indicated by an arrow

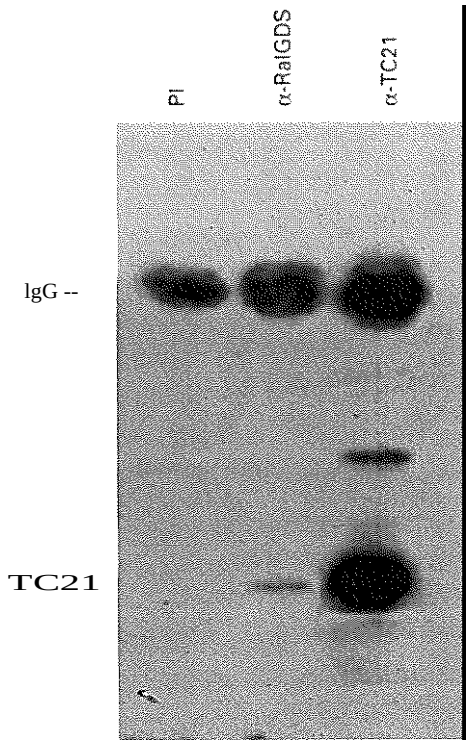

Figure 5 Ra!GDS interacts with TC21 *in vivo.* Lysates from B22- 41 l cells transfonned with the oncogcnic TC21Va122 protein were incubated with either pre-immune serum (PI), or antisera e!icited against TC21 (ct:-TC2l) or RalGDS (a-RalGDS). The resultíng immunocomp!exes were submitted to Western blot analysis with anti-TC2! antibodies. The migration of the TC2l protein is indicated by an arrow

illustrated in Figure 5, the TC2lVal22 oncoprotein coimmunoprecipitated with the endogenous RalGDS, thus indicating that TC2l can interact with RalGDS *in vivo.*

The carboxy-terminal domain of RalGDS has no effect on the transforming activity of TC21

The specific interaction of the carboxy-terminal region of RalGDS with the active, GTP-bound form of TC2l raised the possibility that this domain may modulate its mitogenic activity either acting as a downstream activator or more likely as a dominant negative inhibitor. To examine this possibility, we co-trans- fected NIH3T3 cells with a plasmid, pZIP-TC2lVal22 which encodes a highly transforming TC2 l oncopro- tein and pMLB32, an expression plasmid that encades the 128 carboxy-terminal residues of RalGDS (!,.RalGDS). Expression of the !,.RalGDS protein was monitored by immunoprecipitation of extracts derived from COS cells transiently transfected with pMLB32 in the presence of anti-fi-galactosidase antibodies (data not shown). NIH3T3 cells transfected with pMLB32 <lid not depict auy significan! morphological abnormalities or altered growth properties (data not shown). As controls we used pAL8, a plasmid encoding a transforming H-Ras protein as well as pCMV/i, the vector used to generate pMLB32. As depicted in Figure 6, co-expression of !,.RalGDS with transforming Ras or TC2l proteins had no significan! inhibitory or stimulating activity, suggesting that RalGDS may participa te in TC21 and Ras signaling patlnivays distinct from thost: responsiblc for tht:ir mitogcmc propenics.

Discussion

Mcmbers of the Ras supcrfamily of prntcins play a rnlc in a varicty of ccllular functions such as mitogcncsis. transport, cytoskeleton organization, cte. Most of thesc functions have been unvcilcd by using constitutivcly activated mutant isoforms. These activated proteins are gcnerated by miscoding mutations that favor the formation of GTP-bound complexcs either by inacti- vating their intrinsic GTPase activity or by promoting the unregulated exchange of GDP by GTP. Until recently, the only members of this superfamily of monomeric G protcins known to effciently induce oncogenic transformation are the three Ras proteins, H-Ras, K-Ras and N-Ras. Recent studies have indicated that the activatcd form of TC2 l is al lcast as oncogenic as the Ras oncoprotcins, thus suggesting that TC21 mighl play a related role in mitogenesis (Graham *et al.,* l994; Chan *et al.,* l 994). Indeed, overexpression of wild typc TC2l elicits the transfor- mation of rodent fibroblasts even more efficiently than the related Ras proteins (Lópcz-Barahona *et al.,* unpublished observations).

Biochemical studies have failed so far, to provide évidence for a distinctive functional role of each of the three known Ras proteins. Preliminary evidencc, however, suggests that TC2 l may use at least sorne distinct signaling pathways since this protein interacts poorly with c-Raf and, unlike Ras, cannot actívate its serine/threonine kinase activity (López-Barahona *et al.,* unpublished observations). In the present study, we have used the yeast two hybrid system to isolate proteins that interact with TC21 and therefore, may play a role in mediating sorne of its biological properties. One of these proteins is RalGDS, a GDP dissociating factor specific for the related RalA and RalB proteins (Albright *et al.,* l993). RalGDS was originally isolated by PCR-aided amplification using sequences conserved between the Ras-specific exchange factors CDC25 and Ste-6 (Albright *et al.,* 1993).

RalGDS has recently been shown to bind to each of the three Ras proteins (Hofer *et al.,* 1994; Kikuchi *et al.,* !994) as well as to R-ras and Rap (Spaargaren and Bischoff, l 994), a protein of unknown function that can revert Ras-induced transformation in culture (Kitayama *et al.,* !989). However, RalGDS <loes not interact with other Ras superfamily members such as Rae and Rho (Spaargaren el *al.,* 1994) suggesting a specific role in the Ras/Rap subfamily. The region of RalGDS involved in binding to TC2 l has been mapped to its carboxy-terminal end, not unexpectedly, the same domain involved in Ras binding. The region of RalGDS involved in TC2l and Ras binding <loes not exhibit any structural homology with the Ras binding domains of other effector molecules such as Raf or GAP. This observation indicates that there is not an apparent consensus motif for binding to the effector domain shared by Ras and TC2 l proteins. The TC2 l interacts with RalGDS through its effector domain, a region identical to that present in the Ras proteins. Interestingly, replacement of Thr35 by Ala in Ras

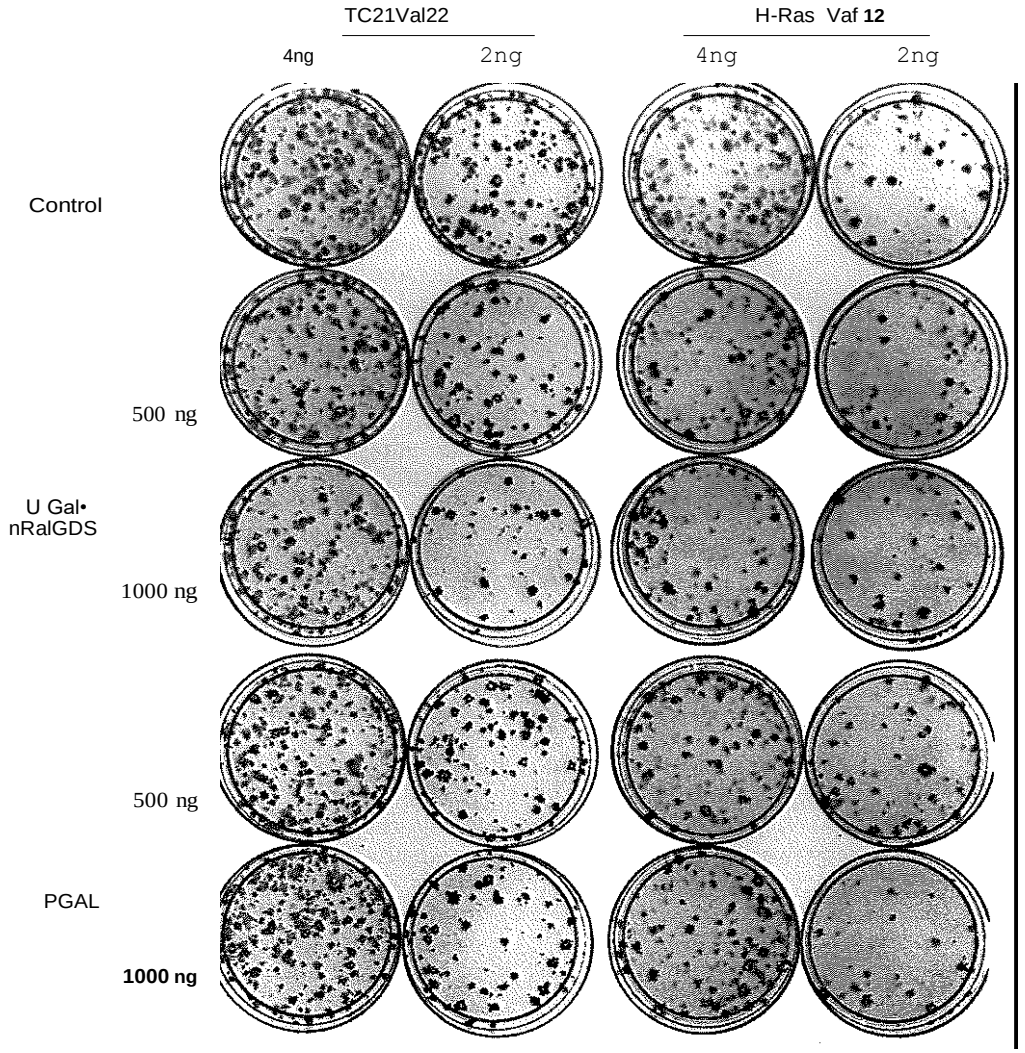

Figure 6 E!Tect of Ra!GDS on the transfonning activity of TC21 and Ras oncogenes. NIH3T3 cells were transfected wíth plasmids encoding the H-ras (pAL8) and the TC21 (pZIP-TC2JVa122) oncogenes, either alone or in the presence of the indicated amounts of an expression plasmid encoding the carboxy-temllnal region of RalGDS fused to /3-Gal (pMLB32) or with the pCMV/J expression vector encoding /J-Gal alone, as previously described (Alan, 1990). Cells were cultured for 15 days, fixed and stained with Giemsa solution

abolished binding to Ra!GDS (Hofer *et a/.,* 1994; Kikuchi *et al.,* 1994). However, mutation of the equivalent residue, Thr45, in TC21 has no effect on its ability to interact with Ra!GDS. These observations **indicate that** sequences outside the conserved effector domain shared by Ras and TC21 proteins might also play a role in their interaction with Ra!GDS.

Imrnunoprecipitation expcrirnents using TC21 transformed NIH3T3 cells have demonstrated that TC21 can bind to Ra!GDS *in vivo.* To our knowledge this is the first rcport indicating an *in vivo* interaction between Ra!GDS and any of the Ras superfamily members. Biochemical analysis of this interaction indicates that TC21 recognizes Ra!GDS directly, without participa- tion of other proteins. More importantly, such interaction only takes place when TC2! is loaded with GTP. These observations argue against the possibility that Ra!GDS may act as an exchange factor for TC2I since an essential feature of GDP/ GTP exchangers is to bind to either the GDP-bound or the nucleotide-free form of their substrates (Lai *et al.,* 1993). In support of this hypothesis, Ra!GDS, unlike Ras exchange factors such as SOS and RasGRF, is not capable of transforming NIH3T3 cells even in the presence of either Ras or TC2I.

Recent studies suggest the existence of cross-talk signaling between families of Ras-like proteins (Qui *et al.,* 1995). Therefore, it is possible that Ra!GDS might be involved in mediating sorne of the downstrearn signaling activities of TC2l. Evidence supporting this hypothesis is tenuous at the present time. However, the fact that the interaction between TC21 and Ras with Ra!GDS is mediated by their respective effector domains suggest that Ra!GDS might be a downstream effeetor. If this is the case, Ral proteins must be involved in mediating TC2 I activities other than mitogenesis. As indicated above, Ra!GDS cannol induce morphologic transforrnation of mouse fibroblasts even in the presence of wild type Ras. Moreover, co-expression of the carboxy-terminal dornain of Ra!GDS responsible for its interaction with TC2l does not result in significan! inhibition of the

transforming propcnics of this oncoprotdn. Finaliy, mutation of Thr45 in the dfcdor domain of TC21, a mutation known to aho!ish its transforming activity (Graham *('! al.*. 1994). lfocs not alfrct RalGDS binding. Littk is klllnvn ahout the hiological functitrn(s) of Ral protL'ins. It has hccn rcportcd that Ra!A and RalB protcins are ubiquiwusly cxprcsscd (Olofsson *et al.*, 1988) and that constitutivdy active Ral mutants do not transform or alter the phcnotypc of cclls in culture (Feig and Emkcy, !993). Likc other mcmbcrs of the Ras supcrfamily of proteins, Ral proteins bccome associatcd with membranes upon prcnylation (Feig and Emkcy, 1993). In addition they havc been found associated with synaptic and clathrin-coated vesicles suggesting a role in transpon (Feig and Emkcy, !993). Unveiling the physiological role of Ral proteins should help to elucida te whether TC21 p!ays an upstrcam regulatory role by modulating Ra!GDS activity.

Materials and methods

Plasmids

pMLB6 is a modificd version of pGBTS (Fields and Song, 1989) which contains the influenza hacmag\utininc pcptidc in framc with the Gal4 binding domain. pMLB24 and pMLBI4 are, rcspectivcly, pMLB6 and pGBT8 dcrivatives containing either the oncogenic TC21Val22 protein without the CAAX box motif rcquircd for isoprenylation (pMLB24) or the N-terminal region of Vav (pMLB!4). pMLB25 is a pGAD-GH-derivative that contains sc- quences encoding residues 767 to 895 of the human RalGDS protein. Plasmids encoding the yeast SNFI and SNF4 proteins have bccn prcviously describcd (Fields and Song, 1989). Bacteria! expression vectors pMLB28, pMLB29, and pMLB3! are pGEX-5X derivatives that encode TC2 l Val22 deletion mutants lacking either 20 (pMLB28), 40 (pMLB29) or 49 (pMLB3 l) amino terminal residues. pMLB30 is identical to pMLB29 cxccpt for the replacement of the Thr45 residue by Ala45, a mutation known to inhibit the transforming activity of TC21 (Graham e1 *al.*, !994). pGEX-TC2! is a pGEX-2T derivative that encodes the wild type TC2! (TC2!Gly22) protein fused to GST. pXRB94 is a pGEX-4T3 derivative that encades a GST-CDC42 fusion protein. pMLB27 is a pMALc derivative that contains sequences encoding residues 767 to 895 of Ra!GDS. pML52 is a pGEX-2T derivative that encades the catalytic domain of MEKK fused to GST. MSV-derived marnmalian expression vectors include pAL8 (H-ras oncogene) (Cuadrado el *al.*, 1990), pbeN l (H-ras proto-oncogene) (Santos el *al.*, 1982), pZIP- TC2! (TC2!Val22 oncogene), pZIP-TC2! (TC2! proto- oncogene) (Graham el *al.*, 1994). pMLB32 is a pCMVP- derived expression vector (Clonetcch) that contains residues 767 to 895 of RalGDS in frame with the *{J-* galactosidase gene.

GTP loading

Five *µg* of purified fusion proteins from either TC21 mutants (pMLB28, pMLB29, pMLB30 and pMLB31), the wild type version of the protein (pGEX-TC2!), GST alone or GST fused to the catalytic domain of MEKK (pML52) were incubated with 10 *µCi* of [y-nP]GTP for 45 min at 32°C in a buffer containing 20 mM Tris-HCI (pH 7.5), l mM MgC!,, I mM DTT, !00 mM NaCI and 40 ¡ig/ml of BSA, as previously described (Bustelo *et al.*, I 994). Afterwards reactions were filtered in a Millipore filtration unit. The GTP bound to each protein was determined by scintillation counting.

Library screening

YPB2 cd!s WCTL' transformcd to tryptnphan prntotrnph;, with pMLB2..t by the alkalinc lllL'thod tBrccdt."n and Nasrnyth, ! 985; and uscd lú scrccn lkL:t and human B-cd! cDNA !ibrarics by standard proccdurcs (Brccdcn and Nasmyth, 1985). Positin:- dones wcrc du.racICri1cd by scqucncing usmg a Taq po!ymcrasc-d;,·c tcrminatnr (Pcrkln-Elmcr) PCR amplification tcchnlquc and a J7J DNA autnmatic scqucnccr (Applicd Biosystcms).

Protein purification and binding assays

Bacteria! expression plasmids wcrc introduccd in *E coli* according to standard protocols. Fusion protcins wcre purificd from IPTG-induccd *E coli* cultures by affinity chromatography using cither amylosc matrix (for MBP fusion protcins, NE Biolabs) or glutathionc-Scpharose beads (for GST fusion protcins, Pharmacia) as previously dcscribed (Maina *et al.*, 1988; Smith and Johnson, 1988). For binding assays, 5 *¡,g* of GST-TC21Gly22 or GST- CDC42 fusion protcins wcre pre-incubated with cither 1 mM Guanosine 5'- (3-O-thio-triphosphate) (GTP-}·-S) or GDP or with EDTA !O rnM l h at 3o•c in 50 ¡,I of reaction mixture (20 mM Tris-HCI, pH 7.6, 10 mM EDTA, 20 mM $MgCl_2$ and 10°;;) Glycerol). The GTP-·;i-S, GDP- bound or unloadcd forms of TC21 or CDC42 proteins were incubated at 4"C during 2 h with a MBP fusion protcin containing the carboxy-tcnninal region (residues 767 to 895) of RalGDS immobilized to amylosc-beads. Alterna- tivcly, *E coli* cells transformed with the expression plasrnid encoding the MBP-Ra!GDS fusion protein (pMLB27) were induced with IPTG 0.3 mM at *3TC* for 2 h. After induction, bacteria! pcllet was resuspended in radiommu- noprecipitation assay buffer (IO rnM Tris-HCl (pH 8.0), 150 mM NaCI, 1% Triton X-100, 1% Aprotinin, 250 pM PMSF, 1 mM NaF and 100 *µM* sodium orthovanadate), disrupted by brief sonication, and centrifuged at 11 000 *g* for 30 min at 4cc. Clarificd supernatant was incubated with the correspondíng GST-fusion proteins of the TC21 mutants bound to gluthatione sepharose beads as describcd above. After incubation, bound proteins were washed four times with a 20 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl, 1% Nonidet P-40, and 0.3% sodium dodecyl sulfate (SDS). Washed beads were resuspended in l x SDS sample buffer (80 mM Tris-HC!, pH 6.8, 2% SDS, IO¾ glycerol, 0.5% /i-mcrcaptoethanol and 0.02% bromophenol blue), fractionated by SDS-polyacrylamidc gel electrophoresis (SDS PAGE), transferred to a nitro- cellulose membrane and incubated with either anti-GST monoclonal or anti-MBP polyclonal antibodies (Santa Cruz Biotechnology) as previously described (Bustelo *er al.*, 1992). For immunoprecipitation, cellular lysates were incubated with the appropriatc antibodies for 2 h at 4ºC. After incubation, immunocomplexes were collected with protein A sepharose (Pharmacia/LKB), washed four times in a buffer containing lO rnM Tris-HCI (pH 8.0), 150 mM NaCl and 0.1 % Triton-Xl00; dissolved in l x SDS sarnple buffer and separated by SDS-"PAGE. Immunoblot analysis of the elcctrophorctically fractionated proteins was performed as described elsewhere (Bustelo and Barbacid, 1992).

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