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30. Wisconsin Sequence Analysis Package, version 8, Genetics Computer Group, Madison, WI.
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The p21^{RAS} Farnesyltransferase α Subunit in TGF-β and Activin Signaling

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The α subunit of p21^{RAS} farnesyltransferase (FNTA), which is also shared by geranylgeranyltransferase, was isolated as a specific cytoplasmic interactor of the transforming growth factor–β (TGF-β) and activin type I receptors with the use of the yeast two-hybrid system. FNTA interacts specifically with ligand-free TGF-β type I receptor but is phosphorylated and released upon ligand binding. Furthermore, the release is dependent on the kinase activity of the TGF-β type II receptor. Thus, the growth inhibitory and differentiative pathways activated by TGF-β and activin involve novel mechanisms of serine-threonine receptor phosphorylation–dependent release of cytoplasmic interactors and regulation of the activation of small G proteins, such as p21^{RAS}.

Cell growth and differentiation are regulated and delicately balanced by the activities of growth stimulators and suppressors. Although much is known about growth stimulatory pathways that act by means of tyrosine kinase receptors (*1*), little is known about the growth inhibitory pathways exemplified by the serine-threonine kinase receptors of the TGF-β family. Recent progress in cloning and characterization of the TGF-β family receptors revealed that two membrane serine-threonine kinases, the type I and type II receptors, form heteromeric complexes. In this functional signaling unit, the TGF-β type II receptor phosphorylates and possibly thereby activates the type I receptor to signal downstream pathways (*2*, *3*). However, the molecular mechanisms involved in the activation of type I receptor–mediated signaling will remain unknown until direct downstream cytoplasmic interactors are identified.

Because conventional biochemical methods to isolate cytoplasmic proteins in tyrosine kinase receptor downstream pathways have not identified intracellular interactors of the serine-threonine kinase receptors, we used a modified version of the yeast two-hybrid system (*4*, *5*). As determined by binding and functional assays (*6*, *7*), the cytoplasmic domain of the TGF-β type I receptor, also known as ALK5 (*6*) and R4 (*8*), was used as a bait to screen a human fetal brain library (Fig. 1A). Three groups of interactors were identified: human immunophilin FKBP12 (*9*) and two versions of the human FNTA (*10*) (Fig. 1B). The latter differ within the 10 COOH-terminal amino acids, the region least conserved among species (*10*) and critical for farnesyltransferase enzyme activity (*11*). Regulation of the expression of these two variants may be important in controlling the activities of the enzyme in vivo.

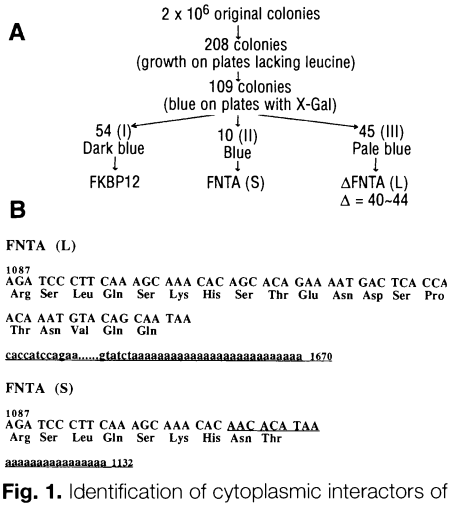

Fig. 1. Identification of cytoplasmic interactors of the TGF-β type I receptor (R4) with the use of a modified yeast two-hybrid system (*4*, *5*). (**A**) Summary of the library screening. The entire cytoplasmic domain of R4 was fused in-frame to the COOH-terminus of the DNA binding domain of LexA to serve as the bait (*5*). A human fetal brain complementary DNA (cDNA) library in the yeast expression vector pJG4-5 was used in the library screening. (**B**) Difference of the nucleotide sequences and their encoded amino acid sequences of the two isolated FNTA cDNAs. L, long; S, short.

When tested in the yeast system (Fig. 2A), the α subunit interacted with the β subunit of farnesyltransferase (FNTB) as expected and also interacted specifically with the functional type I receptors of TGF-β (R4) and activin (R2) among all tested type I receptors (*6*–*8*, *15*). The R4-FNTA interaction appeared not to be dependent on the kinase activity of R4 and was specific for type I receptors, because a kinase-deficient R4 [Fig. 2A, R4(K230R)] (*16*) was still capable of FNTA binding. Neither of the type II receptors of TGF-β and activin exhibited FNTA binding (*17*). The NH_2-terminal 81 amino acids of the α subunit, important for the enzyme activity of FTase in mammalian cells (*11*), were also essential for R4 binding [Fig. 2A, R4(Δ81)FNTA].

The cytoplasmic region of R4 (R4C) contains the juxtamembrane (JM), the serine-threonine kinase (K), and the tail

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(T) domains. Within the JM domain is a characteristic motif specific for, and highly conserved among, all known type I receptors, the GS box (*18*), with a core sequence of SGSGSGLPL/F (*16*). Deletions and mutations of R4C were made to dissect the molecular details of its interaction with FNTA (Fig. 2, B through D). Neither the JM region nor the tail contains the direct FNTA binding motif because deletion of neither alone affected the interaction. Deletion of the last 17 amino acids of the COOH-terminus of the kinase domain (JMΔK), however, completely abolished FNTA binding, which suggests that this sequence may contain a binding site. Deletion of the JM region NH_2-terminal to the GS box core sequence [(+GS)KT] resulted in a significant increase in FNTA binding, suggesting a negative regulatory role for the deleted region. Further deletion of the six amino acids of the GS core sequence (SGSGSG) [(−GS+P)KT] did not affect the interaction, but an additional deletion of the tail [(−GS+P)K] completely abolished the interaction. This result implies that the tail has a positive regulatory role and also indicates that the JM region and the tail may cooperatively regulate the interaction. Deletion or mutation of the proline within the GS core sequence significantly increased FNTA binding [(−GS−P)K, R4(PG)], which suggests that the proline has a negative regulatory role. Point mutations of the serines within the GS box core sequence increased FNTA binding (Fig. 2, B and D), indicating that phosphorylation of this region may regulate FNTA binding.

FNTA specifically co-precipitated with R4C (Fig. 3) from yeast cell lysates containing B42 and LexA fusion proteins of FNTA and R4C, respectively, but not with the cytoplasmic domain of R1 (*8*), a candidate shared receptor of TGF-β and activin (*17*). Histidine-tagged FNTA also co-precipitated with ligand-free R4 and FNTB when transiently expressed in COS cells (Fig. 4, A and B). The interaction of R4 and FNTA was also detected by co-immunoprecipitation experiments with either antibodies to FNTA or R4 in COS cells transfected with R4 and FNTA (*17*).

Because mutation studies of R4 suggested a regulatory role of the GS box on R4-FNTA interaction [Fig. 2, R4(GS)] and the GS box is phosphorylated only by the type II receptor upon ligand binding, we tested the effect of ligand binding on the R4-FNTA interaction. As shown in Fig. 4C, ligand-bound wild-type R4-RII complexes failed to co-precipitate with FNTA (lane 3), indicating that ligand binding to the receptors can release FNTA. Abolishing the kinase activity of the type II receptor (lane 5), but not of the type I receptor (lane 4), prevented the release. These results suggest that ligand-induced type I receptor phosphorylation mediated by the type II receptor releases FNTA.

To test whether FNTA is a substrate for the receptor serine-threonine kinases, we measured FNTA phosphorylation when it was co-expressed either with R4 alone or with R4 and the TGF-β type II receptor (tRII) in the presence of TGF-β. A basal level of FNTA phosphorylation was detected in COS cells expressing R4 alone (Fig. 4D, lane 1) or tRII alone, but a significant increase of FNTA phosphorylation was detected in COS cells expressing both R4 and RII in the presence of TGF-β (Fig. 4D, lane 2). Such an increase is not dependent on R4 kinase activity because a point mutation (K230R) that abolished R4 kinase activity did not prevent the ligand-dependent increase of FNTA phosphorylation (Fig. 4D, lane 3). Thus, the increased FNTA phosphorylation may be mediated by either tRII or receptor-associated kinases. Without overexpressing FNTA or the TGF-β receptors, we also observed a ligand-dependent increase of phosphorylation of the endogenous FNTA in the TGF-β–responsive mink lung epithelial cell line (Mv1Lu) (Fig. 4E).

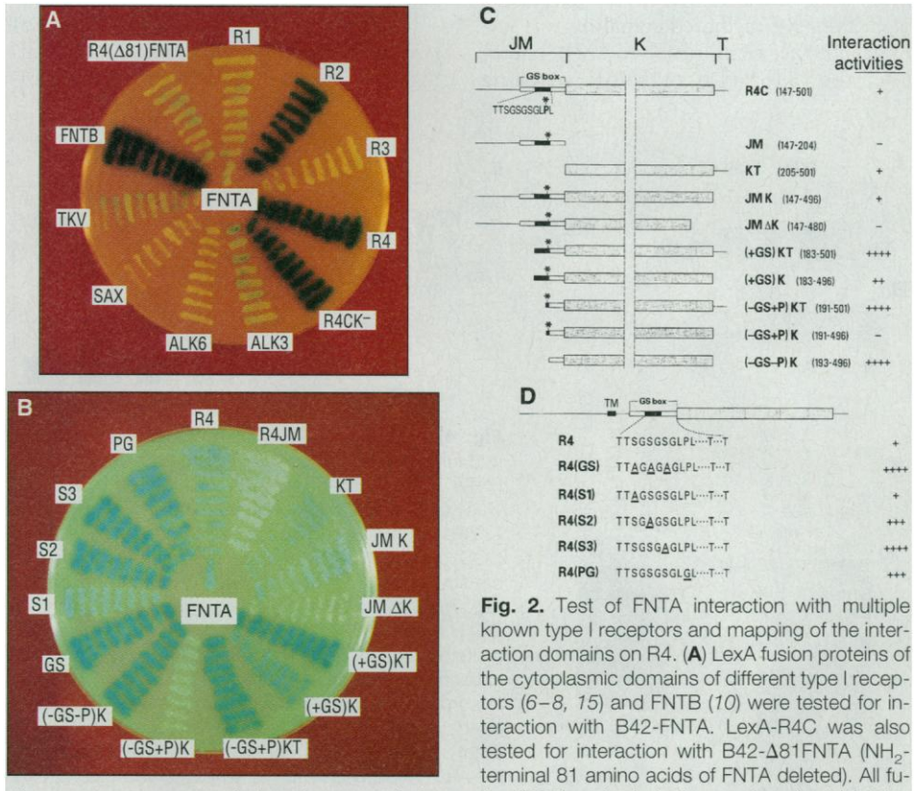

Fig. 2. Test of FNTA interaction with multiple known type I receptors and mapping of the interaction domains on R4. (**A**) LexA fusion proteins of the cytoplasmic domains of different type I receptors (*6–8, 15*) and FNTB (*10*) were tested for interaction with B42-FNTA. LexA-R4C was also tested for interaction with B42-Δ81FNTA (NH_2-terminal 81 amino acids of FNTA deleted). All fusion constructs were made by polymerase chain reaction (PCR) and subcloning, as described (*5*). Nine to 10 individual colonies of yeast transformants were streaked onto a fresh $Ura^-His^-Trp^-$ glucose plate, replica-plated onto both glucose and galactose $Ura^-His^-Trp^-$ plates with X-Gal, and incubated at 37°C for 72 hours. Only the galactose plates are shown here. (**B**) LexA fusion proteins of R4 deletions and mutations were tested for interaction with B42-FNTA as described in (A). To show the different interaction affinities of R4 mutants, the galactose X-Gal plates were incubated at 37°C for 12 hours. (**C**) Schematic drawings of the R4 deletions and summaries of their interaction activities. The core sequence of the GS box (filled) and the proline residue (asterisk) are indicated. (**D**) Schematic drawings of R4 mutations within the GS box and summaries of their interaction affinities (*16*). TM, transmembrane.

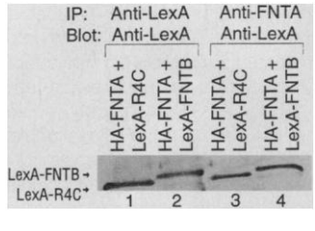

Fig. 3. Co-immunoprecipitation of FNTA (the short variant) and the cytoplasmic domain of R4 from yeast cell lysates. Yeast co-expressing either hemagglutinin (HA)-tagged B42-FNTA (HA-FNTA) and LexA-R4C or HA-FNTA and LexA-FNTB, as indicated, were lysed in lysis buffer (20 mM tris-HCl at pH 7.4, 150 mM NaCl, and 0.5% Triton X-100) with 1 mM phenylmethylsulfonyl fluoride. The obtained cell lysates were immunoprecipitated (IP) with antibodies to either LexA (lanes 1 and 2) or FNTA (lanes 3 and 4), and the precipitated proteins were analyzed by protein immunoblot with LexA antibody.

To explore the functional significance of the interaction between FNTA and R4, we made deletion mutants of R4. Deletion of the COOH-terminal tail region of R4 (last five amino acids) did not affect FNTA binding (Fig. 2B, JMK) nor R4 signaling activity (Fig. 4F, tail-less); however, further deletion of the COOH-terminal 17 amino acids of the R4 kinase domain completely abolished both FNTA binding (Fig. 2B, R4ΔK) and R4 signaling (Fig. 4F, R4ΔK). R4ΔK binds the ligand normally and has intact autophosporylation activity (*17*) but is also defective in binding to the other cytoplasmic interactor FKBP12 (*12*). However, if we blocked FKBP12 binding to R4 with 15-*O*-desmethyl-FK520, a nonfunctional derivative of FK506, R4-mediated signaling was enhanced rather than inhibited (*12*). Therefore, the abrogation of R4ΔK signaling activity might result from loss of binding to FNTA.

Thus, FNTA is a specific cytoplasmic interactor of the TGF-β and activin type I receptors whose binding to the TGF-β type I receptor appears to be essential for the signaling activity of the type I receptor. The ligand-induced release of FNTA, also observed in FKBP12 binding to the type I receptor (*12*), stands in contrast to what is known about tyrosine kinase receptor–activated signaling and highlights the uniqueness of TGF-β family signaling. Because the α subunit is a regulatory subunit shared by the two prenyltransferases, the observed ligand-dependent phosphorylation of the α subunit may affect the activity of the enzyme. An important substrate of farnesyltransferase, $p21^{RAS}$ is involved in both TGF-β and activin signaling (*19*, *20*). Farnesylation of RAS mediates RAS membrane localization, which is critical for both wild-type RAS activity and oncogenic RAS-mediated cell transformation (*13*). TGF-β and activin may therefore mediate their growth inhibitory pathways by direct regulation of RAS farnesyltransferase.

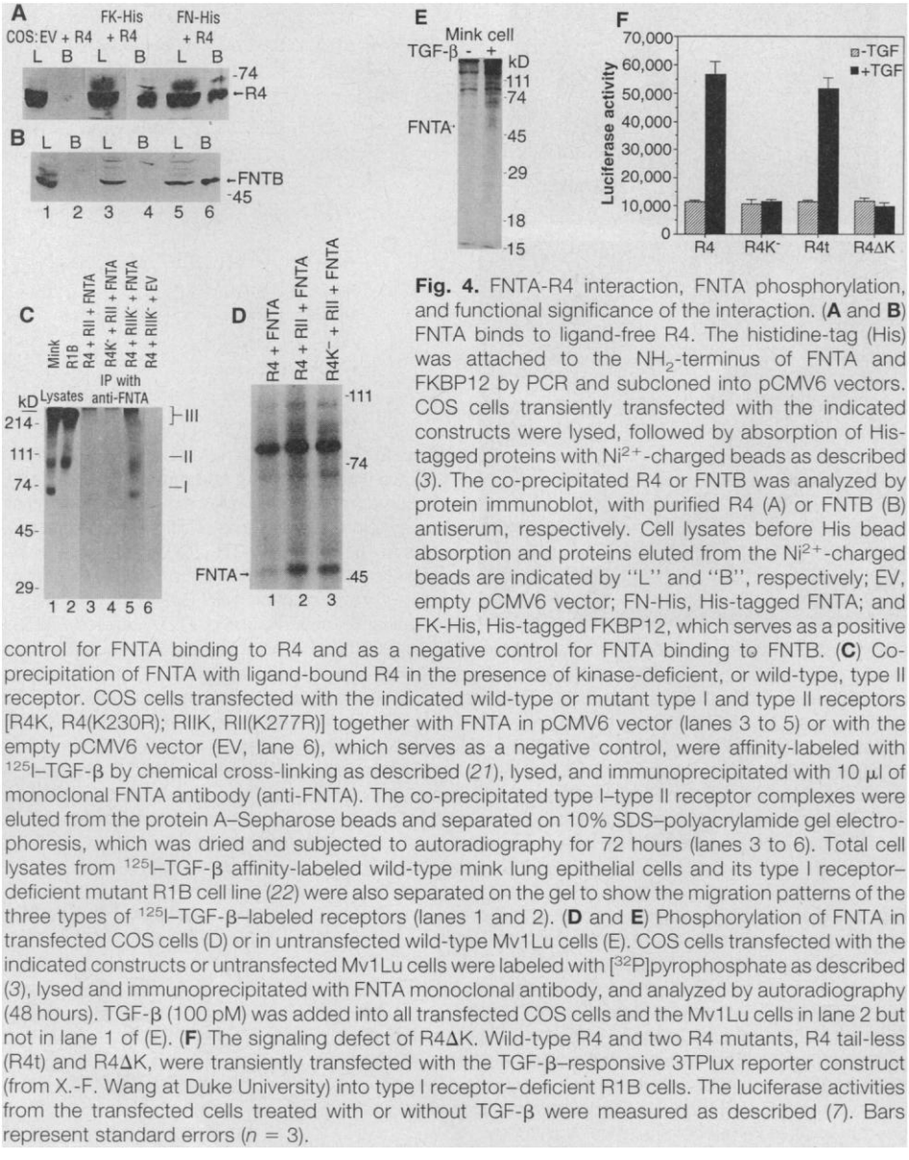

Fig. 4. FNTA-R4 interaction, FNTA phosphorylation, and functional significance of the interaction. (**A** and **B**) FNTA binds to ligand-free R4. The histidine-tag (His) was attached to the NH_2-terminus of FNTA and FKBP12 by PCR and subcloned into pCMV6 vectors. COS cells transiently transfected with the indicated constructs were lysed, followed by absorption of His-tagged proteins with Ni^{2+}-charged beads as described (*3*). The co-precipitated R4 or FNTB was analyzed by protein immunoblot, with purified R4 (A) or FNTB (B) antiserum, respectively. Cell lysates before His bead absorption and proteins eluted from the Ni^{2+}-charged beads are indicated by "L" and "B", respectively; EV, empty pCMV6 vector; FN-His, His-tagged FNTA; and FK-His, His-tagged FKBP12, which serves as a positive control for FNTA binding to R4 and as a negative control for FNTA binding to FNTB. (**C**) Co-precipitation of FNTA with ligand-bound R4 in the presence of kinase-deficient, or wild-type, type II receptor. COS cells transfected with the indicated wild-type or mutant type I and type II receptors [R4K, R4(K230R); RIIK, RII(K277R)] together with FNTA in pCMV6 vector (lanes 3 to 5) or with the empty pCMV6 vector (EV, lane 6), which serves as a negative control, were affinity-labeled with ^{125}I–TGF-β by chemical cross-linking as described (*21*), lysed, and immunoprecipitated with 10 μl of monoclonal FNTA antibody (anti-FNTA). The co-precipitated type I–type II receptor complexes were eluted from the protein A–Sepharose beads and separated on 10% SDS–polyacrylamide gel electrophoresis, which was dried and subjected to autoradiography for 72 hours (lanes 3 to 6). Total cell lysates from ^{125}I–TGF-β affinity-labeled wild-type mink lung epithelial cells and its type I receptor–deficient mutant R1B cell line (*22*) were also separated on the gel to show the migration patterns of the three types of ^{125}I–TGF-β–labeled receptors (lanes 1 and 2). (**D** and **E**) Phosphorylation of FNTA in transfected COS cells (D) or in untransfected wild-type Mv1Lu cells (E). COS cells transfected with the indicated constructs or untransfected Mv1Lu cells were labeled with [^{32}P]pyrophosphate as described (*3*), lysed and immunoprecipitated with FNTA monoclonal antibody, and analyzed by autoradiography (48 hours). TGF-β (100 pM) was added into all transfected COS cells and the Mv1Lu cells in lane 2 but not in lane 1 of (E). (**F**) The signaling defect of R4ΔK. Wild-type R4 and two R4 mutants, R4 tail-less (R4t) and R4ΔK, were transiently transfected with the TGF-β–responsive 3TPlux reporter construct (from X.-F. Wang at Duke University) into type I receptor–deficient R1B cells. The luciferase activities from the transfected cells treated with or without TGF-β were measured as described (*7*). Bars represent standard errors (n = 3).

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