Identification and analysis of two splice variants of human G2A generated by alternative splicing

Ai Ogawa, Hideru Obinata, Tomoyasu Hattori, Mikiko Kishi, Kazuaki Tatei, Osamu Ishikawa and Takashi Izumi

Department of Biochemistry, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan (A.O., H.O., T.H., M.K., K.T., T.I.)

Department of Dermatology, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan (A.O., T.H., O.I.)
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Corresponding author: Dr. Takashi Izumi, Department of Biochemistry, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. Tel: 81-27-220-7940; Fax 81-27-220-7948; E-mail: takizumi@med.gunma-u.ac.jp

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Abbreviations: HU, hydroxyurea; Ara-C, cytosine arabinoside; ATRA, all-trans-retinoic acid; SRE, serum response element; CRE, cAMP response element
Abstract

G2A is a G protein-coupled receptor that can be induced by various stressors. G2A is reported to have proton-sensing activity that mediates intracellular inositol phosphate (IP) accumulation with decreasing pH. Previously, we showed that G2A is also activated by some oxidized free fatty acids such as 9-hydroxyoctadecadienoic acid (9-HODE). In this study, we identified a novel alternative splice variant of G2A (G2A-b) that has a partially different N-terminus compared with the G2A originally reported (G2A-a). The two splice variants of G2A show similar tissue distributions, but G2A-b is expressed more abundantly. There was no difference between the two variants in 9-HODE-induced cellular responses such as intracellular calcium mobilization and GDP/GTP exchange of Gα protein, and in proton-sensitive IP accumulation. However, G2A-b showed a higher basal activity in terms of IP accumulation. Mutagenesis study revealed that the difference in the basal activity is attributable to the K7 residue that exists only in G2A-a. We further demonstrated that an R42A mutation largely impaired both the basal and proton-sensing activities, but did not affect the 9-HODE-induced intracellular calcium increase. Taken together, we found an additional novel G2A variant (G2A-b) that is the major transcript with functional response to ligand stimulation as well as G2A-a, and succeeded in discriminating proton-sensing and oxidized fatty acid-sensing activities of G2A.
Introduction

G2A is a G protein-coupled receptor (GPCR) that forms a homologous cluster with ovarian cancer G protein-coupled receptor 1 (OGR1), G protein-coupled receptor 4 (GPR4) and T cell death-associated gene 8 (TDAG8). Although these receptors were once reported to recognize lysophospholipids as ligands, most of the reports were retracted due to problems in reproducibility of the data. Although lysophosphatidylcholine (LPC) was first reported as a ligand for G2A, its direct interaction with G2A has not been shown yet. However, many studies have been reported that LPC affects plasma membrane redistribution of G2A and cellular responses such as chemotaxis in a G2A-dependent manner (Radu et al., 2004; Yang et al., 2005; Wang et al., 2005; Ikeno et al., 2005). On the other hand, Ludwig et al. (2003) reported that OGR1 and GPR4 are proton-sensing receptors that enhance turnover of inositol phosphate (IP) and production of cAMP, respectively, in response to acidic pH. Following this, TDAG8 and G2A were also reported to have proton-sensing activity (Ishii et al., 2005; Murakami et al., 2004; Wang et al., 2004). G2A mediates acidic pH-sensitive IP accumulation and activation of the zif268 promoter that is antagonized by lysophosphatidylcholine (Murakami et al., 2004). However, G2A is less sensitive to a decrease in pH than the other three proton-sensing receptors (Radu et al., 2005), and any physiological function of human G2A related to proton-sensitivity has not been reported.
We reported that G2A also functions as a receptor for some oxidized free fatty acids such as 9-hydroxyoctadecadienoic acid (9-HODE) and 11-hydroxyeicosatetraenoic acid (11-HETE) (Obinata et al., 2005). When overexpressed in CHO-K1 or HEK293 cells, G2A mediated various 9-HODE-evoked intracellular signals such as intracellular calcium mobilization and activation of JNK. Acidic pH had merely an additive effect on the 9-HODE-induced IP accumulation. We have further demonstrated that 9-HODE induces secretion of some cytokines and inhibition of proliferation via G2A in keratinocytes that endogenously express G2A (Hattori et al., 2008).

G2A is also known to have ligand-independent activities. Overexpression of G2A causes cell cycle arrest in the G2/M-phase (Weng et al., 1998), stress fiber formation in NIH3T3 cells (Kabarowski et al., 2000), accumulation of IP and cAMP in HeLa cells (Lin and Ye, 2003) and cytokine production in human keratinocytes (Hattori et al., 2008). G2A was first identified as a stress-inducible GPCR in lymphocytes (Weng et al., 1998). Various stressors, including chemical DNA-damaging agents, X-rays and UV light induce G2A expression. In human keratinocytes, UV and hydrogen peroxide induce G2A expression, and enhance cytokine production by 9-HODE (Hattori et al., 2008). This suggests that oxidative stress regulates the expression level of functional G2A. Considering the basal activity of G2A, it is important to clarify how G2A expression is regulated.
Alternative splicing can produce multiple protein isoforms from a single gene (Fedor, 2008; Woodley and Valcárcel, 2002). In many cases, alternative splicing events affect the coding sequence, leading to the production of diverse proteins (Black, 2000). Alternative splicing is one of the major mechanisms that regulate gene expression and function. Utilization of different reading frames may produce different proteins, or introduce a premature termination codon that triggers nonsense-mediated mRNA decay. In some cases, partially different proteins may have additional functions, lack a particular function or have a dominant negative function.

In this study, we have identified and characterized a novel G2A splice variant. We designated the novel variant G2A-b, and the original one G2A-a. G2A-a and G2A-b utilize a different translation start codon, and thus have different amino acid sequences in the N-terminus. We examined the differences between the two variants in tissue distribution, 9-HODE-evoked intracellular signals and proton sensitivity. We further performed mutagenesis analysis of some positively charged amino acids in the N-terminal region that are thought to contribute to the basal activity and proton-sensing activity of G2A.
Methods

Materials Linoleic acid was purchased from Sigma-Aldrich (St Louis, MO). 9(S)-HODE was synthesized from linoleic acid with potato 5-lipoxygenase as described previously (Obinata et al., 2005). [α-32P]dCTP was from PerkinElmer (Waltham, MA). Human Multiple Tissue cDNA Panels 1 and 2 were from Clontech (Mountain View, CA).

RNA preparation Total RNA was extracted from human peripheral blood leukocytes or HL-60 cells using a RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK) with on-column DNase digestion, followed by poly(A)^+ RNA isolation using a μMACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

5'- and 3'-rapid amplification of cDNA ends (RACE) analysis First-strand cDNA was synthesized from HL-60 poly(A)^+ RNA using Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany) and a G2A specific primer (5’-GTCTGGAACACCGGGTAGT-3’ designed within exon 4), followed by polyadenylation of the 3’-end using dATP and terminal deoxynucleotidyl transferase. 5’-RACE PCR was performed with KOD DNA polymerase (TOYOBO, Osaka, Japan) using a dT17-adapter sense primer (5’-GACTCGAGTCTGACATCGATTTTTTTTTTTTTTTTTT-3’) and the nested antisense primer (5’-AGATGTCGACGTCACCTTG-3’ designed within exon 4).

For 3’-RACE, first strand cDNA was synthesized from HL-60 poly(A)^+ RNA using an oligo dT
adapter primer (5'-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)_{24} -3'). 3'-RACE

PCR was performed with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using a G2A specific
sense primer 1 (5'-GACCGACAAGACGCATGAC-3' designed within exon 2) or primer 2
(5'-GATGCTGGCAGATGGGTGCAGAAGAA-3' designed within exon 3), and an antisense adapter
primer (5'-CGCTACGTAACCGCATGACAGTG-3'). PCR products were cloned into the
pBluescript vector (Stratagene) and the sequence was confirmed using a LIC-4200L DNA
sequencing system (LI-COR, Lincoln, NE).

**Plasmids** G2A-a (NCBI accession number: NM_013345), FLAG-tagged G2A-a, G2A-b, and
FLAG-tagged G2A-b were cloned into the pcDNA3.1 (Life Technologies, Carlsbad, CA) or
pCXN2.1 vector as described previously (Obinata et al., 2005) using sense primers
(5'-CGGGTACCACCATGTGCCCAATGCTACTGAAAAAC-3' for G2A-a,
5'-CGGGATCCACCATGGATTACAAGGACGACGATGACAGTGCCCAATGCTACTGAAA
AAC-3' for FLAG-G2A-a, 5'-GGGGTACCACCATGCCAGGAAACGCCACCCCGTGG-3' for
G2A-b,
5'-GGGGTACCACCATGGATTACAAGGACGACGATGACAGGCCAAGCCACCCCGTGG-3' for
FLAG-G2A-b) and an antisense primer
(5'-GGAATTCTCAGCAGGACTCCTCAATC-3').
Site-directed mutagenesis was performed according to the method described in Braman et al. (1996) using Pfu DNA polymerase. In brief, PCR was carried out using pcDNA3.1-G2A-a as a template and the following primers for single amino acid changes:

5’-CCATGTGCCCAATGCTACTGGCAACGGTTACAATGGAAACG-3’ for K7A,

5’-TGGGCCTCTCCGCCGCGACCTGCAACAACG for K31A and

5’-GTCCTTCGAAGAGAGCGCGATAGTCCTGGTCGTG-3’ for R42A, and their complementary antisense primers. After digestion of the methylated parental DNA by Dpn I, PCR products were cloned into pcDNA3.1 and the sequence was confirmed.

**Cell culture, transfection and flow cytometry**

HL-60, Chinese hamster ovary-K1 (CHO-K1) and COS-7 cells were maintained in RPMI-1640 medium, Ham’s F-12 medium and Dulbecco's Modified Eagle's Medium (Wako, Osaka, Japan), respectively, containing 10% fetal bovine serum. Cells were cultured at 37°C in a humidified incubator with 5% CO₂. For transient expression, CHO-K1 or COS-7 cells were transfected with plasmid DNAs using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer’s instructions. To observe the transient expression of G2A proteins, cells were incubated with 5 µg/ml anti-G2A antibody (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at RT, followed by incubation with 8 µg/ml donkey anti-goat IgG conjugated with Alexa Fluor 488 (Life Technologies) for 1 h at RT. For stable
expression, the transfected CHO-K1 cells were selected with 1 mg/ml neomycin and the expression level of FLAG-tagged G2A proteins on the cell surface was examined by flow cytometry. For the flow cytometry, the cells were incubated with 10 µg/ml M5 anti-FLAG antibody (Sigma-Aldrich) for 1 h at RT, followed by incubation with 8 µg/ml goat anti-mouse IgG conjugated with Alexa Fluor 488 (Life Technologies) for 1 h at RT. The flow cytometry analyses were performed using an EPICS XL flow cytometry system (Beckman Coulter, Fullerton, CA).

**Preparation of peripheral blood leukocytes** Peripheral blood was obtained from healthy volunteers with written informed consent. The study was performed in accordance with the institutional guidelines of Gunma University School of Medicine. Heparinized peripheral blood was collected and mixed with the same volume of 2% dextran solution. Leukocyte-rich supernatant (buffy coat) was obtained, and washed twice with PBS. After removal of residual erythrocytes by hypotonic shock, leukocytes were lysed for total RNA preparation.

**Northern blot analysis** Poly(A)^+ RNA from human peripheral blood leukocytes or HL-60 cells (2 µg/lane) was electrophoresed on 1% formaldehyde-agarose gels, and transferred onto a nylon membrane (Biodyne A; Pall, East Hills, NY). The membrane was pre-hybridized for 30 min at 68°C, and hybridized with ^32^P-labeled probes (1×10^6^ cpm/ml) for 1 h at 68°C in Express Hyb Hybridization solution (Clontech). Probes were generated using Megaprime DNA labeling systems.
(GE Healthcare) and [\(\alpha^{-32}\text{P}\)]dCTP (111 TBq/mmol). For the detection of G2A mRNA, probe 1 was designed to hybridize within exon 4, and probe 2 within exon 3. For the detection of BLT1 (leukotriene B\(_4\) receptor) mRNA, the probe was designed to hybridize within the coding sequence of BLT1. After hybridization, the membrane was washed with 2 \(\times\) SSC / 0.1% SDS for 30 min at RT, then 0.2 \(\times\) SSC / 0.1% SDS for 1 h at 65°C. The membrane was exposed to an imaging plate and visualized using a Typhoon 9210 System (GE Healthcare).

**Analysis of mRNA stability** HL-60 cells were exposed to Actinomycin D (1 \(\mu\)g/ml) for 0, 0.5, 1, 2 or 3 h. Two \(\mu\)g of poly(A)\(^+\) RNA was subjected to Northern blot analysis using the \([\alpha^{-32}\text{P}]\)dCTP-labeled probes specific for G2A (probe 1) and BLT1.

**Real time PCR analysis** TaqMan\(^\text{\textcopyright}\) PCR analysis was performed to evaluate G2A-a and G2A-b expression levels. TaqMan\(^\text{\textcopyright}\) probes (Sigma-Aldrich) were labeled with the fluorescent dye FAM at the 5' end and with the quencher dye BHQ1 at the 3' end. For G2A-a detection, the probe and primers were designed within exon 3 (probe:

5’-FAM-TGAGCAGAACACGCCACACGCCAC-BHQ-3’, sense primer:

5’-GACGCTCTCTAGCAGCCGAGTC-3’, antisense primer:

5’-GCCCTTCCATCTTGAGCAATCC-3’). For G2A-b detection, the probe was designed at the linkage site of exon 2 and exon 4, the sense primer within exon 2, and the antisense primer within
exon 4 (probe: 5'-FAM-ATGCCAGGAAACGCCACCCCAGT-BHQ-3', sense primer:
5'-AGCCACACTGAGATTGGAACC-3', antisense primer:
5'-GTACACCACGACCAGGACTATC-3'). Quantitative PCR analysis was performed using a DNA Engine Opticon system (MJ Research, Waltham, MA).

**Measurement of intracellular calcium concentration** CHO-K1 cells were loaded with 5 µM Fura 2-AM (Dojindo, Kumamoto, Japan) in HEPES-Tyrode’s-BSA buffer (25 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.8 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM D-glucose, 0.37 mM NaH₂PO₄, 0.49 mM MgCl₂ and 0.01% fatty acid-free BSA) containing 1.25 mM probenecid and 0.02% pluronic F127 for 1 h at 37°C. Cells were washed with HEPES-Tyrode’s-BSA buffer, and changes in intracellular calcium concentration upon 9-HODE stimulation were monitored with a FLEX station scanning fluorometry system (Molecular Devices, Sunnyvale, CA) or a RF5300PC spectrofluorometer (Shimadzu, Kyoto, Japan).

**Preparation of membrane fractions** CHO-K1 or COS-7 cells were disrupted by sonication in homogenizing buffer (20 mM Tris-HCl, pH7.4, 0.25 M sucrose, 10 mM MgCl₂, 1 mM EDTA and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland)). The homogenates were centrifuged for 5 min at 800g, and the resulting supernatants were further centrifuged for 60 min at 100,000g. The precipitates were resuspended in the homogenizing buffer containing 0.5%
dodecylmaltoside, and protein concentrations were determined using the BCA protein assay reagent (Pierce, Rockford, IL) using BSA as a standard.

**GTP binding assay** The GTP binding assay was performed using a DELFIA GTP-Binding Kit (Perkin Elmer) according to the manufacturer’s instructions. The membrane fractions of CHO cells (4 µg protein) were incubated with various concentrations of 9-HODE in 100 µl of reaction buffer (50 mM HEPES-NaOH, pH 7.4, 0.1 µM GDP, 1 mM MgCl₂, 10 mM NaCl and 0.1 mg/ml saponin) in a 96 well AcroWell Filter Plate (Pall). After 30 min incubation at RT, 10 nM Europium-labeled GTP (a non-hydrolysable analog of GTP) was added, and incubated for another 30 min. The reaction was terminated by vacuum filtration using a Multiscreen Vacuum Manifold (Millipore, Billerica, MA), followed by rinses with the wash solution supplied with the kit. The fluorescence intensity of the Europium trapped in the filter was measured in a time-resolved fluorometer (ARVO HTS; PerkinElmer), with the excitation wavelength at 340 nm and the emission at 615 nm.

**Inositol 1- phosphate accumulation assay** COS-7 cells were exposed to various pH of HEPES/EPPS/MES-buffered assay solution (1 mM CaCl₂, 0.5 mM MgCl₂, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose and 0.01% fatty acid-free BSA) for 1 h at 37°C in the presence of 0.5 M LiCl. Accumulated inositol 1- phosphate (IP₁) was quantified using the IP-One ELISA kit (Cisbio, Bagnols-sur-Cèze, France) according to the manufacturer’s instructions.
**Reporter gene assay** The reporter gene assays for serum response element (SRE) and cAMP response element (CRE) were performed in COS-7 cells by co-transfection with G2A expression vector, pTAL-firefly luciferase vector containing specific *cis*-acting DNA sequence (SRE-luciferase reporter gene or CRE-luciferase reporter gene, Clontech), and CMV promoter-driven Renilla luciferase vector (Clontech). Twenty four hours after transfection, Firefly and Renilla luciferase activities were measured using Dual-Glo Luciferase Assay System (Promega, Madison, WI).
Results

Identification of a novel G2A splice variant According to the NCBI database, the human G2A gene (Gene ID: 29933) is located on chromosome 14q32.3, and consists of four exons and three introns (Fig. 1A). The putative translation start codon is located in exon 3, and the stop codon in exon 4. The deduced amino acid sequence reveals that G2A encodes a 380-amino acid protein with seven transmembrane regions. To investigate the existence of G2A splice variants, 5’- and 3’-RACE analysis were performed using poly(A)+ RNA from HL-60 cells. 5’-RACE analysis revealed the presence of two alternatively spliced transcripts; one for the original G2A and the other for a novel G2A splice variant that lacked the sequence of exon 3. 5’-RACE also revealed that the two G2A splice variants utilize the same transcription initiation site, which is 13 bp upstream of the reported G2A transcript (NCBI accession number: NM_013345). A TATA box-like sequence exists in the region between -34 and -26 (Fig. 1B). 3’-RACE revealed that the two variants utilized the same polyA signal. We designated the original variant G2A-a and the novel variant G2A-b (Fig. 1A). G2A-b utilizes a different in-frame start codon in exon 2 and thus has a different amino acid sequence in the N-terminus from G2A-a (Fig. 1C). As a result, the N-terminal eleven amino acids from exon 3 in G2A-a are replaced with two amino acids from exon 2 in G2A-b. Northern blot analysis was also performed in HL-60 cells to confirm whether these two G2A splice variants were
expressed. Two transcripts (3.6 kb and 2.8 kb) were observed with probe 1, which hybridizes to the sequences in exon 4 (Fig. 1D). These transcripts correspond to G2A-a and G2A-b, respectively, and were expressed at nearly equal levels in HL-60 cells. A 3.6 kb transcript only was observed with probe 2, which hybridizes to the sequences in exon 3. Thus, we confirmed the expression of mRNAs for two G2A splice variants by both RACE and Northern blot analysis in HL-60 cells.

Tissue distribution of G2A-a and G2A-b To determine whether the two splice variants exhibit a differential tissue distribution, we performed quantitative PCR using TaqMan® probes that were specific for G2A-a or G2A-b. However, direct quantitative comparison was difficult because the primer set for G2A-b also recognizes G2A-a, and the efficiency of PCR amplification was significantly different between G2A-a and G2A-b. For this reason, the expression level is indicated as a percentage of that in peripheral blood leukocytes. The expression pattern of G2A-a and G2A-b was similar as a whole, with the highest expression in peripheral blood leukocytes, followed by spleen, lung and liver (Fig. 2A). The expression pattern was slightly different between the two variants in kidney, placenta, heart and brain, though the expression level was rather low. To compare the expression levels of G2A-a and G2A-b, Northern blot analysis was performed in peripheral blood leukocytes poly(A)⁺ RNA (Fig. 2B). Analysis using probe 1 revealed that G2A-b was more abundant than G2A-a in leukocytes by about three-fold. These results indicate that G2A-a
and G2A-b are similarly distributed, and that G2A-b is expressed more abundantly than G2A-a.

Induction by DNA-damaging stress and a differentiation inducer G2A was first identified as a GPCR induced by various classes of DNA damaging agents (Weng et al., 1998). To examine whether the expression levels of the G2A splice variants are differently affected by chemical DNA-damaging agents, HL-60 cells were treated with hydroxyurea (an inhibitor of \textit{de novo} synthesis of DNA precursors) or cytosine arabinoside (a direct inhibitor of DNA synthesis) for 16 h. Northern blot analysis revealed that the two G2A splice variants were expressed at nearly equal levels in HL-60 cells, and the expression levels of both variants were increased approximately 1.5-fold by these DNA-damaging agents (Fig. 3A). There was no difference in the degree of increase between the two variants.

Next, we examined changes in the expression level of the G2A variants during neutrophilic differentiation of HL-60 cells. HL-60 cells are derived from acute promyelocytic leukemia cells, and will differentiate into a neutrophilic phenotype when treated with all-\textit{trans}-retinoic acid (ATRA) (Breitman et al., 1980). As shown in Fig. 3B, the expression levels of the two splice variants were increased by ATRA. The degrees of increase were approximately 1.4-fold in G2A-a and 1.8-fold in G2A-b.

\textit{mRNA stability of G2A splice variants} The turnover of mRNA is an important factor in the
regulation of gene expression. To examine the mRNA stability of G2A-a and G2A-b, Northern blot analysis was performed using poly(A)+ RNA prepared from HL-60 cells treated with Actinomycin D (a transcriptional inhibitor). As shown in Fig. 3C, the both transcripts of the two G2A variants rapidly decayed during the initial 1 h after Actinomycin D treatment, while the transcript of BLT1 did not decay within 2 h. The half-lives of two G2A transcripts were shorter than 1 h, and there was no obvious difference between them.

Difference in 9-HODE-induced responses between G2A-a and G2A-b

It is possible that the two G2A variants have different functional properties because of their different N-terminal extracellular regions. First, we established several stable clones that expressed FLAG-tagged G2A-a or G2A-b in CHO-K1 cells. There was no obvious difference in the level of cell surface expression between FLAG-G2A-a and FLAG-G2A-b, when examined by flow cytometry using an anti-FLAG antibody (data not shown).

Using these stable clones, we examined intracellular calcium mobilization evoked by 9-HODE. Fig. 4A shows intracellular calcium mobilization in each representative clone. 9-HODE evoked intracellular calcium mobilization almost equally in both the clones. We performed the assay for each of four clones and obtained essentially the same results. A maximal response was obtained at approximately 3 µM, and the averages of the EC50 values for the four clones were 345
nM for G2A-a and 370 nM for G2A-b (Fig. 4A). 9-HODE did not evoke the calcium response in parental CHO-K1 cells at all (data not shown).

To exclude the possibility of artifacts arising during the selection of stable clones, we analyzed the intracellular calcium mobilization in a transient expression system co-expressed with a Gqi chimeric protein (Obinata et al., 2005). We also examined the influence of the FLAG tag sequence that was inserted between the first methionine and the second amino acid of the G2A variants, as it was possible that the FLAG sequence affects the difference between G2A-a and G2A-b. 9-HODE evoked intracellular calcium mobilization at nearly equal levels in all cells (data not shown). These results indicate that 9-HODE induced equivalent intracellular calcium mobilization both in CHO-G2A-a and CHO-G2A-b cells, irrespective of the additional FLAG sequence.

Next, we examined 9-HODE-induced GDP/GTP exchange of Gα protein to compare the affinity of the G2A variants for 9-HODE. G2A-a or G2A-b was co-expressed with Gαi in CHO-K1 cells, and the membrane fractions were prepared. As shown in Fig. 4B, G2A-a and G2A-b exhibit similar GDP/GTP exchange in response to 9-HODE. The averages of EC50 values from four independent measurements were 1.5 µM for G2A-a and 1.7 µM for G2A-b (Fig. 4B). There was no statistically significant difference between them. Taken together, these results indicate that G2A-b
encodes a functional protein that is equivalent to G2A-a with respect to the response to 9-HODE.

**Difference in basal activity and proton-sensing activity between G2A-a and G2A-b** Since it was reported that acidic pH evoked accumulation of intracellular inositol phosphates via G2A (Murakami et al., 2004), we compared the proton-sensing activity of G2A-b with that of G2A-a. COS-7 cells were transiently transfected with G2A-a, G2A-b or OGR1, then exposed to various pH conditions for 1 h in the presence of LiCl (an inositol monophosphatase inhibitor), and accumulated IP1 was measured. Fig. 5 illustrates that both G2A-a and G2A-b showed significant IP1 accumulation compared with the vector control at physiological pH (7.4 or 7.0), confirming that G2A has basal activity as well as OGR1 (Ludwig et al., 2003). These basal activities of the G2A variants were observed even in alkaline pH (8.2) in contrast to OGR1 whose activity was decreased to the basal level. Moreover, G2A-b showed higher IP1 accumulation than G2A-a at any pH examined. With decreasing pH, G2A-a and G2A-b showed an increased IP1 accumulation, indicating that both variants have proton-sensing activity, although their activities were much less than that of OGR1. The extent of increase in IP1 accumulation with decreased pH (from 8.2 to 6.2) was almost the same between G2A-a and G2A-b. Taken together, these results demonstrate that G2A-a and G2A-b have similar proton-sensing activity, and that G2A-b has higher basal activity.

This led us to analyze the difference in the N-terminal extracellular region between G2A-a and
G2A-b.

*Mutagenesis study in the N-terminal extracellular region of G2A* Ludwig et al. (2003) reported that five histidine residues are involved in the proton-sensing activity of OGR1, that is, H17 and H20 in the N-terminus, H84 in extracellular loop 1, H169 in extracellular loop 2 and H269 in helix 7. The importance of the two histidine residues in the N-terminus (H10 and H14) was also confirmed by the proton-sensing activity of TDAG8 (Wang et al., 2004). However, G2A lacks these histidine residues. Instead, G2A contains other positively charged amino acids in these positions. R42 in G2A-a corresponds to H20 in OGR1. G2A-a has other two positively charged amino acids in N-terminus, that is K7 and K31 in addition to R42 (Fig. 6A). As G2A-b lacks K7, we first mutated K7 to alanine in G2A-a (K7A) and analyzed the effects on the basal activity. As shown in Fig. 6C, the K7A mutation in G2A-a resulted in almost the same basal activity as in G2A-b. This result suggests that difference in the basal activity of G2A-a and G2A-b is attributable to the K7 residue in G2A-a.

We further analyzed the effects of K31A and R42A mutations on the basal and proton-sensing activities. These mutant proteins were all expressed at a similar level in COS-7 cells, as revealed by flow cytometric analysis (Fig. 6B) and by Western blot analysis (data not shown). As shown in Fig. 6C, IP1 accumulation was decreased in the K31A mutant compared with wild type
G2A-a at any pH. The decrease was more evident in acidic conditions. In the R42A mutant, the basal activity at an alkaline pH decreased to near the basal level, and the proton-sensing activity with pH decrease was almost completely lost. These results suggest that both K31 and R42 are involved in the basal and proton-sensing activities of G2A, and that R42 plays a critical role. On the other hand, all of the mutations had little or no effect on the 9-HODE-induced intracellular calcium mobilization (data not shown). These results suggest that all the mutants are functionally expressed on the cell surface, and that 9-HODE and acidic pH require different mechanisms to activate G2A.

Difference in basal activity between G2A-a, G2A-b and mutants in SRE -and CRE-driven transcriptional activation

To investigate whether the basal activity of G2A-a and G2A-b would activate gene expression regulated by cis-acting elements, we performed reporter gene assay using luciferase driven by SRE and CRE. SRE- and CRE-luciferase activities were increased in both G2A-a- and G2A-b-expressing cells (Fig.6D, E). The activities of both expressing cells were approximately 3-fold in SRE- and 12-fold in CRE-luciferase, compared to those of mock-transfected cells. We also investigate the effects of mutation in the positively charged amino acids in the N-terminal region of G2A-a. K7A mutation resulted in almost the same luciferase activity driven by SRE as G2A-a, and showed a little higher luciferase activity driven by CRE. However, these activities were reduced in K31A and R42A mutations (Fig.6D, E), indicating that
both K31 and R42 residues play some roles in the basal activity of G2A in SRE- and CRE-driven transcriptional activation. These results were compatible with the results observed in the assay of IP1 production at neutral pH.
Discussion

In this study, we identified a novel G2A isoform (G2A-b) that has a partially different N-terminus from the G2A originally reported (G2A-a). G2A-a and G2A-b are alternatively spliced variants; mRNA for G2A-a utilizes all four exons and mRNA for G2A-b lacks the exon 3 sequence (Fig. 1A). Many splice variants have been reported in GPCRs. The most common form is splice variants in the C-terminal intracellular region. Variants in the N-terminal extracellular region and intracellular loop are also found. Splice variants could lead to physiological diversity such as differences in tissue distribution, ligand-binding properties, signaling pathways and coupling efficiency with Gα protein (Kilpatrick et al. 1999). G2A-a and G2A-b are similarly distributed, but G2A-b is more abundantly expressed (Fig. 2). In HL-60 cells, both variants were expressed at nearly equal levels (Fig. 1D). Although both variants were induced similarly by chemical DNA-damaging agents, G2A-b was more inducible during neutrophilic differentiation of HL-60 cells (Fig. 3). Thus, the expression pattern of G2A-a and G2A-b may vary depending on the cellular contexts such as proliferation, cell cycle regulation and differentiation.

Since G2A-a and G2A-b utilize the same transcription initiation site, the ratio of mRNAs for G2A-a and G2A-b may be determined by post-transcriptional regulation such as splicing efficiency and mRNA stability. The stability of the mRNA was not different between G2A-a and
G2A-b (Fig. 3C). Both G2A mRNAs showed very rapid mRNA decay. They share a common 3'-untranslated region that contains AUUUA and a tandem repeat of 5 GUUU (NCBI accession number: NM_013345). Such AU-rich elements were reported to be involved in mRNA instability in the mRNAs for early-response-genes and cytokines (Peng et al., 1996; Xu et al., 1997). These observations on the rapid decay of G2A mRNA are compatible with the inducible expression of G2A.

Splicing is regulated by several factors such as splice site recognition, splicing regulators and RNA secondary structure (Black, 2000; Hertel, 2008; Zheng, 2004). In mammalian genes, splice sites are not well conserved, which allows alternative splicing to occur frequently. Based on a splice site predictor program NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/), both the 3’ splice site of intron 2 and the 5’ splice site of intron 3 of G2A are “weak” splice sites compared with the other splice sites, therefore exon 3 is susceptible to exon skipping. Recognition of splice sites can be altered by external stimuli such as heat shock, pH, hypoxia and oxidative stress (Manabe et al., 2003; Stamm, 2002). Another factor that affects splicing efficiency is the secondary structure of pre-mRNA. Local RNA structures can affect splice-site recognition to promote or interfere with spliceosome assembly. G2A has a tandem repeat of a sequence, 4 repeats of GCCAC, that is likely to form a hairpin structure with GUGGC in the exon 3-derived pre-mRNA (NCBI accession
number: NC_000014). It is possible that this structure may interfere with splice-site recognition at both ends of exon 3. Taken together, G2A-b is more likely to be produced than G2A-a as a result of alternative splicing, although both variants have a similar tissue distribution due to use of the same transcription initiation site. We also investigated the stability of G2A-a and G2A-b proteins using a protein synthesis inhibitor. In the presence of cycloheximide, G2A-a and G2A-b gradually decreased, but more than 50% of both proteins were retained at 24h. The degradation rates of G2A-a and G2A-b were similar (data not shown).

As mouse G2A gene (NCBI gene ID: 56696) consists of three exons and two introns, mouse G2A corresponds to human G2A-b. There has been no splice variant of mouse G2A reported in EST databases. While mouse G2A and human G2A-b share 67% amino acid identity, mouse N-terminus (1MRSEPNAAGNTTLGVTSLQSTSVPSETCHVSYEESR39) shows only 23% identity (9/39) with that of human G2A-b. In contrast to human G2A, mouse G2A did not respond to 9-HODE (Obinata and Izumi, 2009), and not to acidic pH (Radu et al., 2005). In evolutionary processes, human and mouse G2A might acquire different mechanisms for ligand recognition and receptor activation.

There was no difference between G2A-a and G2A-b in 9-HODE-induced cellular responses (Fig. 4), indicating that the difference in the N-terminus does not alter the response to
9-HODE. In contrast, G2A-b showed a higher IP accumulation than G2A-a at every pH examined (Fig. 5). Thus, G2A-b has higher basal activity than G2A-a, though G2A-a and G2A-b have similar proton-sensing activity. Previous reports have shown that certain histidine residues in the N-terminus are critical for the proton-sensing activity of OGR1 and TDAG8 (Ludwig et al., 2003; Wang et al., 2004). However, these histidine residues are not conserved in G2A. Instead, G2A-a has three other positively charged amino acids in the N-terminus, that is K7, K31 and R42 (Fig. 6A).

Using mutagenesis, we demonstrated that the difference in the basal activity between the two variants could be attributed to the K7 that is absent in G2A-b (Fig. 6C).

K31A and R42A also altered the basal activity and proton-sensing activity of G2A. Particularly, mutation of R42A showed a marked reduction in the basal activity in terms of IP turnover, transcriptional activation and also in the proton-sensing activity (Fig. 6). In OGR1, histidine pairs between the N-terminus and the extracellular loop are suggested to stabilize OGR1 in the inactive conformation in alkaline conditions (Ludwig et al., 2003). In acidic conditions, these histidine pairs are thought to dissociate as a result of protonation, which leads to conformational change in OGR1. Since the pKa of histidine is pH 6.0, histidine residues can sense changes in the concentration of protons within a physiological range. In contrast, the pKa of lysine and of arginine is much higher (pH 10.0 and 12.5, respectively), indicating that these residues are always
protonated in physiological conditions. We assume that R42 in G2A acts like the protonated H2O in OGR1, and keeps G2A in the active conformation. R42 might interact with some negatively charged amino acids in the extracellular loop, and K7 and K31 might modulate this interaction.

In contrast to significant impacts on the basal and proton-sensing activities, none of the mutants affected 9-HODE-induced intracellular calcium mobilization. This suggests that 9-HODE and proton use different mechanisms to activate G2A. Generally, hydrophobic lipid mediators are thought to interact with GPCRs in binding pockets formed by several transmembrane helices. Two potential ligands of G2A, 9-HODE and 11-HETE, share the same structure from the ω end to the hydroxy group. We speculate that 9-HODE/11-HETE sticks into the hydrophobic pocket, and its hydroxy group plays a role in inducing conformational changes in G2A. Crucial residues responsible for 9-HODE/11-HETE binding should be clarified by further mutagenesis.

There is no achieved consensus on the ligands for G2A. LPC was reported to act as a modulator of cellular signaling via G2A (Radu et al., 2004; Wang et al., 2005; Yang et al., 2005; Ikeno et al., 2005). A proton-sensitive activity was shown in G2A, but was inhibited by LPC (Murakami et al., 2004). We think that 9-HODE and 11-HETE are potential ligands for G2A. 9-HODE induced GTP binding to the membrane fractions and rapid cellular response such as calcium mobilization in G2A-expressing cells (Fig. 4), indicating that 9-HODE acts as a ligand of
GPCR. We have also showed that 9-HODE induces cytokine secretion and proliferation inhibition via G2A in keratinocytes (Hattori et al., 2008). One criticism of 9-HODE as a G2A ligand is that the concentration of 9-HODE required for cellular responses is high. Actually, EC_{50} values of 9-HODE in cellular signaling were as high as sub-µM or µM orders (Fig. 4). It can be attributed to BSA used in the assay buffer in which the large part of 9-HODE exists as BSA-bound form. An important issue to be clarified is whether enough amount of 9-HODE is produced in situations where G2A plays a role. It must be kept in mind that 9-HODE may cause non-specific detergent effects or act as an endogenous activator of PPARgamma (Nagy et al., 1998), especially at higher concentrations than 10 µM orders.

Both oxidized fatty acids like 9-HODE and LPC are continually produced by cell or in serum lipoprotein, and they could activate G2A. So, the basal activity of G2A may not be necessarily ligand-independent, but involves interaction of G2A with endogenously produced lipids. Further mutagenesis analysis of the important residues that interact with ligand/modulator will provide an opportunity to dissect precise mechanisms of G2A activation.

In summary, we identified a novel G2A splice variant termed G2A-b as a result of exon skipping. G2A-b showed a similar tissue distribution to G2A-a, but was more abundantly expressed than G2A-a. Although both variants showed similar 9-HODE-induced cellular responses, G2A-b
showed a higher basal activity in terms of IP1 accumulation, which is attributable to the lack of K7.

We showed that R42 plays a critical role in proton-sensing activity. However, the response of the R42A mutant to 9-HODE remains, indicating that there are different mechanisms of G2A activation by 9-HODE and protons. Our study will contribute to further consideration of the molecular mechanisms and the cellular functions of G2A-mediated signaling.
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Footnotes

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The entire sequences of G2A-a and G2A-b mRNAs were deposited in the DNA Data Bank of Japan, accession number: AB465599 for G2A-a and AB465600 for G2A-b.

Corresponding author: Dr. Takashi Izumi, Department of Biochemistry, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. Tel: 81-27-220-7940; Fax 81-27-220-7948; E-mail: takizumi@med.gunma-u.ac.jp
Legends for Figures

**Fig. 1.** Identification of a novel splice variant of human G2A. (A) Schematic representation of the G2A gene and the intron-exon structures for the two alternatively spliced variants. Exons are shown as closed boxes and introns as lines. The 5’ and 3’ untranslated regions are shown in white and the coding sequences are shown in gray. G2A-a utilizes four exons, whereas the novel splice variant, G2A-b, does not utilize exon 3 as a result of exon skipping. The locations of probes for Northern blot analysis are also indicated. (B) Nucleotide sequence around the putative transcription initiation site for G2A. The transcription initiation site indicated by an arrow was determined by 5’ RACE, and was shared among the variants. The TATA like sequence is underlined. (C) Amino acid sequence of the N-terminal region of G2A. The residues different between the variants are shown in *italics*. (D) Northern blot analysis of G2A expression in HL-60 cells. Two µg of poly(A)+ RNA was hybridized with [α-^32^P]dCTP-labeled probes. Two bands corresponding to G2A-a (3.6 kb) and G2A-b (2.8 kb) were observed with probe 1, and only G2A-a (3.6 kb) was observed with probe 2. Experiments were performed twice with similar results.

**Fig. 2.** Tissue distribution of G2A splice variants. (A) Expression profile determined by TaqMan® real time PCR using cDNAs from various human tissues. The data are represented as the expression...
level relative to that in peripheral blood leukocytes (mean + S.D., n = 3). (B) Northern blot analysis in peripheral blood leukocytes from two healthy volunteers (lanes 1 and 2). Two µg of poly(A)+ RNA was prepared, and Northern blot analysis was performed using \( [\alpha -^{32}P]dCTP \)-labeled probe 1. Experiments were performed twice with similar results. Densitometric analysis is shown in the right panel.

**Fig. 3.** Induction and stability of G2A mRNA. (A, B) HL-60 cells were treated with hydroxyurea (HU: 1 mM) or cytosine arabinoside (Ara-C: 10 µM) for 16 h (A) or differentiated with all-trans-retinoic acid (ATRA: 1 µM) for 24 h (B). Two µg of poly(A)+ RNA was prepared and Northern blot analysis was performed using \( [\alpha -^{32}P]dCTP \)-labeled probe 1. Densitometric quantification of the expression levels of G2A-a (filled bars) and G2A-b (open bars) is shown. (C) HL-60 cells were exposed to Actinomycin D (1 µg/ml) for the indicated time. Two µg of poly(A)+ RNA was prepared and Northern blot analysis was performed using the \( [\alpha -^{32}P]dCTP \)-labeled probes specific for G2A (probe 1) and BLT1. Densitometric quantification of the expression levels of each transcript for G2A-a (filled circles), G2A-b (open circles) and BLT1 (open squares) is also shown. The results are shown as percentage of the value at 0 h. All experiments were performed twice with similar results.
Fig. 4. 9-HODE-evoked intracellular calcium mobilization and GDP/GTP exchange via G2A-a and G2A-b. (A) CHO cells stably expressing G2A-a (filled circles) and G2A-b (open circles) were loaded with Fura 2-AM and stimulated with increasing concentrations of 9-HODE. Increases in the intracellular calcium concentration were measured by a FLEXstation system (mean ± S.D., n = 4). Data are typical of four independent experiments. Inset, averages of the EC$_{50}$ values of 9-HODE in the intracellular calcium mobilization assay, calculated from four independent stable clones of G2A-a or G2A-b in CHO cells. (mean ± S.D.). (B) The membrane fractions (4 µg) of CHO cells transiently expressing G2A-a (filled circles) or G2A-b (open circles) were incubated with increasing concentrations of 9-HODE. After 30 min incubation, 10 nM Europium labeled-GTP was added and incubated for another 30 min. After filtration of unbound Europium labeled-GTP using a Multiscreen Vacuum Manifold, the fluorescence intensity of Europium trapped in the filter was measured (mean ± S.D., n = 4). Data are typical of three independent experiments. Inset, the averages of the EC$_{50}$ values of 9-HODE in GDP/GTP exchange assays were calculated from four independent experiments (mean ± S.D.).

Fig. 5. pH-dependent inositol 1- phosphate (IP1) accumulation via G2A-a and G2A-b. COS-7 cells
were transiently transfected with vector (triangles), G2A-a (filled circles), G2A-b (open circles) or OGR1 (squares). Cells were exposed to various pH of HEPES/EPPS/MES-buffer for 1 h in the presence of 0.5 M LiCl. Accumulated IP1 was quantified using an IP-One ELISA kit (mean ± S.D., n = 3). Data are typical of three independent experiments.

**Fig. 6.** Role of positively charged amino acids in the N-terminal region of G2A in proton-sensing and basal activity. (A) Amino acid sequences of the N-terminal region of G2A and the other three proton-sensing GPCRs are aligned. Histidine residues (H) that have been shown to be important in proton-sensing activity are marked in bold. Lysine (K) and arginine (R) residues mutated in this study are indicated by arrowheads. (B) COS-7 cells were transiently overexpressed with each G2A expression plasmid, and the expression levels were confirmed by flow cytometric analysis using anti-G2A antibody. (C) Proton-sensing activity of G2A-a, G2A-b and G2A-a mutants. COS-7 cells were transiently overexpressed with wild-type G2A-a, G2A-a (K7A), G2A-a (K31A), G2A-a (R42A) or wild-type G2A-b. Cells were exposed to various pH of HEPES/EPPS/MES-buffer for 1 h in the presence of 0.5 M LiCl. Accumulated IP1 was quantified using an IP-One ELISA kit (mean ± S.D., n = 3). Data are typical of three independent experiments. (D, E) Basal activity of G2A-a, G2A-b and G2A-a mutants. COS-7 cells were transiently transfected with G2A expression plasmid
(wild-type G2A-a, wild-type G2A-b, G2A-a (K7A), G2A-a (K31A) or G2A-a (R42A)),
pTAL-Firefly luciferase vector (SRE (D) or CRE (E)), and CMV promoter-driven Renilla luciferase
vector. Firefly and Renilla luciferase activities were measured using Dual-Glo Luciferase Assay
System, and each Firefly luciferase activity was divided by Renilla’s one to normalize luciferase
activity for each transfection efficiency (mean + S.D., n = 4). Data are typical of three independent
experiments. * $p < 0.05$ (Student’s $t$-test, compared with pcDNA3.1 vector). # $p < 0.05$ (Student’s
$t$-test, compared with wild-type G2A-a).
Fig. 1

A

G2A gene

exon 1  exon 2  exon 3  exon 4

G2A-a

probe 2

probe 1

G2A-b

B

agtcttagac agaacataat tagacctcctg ctaacttcctg aaacctcagc taggactgca

TATA like sequence

putative transcription initiation site

gggaggggtg cgaggctagc cacgcagggc ggccctggg tcatttttaa ctctcagagt

C

G2A-a: MCPMLLKNGTNGNATPVTTPAPVSLGSAKTNCNVSFEERS IVL
G2A-b: MPGNATPVTTPAPVSLGSAKTNCNVSFEERS IVL

N-terminal extracellular region  TM 1

D

probe 1

probe 2

9.49
7.46
4.40
2.37
1.35

G2A-a

G2A-b
Fig. 2

A

G2A-a

Amount of mRNA (% of peripheral blood leukocytes)

G2A-b

Amount of mRNA (% of peripheral blood leukocytes)

B

kb

9.49

7.46

4.40

2.37

G2A-a

G2A-b

Densitometric units

250,000

200,000

150,000

100,000

50,000

0

1

2
Fig. 6

A

OGR1: MGNITADNSSMSCTIDHTIHQ TLA •
TDAG8: MNSTCIEBQHDLDH YLF •
GPR4: MGNTWEGCHVDSRVDH LFP •
G2A-a: MCPMLKNGTNATPVTTPAPWASLGLS A KTCNNV8FEESR IVL •
G2A-b: MPGNATPVTTPAPWASLGLS A KTCNNV8FEESR IVL •

N-terminal extracellular region

TM 1

B

Cell counts

mock  
G2A-a  
G2A-b  
G2A-a (K7A)  
G2A-a (K31A)  
G2A-a (R42A)

Fluorescence intensity

C

Inositol 1-phosphate (%vector[pH 8.2])

pH

control  
G2A-a  
G2A-b  
G2A-a (K7A)  
G2A-a (K31A)  
G2A-a (R42A)

D

SRE

Ratio

vector  
G2A-a  
G2A-b  
G2A-a (K7A)  
G2A-a (K31A)  
G2A-a (R42A)

E

CRE

Ratio

vector  
G2A-a  
G2A-b  
G2A-a (K7A)  
G2A-a (K31A)  
G2A-a (R42A)