Truncated IRAG variants modulate cGMP-mediated inhibition of human colonic smooth muscle cell contraction

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Truncated IRAG variants modulate cGMP-mediated inhibition of human colonic smooth muscle cell contraction. Am J Physiol Cell Physiol 301: C1445–C1457, 2011. First published August 24, 2011; doi:10.1152/ajpcell.00304.2010.—Nitric oxide (NO) induces relaxation of colonic smooth muscle cells predominantly by cGMP/cGMP-dependent protein kinase I (cGKI)-induced phosphorylation of the inositol 1,4,5-trisphosphate receptor (IP3R)-associated cGMP kinase substrate (IRAG), to block store-dependent calcium signaling. In the present study we analyzed the structure and function of the human IRAG/MRVI1 gene. We describe four unique first exon variants transcribed from individual promoters in diverse human tissues. Tissue-specific alternative splicing with exon skipping and alternative splice donor and acceptor site usage further increases diversity of IRAG mRNA variants that encode for NH2- and COOH-terminally truncated proteins. At the functional level, COOH-terminally truncated IRAG variants lacking both the cGKI phosphorylation and the IP3R interaction site counteract cGMP-mediated inhibition of calcium transients and relaxation of human colonic smooth muscle cells. Since COOH-terminally truncated IRAG mRNA isoforms are widely expressed in human tissues, our results point to an important role of IRAG variants as negative modulators of nitric oxide/cGKI-dependent signaling. The complexity of alternative splicing of the IRAG gene impressively demonstrates how posttranscriptional processing generates functionally distinct proteins from a single gene.

Nitric oxide; cGMP-dependent protein kinase I; splicing; gene regulation; smooth muscle relaxation

THE NONADRENERGIC, NONCHOLINERGIC neurotransmitter nitric oxide (NO), generated in enteric neurons by neuronal nitric oxide synthase (nNOS), induces smooth muscle relaxation in the gastrointestinal tract (10, 17, 25). NO bioactivity diffuses from enteric neurons directly into target cells and activates its receptor, soluble guanylyl cyclase (sGC) (16, 23). Activated sGC increases intracellular levels of cGMP which mediate smooth muscle relaxation mainly via activation of the cGMP-dependent protein kinase I (cGKI) (5, 18). Consistently, cGKI-deficient mice show a selective lack of NO-dependent smooth muscle relaxation associated with severe gastrointestinal dysfunction (31).

cGKI-dependent smooth muscle relaxation is mediated by calcium-dependent and -independent mechanisms (10, 12, 25, 42, 46). Calcium-independent mechanisms involve inhibition of Rho activation, phosphorylation of heat shock protein 22, and phosphorylation of myosin phosphatase targeting subunit 1 with a subsequent increase of myosin light chain phosphatase activity. Calcium-dependent downstream mechanisms include inhibition of inositol 1,4,5-trisphosphate (IP3) synthesis, increase of the open probability of calcium-activated potassium channels, and inhibition of calcium release from IP3-sensitive intracellular stores (17).

Intestinal hormones, like bradykinin, induce calcium release from intracellular stores in enteric smooth muscle cells, a process inhibited by the cGMP/cGKI-signaling pathway. This pathway depends on the physical interaction of cGKIB, IP3 receptor I (IP3RI) and the recently identified protein IP3R-associated cGMP kinase substrate (IRAG) (1, 37). cGKI phosphorylates IRAG at S696 (1), and, functionally, the essential role of IRAG for cGMP/cGKI-dependent inhibition of IP3-elicted calcium release was demonstrated by targeted deletion of the IP3R interaction domain of IRAG by homologous recombination in mice or knockdown of IRAG expression in human colonic smooth muscle cells (CoSMC) (11, 14). Interestingly, cGMP/cGKI signaling inhibits enteric smooth muscle contraction differentially in a site-specific fashion in mice. In the small intestine, activation of myosin phosphatase by cGKI mediates smooth muscle relaxation without changing intracellular calcium levels, whereas interaction of cGKI with IRAG and subsequent inhibition of intracellular calcium signaling is essential for relaxation of the large intestine (10).

The human IRAG/MRVI1 gene is localized on chromosome 11 and spans a region of 120 kb of genomic DNA with so far 21 known exons and 20 introns. Alternative promoter usage generates two different IRAG mRNA isoforms (37, 41). Alternative splicing at the 5′-end of the IRAG mRNA generates five additional IRAG mRNA variants with unknown functions (41).

Here we show that several novel IRAG mRNA variants are expressed in human tissues, which encode for distinct COOH- and NH2-terminally truncated proteins. Expression of COOH-terminally truncated IRAG variants lacking both the cGKI phosphorylation and the IP3R interaction site in human CoSMC cells abolishes the inhibitory effect of 8-pCPT-cGMP on bradykinin-induced calcium release and smooth muscle relaxation. These data point to a dominant negative function of COOH-terminally truncated IRAG variants and suggest that...
alternative splicing of the IRAG mRNA is a novel mechanism to regulate cGMP/cGKI-dependent signaling in the human gut.

MATERIALS AND METHODS

Reagents, cell culture, and transfection of cells. DEA-NO, 8-pCPT-cGMP, Rp-8-pCPT-cGMPS, bradykinin, carbachol, 2-aminoethoxydiphenyl borate (2-APB), and MG-132 were purchased from Calbiochem (San Diego, CA), and Fluo-3 AM was from Molecular Probes (Leiden, Netherlands). All other materials were purchased as indicated.

Primary human CoSMC cells (Clonetics, San Diego, CA) were cultivated as described (11). For all experiments, low passaged cells (P < 6) were used. Cells were grown to 60% confluence before they were used for calcium imaging experiments. The cells preserved the typical morphology of smooth muscle cells, expressed cGKIß and IRAG, and characteristic cellular transitions were elicited by high potassium depolarization or cholinergic stimulation with carbachol as described previously (11).

SW480, HT1080, COS7, human embryonic kidney 293 (HEK293), and HeLa cells were cultivated as described (28, 35, 36). MCF-7 cells were also used, as described before (11, 35) and harvested 24 or 48 h after transfection as indicated. Some experiments were performed in the presence of the proteasome inhibitor MG-132 (10 μM; incubation time 3 h).

Tissue preparation. Tissues from human esophagus, stomach, pylorus, duodenum, colon, sigma, and rectum were obtained from surgical resections for malignant disease. The tissues were macroscopically and microscopically free of tumor. The muscle layer containing the nerve plexus was separated from the mucosa by sharp dissection and prepared as described previously (33, 34). All tissue samples were obtained with the approval of the Local Research Ethics Committee and the written consent of the patients.

Rapid amplification of 5' and 3'-cDNA ends. RNA was isolated from liquid nitrogen frozen tissues as previously described (33, 36). Poly (A) RNA was isolated from total RNA with the Oligotex mRNA Kit (Qiagen, Hilden, Germany) or purchased from Clontech (Clontech, Heidelberg, Germany).

To determine 5'-end mRNA splice variants of IRAG, a thermal rapid amplification of cDNA ends (RACE) protocol starting from exon 4 was performed as previously described (36). Poly (A) RNA was reverse transcribed with exon 4 antisense generic-specific primer (GSP1/ex 4-AS; for primers see Supplemental Table S1; Supplemental Material for this article is available online at the Journal website). First round of PCR was performed with nested gene-specific primer GSP2/ex 4-AS and sense generic primers Q and Q1 using the touchdown PCR technique (annealing at 66°C with a decrease of 0.4°C each cycle). A second round of amplification (annealing at 58°C, 30 cycles) was performed with nested primers GSP3/ex 4-AS and sense generic primer Q2. To determine 3'-end and internal IRAG mRNA variants, 3'-RACE PCRs starting from exon 4 and exon 15 were performed using oligo(dT) primed cDNA (primer Q), gene-specific primers GSP1/ex 4-S or GSP1/ex 15-S and antisense generic primer Q1, followed by a second round of PCR with nested gene-specific sense primers GSP2/ex 4-S or GSP2/ex 15/16-S and antisense generic primer Q2. Amplification products were cloned into pCRII plasmid (Invitrogen, Groningen, Netherlands). Clones with different PCR product inserts were identified by PCR and restriction mapping and subjected to DNA sequence analysis.

Reverse transcriptase PCR and quantitative RT-PCR. Total RNA was isolated using the RNeasy kit (Qiagen) following the manufacturer's instructions. Total RNA (5 μg) was used for cDNA synthesis as previously described (34). Site-specific expression of IRAG splice variants was investigated by Reverse transcriptase-PCR (RT-PCR) using isoform-specific primers (for primers see Supplemental Table S1). Quantitative real-time RT-PCR analysis was performed using standard curves generated by known copy numbers of a plasmid carrying full-length IRAGex cDNA (containing exon 5/6 and 15/16) or the mRNA splice variant IRAG-c4 (Fig. 2B) as previously described for nNOS variants (34, 36). To exclude amplification of genomic DNA, intron spanning IRAG primers and probes (see Supplemental Table S1) were used. GAPDH primers were purchased from Applied Biosystems (Norwalk, CT).

Preparation of total cell lysates. Whole cell lysates were prepared by incubating cell pellets for 30 min at 4°C in immunoprecipitation buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 mM NaF). Insoluble material was removed by centrifugation, and lysates were aliquoted and stored at −80°C.

Western blot analysis. Extracts were normalized for protein and heated at 95°C for 5 min in Lämmli buffer. Proteins were resolved on 7.5% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) in a semidy blotting system as described (32). Membranes were blocked in phosphate-buffered saline (PBS) supplemented with 5% skim milk and 0.1% NP-40. Membranes were probed for 2 h with a polyclonal rabbit anti-IRAG antibody [raised against recombinant IRAGex5–499 (encoded by exon 1d–exon 8) and the cGKIß binding site; dilution: 1:2,000 (1)], V5 antibody (Invitrogen), cGKIß antibody (21), and β-actin antibody (Sigma-Aldrich, Munich, Germany) as described (11). Purified IRAG protein was used as positive control as described previously (1). Proteins recognized by the antibodies were detected by the Odyssey Infrared Imaging System (Lico, Bad Homburg, Germany) using Alexa680-coupled (Molecular Probes) or IRDeye800-coupled (Rockland, Gilbertsville, PA) secondary antibodies.

Immunoprecipitation. Commonmunoprecipitation assays were performed as described (3, 44). In brief, transfected HEK293 cells were washed twice with PBS and 800 μl lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet-P 40) containing protease inhibitors (Roche, Indianapolis, IN) was added. One milligram protein in a total volume of 1,000 μl was preclarified for 1 h at 4°C by adding 40 μl of a 50% slurry of washed protein A agarose beads (Invitrogen). Preclarified lysates were then incubated overnight at 4°C with one of the following antibodies: 3 μg anti-IRAG (1) or 3 μg cGKIß antibody (21) or 4 μg nonimmunogenic purified IgG (Invitrogen). After 24 h, antibody-protein complexes were precipitated by incubation with protein A agarose beads for 2 h and washed three times with lysis buffer. Precipitated proteins were eluted with 2× sample buffer (120 mM Tris-HCl, pH 6.8, 500 mM NaCl, 1% SDS, 10% glycerol, 40 μg/ml bromphenol blue) for 3 min at 95°C, followed by SDS-PAGE and Western blot analysis with anti-IRAG or anti-cGKIß antibody. Expression of proteins in input samples was confirmed by Western blot using 5% of the starting material.

Plasmid constructions. The cDNA sequences of the different IRAG variants were PCR amplified using a proofreading polymerase (UltraPfu, Stratagene, Heidelberg, Germany) and cloned into pEEntr plasmid (Invitrogen) with and without an EF1α-driven enhanced green fluorescent protein (EGFP) expression cassette. IRAG cDNAs were fused to the V5 epitope present in pEEntr to facilitate detection of the recombinant proteins. Subsequently, the expression cassettes were transfected into pcDNA3.2 (without EF1α-EGFP cassette) or pLEnti6 (with EF1α-EGFP cassette) using recombinemming (Gateway system, Invitrogen) as described (39). The 5'-regulatory regions of IRAG exon 1a–d were PCR amplified (UltraPfu) and cloned blunt end into the promoterless firefly luciferase reporter gene vector pGL3 (Promega, Mannheim, Germany). Integrity of all cloned sequences was confirmed by automated DNA sequencing (GATC, Konstanz, Germany) using an ABI Prism 377 DNA sequencer (Applied Biosystems). Potential cis-acting DNA sequences were identified by analysis with MatInspector (http://www.genomatix.de).

Luciferase assays. CosSMC cells were transiently cotransfected with the different pGL3 firefly luciferase reporter gene plasmids (950
The putative transcription start site and nucleotide sequence of all first exons was verified by independent RACE-PCR protocols in human brain, stomach, and large intestine (data not shown).

Analysis of the 5' termini revealed intriguing features of IRAG primary RNA processing. Alternative splicing generates mRNA transcripts with cassette exon insertions or deletions. Exon 1b, 1c, and 1d were interposed in various combinations between exon 1a and the common exon 2 (Fig. 1A). Cassette deletion of exon 1b was observed in variants containing exon 1b. Usage of alternative splice donor and acceptor sites further increased diversity of IRAG 5'-mRNA termini. An alternative splice donor site results in deletion of 83 nt of the 3'-end of exon 1a, an alternative splice acceptor site results in deletion of 196 bp of the 5'-end of exon 1d and an alternative splice acceptor site within exon 2 results in deletion of the first 77 bp of exon 2 (Fig. 1, A and B). All 5'- and 3'-splice junctions conformed to the GT/AG donor/acceptor splice rule (Fig. 1B). Thus, alternative splicing at the 5'-mRNA end generates a total of 24 IRAG variants with structural alterations of the 5'-untranslated region (UTR) or the IRAG protein-coding region. Open reading frame analysis revealed putative consensus translational initiation sites in exon 1b (CUG), 1c (CUG), 1d (AUG), 1d' (AUG), or exon 2 (CUG) (Supplemental Fig. S1). Translation of IRAG mRNA forms lacking full-length exon 1d would generate alternative truncated IRAG proteins lacking the putative NH2-terminal transmembrane domain (Supplemental Fig. S1).

To compare our findings with IRAG variants deposited in the GenBank Expressed Sequence Tag (EST) database, we used the IRAG full-length cDNA sequence (NM_001098579.1) and the 24 IRAG 5'-mRNA sequences identified by 5'-RACE-PCR (Fig. 1A). This in silico analysis verified the expression of exon 1a (present in 55 of 268 IRAG containing EST sequences), 1b (28/268), 1c (49/268), and 1d (48/268). Furthermore, presence of alternative splice donor and acceptor site usage for exons 1a, 1d, and 2 as well as cassette deletion of exon 2 was confirmed by the EST search (Fig. 1A). The number of ESTs representing the respective individual 5'-IRAG mRNA variant is given in Fig. 1A.

Expression of IRAG alternative 5'-mRNA variants in the human gastrointestinal tract. We next investigated the relative expression ratio and site specificity of 5'-mRNA IRAG variants in the human gastrointestinal tract. A series of RT-PCR experiments using primer sets spanning all alternative first exons and alternatively spliced regions using exon 1a, 1b, 1c, and 1d forward and exon 5 reverse primers confirmed the presence of a wide range of alternative first exon variants and splicing events in the human gastrointestinal tract (Fig. 2, A and B). Interestingly, we were able to identify five additional variants that were not identified by 5'-RACE-PCR most likely due to very low expression levels (Fig. 2B). To proof specificity of our RT-PCR experiments, all RT-PCR products were cloned and sequenced. In addition, expression of IRAG-c4 mRNA was proofed by quantitative RT-PCR (data not shown). Thus, we were able to validate site-specific expression of IRAG 5'-mRNA variants in the human gastrointestinal tract.

Genomic organization of alternative first exons and basal promoter analysis of the human IRAG gene. Screening of the human genome database with sequences of the four different first exons revealed clustering of IRAG exon 1a, 1b, and 1c.
within a 3-kb upstream genomic region whereas the genomic region encoding for exon 1d is localized 37 kb further downstream (Fig. 3A).

To test whether each variable first exon is transcribed from its own promoter, we cloned the 5′-flanking regions of the four different first exons 5′ of a firefly luciferase and generated 5′-deletions of the 5′-flanking regions, respectively. The 5′-deletions were generated according to putative transcription factor binding sites identified in silico by using the MatInspector software package. Examples of putative cis-regulatory elements, like Sp1, GATA1, nuclear factor of activated T cells (NFAT), MyoD, AP1, and NF-H260B are shown in Fig. 3A. The respective reporter plasmids were transiently transfected into IRAG positive primary human CoSMC (Fig. 3B–E). The full-length 5′-flanking regions of exon 1a, 1b, 1c, and 1d showed all promoter activity. Notably, the 5′-regulatory region
of exon 1d showed highest activity in CoSMC, which correlates well with the high exon 1d mRNA expression levels observed in these cells (Fig. 3E and data not shown). Since in silico analysis of the 5′-regulatory region of exon 1d revealed a distinct subset of putative transcription factor binding sites, factors like GATA1, retinoid X receptor (RXR), and NF-κB may account at least in part for the observed differential promoter activities (Fig. 3, B–E).

Identification of internal and 3′-mRNA variants of the human IRAG gene. To characterize the internal and 3′-mRNA structure of the human IRAG gene, we performed 3′-RACE-PCRs starting from exon 4, which encodes for the cGKI...
interaction site, and exon 15, which encodes for the eGKI phosphorylation site of IRAG, respectively (1). Sequencing of 3’-RACE products revealed a large variety of IRAG transcripts arising from cassette exon insertions/deletions, alternative splice donor and/or acceptor site usage and/or alternative polyadenylation sites (Fig. 4A).

The alternative variants were termed IRAGα, β, γ, δ, ε, ζ, and η mRNA. Human IRAGα mRNA has been described before and represents full-length IRAG (41). We were able to detect this mRNA variant in all tissues and cells analyzed by 3’-RACE-PCR and RT-PCR (Fig. 4, A and C, and data not shown). IRAGβ mRNA is generated due to skipping of

**Fig. 3.** Genomic organization of alternative first exons and basal promoter analysis of the human IRAG gene. **A:** schematic illustration of the genomic organization of the alternative first exons of the human IRAG gene. **B–E:** functional analysis of the human IRAG exon 1a, 1b, 1c, and 1d 5’-regulatory region. 5’-Deletions of the IRAG exon 1a–d promoters are shown, and putative cis-acting regulatory elements identified in silico by MatInspector analysis are depicted. **B:** IRAG exon 1a promoter luciferase reporter gene constructs (−2,121/+23, −1,252/+23, −451/+23, −63/+23, and +12/+23) were transiently transfected into CoSMC cells. At 24 h after transfection, luciferase activity was determined. NFAT, nuclear factor of activated T cells. **C:** IRAG exon 1a and exon 1b promoter luciferase reporter gene constructs (−789/+44, −111/+44, −21/+44, and +19/+44) were transiently transfected into CoSMC cells. At 24 h after transfection, luciferase activity was determined. **D:** IRAG exon 1c promoter luciferase reporter gene constructs (−956/+37 and +14/+37) were transiently transfected into CoSMC cells. At 24 h after transfection, luciferase activity was determined. RXR, retinoid X receptor. Data are expressed as means ± SD of three independent experiments in triplicate. (Student’s t-test: ***P < 0.001.)
exon 6 (52 bp) and is expressed exclusively in the esophagus within the gastrointestinal tract. IRAGγ is composed of exon 5, 6, 7, 9, and a novel 3'-terminus containing a polyadenylation sequence. The novel 3'-end is termed exon 9a according to its genomic localization (Figs. 4A and 5A).

Interestingly, this exon with a length of 667 nt was not found in human EST databases. The 3'-terminus of IRAG/H9254 mRNA is also composed of exon 9a. However, all internal exons (exon 5, 6, 7, 8, and 9) are expressed. IRAGε displays the same structure as IRAG/H9254 and contains exon 9a, but lacks exon 7 due to exon skipping. IRAG/H9256 mRNA variants are generated by usage of an alternative splice donor site within exon 8 resulting in deletion of 526 nt of its 3'-end and alternative splicing to a so far unknown last exon with a polyadenylation sequence. This novel last exon was termed exon 21 according to its genomic localization of exon 20 (Figs. 4A and 5A).

Taken together, alternative splicing with exon skipping and alternative translation termination sequences in exon 9a and 21 generates one internal and five different 3'-truncated mRNA variants with structural alterations of the protein coding sequence. Expression of alternative internal and 3'-IRAG mRNA variants in the human gastrointestinal tract.

Next, we investigated site-specific expression of internal and 3'-mRNA IRAG variants in the human gastrointestinal tract. A series of RT-PCR experiments using primer sets spanning all alternative spliced regions confirmed to the GT/AG splice donor/acceptor rule (Fig. 4i). The novel sequences were submitted to the GenBank database (AM000024).

Expression of alternative internal and 3'-IRAG mRNA variants in the human gastrointestinal tract. Next, we investigated site-specific expression of internal and 3'-mRNA IRAG variants in the human gastrointestinal tract. A series of RT-PCR experiments using primer sets spanning all alternative spliced regions using exon 4 and exon 15. Gray boxes mark the novel exons 9a and 21, respectively. Stop codons are marked by a vertical arrow. B: sequence of human IRAG exons 8, 9a, and 21 as determined by 3'-RACE-PCR. Exonic sequences are given in uppercase letters. Splice donor and acceptor sites are given in lowercase letters and are underlined. Boldface uppercase letters mark the shortened exon variants generated by alternative splice donor and acceptor site usage (exon 8: nt 3–620 = exon 8, nt 3–94 = exon 9a). Stop codons are bold and underlined. C: IRAG 3'-variants mRNA expression in esophagus, stomach, pylorus, duodenum, colon, and CoSMC cells was determined using RT-PCR with primers spanning all alternative spliced regions.
To quantify the relative expression ratio of 3′-truncated IRAG variants that lack exon 10–20 encoding the coiled-coil domain for interaction with the IP₃ receptor and the cGKI phosphorylation site (Fig. 5, A and B), we designed quantitative RT-PCR assays based on the sequence of full-length IRAGα with standard curves containing known copy numbers of full-length IRAGα. Total IRAG mRNA expression was evaluated by quantification of exon 4/5, which is present in all known IRAG mRNA variants (Refs. 37 and 41 and this study). Using a panel of human tissues, we detected total IRAG mRNA (exon 4/5) and full-length IRAGα mRNA (exon 15/16) in all tested samples (Fig. 5C). Interest-

Fig. 5. Genomic organization of the human IRAG gene and tissue-specific expression of IRAG variants. A: illustration of the genomic organization of the human IRAG gene with 25 exons and 24 introns. The length in nucleotides of each individual exon and intron is given above (exons) and below (introns) the boxes, respectively. The transmembrane domains (TMD), the cGMP-dependent protein kinase I (cGKI) binding site (B), the coiled coil domain, and the cGKI phosphorylation site (P) are indicated. B: schematic illustration of the exon structure resulting in IRAG protein α, β, δ, ε, ζ, and η. Predicted molecular weight of the various IRAG proteins is indicated. C: quantitative RT-PCR of full-length IRAG mRNA (exon 15/16) (top, left graph) and total IRAG mRNA (exon 4/5) (top, right graph) determined by using standard curves with known copy numbers and intron spanning primers and probes in various human tissues as indicated. Bottom graph demonstrates the ratio of full-length (exon 15/16) to total (exon 4/5) IRAG mRNA expression. LES, lower esophageal sphincter; ML, muscle layer. D: IRAGα, δ, ζ, and η were transfected into COS7 cells and expression was determined in Western blots using a polyclonal IRAG antibody [raised against recombinant IRAGα53–499 that contains the NH₂ terminus of IRAG (encoded by exon 1d–exon 8; see A) and the common cGKI binding site (1)]. E: cGKIβ, IRAGγ, δ, and ζ were transfected alone or in combination into human embryonic kidney 293 (HEK293) cells as indicated. At 48 h after transfection, the interaction of cGKIβ with IRAGγ, δ, and ζ was determined in immunoprecipitations (IP) as indicated. Note that IRAGδ migrates with the IgG heavy chain and is therefore not presentable in the reprobes. Top: Western blot (WB) no. 1 was probed with cGKIβ antibody and then reprobed with IRAG antibody (Western blot no. 2). Therefore, cGKIβ immunoreactivity is visible in the second upper blot. Depicted are composites of different lanes from the same gel.
ingly, some tissues, like heart and skeletal muscle, showed extraordinary high expression levels of 3'-truncated mRNA species demonstrated by a ratio of full-length IRAG vs. total IRAG in these tissues below 20% (Fig. 5C).

**Genomic organization of the human IRAG gene.** Figure 5A summarizes the genomic organization of the human IRAG gene with 25 exons. Full-length exon 1d and exon 20 encode for transmembrane domains (TMD); exon 4 for the cGKI binding site (B); and exon 15 for the cGKI phosphorylation site (P). Exons 11–14 encode a coiled-coil domain, which is essential for interaction of IRAG with the IP3 receptor (14, 37).

3'-IRAG mRNA variants encode for COOH-terminally truncated proteins. Open reading frame analysis of IRAGα, β, γ, δ, ε, ζ, and η mRNA variants revealed alternative stop codons within the novel last exons 9a and 21 (IRAGγ-η), or a frameshift of the open reading frame with a premature stop codon within exon 7 due to skipping of exon 6 (IRAGβ) (Figs. 4B and 5B). Thus 3'-IRAG mRNA species generated by exon skipping or alternative splice donor/acceptor site usage encode for COOH-terminally truncated IRAG protein variants (Fig. 5B). IRAGα mRNA encodes for a full-length IRAG protein with 903 amino acids and a predicted molecular mass of 99.36 kDa. IRAGβ, IRAGγ, IRAGδ, IRAGε, IRAGζ, and IRAGη mRNA variants encode for truncated proteins with a predicted molecular mass of 23.2, 30.81, 52.73, 48.99, 40.3, and 36.56 kDa, respectively. The predicted protease IRAGβ-η lack the coiled-coil domain for the interaction with the IP3 receptor, the cGKI phosphorylation site and the C-terminal transmembrane domain, but retain the cGKI binding site (Fig. 5B).

**Overexpression of truncated IRAG variants and direct interaction with cGKβ.** Expression of IRAGα, γ, δ, and ζ in COS7 cells revealed immunoreactive bands at 125 kDa, 55 kDa, 80 kDa, and 60 kDa, respectively (Fig. 5D). Interestingly, these molecular weights (MW) differ from the predicted MW of the IRAG variants significantly, suggesting distinct post-translational modifications. Consistently, significant difference between the predicted and the actual MW has been described for full-length IRAGα before (37, 41).

Cotransfection of the truncated IRAG variants γ, δ, and ζ with cGKβ in HEK293 cells and subsequent coinmunoprecipitation using polyclonal IRAG or cGKβ antibody provide evidence for direct physical interaction of IRAG γ, δ, and ζ with cGKβ (Fig. 5E).

**Inhibition of bradykinin-induced calcium transients by cGMP-dependent pathways.** To investigate the functionality of the COOH-terminal IRAG variants, we first characterized a model to monitor changes in [Ca2+]i in cultured primary human CoSMC. Addition of 5 × 10^{-7} M bradykinin or 1 × 10^{-5} M carbachol to the cell culture medium using a tempered fluorometer evoked a rapid increase in intracellular calcium concentration followed by a slow decrease to baseline levels in CoSMC (Fig. 6A and data not shown). Importantly, almost identical calcium signals were observed after manual bradykinin washout and restimulation (data not shown).

To demonstrate a cGMP-dependent mechanisms in bradykinin-induced calcium transients in CoSMC cells, DEA-NO, the selective and membrane-permeable inhibitor of cGK, Rp-8-pCPT-cGMPS (5 × 10^{-5} M), and the membrane-permeable direct activator of cGK, 8-pCPT-cGMP, were used as described (11). Increasing concentrations of DEA-NO blocked bradykinin-induced calcium release in a dose-dependent manner (Fig. 6B). This NO-dependent effect was blocked by coinubation with Rp-8-pCPT-cGMPS. Furthermore, addition of 8-pCPT-cGMP blocked dose dependently bradykinin-induced calcium transients with a maximum effect at 10^{-4} M (Fig. 6B). These data show that activation of the cGK-signaling pathway induces a significant inhibition of bradykinin-induced calcium transients in CoSMC.

**Dominant negative effect of COOH-terminally truncated IRAG variants on IRAG function in CoSMC.** Since previous experiments showed that cGK-dependent inhibition of bradykinin-induced [Ca2+]i depends on expression of functional IRAG in CoSMC cells (11), we tested whether the COOH-terminally truncated IRAG variants modulate cGK-dependent inhibition of Ca2+ signaling. After transfection of CoSMC with the pcDNA3-EGFP reporter plasmid, transfection efficiencies of ~60–70% were observed by fluorescence microscopy (data not shown). Similar transfection efficiencies have been reported by other groups for CoSMC cells (20). As expected, bradykinin induced similar calcium transients in mock (Fig. 6C) and IRAGα, γ, δ, and ζ transfected cells (data not shown). In mock-transfected cells, calcium transients were reduced by 8-pCPT-cGMP coinubation (Fig. 6C). Overexpression of full-length IRAGα further increased inhibition of bradykinin-induced calcium transients by 8-pCPT-cGMP treatment (Fig. 6C). In contrast, overexpression of the COOH-terminally truncated variants IRAGγ, δ, and ζ blocked 8-pCPT-cGMP induced inhibition of calcium entry from intracellular stores (Fig. 6C). These effects of COOH-terminally truncated IRAG variants were observed in all experiments and were not due to a decreased expression of endogenous full-length IRAGα in CoSMC (see Fig. 6E).

Expression levels of endogenous full-length IRAG and the putative truncated IRAG proteins in relation to transfected recombinant IRAG variants were assessed in CoSMC by Western blot analysis. These experiments showed similar expression levels of endogenous and transfected IRAG proteins, excluding an extreme overexpression of the recombinant proteins and nonspecific effects (Fig. 6E).

**Effect of expression of COOH-terminally truncated IRAG variants on CoSMC contraction.** To evaluate the effects of C-terminally truncated IRAG variants on cGK-dependent inhibition of smooth muscle contraction, CoSMC contraction was measured in culture by scanning microscopy as described by Dr. K. S. Murthy’s group (20, 24). Previous studies showed that the activation of cGK by sodium nitroprusside, 8-pCPT-cGMPS, and eBIMPS blocks acetylcholine-induced contraction of dispersed smooth muscle cells (27). Accordingly, 8-pCPT-cGMPS blocked bradykinin-induced CoSMC contraction in mock and IRAGα transduced cells in our study (Fig. 6D). Consistent with the effect of the COOH-terminally truncated IRAG variants on [Ca2+]i, overexpression of IRAGγ, δ, and ζ completely abolished cGK-dependent inhibition of smooth muscle contraction (Fig. 6D).

**DISCUSSION**

Here we describe novel 5' and 3'-mRNA variants of human IRAG, pointing to an important role of alternative splicing and promoter usage for the control of IRAG expression and function. Translation of 3'-IRAG mRNA isoforms generates COOH-terminally truncated IRAG protein variants which neg-
Fig. 6. Effect of expression of COOH-terminally truncated IRAG variants on IRAG function in CoSMC. A: CoSMC cells were treated with 5 × 10^{-7} M bradykinin or were left as vehicle-treated controls, and intracellular calcium concentration ([Ca^{2+}]_i) was measured using Fluo-3 AM as calcium-sensitive fluorescent dye over time. B: CoSMC cells were treated in separate wells in triplicate with 5 × 10^{-7} M bradykinin and DEA-NO, 5 × 10^{-7} M bradykinin and DEA-NO and Rp-8-pCTP cGMP as indicated, or were left as vehicle-treated controls (left graph). CoSMC were treated with 5 × 10^{-7} M bradykinin, 5 × 10^{-7} M bradykinin and 8-pCTP-cGMP, or were left as vehicle-treated controls (right graph). [Ca^{2+}]_i was measured as in A. Data are expressed as means ± SD of three independent experiments in triplicate. C: CoSMC cells were transfected with IRAGα, γ, δ, and ζ expression plasmids or were left as mock-transfected controls. At 24 h after transfection, cells were treated with 5 × 10^{-7} M bradykinin (BK) or 5 × 10^{-7} M bradykinin and 8-pCTP-cGMP. [Ca^{2+}]_i was measured as in A. (Student’s t-test: *P < 0.001 vs. IRAGα.) D: CoSMC cells were transduced with IRAGα, γ, δ, and ζ or were left as mock-transduced controls. At 24 h after transduction, cells were treated with 5 × 10^{-7} M bradykinin or 5 × 10^{-7} M bradykinin and 8-pCTP-cGMP. Contraction was measured by scanning micrometry of 50 cells per experiment in triplicate as described in MATERIALS AND METHODS. (Student’s t-test: *P < 0.001 vs. IRAGα.) E: expression levels of endogenous IRAGα and putative IRAG variants in CoSMC cells and the ratio between endogenous and transfected recombinant IRAG variants were assessed by Western blot analysis. CoSMC cells were transfected with IRAGα, γ, δ, and ζ or were left as mock-transfected controls. At 24 h after transfection, cells were incubated with the proteasome inhibitor MG-132 (10 μM) for 3 h. MG-132 was used to avoid tempering of expression analysis by proteasomal degradation. IRAG and cGKIβ expression was determined in Western blots using a polyclonal IRAG antibody raised against recombinant IRAGα53-499 (1) or a cGKIβ antibody (21). Results shown are representative images of three independent experiments.

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that other mechanisms are required to generate protein diversity. In the last years it became clear that alternative splicing is one of the main sources of protein variation. An intriguing example is the gene *Drosophila syndrome cell adhesion molecule* (*Dscam*) in *Drosophila melanogaster*. It can generate 38,016 distinct mRNA variants, which is far in excess of the total number of genes in *Drosophila* (14,500 estimated genes) (38).

Several reports demonstrate that alternative splicing is essentially involved in the regulation of the NO/cGMP/cGKI signaling pathway. For example, alternative splicing of the nNOS gene in the human gastrointestinal tract generates multiple nNOS isoforms with distinct functional properties (33, 34, 45). Interestingly, several truncated splice forms of soluble guanylyl cyclase (sGC) with defined tissue distribution have been identified and it has been suggested that these truncated versions functionally interfere with full-length sGC (40, 43). For cGKI it was shown that splice-dependent deletion of exons encoding the NH2-terminus of cGKI produces two distinct variants, cGKIO and cGKIB, which have different tissue distribution and target specificity (19). cGKIB selectively binds IRAG and responds differently to activation by hydrogen peroxide (4).

In this study, we add 29 novel IRAG isoforms to the previously identified seven 5′-mRNA variants (37, 41), resulting in a total of 36 IRAG versions, all produced by alternative promoter usage, exon insertions/deletions, alternative splice donor/acceptor site usage or alternative polyadenylation sites, making human IRAG one of the most complex genes known in terms of transcriptional and posttranscriptional regulation (45). Physiological or pathological stimuli are likely to differentially regulate the different IRAG promoters and splicing events, leading to the generation of distinct IRAG transcripts in a cell and tissue-specific fashion. Similar to the human nNOS gene (36), individual IRAG transcripts may well play a role in the development of pathophysiological conditions associated with inappropriate regulation of IRAG expression, resulting in defective gastrointestinal smooth muscle contraction (2).

Most of the IRAG transcripts are produced by distinct sequence rearrangements in the 5′-UTR. Such variations are usually associated with altered posttranscriptional regulation of gene expression like mRNA localization, stability, and translation efficiency (9, 45). Within the NO/sGC/cGK pathway, the tissue-specific RNA-binding protein HuR has been shown as an important regulator of the sGC-α1 splice variant in pulmonary vascular smooth muscle during chronic hypoxia-induced pulmonary hypertension (8). In addition, variations in the 5′-UTR have been shown to regulate translational efficiency of the nNOS gene differentially (45). Thus, the diversity of the 5′-UTR of IRAG reflects most likely multiple tissue- and site-specific mechanisms regulating stability, localization, and translation efficiency of distinct IRAG transcript variants in response to various extra- or intracellular stimuli in different cell types or at different developmental stages. The biological consequences of these alternative 5′-mRNA splicing events affecting the 5′-UTR of IRAG were not addressed experimentally in our current study. However, they are intriguing and merit further exploration. In addition, splicing at the 5′-end of the IRAG gene generates IRAG variants lacking full-length exon 1d. Expression of such isoforms would result in proteins lacking the putative NH2-terminal transmembrane domain, suggesting regulation of subcellular IRAG localization and function by these splicing events (37, 41). Further studies are necessary to address this important issue.

In addition to the novel 5′-termini, we identified one internal mRNA variant lacking exon 6 and five mRNA variants differing at their 3′-terminal end. Theses variants encode for COOH-terminally truncated proteins lacking important functional domains of full-length IRAGo.

In silico analysis of the National Center for Biotechnology Information database confirmed the expression of most individual IRAG mRNA variants. This diversity does not appear to be unique for humans. Analysis of the mouse genome database also identified eight IRAG splice variants and the presence of alternative first and last exons like exon 1d (nucleotide homology mouse/human: 78%) and exon 21 (nucleotide homology mouse/human: 73%). Conservation of these splice forms points to a functional importance of the respective IRAG variants in mammals (30).

Interestingly, although we found expression of exon 9a variants in a broad range of tissues, no ESTs corresponding to these splice variants have been enrolled, probably because IRAG containing ESTs are enriched for the 5′-mRNA end (215 of 268 human IRAG ESTs). Only a small amount of all ESTs that harbor IRAG sequence fragments contain internal exons like exon 7 (15 of 268 ESTs) and exon 8 (16 of 268 ESTs).

Because of the 1) relatively low and/or restricted tissue specific expression of IRAGβ, ε, and η mRNA and because of the 2) structural similarities of IRAGε with IRAGβ, and of IRAGγ with IRAGγ and ζ, we focused on three major novel splice variants—IRAGγ, δ, and ζ—with COOH-terminal deletion of the cGKI phosphorylation and the IP3RI interaction site. When expressed in colonic smooth muscle cells, these variants exhibited a profound dominant-negative effect on both NO/cGMP/cGKI-mediated smooth muscle relaxation and inhibition of store-dependent calcium release. On the molecular level, it is tempting to speculate that these effects are triggered by binding of cGKI to the truncated, nonfunctional IRAG variants lacking the cGKI phosphorylation and the IP3RI interaction site. Consistently, we observed direct interaction of cGKIB with IRAGγ, δ, and ζ in a heterologous expression system.

Remarkably, IRAG is not only an effector of cGKI. Since IRAG anchors cGKIB, it dictates the subcellular localization of the kinase and affects cGMP signaling to the nucleus in a heterologous expression system (6). In that study, a phosphorylation-deficient IRAG mutant that is not functionally regulated by cGKIB phosphorylation suppressed the observed transcriptional activity of cGKIB in a similar manner as wild-type full-length IRAG. These data suggest that COOH-terminally truncated IRAG variants not only have the potential to interfere with intracellular calcium signaling, but may also affect the transcriptional activity of cGKIB.

In summary, we have identified new splice variants of the human IRAG gene and characterized the role of COOH-terminally truncated IRAG variants for cGKI-dependent signaling. Our results demonstrate that IRAG variants can function in a dominant-negative manner to counteract cGMP/cGKI signaling. These findings point to a novel mechanism modulating the NO/cGMP/cGKI signaling pathway, which might play an important role in health and disease.
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DISCLOSURES

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