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Retinoic Acid-Inducible G Protein-Coupled Receptors Bind to Frizzled Receptors and May Activate Non-canonical Wnt Signaling

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Abstract

Frizzled (Fz) seven-pass transmembrane receptors are Wnt receptors and function in a variety of developmental pathways. Here we identify retinoic acid-inducible gene-1, 2, 3, and 4 (RAIG1, 2, 3, and 4) as potential Fz binding proteins. RAIG proteins are seven-pass transmembrane receptors, and *Xenopus* RAIG2, 3, and 4 are expressed in early gastrula. XRAIG2 can activate small GTPases, such as RhoA, Rac, and Cdc42, and Jun N-terminal kinase, thus exhibit activities that overlap with non-canonical Wnt/Fz signaling. Injection of XRAIG2 mRNA into *Xenopus* embryo causes a severe shortened and bent body axis due to defective gastrulation movements, reminiscent of abnormal non-canonical Wnt signaling. XRAIG2 affects convergent extension in activin-treated animal caps, which can be partially rescued by co-injection of a dominant-negative form of Cdc42. In zebrafish embryo, XRAIG2 also causes Ca²⁺ flux, one of the consequences of non-canonical Wnt signaling. These results suggest a possible crosstalk/integration between Wnt/Frizzled and RAIG signal transduction pathways.

Keywords

RAIG; Frizzled; G protein-coupled receptor; RhoA; Rac; Cdc42; JNK; Ca²⁺ flux; convergent extension; gastrulation

Introduction

Signaling by the Wnt family of secreted growth factors has diverse roles in developmental processes such as cell proliferation, differentiation, polarity and migration [1]. Wnt signaling is mediated via members of the Frizzled (Fz) family of seven-pass transmembrane receptors, and Wnt-Fz interaction activates different intracellular signaling cascades, such as the Wnt/ β -catenin signaling pathway and non-canonical Wnt signaling pathways. Canonical Wnt/ β -catenin signaling leads to the accumulation of β -catenin protein that associates with the TCF/LEF (T cell factor/Lymphoid enhancer factor) family of transcription factors, resulting in the

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activation of Wnt-responsive transcription [1]. In *Xenopus laevis* embryos, Wnt/ β -catenin signaling contributes to the establishment of the dorsal-ventral axis. In contrast, non-canonical Wnt signaling controls morphogenetic movements in vertebrates [reviewed in ref. 2]. During gastrulation, dorsal mesodermal cells intercalate in the mediolateral axis (convergence), resulting in anteroposterior extension of the body axis. This convergent extension movement is under the control of non-canonical Wnt signaling involving Wnt-11 and Fz7 [3–5], which bears significant similarity with the planar cell polarity (PCP) signaling in *Drosophila* [6]. In this so-called Wnt/PCP signaling pathway, Wnt binding to Fz results in the activation of small GTPases such as RhoA and Rac, and of c-jun N-terminal kinase (JNK) [2,7,8], leading to cytoskeletal rearrangements and cell polarization and movements [9,10]. In addition to Fz, other transmembrane proteins, such as paraxial protocadherin and neurotrophin receptor homologue 1, are also known to activate the Rho family GTPases and JNK [11–13] and to regulate convergent extension movements. Non-canonical Wnt signaling also triggers the release of Ca^{2+} influx [14,15], resulting in the activation of Ca^{2+} sensitive protein kinases, such as protein kinase C and calcium/calmodulin-dependent kinase II [reviewed in ref. 2].

In the present study, we screened for binding proteins for Fz by a proteomic analysis, and demonstrated the specific binding between Fz and the protein product of retinoic acid-inducible genes (RAIGs), which encode G protein-coupled seven-pass transmembrane receptor. Interestingly, RAIG can activate Rho family GTPases and JNK and the intracellular Ca^{2+} release, and perturb convergent extension movements in *Xenopus* and zebrafish.

Materials and methods

Purification of hFz5-containing protein complex and mass spectrometry

HA-Flag-tagged hFz5 subcloned in pcDNA3 vector was transfected into HeLa cells, and cells stably expressing HA-Flag-hFz5 were isolated in the presence of 400 $\mu\text{g}/\text{mL}$ Geneticin. Stable cell lines were propagated as suspension cells, and plasma membrane fraction was purified as described [16]. The plasma membrane fraction was applied to the anti-Flag (M2)-conjugated agarose beads (Sigma), and the hFz5-containing protein complex was eluted with Flag peptide. The eluted fraction was applied to the anti-HA (3F10)-conjugated agarose beads (Roche), and the hFz5-containing protein complex was eluted with HA peptide. The proteins were separated by SDS-PAGE and stained with Coomassie blue. The protein band was excised and analyses by mass spectrometry at the Harvard Medical School Taplin Biological Mass Spectrometry Facility.

RT-PCR—RNA from *Xenopus* embryo was prepared with TRIzol (Invitrogen), and RT-PCR was performed as described [17] using the following primers; XRAIG1 (Fw primer: TCTTTATGTATTGCTGAACCC ; Rv primer: GATGATAATTAGTAGCGCC), XRAIG2 (Fw primer: TTTGTGATTGGCTATGGCTC ; Rv primer: GCAAGCCGGCTTCCTGTC), XRAIG3 (Fw primer: CCATGGCTTGTTCATC ; Rv primer: TTGACAATCGAGCAAGGT), XRAIG4 (Fw primer: AGTCGGGATGTCCCGTCC ; Rv primer: GATAGCTGATGGAAGGTTATG).

Embryo manipulations and animal cap explant assays

In vitro transcribed capped RNAs (mMessageMachine, Ambion) were injected into *Xenopus* embryos at 2 or 4-cell stage as described [17]. Animal cap explant assays were performed using 20 ng/mL activin as described [18].

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed by using digoxigenin-labeled RNA probe and alkaline phosphatase substrate (NBT/BCIP, Roche) as described [19].

RhoA, Rac, and Cdc42 activity assay

These are performed as described [7].

JNK activity assay

Activation of JNK was determined by monitoring c-Jun phosphorylation. The Myc-tag was introduced at the amino terminus of *Xenopus* c-Jun in pCS2+ (Myc-c-Jun/pCS2+). RAIG or Fz mRNAs together with myc-tagged c-Jun mRNA (200 pg) was injected into two-cell embryos. At stage 11.5, twenty injected embryos were solubilized with 800 μ L of buffer A [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 50 mM, NaF, 1 mM Na₃VO₄, protease inhibitor cocktail (Roche)]. The extracts were centrifuged for 5 min at 18,400 $\times g$. The supernatant was incubated with anti-myc antibody (clone 9E10, Santa Cruz) at 4°C for 2 h, followed by incubation with GammaBind G Sepharose at 4°C for 1 h. The beads were washed four times with buffer A. Immunoblotting was performed using an anti-phospho-c-Jun(Ser63) antibody (Cell Signaling) and anti-c-Jun antibody (Santa Cruz).

Results

Human and *Xenopus* retinoic acid-inducible genes (RAIGs)

To identify proteins potentially involved in Fz function, we designed a strategy to isolate Fz-interacting proteins in the plasma membrane. Fz-containing protein complexes were purified from HeLa cells that stably express human Frizzled 5 (hFz5) tagged with both the Flag and hemagglutinin (HA) epitopes via affinity chromatography (see materials and methods). Mass spectrometric analysis revealed that retinoic acid-inducible gene-1 (RAIG1, HGMW-approved symbol RAIG1; ref. 20) is one of the proteins co-purified in the hFz5 precipitates.

Besides RAIG1, three other closely related G protein-coupled receptors (GPCRs) - RAIG2 (HGMW-approved symbol GPRC5B), RAIG3 (HGMW-approved symbol GPRC5C), GPRC5D - have been reported in the human genome, and they form the group 5 of family C GPCRs [20–23]. By performing a homology search in the *Xenopus* EST databases using these human RAIG cDNA sequences, we identified five *Xenopus* RAIG orthologs, XRAIG1, XRAIG2 (GenBank accession no. **EF456760**), XRAIG3 (GenBank accession no. **EF456761**), XRAIG4a (GenBank accession no. **EF456762**), and XRAIG4b (GenBank accession no. **EF456763**; Fig. 1A and B). XRAIG1 protein sequence was predicted from an EST clone (IMAGE Consortium clone 4057778). XRAIG4a and XRAIG4b likely represent pseudoalleles due to gene duplication in *Xenopus laevis* lineage, since they show 96% identity on the amino acid level.

To determine the temporal expression pattern of XRAIGs during embryogenesis, we executed RT-PCR analysis with cDNA derived from *Xenopus* embryos at various developmental stages. None of the XRAIG genes was expressed maternally (Fig. 1C). XRAIG2, XRAIG3 and XRAIG4 were expressed at the gastrulation and the later stages (Fig. 1C). In contrast, XRAIG1 was not expressed during gastrulation, and the transcript became observed at stage 28 (data not shown) and stage 41 (Fig. 1C). In the early gastrula, XRAIG2 exhibited relatively ubiquitous expression, whereas XRAIG3 and XRAIG4 were predominantly expressed in the ectodermal and dorsal mesodermal tissues (Fig. 1D and E).

Fz-RAIG interaction

To examine the interaction between Fz and RAIG proteins, co-immunoprecipitation analyses were performed in mammalian cell lines. In HEK 293T cells, Flag-tagged hRAIG1 was coexpressed with HA-hFz5 or HA-Smoothed (HA-Smo, ref. 24), which is a seven-transmembrane protein related to the Fz family. HA-hFz5 or HA-Smo was immunoprecipitated

from cell lysates with anti-HA antibody. As shown in Fig. 2A, hRAIG1-Flag was co-immunoprecipitated with HA-hFz5 (lane 4), but not with HA-Smo (lane 3), indicating specific interaction between hFz5 and hRAIG1 in several types of human cells.

Xenopus Frizzled7 (XFz7) plays important roles in dorsal development and gastrulation [4,5, 25]. XFz7 transcripts are expressed on the dorsal side at the early gastrula stage [4,5], thus are coexpressed with XRAIG2, 3, and 4 (Fig. 1E). We therefore examined a possible interaction between XFz7 and XRAIG2 in *Xenopus* embryos. XRAIG2-Flag mRNA was co-injected with HA-hFz5, HA-XFz7, or HA-Smo mRNA into *Xenopus* embryos, and HA-tagged proteins were immunoprecipitated with anti-HA antibody. As shown in Fig. 2B, Flag-XRAIG2 was co-immunoprecipitated with either HA-hFz5 (Fig. 2B, lane 9) or HA-XFz7 (Fig. 2B, lane 11), but not with HA-Smo (Fig. 2B, lane 8). A truncated form of XRAIG2 lacking the carboxy-terminal cytoplasmic region (XRAIG2 Δ C-Flag) also associated with either HA-hFz5 (Fig. 2B, lane 10) or HA-XFz7 (Fig. 2B, lane 12), indicating that the carboxy-terminal cytoplasmic region of XRAIG2 is not necessary for the binding with Fz. Therefore XRAIG2 associates with XFz7 in *Xenopus* embryos.

Expression of hRAIG1 or XRAIG2 perturbs *Xenopus* gastrulation

Since hFz5 is known to activate canonical Wnt/ β -catenin signaling [26], we next investigated whether hRAIG1 has any effect on this pathway. By RT-PCR analysis, we examined the expression of nodal-related 3 (Xnr3) and siamois, two well-known target genes of Wnt/ β -catenin signaling. Injection of hRAIG1 mRNA into two-cell stage embryos induce the expression of neither Xnr3 nor siamois (Fig. 3A, lane 2), and injection of hRAIG1 mRNA into ventral blastomeres at the four-cell stage did not induce any ectopic axis formation (Fig. 3E). Conversely, hRAIG1 mRNA did not inhibit the expression of Xnr3 or siamois induced by Wnt3a or by the Wnt5a/hFz5 combination (Fig. 3B). These results suggest that hRAIG1 is not involved in Wnt/ β -catenin signaling. Interestingly, however, embryos injected with hRAIG1 mRNA (1 ng) dorsally showed a reduction of trunk and tail structures and exhibited a spina bifida (64%, $n = 53$; Fig. 3D), implying the possible role of hRAIG1 in morphogenetic movements, which are regulated through non-canonical Wnt signaling pathway. Indeed, the hRAIG1 overexpression phenotype is very similar to that caused by XFz7 overexpression [4, 5].

Similar to hRAIG1, dorsal injection of 2 ng of either XRAIG2 or XRAIG3 mRNA also resulted in shortening of the body axis (XRAIG2; 100%, $n = 66$ and XRAIG3; 74%, $n = 54$; data not shown). To understand whether this phenotype was caused by impaired convergent extension movements, we next executed the animal cap elongation assay. As reported, animal caps treated with activin formed dorsal type mesodermal tissue, which exhibited typical morphogenetic elongation (Fig. 3G). This elongation depended on the non-canonical Wnt-11 signaling and was suppressed by Xdd1, a dominant negative Dishevelled mutant, which is known to inhibit the convergent extension movements (Fig. 3H, ref. 18). XRAIG2 injection also inhibited the activin-induced elongation (Fig. 3I).

Whole mount in situ hybridization analysis showed that XRAIG2 did not affect the induction of mesodermal genes, such as *Brachyury* (*Xbra*) and *Goosecoid* (*Gsc*) (Fig. 3J-U), but rather altered their expression domain/positioning due to defective gastrulation movements. These results together indicate that XRAIG2 overexpression disrupts convergent extension movements without inhibiting mesoderm formation.

XRAIG activation of small GTPases and JNK

Convergent extension movements are regulated by the non-canonical Wnt signaling pathway through the small GTPases such as RhoA and Rac. Therefore, we examined whether XRAIG2

activated these effectors. As reported, overexpression of mouse Dishevelled 2 (mDvl2) in HEK 293T cells increased the level of GTP-bound active forms of RhoA and Rac, but not Cdc42 (Fig. 4A, ref. 7). Interestingly, overexpression of XRAIG2 in HEK 293T cells increased the level of active form of RhoA and Rac, and also Cdc42 (Fig. 4A). Importantly, XRAIG2 inhibition of elongation in activin-treated animal caps (Fig. 4D) was partially reversed by co-injection of mRNA for Cdc42(N17), a dominant negative form of Cdc42 (Fig. 4E), indicating that Cdc42 is indeed one of the downstream effectors of XRAIG2 signal transduction pathway *in vivo*.

Convergent extension movement is also regulated by the JNK pathway [27], and components of non-canonical Wnt pathway are known to activate JNK in both *Xenopus* embryo and mammalian cells [27–29]. To examine whether XRAIGs activate JNK, we monitored the phosphorylation level of c-Jun, one of the downstream targets of JNK. Overexpression of XFz7 in *Xenopus* embryos slightly activates JNK as reported (Fig. 4F, lane 3; ref. 28), and injection of XRAIG2 or XRAIG3 mRNA also increased the phosphorylation level of c-Jun (Fig. 4F, lanes 4 and 5). Interestingly, the phosphorylation level was enhanced by the co-injection of XFz7 and XRAIG3 (Fig. 4F, lane 6), suggesting that XFz7 and XRAIG3 collaboratively activate the same pathway, such as the Rho family GTPases and JNK.

Activation of intracellular calcium flux by XRAIG2

In zebrafish embryo, overexpression of XRAIG2 also caused the multiple defects consistent with alterations in convergent extension movements, including short anteroposterior axis (Fig. 4H) and cyclopia (Fig. 4J). Since activation of Wnt- Ca^{2+} pathway is also implicated in the regulation of convergent extension movements [30], we investigated the possible stimulation of Ca^{2+} flux by XRAIG2. *In vivo* image analysis in zebrafish showed that XRAIG2 overexpression increased Ca^{2+} release frequency (Fig. 4L), indicating that the phenotypic defects are in part due to the altered Ca^{2+} flux.

Discussion

RAIG1 was originally identified as a retinoic acid responsive gene in human carcinoma cells [20]. Recently, human RAIG2, RAIG3, and GPCR5D were also identified [21–23], and these four genes belong to group 5 of family C GPCRs. Other family C members, such as metabotropic glutamate receptors, calcium sensing receptor, and γ -aminobutyric acid type B receptors, have a large amino terminal extracellular domain of 500–600 amino acids, and the amino terminal domain is shown to be the agonist-binding site. In contrast, human RAIGs have short amino terminal domains of 30–50 amino acids. In the present study, we identified RAIGs as potential binding partners for Fz proteins. We report the sequence of five *Xenopus* orthologs of RAIGs, which also have characteristically short amino terminal domains. The endogenous ligands for RAIGs remain unknown, but the ligand-binding domain may lie in the extracellular loops of transmembrane domain as well as the short amino terminal domain [20,31].

Dorsal injection of hRAIG1 or XRAIG2 mRNA induced a shortened/kinked axis and severe gastrulation defects, and XRAIG2 also prevented elongation of activin-induced animal caps without affecting expression of mesodermal marker genes. These results indicate that RAIG causes perturbation of cell movements, rather than change in cell specification. These phenotypes are highly reminiscent of those caused by perturbation of Wnt/Fz PCP signaling [3,4,5]. Indeed XFz7 and XRAIG can activate a very similar set of effectors, such as RhoA, Rac, Cdc42, and JNK (Fig. 4, refs. 2,7,8,28), and to trigger Ca^{2+} flux. The physical interaction between RAIG and Fz is rather specific, as hRAIG1-hFz5 and XRAIG2-XFz7 associations are readily demonstrated in mammalian cells and *Xenopus* embryos, whereas neither hRAIG1 nor XRAIG2 exhibits detectable association with Smo, a more distantly related member of the Fz family. The biological significance of Fz-RAIG interaction, however, remains to be elucidated.

As XRAIG genes, like XFz7, are expressed in dorsal tissues during gastrulation, and their overexpression phenotypes are similar to those of XFz7 overexpression, we attempted to carry out loss-of-function studies using morpholino antisense oligonucleotides against XRAIG2, XRAIG3, and XRAIG4a and XRAIG4b. However, injection of morpholino oligos alone or in combination has thus far failed to cause any severe defects in gastrulation or animal cap elongation (data not shown). We note, however, that it remains possible that our depletion of four XRAIG proteins, which are likely to have redundant functions, was incomplete, thus precluding us from a clear 'loss-of-function' phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Amino acid sequence of XRAIGs and the expression pattern of XRAIG mRNAs during embryogenesis

(A) Alignment of the predicted amino acid sequence of *Xenopus* RAIG1, RAIG2, RAIG3, RAIG4a and RAIG4b. Amino acid residues conserved between all proteins are shaded in black, and putative transmembrane segments are overlined. Only partial sequences of XRAIG4a and XRAIG4b are available from EST databases. (B) Phylogenetic analysis of human and *Xenopus* RAIGs. The full-length hRAIGs and XRAIG1–3 and the transmembrane domains (I–IV) of XRAIG4a and XRAIG4b were aligned using CLUSTALW with the point accepted mutation (PAM) series substitution matrix. The tree was constructed using CLUSTALW with standard parameters. (C) RT-PCR analysis of XRAIGs during *Xenopus* development. -RT: without

reverse transcriptase. EF-1 α was used as an internal control. (D) A schematic diagram shows the dissection of blastula at stage 10.5 into six regions. (E) RT-PCR analysis of XRAIG2, 3, and 4. *Xnr3* and *Cerberus* (*Cer*) are used as control markers for dissection.

Fig. 2. Interaction between Frizzled and RAIG proteins
(A) HEK 293T cells were transiently transfected with plasmids expressing Flag-hRAIG1, HA-Smo, and HA-hFz5 as indicated. The cell lysate was subjected to immunoprecipitation with anti-HA antibody, and the immunocomplex was blotted with anti-Flag antibody (first panel) or anti-HA antibody (third panel). (B) Both blastomeres of two-cell embryos were injected with XRAIG2-Flag mRNA (2 ng), XRAIG2ΔC-Flag mRNA (2 ng), HA-SMO mRNA (2 ng), HA-hFz5 mRNA (2 ng), or HA-XFz7 mRNA (2 ng) in various combinations as indicated. Extracts were prepared from 20 embryos at stage 9, and subjected to immunoprecipitation with anti-HA antibody. The immunocomplex was blotted with anti-Flag antibody (top panel) or anti-HA antibody (bottom panel).

Fig. 3. Functional analysis of overexpression of RAIG in *Xenopus* embryos

(A, B) Animal caps were excised from injected with various combinations of mRNA as indicated. The explants were cultured until control sibling embryos reached stage 10.5, and total RNAs were isolated and subjected to RT-PCR analysis. (C) Normal un-injected tadpoles. (D) Both dorsal blastomeres of four-cell embryos were injected with hRAIG1 mRNA (1 ng). Embryos display severely reduced tail structures and kinked trunks. (E) Ventral injection of hRAIG1 mRNA (1 ng). (F-I) Activin-dependent animal cap elongation assay. Animal cap control explants from uninjected embryos were treated with (G) or without (F) activin. 1 ng of Xdd1 mRNA (H) or 200 pg of XRAIG2 mRNA (I) were injected into embryos, and the animal cap explants were treated with activin. (J-U) Expression of *Xbra* (J-O) and *Gsc* (P-U) in

uninjected (J, M, P, S), 1 ng of Xdd1-injected (K, N, Q, T), and 1 ng of XRAIG2-injected (L, O, R, U) embryos was examined at the indicated stage by whole-mount in situ hybridization. XRAIG2-injected embryos showed normal expression of mesodermal *Xbra* and dorsal *Gsc* at stage 10.5. At stage 12, the blastopore size of XRAIG2-injected embryos (O) was larger than that of uninjected (M), and the *Xbra*-positive cells at the involuting notochord (M) was missing in the XRAIG2-injected embryos (O). *Gsc* expression in control embryos at stage 13 is observed in anterior mesoendoderm far from the closed blastopore (S), but remained in a dorsal region close to the open blastopore in XRAIG2-injected embryos.

Fig. 4. Signal transduction by XRAIGs

(A) Flag-Dvl2 or XRAIG2 was transfected to HEK 293T cells. GTP-bound RhoA in cell lysates was precipitated using GST-RBD and detected by an anti-RhoA antibody. GTP-bound Rac and Cdc42 were precipitated using GST-PBD and detected by an anti-Rac or an anti-Cdc42 antibody, respectively. (B–E) Activin-dependent animal cap elongation assay. Animal cap control explants from uninjected embryos were treated with (C) or without (B) activin. Injection of XRAIG2 mRNA (200 pg) blocked the activin-induced elongation of animal cap explants (D). Dominant negative form of Cdc42 (200 pg) partially rescued the elongation inhibited by XRAIG2 injection (E). (F) The Myc-tagged c-Jun mRNA (200 pg) was co-injected with XFz7 mRNA (1 ng), XRAIG2 mRNA (500 pg), or XRAIG3 mRNA (500 pg). Myc-c-Jun was

immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were immunoblotted with anti-phospho-c-Jun antibody (top panel) or anti-c-Jun antibody (middle panel). (G–H) Phenotypes of wild-type (G, I) and XRAIG2-injected (H, J) zebrafish. (K, L) Ca^{2+} release dynamics in un-injected (K) and XRAIG2-injected (L) zebrafish embryos.