

Gab2 antisense oligonucleotide blocks rat basophilic leukemic cell functions[☆]

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Received 22 January 2007; received in revised form 2 March 2007; accepted 5 March 2007

Abstract

Adapter molecule Grb2-associated binder-like protein 2 (Gab2) plays a critical role in FcεRI-induced mast cell degranulation and activation. The present study aimed to investigate the pharmacological effects of an antisense oligonucleotide (ASO) targeted at Gab2 on the immune responses of rat basophilic leukemic (RBL)-2H3 cells. Gab2 ASOs were rationally designed and transfected into RBL-2H3 cells. Gab2 mRNA and protein knockdown was confirmed by real-time RT–PCR and immunoblotting, respectively. Effects of Gab2 ASO on FcεRI-induced release of histamine and β-hexosaminidase was measured by EIA and an enzymatic assay, respectively; signaling events by immunoblotting; and cytokine mRNA expression by RT–PCR. Effects of Gab2 ASO on cell adhesion and migration were performed on fibronectin-coated 96-well plate and transwells cell culture chambers, respectively. We have characterized a phosphorothioate-modified ASO targeted at Gab2 mRNA that was able to knockdown Gab2 mRNA and protein in RBL-2H3 cells. Gab2 ASO significantly blocked IgE-mediated mast cell release of β-hexosaminidase and histamine; phosphorylation of Akt, p38 mitogen-activated protein kinase and PKCδ; and up-regulation of cytokine mRNA levels (e.g. IL-4, -6, -9 and -13, and TNF-α). In addition, Gab2 ASO markedly prevented mast cell adhesion to fibronectin-coated plates and restrained random migration of RBL-2H3 cells in cell culture chambers. Our findings show that Gab2 knockdown in RBL-2H3 cells by ASO strategy can suppress many aspects of the mast cell functions and, therefore, a selective Gab2 ASO may have therapeutic potential for mast cell-dependent allergic disorders.

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Keywords: Adhesion; Akt; FcεRI; p38 MAPK; RBL-2H3

1. Introduction

Mast cell plays an important role in allergic disorders. Upon aggregation of the high-affinity IgE receptors (FcεRI) by multivalent allergen on mast cells, a diverse array of mediators including histamine, leukotrienes, cytokines and chemokines, is released that ultimately contributes to allergic responses [1]. It is believed that FcεRI-mediated signaling events in mast cells can be divided into two independent but

[☆] This work was supported by a BioMedical Research Council of Singapore grant (BMRC/01/1/21/17/046).

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Table 1

Sequences of Gab2 ASO and inverted oligonucleotides

Name	Gab2 ASO	Inverted ODNs
ASO1	5'-TCGCCGCCGCCGCCGCTCAT-3'	5'-TACTCGCCGCCGCCGCCGCT-3'
ASO2	5'-CTTTTCCGGAGGCGATTTCCT-3'	5'-TCCTTTAGCGAGGCCTTTTC-3'
ASO3	5'-CGCCTCAACTTCTTTCCGG-3'	5'-GGCCTTTTCTTCAACTCCGC-3'
ASO4	5'-TGCTGGTGATGCTCTGTGTG-3'	5'-GTGTGTCTCGTAGTGGTCGT-3'

complementary pathways. The first pathway is mediated through proximal signaling molecules Lyn/Syk/LAT (linker for activation of T cells), leading to activation of phospholipase C γ (PLC γ) critical for increasing intracellular Ca²⁺ level and protein kinase C (PKC) activity [2,3]. This is referred to as the Ca²⁺-dependent pathway. The second pathway is Ca²⁺-independent and mediated through Fyn/Gab2 (Grb2-associated binder-like protein 2)/PI3K (phosphoinositide 3-kinase) signaling cascade [4,5]. Fyn-null bone marrow-derived mast cells (BMMCs) revealed marked impairment in IgE-induced mast cell degranulation together with substantial drop in Gab2 phosphorylation [5]. Likewise, in Gab2-null BMMCs, IgE-induced mast cell degranulation was severely impaired and the activity of downstream signaling molecules PI3K and Akt were largely abolished [4]. More recent findings revealed a critical role of Syk in activating Gab2 leading to mast cell activation [6]. These findings indicate that Gab2 plays an important role in relaying upstream Fc ϵ RI activation signal to downstream kinases to induce mast cell degranulation and activation in a Ca²⁺-independent manner. Gab2 appears to be a very attractive target for the modulation of mast cell activity.

As Gab2 is an intracellular adapter protein, one of the best ways to regulate the function of adapter molecule pharmacologically is to use antisense oligonucleotide (ASO) technology [7,8]. ASO is usually a short strand of nucleic acid (typically 20-bp) that is complementary to the target mRNA. Hybridization of ASO to the intended mRNA can result in specific inhibition of target gene and protein expression. The purpose of this study was to design and develop a Gab2-specific ASO

and to investigate its potential immunopharmacological actions in RBL-2H3 mast cell line.

2. Materials and methods

2.1. Materials

The following materials were used: RBL-2H3 cell line (American Type Culture Collection, Rockville, MD); Eagle's minimal essential medium (MEM), lipofectin, Opti-MEM and random primer (Invitrogen, Carlsbad, CA); RNAqueous-4PCR mRNA isolation kit (Ambion, Austin, TX); Taqman universal master mix, and assay-on-demand real-time Gab2 primer and probe (ABI Biosystems, Foster City, CA); Gab2 PS-modified ASOs and corresponding inverted oligodeoxynucleotides control (ODN), and IL-4, IL-6, IL-9, IL-13, TNF- α and β -actin primer sets (Proligo, Boulder, CO); anti-Gab2 mAb (Upstate Biotechnology, Charlottesville, VA); dinitrophenyl (DNP)-specific mouse IgE, DNP-human serum albumin (DNP-HSA) and fibronectin (Sigma, St. Louis, MO); histamine EIA kit (SPI bio, Massy Cedex, France); anti-ERK and -phospho-ERK (Thr²⁰²/Tyr²⁰⁴) mAbs, anti-Akt and -phospho-Akt (Ser⁴⁷³) mAbs, anti-PKC δ and -phospho-PKC δ (Tyr³¹¹) Abs, and anti-p38 MAPK and -phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) Abs (Cell Signaling Technology, Beverly, MA); transwells cell culture chambers (Corning Costa, Cambridge, MA); and ECL reagent (Amersham, Piscataway, NJ).

2.2. Design and synthesis of ASOs

Four Gab2 ASOs were designed to target at different sites of the coding region of rat Gab2 mRNA using published mRNA sequence (GenBank accession number AF230367). The ASO design strategy was based on a combined consideration of free energy for duplex ASO/mRNA

Table 2

Primer sets for cytokine RT-PCR

Cytokine	Forward primer	Reverse primer
IL-4	5'-ACCTTGCTGTACACCTGTTC-3'	5'-TTGTGAGCGTGGACTCATTC-3'
IL-6	5'-GAAATGATGGATGCTTCCAACTGG-3'	5'-GGATATATTTCTGACACAGTGAGG-3'
IL-13	5'-GCTCTCGCTTGCCTTGGTGGTC-3'	5'-CATCCGAGGCCTTTTGGTTACAG-3'
IL-9	5'-TCCAACACACCTCTTACC-3'	5'-AGTATCTGTCTTACGCC-3'
TNF- α	5'-CAAGGAGGAGAAGTTCCCAA-3'	5'-CGGACTCCGTGATGTCTAAG-3'
β -Actin	5'-TAACCAACTGGGACGATATG-3'	5'-ATACAGGGACAGCACAGCCT-3'

formation, G–C contents, specific sequence motifs, and secondary mRNA structures [9–11]. ASOs were synthesized with full backbone modification with PS to resist endonuclease degradation and promote RNase H-mediated mRNA degradation [7,8]. The sequences of these Gab2 ASOs and their corresponding inverted ODN controls are shown in Table 1.

2.3. Gab2 ASO transfection into RBL-2H3 cells

RBL-2H3 cells were maintained as monolayer in Eagle's MEM. To study Gab2 mRNA and protein knockdown, RBL-

2H3 cells were transfected with Gab2 ASO or inverted ODN in the form of a complex with lipofectin (10 µg/ml) in Opti-MEM for 4 h. Transfected cells were allowed to recover for 24 h in complete Eagle's MEM. Cells were then analyzed for mRNA and protein expression. To investigate inhibitory actions of Gab2 ASO on mast cell functions, the transfected RBL-2H3 cells were allowed to recover for 24 h in the presence of anti-DNP IgE (1:5000). The sensitized cells were then activated with DNP-HSA (300 ng/ml) for indicated time points at 37 °C. Cells were lysed for immunoblotting and mRNA expression analyses.

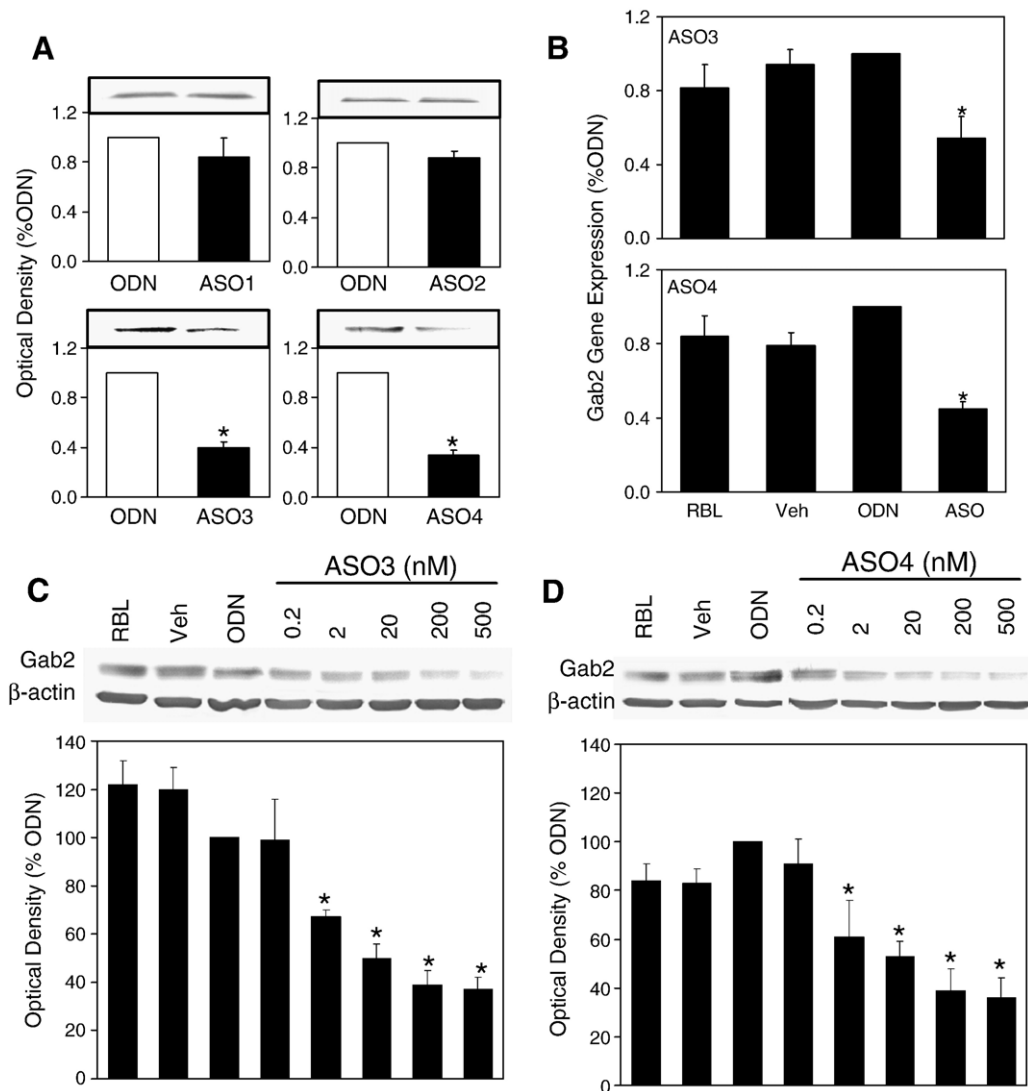


Fig. 1. Characterization of Gab2 ASO. RBL-2H3 cells were transfected with 200 nM ASO1, ASO2, ASO3 or ASO4 for 4 h. Corresponding inverted ODNs were used as controls. Whole cell extracts were prepared 24 h later, and proteins (40 µg per lane) were separated by SDS–PAGE and probed with anti-Gab2 mAb (A). Gab2 mRNA levels measured by real-time PCR were significantly suppressed by ASO3 and ASO4 (B). Gab2 protein expression in RBL-2H3 cells was inhibited in a concentration-dependent manner (0.2–500 nM) by ASO3 (C) and ASO4 (D). Results are expressed as percentage of inverted ODN controls. Values are expressed as mean ± S.E.M. of 4 separate experiments. Veh, lipofectin. *Significant difference from inverted ODN control, $P < 0.05$.

2.4. Immunoblotting

Protein lysates were subjected to SDS-PAGE and immunoblotting with anti-Gab2 mAb, or with Abs raised against PKC δ and phospho-PKC δ , Akt and phospho-Akt, ERK and phospho-ERK, and p38 MAPK and phospho-p38 MAPK. Immunoblots were developed using ECL reagent and quantitated using Gel-Pro imaging software (Media Cybernetics, Silver Spring, MD).

2.5. Gene expression

Total mRNA from Gab2 ASO-transfected cells was extracted using RNA isolation kit. Quantitative real-time PCR was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems). Gab2 mRNA expres-

sion was normalized with ribosomal 18S endogenous control. To investigate inhibitory actions of Gab2 ASO on cytokine mRNA production from activated mast cells, total RNA was isolated from transfected RBL-2H3 cells sensitized with DNP-specific IgE overnight and stimulated with DNP-HSA for 2 h. The primer sets for cytokines are summarized in Table 2. PCR products were run in a 2% agarose gel, visualized under UV light and analyzed using the Gel-Pro imaging software.

2.6. β -Hexosaminidase and histamine measurements

Transfected RBL-2H3 cells were sensitized with DNP-specific IgE overnight and challenged with DNP-HSA to induce mast cell degranulation for 1 h before supernatants were collected. β -Hexosaminidase enzymatic assay was performed as previously described [12]. β -Hexosaminidase

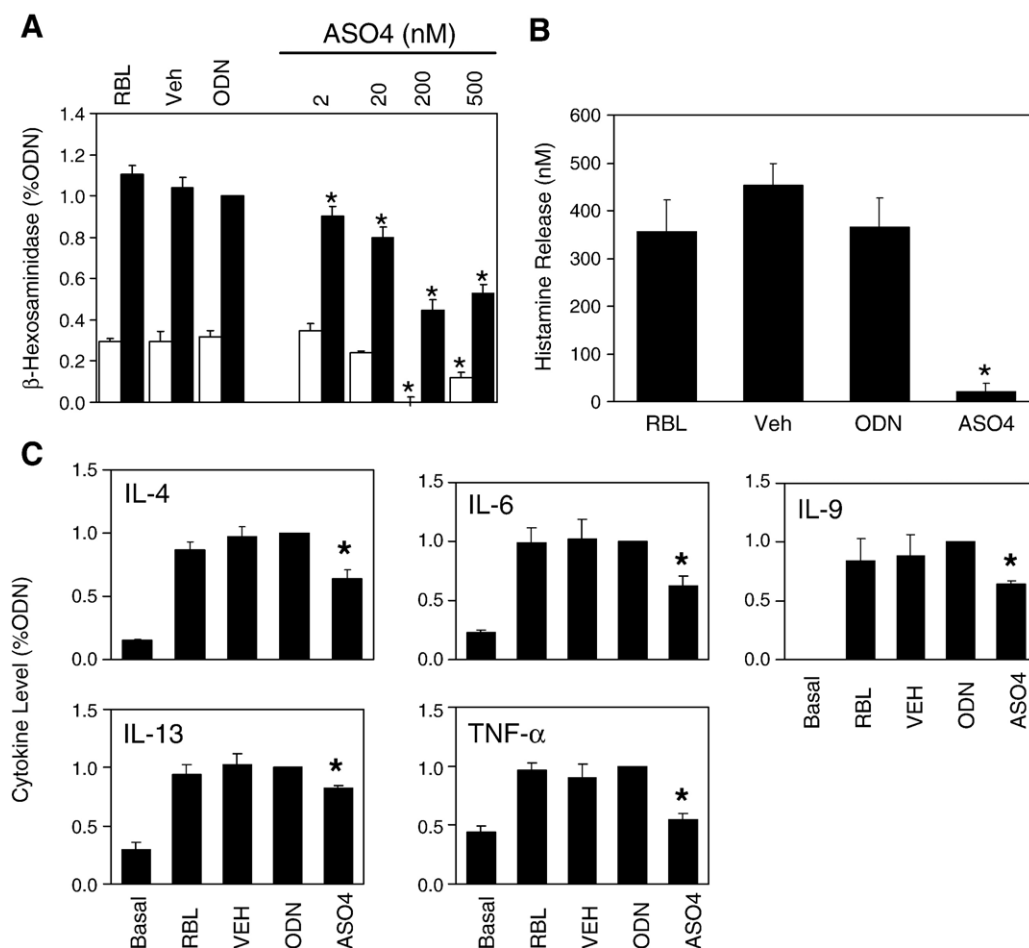


Fig. 2. Effects of Gab2 ASO on Fc ϵ RI-mediated mast cell degranulation (A and B) and cytokine mRNA expression (C). (A) β -Hexosaminidase release was measured using a colorimetric enzymatic assay as described (12). Open bar indicates β -hexosaminidase basal release and filled bar indicates DNP-HSA-induced release. Results are expressed as percentage of inverted ODN control. (B) Histamine release was measured using a colorimetric EIA system. (C) Cytokine mRNA productions were determined by RT-PCR. Results are expressed as percentage of inverted ODN control. Data are expressed as mean $\pm$ S.E.M. of 3–5 separate experiments. RBL, RBL-2H3; Veh, lipofectin. *Significant difference from inverted ODN control, $P < 0.05$.

release was expressed as absorbance in the supernatant divided by total absorbance in both supernatant and cell lysate. Histamine release was determined using an EIA system. Samples were assayed in duplicate.

2.7. Adhesion and migration assays

Mast cell adhesion to fibronectin-coated wells was performed as previously described [13]. Adherent cells were fixed with 2.5% formaldehyde and stained with 0.1% crystal violet before lysis in 10% acetic acid. Absorbance was determined using a microplate reader (Tecan, Austria) at 570 nm. Mast cell migration assay was performed in fibronectin-coated transwells cell culture chambers (8 μ m pore size and 6.5 mm diameter) as described [14]. Cells that had migrated to the lower side of the membrane were fixed, stained and then counted under a light microscope in five randomly selected fields (original magnification $\times$ 400).

2.8. Statistical analysis

Data are presented as mean $\pm$ S.E.M. Statistical differences between inverted ODN and Gab2 ASO groups were analyzed using one way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Significance level was set at $P < 0.05$.

3. Results

3.1. Characterization of Gab2 ASO

We have designed and screened four Gab2 ASOs targeted at different sites of the coding region of rat Gab2 mRNA. These ASOs hybridized with either the start codon sequence 1–20 bp (ASO1), early first stem loop sequence 46–66 bp (ASO2), late first stem loop sequence 58–77 bp (ASO3) or carboxyl-terminal conserved bulge loop 1671–1690 bp (ASO4). Their corresponding ODN controls consist of exactly the same nucleotide composition and chemical makeup but in inverted sequences which have been confirmed by genome-wide search not to bind to any known rat mRNA. Fig. 1A clearly shows that ASO1 and ASO2 failed to knockdown Gab2 protein expression in RBL-2H3 cells. In contrast, ASO3 and ASO4 markedly suppressed Gab2 protein expression 24 h after transfection by about 60% and 66%, respectively, as compared to their corresponding inverted ODNs ($P < 0.05$). Our preliminary time-course study using ASO3 revealed a Gab2 knockdown effect occurred at 10 h, peaked at 24 h, and disappeared at 36 h after transfection (data not shown). Immunoblotting results showed that both ASOs down-regulated Gab2 protein level in a concentration-dependent manner (Fig. 1C and D). The IC_{50} values for Gab2 protein knockdown for ASO3 and ASO4 were 1.5 nM and 1.2 nM, respectively. Quantitative real-time PCR analysis revealed significant reductions ($P < 0.05$) in Gab2 mRNA level by 46% and 55% in RBL-2H3 cells transfected with 200 nM ASO3 or ASO4, respectively, as compared with

inverted ODNs (Fig. 1B). Taken together, ASO4 has demonstrated a consistently superior Gab2 protein and mRNA knockdown activity, and therefore, it was selected for all subsequent mast cell functional studies.

3.2. Gab2 ASO inhibits mast cell degranulation

ASO4 significantly ($P < 0.05$) inhibited DNP-HSA-induced β -hexosaminidase release from RBL-2H3 cells in a concentration-dependent manner as compared with inverted ODN control (Fig. 2A). ASO4 also suppressed basal β -hexosaminidase release. In addition, ASO4 (200 nM) substantially abated DNP-HSA-induced histamine release from RBL-2H3 cells by 94% as compared with inverted ODN control (200 nM) (Fig. 2B).

3.3. Gab2 ASO blocks Fc ϵ RI-induced cytokine production in mast cells

As shown in Fig. 2C, DNP-HSA challenge induced substantial cytokine production as compared to basal condition. ASO4 (200 nM) significantly ($P < 0.05$) reduced IL-4, IL-6, IL-9, IL-13 and TNF- α mRNA expression as compared with inverted ODN control.

3.4. Gab2 ASO blocks Fc ϵ RI-induced signaling events in mast cells

Gab2 has been shown to act directly upstream of PI3K [3–5]. DNP-HSA challenge of RBL-2H3 cells induced sustained serine phosphorylation of Akt, a direct downstream

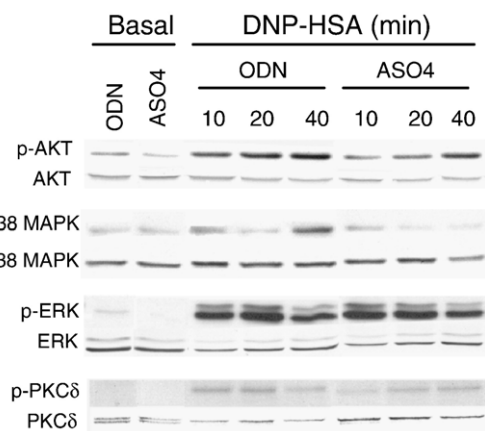


Fig. 3. Effects of Gab2 ASO on Fc ϵ RI-induced signaling in RBL-2H3 cells. ASO4-transfected RBL-2H3 cells were sensitized with DNP-specific IgE and challenged with DNP-HSA for 10, 20 and 40 min to determine the phosphorylation states of Akt, p38 MAPK, ERK and PKC δ . Inverted ODN was used as negative control. Total proteins (20 μ g per lane) were separated by SDS–PAGE, probed with relevant Abs and developed with ECL reagent. Immunoblots shown are representatives of 3–5 separate experiments with similar patterns of results.

substrate of PI3K. This PI3K-dependent phosphorylation of Akt was significantly impaired by ASO4 (200 nM) as compared with the inverted ODN (200 nM). ASO4 also suppressed FcεRI-induced phosphorylation of PKCδ, another downstream molecule of PI3K. In addition, ASO4 markedly abrogated FcεRI-induced p38 MAPK phosphorylation as compared with the inverted ODN (Fig. 3). In contrast, ASO4 did not alter antigen-induced ERK phosphorylation in RBL-2H3 cells. These findings confirm that Gab2 plays a role in the activation of PI3K and p38 MAPK pathways upon FcεRI cross-linking on mast cells.

3.5. Gab2 ASO blocks mast cell adhesion and migration

As shown in Fig. 4A and B, RBL-2H3 cells could readily adhere to fibronectin-coated wells without any stimulation and ASO4 markedly prevented mast cell adhesion by 60% ($P<0.05$) as compared to inverted ODN. As shown in Fig. 4C, RBL-2H3 cells could randomly migrate to the underside of the transwells. ASO4 markedly blocked the random migration by 60% ($P<0.05$) as compared to inverted ODN.

4. Discussion

BMMC derived from Gab2-deficient mice elicited dramatically lower FcεRI-mediated mast cell degranulation and activation [4]. Gab2 is a 97-kDa scaffolding protein belonging to the *Drosophila* daughter of sevenless (Dos)/Gab adapter family, and contains a pleckstrin homology (PH) domain at the N terminus that can bind to phosphoinositide-3,4,5-triphosphate (PIP₃) in the plasma membrane, multiple tyrosine phosphorylation motifs at the C terminus that allow binding with SH2 domain-containing proteins such as p85 subunit of PI3K, PLCγ and SHP2, and two proline-rich motifs that physical interact with SH3-containing proteins such as Grb2, Src tyrosine kinases and PLCγ [15]. Therefore, knocking down Gab2 protein level pharmacologically using ASO is likely to produce immunomodulatory actions on mast cell functions.

ASO1 targeting at 1–20 bp of Gab2 mRNA coding region was designed based on the fact that many effective ASOs are directed at the translation initiation site (AUG codon). By using *mfold* and *sfold* algorithm strategies predicting nucleic acid secondary structures [16,17], we have identified additional accessible single-stranded sites for ASO hybridization with Gab2 mRNA, which include 46–66 bp for ASO2, 58–77 bp for ASO3, and 1671–1690 bp for ASO4. In this study, only ASO3 and ASO4 demonstrated potent Gab2 gene and protein knock-down activities, and since the ASO4 could form a more stable heteroduplex with Gab2 mRNA based on predicted free energy for the complex, together with a consistently stronger inhibition of Gab2 gene and protein expression than ASO3, ASO4 was chosen to investigate its potential immunomodulatory actions in mast cells.

ASO4 markedly suppressed FcεRI-induced mast cell degranulation by a substantial drop in β-hexosaminidase and histamine levels in cell culture supernatants. Recent study demonstrated that Gab2 contributes to Ca²⁺-independent microtubule formation via activation of a small GTPase RhoA, a critical step for translocation of cytoplasmic granules to the plasma membrane before mast cell exocytosis upon FcεRI aggregation

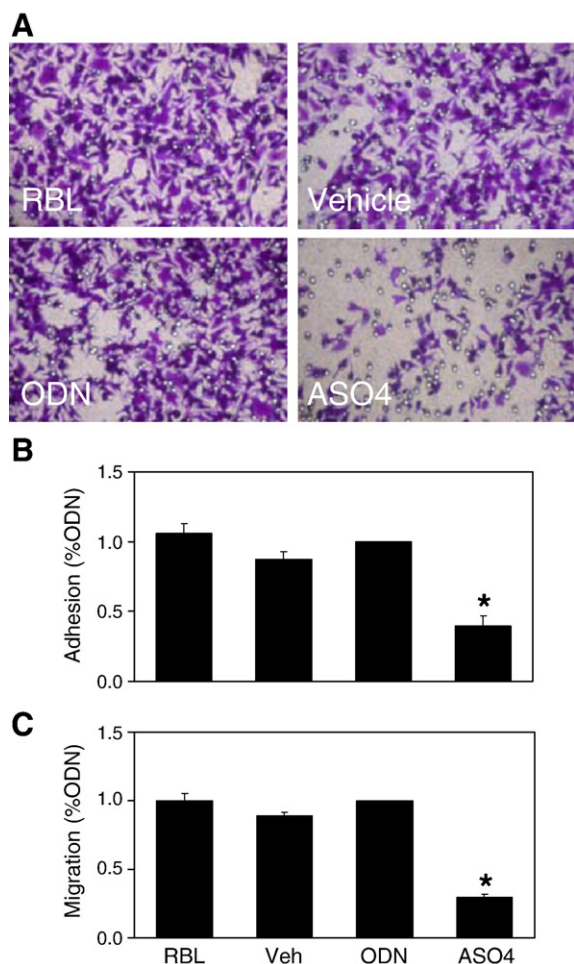


Fig. 4. Effects of Gab2 ASO on RBL-2H3 cell adhesion and migration on fibronectin. (A) Representative photomicrographs showing RBL-2H3 spontaneous adhesion to fibronectin-coated plates with and without vehicle (lipofectin), inverted ODN (200 nM) or ASO4 (200 nM). (B) Quantitative analysis of RBL-2H3 cell adhesion to fibronectin-coated plates. (C) RBL-2H3 cells that had migrated to the lower chamber were counted under a light microscope. Results are expressed as percentage of inverted ODN control. Values shown are mean±S.E.M. of 3–5 separate experiments. Veh, lipofectin. *Significant difference from inverted ODN control, $P<0.05$.

[12]. In addition, Ca^{2+} -independent PKCs (e.g. PKC δ and PKC θ) have been shown to mediate antigen-induced mast cell activation and degranulation [18,19]. Gab2 plays a critical role in the activation of PI3K leading to phosphoinositide-dependent kinase-1 (PDK-1) stimulation of Ca^{2+} -independent PKC δ isoform and mast cell degranulation [4,20–22]. Our findings demonstrate that Gab2 knockdown using ASO can be an effective strategy in blocking Fc ϵ RI-mediated mast cell degranulation.

Gab2-deficient BMMCs showed a severely impaired PI3K as manifested by decreased phosphorylation of PDK-1 and Akt [4]. Akt plays a key role in mast cell survival, proliferation and cytokine production [23]. Activated PDK-1 can selectively stimulate PKC δ activity leading to mast cell exocytosis, cytokine production and cysteinyl leukotriene synthesis [4,18,20,24]. In addition, Gab2-deficient BMMCs showed a marked reduction in phosphorylation of Jun N-terminal kinase (JNK) and p38 MAPK in response to Fc ϵ RI activation. JNK and p38 MAPK have been shown to be important for cytokine production in mast cells [25]. In this study, ASO4 markedly abated Fc ϵ RI-induced phosphorylation of downstream signaling molecules such as Akt, p38 MAPK and PKC δ in RBL-2H3 cells, which may ultimately regulate immune functions of mast cells.

Fc ϵ RI cross-linking on mast cells induces production of pro-inflammatory cytokines which may contribute to the development of Th₂ immune response [1]. In this study, ASO4 significantly suppressed mast cell mRNA levels of IL-4, IL-6, IL-9, IL-13 and TNF α in response to Fc ϵ RI aggregation. In Gab2-deficient BMMCs, Fc ϵ RI-induced production of IL-6 and TNF α has been impaired [4]. In human mast cell (HMC-1) and murine mast cell (MC/9) cultures, JNK and p38 MAPK have been shown to mediate IL-4, IL-6 and TNF α production upon Fc ϵ RI activation [26–29]. Over-expression of PKC δ in RBL-2H3 cells has also been shown to increase production of IL-2, IL-3, IL-4, IL-6, IL-9 and TNF α in response to Fc ϵ RI activation [30]. Taken together, Gab2 knockdown by ASO strategy in mast cells can markedly reduce antigen-induced cytokine production probably via inhibition of downstream signaling molecules like p38 MAPK and PKC δ .

Similar to BMMC, RBL-2H3 cells express high level of cell-surface $\alpha\beta$ 1 integrin that binds selectively to extracellular matrix protein fibronectin [31]. β 1-Integrin cross-linking in RBL-2H3 cells has been shown to induce Gab2-PI3K signaling pathway that lead to activation of Akt and PKC δ , and cell adhesion and migration. Inhibition of Gab2 signaling pathway leads

to inhibition of cell adhesion and migration [14,13]. The present study revealed that Gab2 knockdown by ASO4 dramatically suppressed RBL-2H3 cell adhesion and random migration in fibronectin-coated wells. These observations are likely associated with the ability of ASO4 down-regulating the phosphorylation of Akt and PKC δ in RBL-2H3 cells.

We have characterized a Gab2-selective ASO in RBL-2H3 cells capable of preventing IgE-mediated mast cell degranulation and cytokine production, and blocking RBL-2H3 cell adhesion and migration in fibronectin-coated surface. These inhibitory effects are likely mediated by the ASO4-induced inhibition of Akt, p38 MAPK and PKC δ activation. Therefore, future study of Gab2 ASO in animal models of allergy is warranted and Gab2 ASO pharmacological approach may have therapeutic potential for the treatment of mast cell-associated disorders.

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