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

Alexander von Werder,1✉ Martina Mayr,1✉ Günter Schneider,1✉ Daniela Oesterle,1 Ralph M. Fritsch,1 Barbara Seidler,1 Jens Schlossmann,2 Franz Hofmann,3 Michael Schemann,4 Hans D. Allescher,5 Roland M. Schmid,1 and Dieter Saur1
1II. Medizinische Klinik, 2Institut für Pharmakologie und Toxikologie, and 4Institut für Humanbiologie, Technische Universität München, Munich, Germany; 3Pharmakologie und Toxikologie, Universität Regensburg, Regensburg, Germany; and 5Zentrum für Innere Medizin, Klinikum Garmisch-Partenkirchen, Garmisch-Partenkirchen, Germany

Submitted 3 August 2010; accepted in final form 16 August 2011

vон Werder A, Mayr M, Schneider G, Oesterle D, Fritsch RM, Seidler B, Schlossmann J, Hofmann F, Schemann M, Allescher HD, Schmid RM, Saur D. Truncated IRAG variants modulate cGMP-mediated inhibition of human colonic smooth muscle 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 IRA/GMRVII 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 IRA/G variants lacking both the cGKI phosphorylation and the IP3/IR interaction site counteract cGMP-mediated inhibition of calcium transients and relaxation of human colonic smooth muscle cells. Since COOH-terminally truncated IRA/G mRNA isoforms are widely expressed in human tissues, our results point to an important role of IRA/G variants as negative modulators of nitric oxide/cGKI-dependent signaling. The complexity of alternative splicing of the IRA/G gene impressively demonstrates how posttranscriptional processing generates functionally distinct proteins from a single gene.

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 cGKβ, IP3 receptor I (IP3/RI) 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-elicited calcium release was demonstrated by targeted deletion of the IP3/RI 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 IRA/GMRVII 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 IRA/G mRNA isoforms (37, 41). Alternative splicing at the 5′-end of the IRA/G mRNA generates five additional IRA/G mRNA variants with unknown functions (41).

Here we show that several novel IRA/G mRNA variants are expressed in human tissues, which encode for distinct COOH- and NH2-terminally truncated proteins. Expression of COOH-terminally truncated IRA/G variants lacking both the cGKI phosphorylation and the IP3/RI interaction site in human CoSMC cells abolishes the inhibitory effect of 8-cPT-cGMP on bradykinin-induced calcium release and smooth muscle relaxation. These data point to a dominant negative function of COOH-terminally truncated IRA/G 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 cGKIβ and IRAG, and characteristic calcium transients 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).

Cells were transfected using FuGEN6 (Roche, Mannheim, Germany) 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 gene-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 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-4S or GSP1/ex15-S and antisense generic primer Q1, followed by a second round of PCR with nested gene-specific sense primers GSP2/ex4-S or GSP2/ex15/16-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 IRAG cDNA (containing exon 5/6 and 15/16) or the mRNA splice variant IRAG-c (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 diethiothreitol, 1 mM phenylmethylsulfonylfluoride, 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 Lammlı buffer. Proteins were resolved on 7.5% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) in a semidyblotting 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 that contains the NH2 terminus of IRAG (encoded by exon 1d–exon 8) and the cGKIβ binding site; dilution: 1:2,000 (1)]. V5 antibody (Invitrogen), cGKIβ 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 (Liric, Bad Homburg, Germany) using Alexa680-coupled (Molecular Probes) or IRDeye800-coupled (Rockland, Gilbertsville, PA) secondary antibodies.

Immunoprecipitation. Coimmunoprecipitation 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 precleared for 1 h at 4°C by adding 40 μl of a 50% slurry of washed protein A agarose beads (Invitrogen). Precleared lysates were then incubated overnight at 4°C with one of the following antibodies: 3 μg anti-IRAG (1) or 3 μg cGKIβ antibody (21) or 1 μg non-immune 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 2X sample buffer (120 mM Tris-HCl, pH 6.8, 3.3% SDS, 10% glycerol, 40 μg/ml bromophenol blue) for 3 min at 95°C, followed by SDS-PAGE and Western blot analysis with anti-IRAG or anti-cGKIβ antibody. Expression of proteins in input samples was confirmed by Western blots 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 transferred into pcDNA3.2 (without EF1α-EGFP cassette) or pLent6.3 (with EF1α-EGFP cassette) using recombining (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. CoSMC cells were transiently cotransfected with the different pGL3 firefly luciferase reporter gene plasmids (950
ng/well) and pRL-TK renilla luciferase control plasmids (50 ng/well) in six-well plates using Lipofectamine (Invitrogen) as described (36). After 48 h, cells were incubated in lysis buffer (Promega) for 15 min, harvested, and cleared by centrifugation for 15 min. Firefly and Renilla luciferase activities were determined in a LS 9501 luminometer (Berthold, Bad Wildbad, Germany) using a dual-luciferase assay system (Promega). Relative light units of firefly luciferase were normalized against relative light units of Renilla luciferase.

Retroviral gene transfer. pLenti retroviral vectors containing the coding sequence of the different IRAG variants and the EF1α reporter gene were transiently cotransfected with an optimized packaging plasmid mix (pLP1, pL2, and pLP-VSVG) into HEK293FT cells by using the ViraPower Lentiviral Expression System and Lipofectamine 2000 as recommended by the manufacturer (Invitrogen). After 72 h, supernatants were collected, filtered through a 0.45-μm filter, and used to transduce HT1080 and CoSMC cells in the presence of hexadimethrine bromide (6 μg/ml) for virus titer determination by limiting dilution and smooth muscle contraction assays.

Calcium imaging experiments. Changes in intracellular calcium concentration ([Ca2+]i) in cultured primary human CoSMC cells were measured using Fluo-3 AM (FLIPR Calcium assay kit, Molecular Devices, Sunnyvale, CA) as calcium-sensitive fluorescent dye according to manufacturer’s protocol. Colonic myocytes (104/well) at passage 5 or 6 were grown in 96-well plates with transparent bottom (μclear, Greiner, Frickenhausen, Germany) overnight to ~60% confluence. After being washed once with PBS, cells were loaded with Fluo-3 AM for 60 min at 37°C. Fluorescence measurements were subsequently performed with an excitation wavelength at 485 nm and an emission wavelength of 520 nm at 37°C using the FLUOStar Optima reader with an automated injection device (FLUOStar Optima, BMG Labtechnologies, Offenburg, Germany).

Smooth muscle cell contraction assay. CoSMC cells were transduced with lentivirus containing expression cassettes for IRAG α, β, δ, or ξ and the EF1α reporter gene to identify transduced cells by fluorescence microscopy. Transduction efficiencies varied between 35% and 45% (data not shown). Mean cell length of muscle cells treated with and without bradykinin ± 8-pCPT-cGMP was measured by scanning microscopy as described by Murthy et al. (26). Data were collected from 50 cells within a microscopic field. Control experiments showed that about 70–80% of all cells were reactive to bradykinin. Time course measurements were done at intervals of 15 s. Peak contraction was observed after 30–45 s. The lengths of muscle cells treated with agonist were compared with the lengths of vehicle-treated cells, and contraction was expressed as the percent decrease in mean cell length from control.

Data analysis. Unless otherwise indicated, all data were determined from three independent experiments in triplicate and are expressed as mean values ± SD. Comparisons among data sets were made with analysis of variance, followed by Students t-test. For multiple testing, a Bonferroni correction of the P values was performed. P < 0.05 was considered to be statistically significant.

RESULTS

Identification of human IRAG 5′-mRNA variants. To identify novel IRAG 5′-mRNA variants and to characterize the structure of the 5′-mRNA termini, we performed 5′-RACE-PCR with reverse primers binding to IRAG exon 4, which encodes for the IRAG-cGKI binding site (1). Cloning and sequencing of diverse 5′-RACE-PCR products revealed two novel so far unknown 5′-end mRNA sequences upstream of the exon 1/exon 2 splice junction termed exon 1b and exon 1c as well as the previously described first exons 1a and 1d (Fig. 1A). The nucleotide sequences of the alternative first exons have been deposited in the GenBank database (AM000024). 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 2 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. 1A). 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. Numbers in parentheses behind the name of the IRAG variants indicate the number of GenBank Expressed Sequence Tags (ESTs) present in the GenBank database representing the respective variant vs. the total number of IRAG-containing ESTs (n = 268). Consensus translation initiation sites are marked by an arrow. B: sequence of human IRAG exons 1a, 1b, 1c, 1d, and 2 as determined by 5'-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 1a/1b: nt 9–461 = exon 1a, nt 381–461 = exon 1a’, nt 484–535 = exon 1b; exon 1d: nt 3–358 = exon 1d, nt 201–358 = exon 1d’, exon 2: nt 3–183 = exon 2, nt 80–183 = exon 2’). Putative consensus translational initiation sites are underlined in boldface letters.

Fig. 1. Inositol 1,4,5-trisphosphate receptor (IP3R)-associated GMP kinase substrate (IRAG) 5'-mRNA variants. A: schematic illustration of IRAG exon 1a, 1b, 1c, and 1d mRNA variants as determined by rapid amplification of 5'-cDNA ends (5'-RACE)-PCR with reverse primers binding to IRAG exon 4. Gray boxes mark the novel first exons 1b and 1c, respectively. Numbers in parentheses behind the name of the IRAG variants indicate the number of GenBank Expressed Sequence Tags (ESTs) present in the GenBank database representing the respective variant vs. the total number of IRAG-containing ESTs (n = 268). Consensus translation initiation sites are marked by an arrow. B: sequence of human IRAG exons 1a, 1b, 1c, 1d, and 2 as determined by 5'-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 1a/1b: nt 9–461 = exon 1a, nt 381–461 = exon 1a’, nt 484–535 = exon 1b; exon 1d: nt 3–358 = exon 1d, nt 201–358 = exon 1d’, exon 2: nt 3–183 = exon 2, nt 80–183 = exon 2’). Putative consensus translational initiation sites are underlined in boldface letters.
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

Fig. 2. Expression of IRAG alternative 5'-mRNA variants in the human gastrointestinal tract. A: expression of IRAG 5'-mRNA variants in the human esophagus, stomach, pylorus, duodenum, colon, and colonic smooth muscle cells (CoSMC) was determined using RT-PCR with primers spanning all alternative first exons and alternatively spliced regions using exon 1a, 1b, 1c, and 1d forward and exon 5 reverse primers. IRAG mRNA splice variants expressed in the respective tissues are indicated at the right margin of each lane. B: schematic illustration of alternative first exon variants and splicing events resulting in various IRAG 5'-variants. Length of the PCR products for each of the 5'-IRAG mRNA variants is indicated. Location of forward and reverse primers is given by horizontal arrows. Consensus translation initiation sites are marked by vertical arrows.

IRAG SPLICE VARIANTS
interaction site, and exon 15, which encodes for the cGKI 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
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 9a, 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 (Figs. 4A and 5A). This variant is present in several EST sequences (49/268 IRAG containing ESTs). IRAGγ shows the same structure as IRAGζ, but lacks exon 7 due to exon skipping (Fig. 4A). All alternative 5′- and 3′-splice junctions confirmed to the GT/AG splice donor/acceptor rule (Fig. 4i). The novel sequences were submitted to the GenBank database (AM000024).

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 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 site usage (exon 8: nt 3–620 = exon 8, nt 3–94 = exon 8′). 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 and -β (exon 15/16) 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. 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, and ζ were transfected into COS7 cells and expression was determined in Western blots using a polyclonal IRAG antibody [raised against recombinant IRAGδ3–499 that contains the NH2 terminus of IRAG (encoded by exon 1d–exon 8; see A) and the common cGKIβ 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 IRAGζ 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 9α 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, 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 proteins 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 cGKIβ. 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 cGKIβ in HEK293 cells and subsequent coimmunoprecipitation using polyclonal IRAG or cGKIβ antibody provide evidence for direct physical interaction of IRAG γ, δ, and ζ with cGKIβ (Fig. 5E).

Inhibition of bradykinin-induced calcium transients by cGMP-dependent pathways. To investigate the functionality of the COOH-terminally 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 cells (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 coinubcation 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 cells and IRAGγ, δ, and ζ transfected cells (data not shown). In mock-transfected cells, calcium transients were reduced by 8-pCPT-cGMP coinubcation (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 micrometry as described by Dr. K. S. Murthy’s group (20, 24). Previous studies showed that the activation of cGK by sodium nitroprusside, 8-pCPT-cGMP, and cBIMPS blocks acetylcholine-induced contraction of dispersed smooth muscle cells (27). Accordingly, 8-pCPT-cGMP 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+}]) 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-pCPT-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-pCPT-cGMP, or were left as vehicle-treated controls (right graph). [Ca^{2+}] was measured as in A. Data are expressed as means ± SD of three independent experiments in triplicate. C: CoSMC cells were transfected with IRAγ, δ, and ζ expression plasmids or 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-pCPT-cGMP. [Ca^{2+}] was measured as in A. (Student’s t-test: *P < 0.001 vs. IRAγ). D: CoSMC cells were transduced with IRAγ, δ, and ζ or were left as mock-transfected controls. At 24 h after transduction, cells were treated with 5 × 10^{-7} M bradykinin or 5 × 10^{-7} M bradykinin and 8-pCPT-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. IRAγ). E: expression levels of endogenous IRAγ 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 IRAγ, 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 IRAG γ (1) or a cGKIβ antibody (21). Results shown are representative images of three independent experiments.

**C1454 IRAG SPLICE VARIANTS**

**AJP-Cell Physiol • VOL 301 • DECEMBER 2011 • www.ajpcell.org**

Alternatively interfere with cGMP-mediated inhibition of bradykinin-induced [Ca^{2+}] release and human smooth muscle cell contraction.

Alternative splicing is one of the main sources of transcriptome and proteome diversity, and disturbed regulation of alternative splicing has been implicated in numerous diseases (7, 13, 22, 38). A scan of 20,213 human mRNAs from the RefSeq database revealed that splice acceptor motifs that may cause splice insertion-deletions in transcripts occur in 30% and are functional in at least 5% of all human genes (15).

In 2004, the Human Genome Project reported that the haploid human genome contains about 20,000–25,000 genes, far fewer than had been expected before. Early estimates calculated that mammals need about 100,000 genes (29). The surprising low number of mammalian genes, which is actually just four times the number of genes in budding yeast, indicates
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 \textit{Drosophila melanogaster}. It can generate 38,016 distinct mRNA variants, which is far in excess of the total number of genes in \textit{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 \textit{nNOS} gene in the human gastrointestinal tract generates multiple \textit{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 NH$_2$-terminus of cGKI produces two distinct variants, cGKI$_\alpha$ and cGKI$_\beta$, which have different tissue distribution and target specificity (19). cGKI$_\beta$ 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\textit{ 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 \textit{IRAG} promoters and splicing events, leading to the generation of distinct \textit{IRAG} transcripts in a cell and tissue-specific fashion. Similar to the human \textit{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/cGKI pathway, the tissue-specific RNA-binding protein HuR has been shown as an important regulator of the sGC-\alpha$\scriptscriptstyle I$ 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 \textit{nNOS} gene differentially (45). Thus, the diversity of the 5'-UTR of \textit{IRAG} reflects most likely multiple tissue- and site-specific mechanisms regulating stability, localization, and translation efficiency of distinct \textit{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 \textit{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 \textit{IRAG} gene generates \textit{IRAG} variants lacking full-length exon 1d. Expression of such isoforms would result in proteins lacking the putative NH$_2$-terminal transmembrane domain, suggesting regulation of subcellular \textit{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 IRAGs.

\textit{In silico} analysis of the National Center for Biotechnology Information database confirmed the expression of most individual \textit{IRAG} mRNA variants. This diversity does not appear to be unique for humans. Analysis of the mouse genome database also identified eight \textit{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 \textit{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 \textit{IRAG} containing ESTs are enriched for the 5'-mRNA end (215 of 268 human \textit{IRAG} ESTs). Only a small amount of all ESTs that harbor \textit{IRAG} sequence fragments contain internal exons like exon 7 (15 of 268 ESTs) and exon 8 (16 of 268 ESTs).

Because of the \textit{IRAG} relatively low and/or restricted tissue-specific expression of \textit{IRAG}$_\beta$, -e, and -\eta mRNA and because of the 2) structural similarities of \textit{IRAG}e with \textit{IRAG}8, and of \textit{IRAG}\gamma with \textit{IRAG}\psi and \textit{IRAG}\zeta, we focused on three major novel splice variants---\textit{IRAG}$_\gamma$, \delta, and \zeta---with COOH-terminal deletion of the cGKI phosphorylation and the IP$_3$RI 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 \textit{IRAG} variants lacking the cGKI phosphorylation and the IP$_3$RI interaction site. Consistently, we observed direct interaction of cGKI _\beta with IRAG\gamma, \delta, and \zeta in a heterologous expression system.

Remarkably, \textit{IRAG} is not only an effector of cGKI. Since \textit{IRAG} anchors cGKI, 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 \textit{IRAG} mutant that is not functionally regulated by cGKI phosphorylation suppressed the observed transcriptional activity of cGKI _\beta in a similar manner as wild-type full-length \textit{IRAG}. These data suggest that COOH-terminally truncated \textit{IRAG} variants not only have the potential to interfere with intracellular calcium signaling, but may also affect the transcriptional activity of cGKI _\beta.

In summary, we have identified new splice variants of the \textit{human \textit{IRAG}} gene and characterized the role of COOH-terminally truncated \textit{IRAG} variants for cGKI-dependent signaling. Our results demonstrate that \textit{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.
ACKNOWLEDGMENTS
We thank T. Schmid, U. Götz, and M. Werb for excellent technical assistance.

GRANTS
This work was supported by Deutsche Forschungsgemeinschaft (DFG) (Sonderforschungsbereich 391 CS and Grant SA 1374/1-2) to D. Saur.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


