Upregulation of the transcript level of GTPase activating protein KIAA0603 in T cells from patients with atopic dermatitis

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Abstract We have analyzed transcription profiles in peripheral blood CD3+ cells from patients with allergic diseases to better understand the genes that are involved. Transcription levels of the gene *KIAA0603*/*AS160* in CD3+ cells from patients with atopic dermatitis (AD) were significantly higher than in normal individuals. The *KIAA0603* gene encodes a 1299 amino acid protein with two phosphotyrosine interaction domains at the N-terminal region and a TBC domain at the C-terminal region. The region containing the TBC domain has a 31% homology to human rab6 GTPase activating protein (GAP). When human primary CD3+ cells were stimulated with anti-CD3 or calcium ionophore, the *KIAA0603* transcript level was upregulated. The marked upregulation of KIAA0607 was accompanied by activation induced cell death of primary CD3+ cells. KIAA0603 is likely to be a Rab GAP that participates in the regulation of activated T cells, especially helper memory T cells. Expression of *KIAA0603* in T cells may be involved in pathogenesis of AD.

Keywords: T cell; GTPase activating protein; Atopic dermatitis

1. Introduction

T cells orchestrate immune responses against antigenic challenges. The Th2 subset of helper T cells produces Th2 cytokines, such as IL-4, IL-5 and IL-13, that play critical roles in the pathogenesis of allergic diseases. Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease that is characterized by pruritus, elevated serum IgE levels, and peripheral blood eosinophilia. A two-phase model of the pathogenesis of AD has been proposed, in which T cells play an immunoregulatory role in both the physiologic and pathologic immune responses. An AD episode is initiated by an inflammatory response in which the Th2 cells predominate. The eczematous phase that follows is dominated by Th1 cells, which produce IL-2 and IFN-γ [1,2].

Allergic diseases are complex because multiple genes are thought to be involved, as well as environmental factors. We have analyzed transcription profiles in peripheral blood immune cells from patients with allergic diseases and found many genes that were differentially expressed when patients and healthy individuals were compared [3,4].

During differential gene expression analysis of human peripheral blood CD3+ cells from patients with allergic diseases, such as AD and asthma, we observed that the expression levels of the gene, *KIAA0603*, were higher in AD patients than in healthy controls. The deduced amino acid sequence of KIAA0603 showed homology to GTPase activation proteins (GAP). The Ras superfamily of small GTP-binding proteins (G protein) comprises a large group of proteins involved in signal transduction, proliferation, vesicle trafficking, and regulation of the actin cytoskeleton. The G protein is activated by GTP binding and inactivated when the bound GTP is converted to GDP. The G protein has GTPase activity that is enhanced by GAP. GAP is involved in conversion of the G protein to the inactivated form [5].

We describe the identification and analysis of the expression profiles of *KIAA0603* in CD3+ cells from allergic patients. Transcriptional upregulation of *KIAA0603* after stimulation through the T cell receptor, especially during induction of apoptosis, is discussed.

2. Materials and methods

2.1. Study population

Patients with allergic disease and healthy individuals were both recruited for participation in the study. Patient profiles and clinical parameters have been described in detail [3]. Patients with AD were diagnosed according to the criteria of Hanifin [6]. Diagnosis of atopic asthma and classification of asthma severity were based on the asthma prevention and management guidelines [7]. Written informed consent to participate in the study was obtained from all subjects. The conduct of the study was in accordance with the guidelines established in the National Research Institute for Child Health and Development.

2.2. Identification of KIAA0603 gene by differential display analysis

The methods for differential display analysis (DD) of human peripheral blood T cells from allergic patients have been described in detail [3]. A slightly modified version of the fluorescent differential display method described by Ito et al. [8] was used in this study.

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Abbreviations: AD, atopic dermatitis; GAP, GTPase activating protein; G protein, GTP binding protein; PBMC, peripheral blood mononuclear cell; DD, differential display analysis; AICD, activation induced cell death

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Transcripts in CD3+ cells were analyzed by the DD PCR using different primer sets in a combination of 3′-anchored oligo-dT primers and arbitrary decamer primers. The DNA fragment that was obtained by DD PCR was further elongated by PCR cloning using a kit based on the 5′-RACE method (Clontech, Palo Alto, CA, USA). A human leukocyte cDNA (Clontech) was used as a template for PCR.

2.3. Preparation of leukocyte cDNA samples

Peripheral blood mononuclear cells (PBMCs) were prepared from 10–20 ml of venous blood. After dextran sedimentation, white cells were separated using standard methods for Ficoll-Hypaque gradient centrifugation. CD3+ cells were isolated using a magnetic cell sorter (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The percentage of CD3+ T cells was >97%. CD16-negative eosinophils, obtained from the sedimented granulocyte fraction of the Ficoll-Hypaque gradients, were isolated by negative selection using a magnetic cell sorter. The percentage of eosinophils was >97%. CD14+ monocytes were prepared from cells remaining after separation of CD3+ cells. The cells remaining after separation of CD14+ cells were the source of B cells. CD16+ granulocytes remaining after separation of CD16− cells were the source of neutrophils. Total RNA was prepared using an RNA extraction kit (RNeasy Mini, Qiagen GmbH, Hilden, Germany). DNase-treated total RNAs were mixed with oligo $(dT)_{12-18}$ primer or random primers and first strand cDNAs were synthesized using SuperScript II reverse transcriptase.

2.4. Preparation of T cell subsets

CD8+ cells were prepared from PBMCs of a healthy volunteer by magnetic sorting. CD4+ cells were isolated using CD4 multisort microbeads (Miltenyi Biotec) from cells remaining after separation of CD8+ cells. After removing the magnetic particles from the CD4+ cells, CD45RA+ cells were isolated. The CD45RO+ cells were prepared from the cells remaining after separation of CD45RA+ cells.

2.5. Real time quantitative RT-PCR

Real time RT-PCR for quantitation of gene expression was performed using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, USA). Based on the ORF of *KIAA0603*, primers and a dual-labeled fluorogenic probe (TaqMan Probe) were designed using the computer program Primer Express 1.0 (PE Applied Biosystems, Foster City, USA). The primer sequences were 5′-AAGACAGTGGAGCAACTCCGG-3′ (forward) and 5′-CAGCAACAGGTCACAATTGGC-3′ (reverse) and the probe sequence was 5′-AGCTGCTGCCCGCGGATGCT-3′. The quantitative PCR was performed using a TaqMan PCR Reagent Kit according to the manufacturer's protocol (PE Applied Biosystems). Sample cDNAs, equivalent to 5 ng of starting RNA, were used for each reaction in a 96-well PCR plate. The fragment that was PCR amplified from the human leukocyte cDNA library template by using the forward and reverse primers was cloned into a plasmid. The resulting plasmid DNA was used to prepare absolute standards.

Levels of β-actin mRNA were used as an internal standard for each sample. β-actin was quantified in each freshly isolated sample as an absolute value. The copy number of β-actin was averaged for all such samples and the ratio of absolute to average β-actin values was used to normalize the copy number of the target sequence. For in vitro experiments using primary T cells, ribosomal RNA was used for an internal standard instead of β-actin. Quantity of ribosomal RNA was measured using commercially available control reagents (VIC probe, PE Applied Biosystems).

2.6. Northern blot hybridization

Commercially available Northern blot analysis membranes (human MTN Blot, Clontech) were used to analyze human tissues for *KIAA0603* mRNA. Plasmid clone HG01488 containing the complete coding region of *KIAA0603* was obtained from Kazusa DNA Research Institute. The plasmid DNA was labeled with ^{32}dCTP using a random primer labeling kit (Takara, Kyoto, Japan) and used as probe. Hybridization was carried out using ExpressHyb™ Hybridization Solution (Clontech) as described in the manufacturer's protocol.

2.7. Activation-induced cell death of T cells

CD3+ cells were isolated from PBMCs and maintained in RPMI 1640 medium supplemented with 5% FCS, 2mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml). For the first activation, CD3+ cells (5×10^5 cells/ml) were placed into 6-well plates that had been pre-coated with 10 μg/ml of CD3 monoclonal antibody (mAb, Orthoclone OKT3, Ortho Biotech, South Raritan, USA) and cultured using media both with and without 100 U/ml of IL-2, for various time periods before analysis of gene expression. For the second activation, cells were initially activated at a density of 1×10^5 cells/ml, using media with IL-2 (200 U/ml). After a 5-day expansion period, the cells were harvested and re-incubated for additional 3 days in medium containing 200 U/ml IL-2. For the second activation, cells were harvested and placed into anti-CD3 coated 6-well plates in medium containing 200 U/ml IL-2. For stimulation with ionomycin (1 μg/ml), cells were incubated in plates without anti-CD3. Cell viability was determined by trypan blue exclusion.

2.8. Modeling of tertiary structure of KIAA0603

Models of protein KIAA0603 were generated based on the homology modeling method. PSI-Blast was first used to find templates in the PDB95 database. Modeler [9] was then used to create a model based on the PSI-blast alignment. To evaluate the quality of the model, the scores from Profile 3D and PMF methods were combined to verify the score [10–12].

2.9. Statistical analysis

Statistical analysis was computer-based and used StatView 5 software (SAS Institute, Cary, NC, USA). The *t*-test was used for comparisons between the normal controls and patient groups. When multiple comparisons were made between groups, significant between-group variability was first established using the ANOVA. The Fisher's PLSD was then used for intergroup comparisons. Probability values of $P < 0.05$ were accepted as significant.

3. Results

3.1. Isolation of DNA fragment by differential display analysis

Peripheral blood CD3+ cells were prepared from 59 subjects (12 healthy volunteers, 23 AD patients, and 24 asthmatic patients). A 164 bp DNA fragment that was amplified using an anchor primer 5′-GT_{15}C-3′ and an arbitrary primer 5′-TCTCTGGAGT-3′ was identified as being expressed at a higher level in allergic patients than in normal individuals in the DD PCR. To confirm the result of DD study, the transcription level of the 164 bp DNA fragment in the same RNA samples was examined with quantitative RT-PCR. The 164 bp DNA fragment was expressed at a significantly higher level in the AD patients than in the healthy controls ($P < 0.05$, data not shown).

The 164 bp DNA fragment was further elongated to obtain a DNA fragment of 486 bp. A BLAST search on a public database revealed that position 27–255 of the 486 bp nucleotide sequence had 100% identity to the cDNA of *KIAA0603* (GenBank Accession No. AB011175), previously identified by the Kazusa DNA Research Institute [13]. Furthermore, the 486 bp sequence was completely identical to the nucleotides 128071–127586 (reverse strand) of the genome sequence (Accession No. AL162571) that encodes *KIAA0603* gene on chromosome 13. The 164 bp DD fragment was considered to be derived from the immature mRNA intron region of *KIAA0603* gene. The KIAA0603/AS160 protein is the product of the human TBC1D4 gene that maps to 13q21.33.

3.2. Structure analysis of KIAA0603

The deduced KIAA0603 aa sequence encoded a protein of 1299 residues, which had two phosphotyrosine interaction domains (167–237 and 415–487 aa) at the N-terminal region and a TBC domain (965–1184 aa) at the C-terminal region (Fig. 1A). The full amino acid sequence of KIAA0603/human

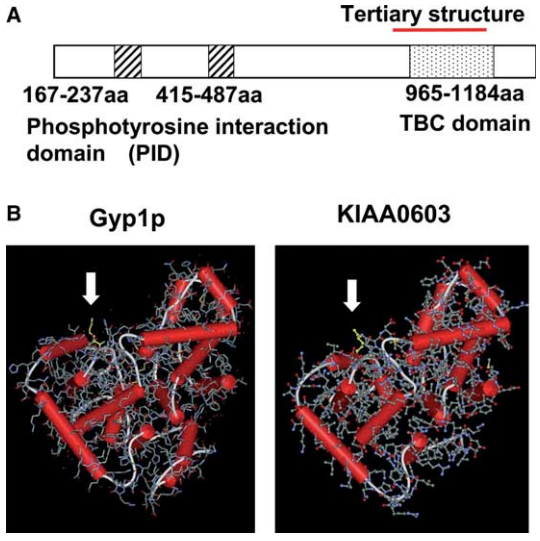

Fig. 1. Domain and tertiary structures of KIAA0603. (A) Two phosphotyrosine interaction domains (PID) are indicated by slashed boxes and a TBC domain present in KIAA0603 is indicated by a dotted box. The region analyzed for the tertiary structure is indicated by a red underline. (B) The tertiary structure of KIAA0603 (913–1167 aa) was generated based on the homology modeling method using the tertiary structure of Gyp1p protein. The Arg973 residue of KIAA0603 protein and the corresponding Arg343 residue of Gyp1p, that is an essential GTAase activating site, are both shown in yellow within the structure model and indicated by arrows within panel B.

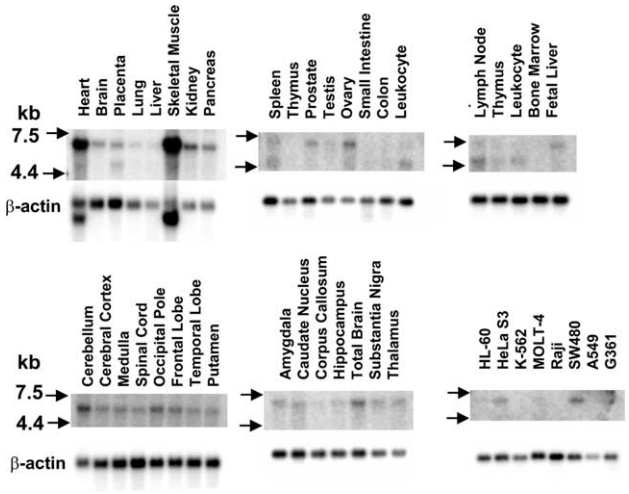

Fig. 2. Expression of *KIAA0603* mRNA in various human tissues. Northern blot analysis of human tissues probed with the ^{32}dCTP labeled plasmid DNA encoding the entire sequence of *KIAA0603*. RNA kb molecular size markers are shown on the left. The β-actin probe was hybridized to the same blot for comparison and is shown at the bottom of each blot.

TBC1D4 showed high homologies to the overall sequences of mouse Tbc1 (Accession No. T29104; 45% identity in 1238 aa residues) and human TBC1D1/KIAA1108 (Accession No. AB029031; 51% identity in 819 aa residues). Tbc1, the mouse ortholog of human TBC1D1, also contains phosphotyrosine interaction domains and a TBC domain, indicating the close relationship of these genes. The TBC domain was first identified as a homologous region commonly present in Tbc1, the tre-2 oncogene and the yeast cell cycle regulators BUB2 and cdc16 [14]. The 52–584 aa region of KIAA0603 and the related 1–478 aa region of mouse Tbc1 have 43% homology. Similarly, the 738–1256 aa region of KIAA0603 and the related 585–1094 aa region of Tbc1 show 58% homology. The region containing the TBC domain (886–1237aa) showed homology of 31% to human rab6 GAP (Accession No. NP 036329).

The 913–1167 aa region had a 21% homology to yeast Gyp1p protein (Accession No. NP 014713), which has a known crystal structure [15]. The tertiary structure of KIAA0603 was generated by the homology modeling method and using the tertiary structure of Gyp1p protein as a basis. KIAA0603 protein (913 – 1167 aa) was predicted to have a tertiary structure similar to the GTPase activating site structure of Gyp1p protein. Arg973 residue of KIAA0603 protein corresponds to Arg343 residue of Gyp1p, which is essential to the GTAase activating site (Fig. 1B).

3.3. Expression of KIAA0603 in tissues

An approximately 6 kb mRNA was detected in a survey of human tissues when the mRNA blots were hybridized with the *KIAA0603* probe. The mRNA was identical in size to the cloned *KIAA0603* cDNA (5922 bp). Abundant expression was observed in heart and skeletal muscle. An additional 5 kb mRNA was also observed in the immune tissues, such as spleen, lymph node and leukocyte. The 5 kb mRNA expression was higher than that of the 6 kb mRNA in tissues from spleen and lymph node (Fig. 2).

3.4. Transcription levels in T cells from AD patients

To reconfirm the result of differential expression analysis of the 164 bp DNA fragment, the transcription levels of *KIAA0603* in the CD3+ cells from AD patients were measured by quantitative PCR using primers and a probe designed from the ORF region. Because the cDNA samples used for the DD study were no longer available, transcription levels were measured in another set of cDNA samples that were prepared previously and independent of the DD study. The set consisted of individual cDNAs from CD3+ cells of 10 healthy volunteers and 30 AD patients [3]. *KIAA0603* mRNA was expressed at a significantly higher level in T cells from a group of 30 AD patients that included all severities of disease than in T cells from healthy controls ($P < 0.05$). In addition, the expression levels were higher in patients with moderate pathology as compared to healthy subjects ($P < 0.01$, Fig. 3A).

3.5. Transcription levels of KIAA0603 in leukocyte subsets

RNA samples were prepared from T cells, B cells, eosinophils, monocytes, and neutrophils of five healthy subjects for quantification of *KIAA0603* transcripts. The expression level was highest in T cells (Fig. 3B). The expression was higher in cells from CD4+ T cell subset than in CD8+ cells. The expression was also higher in the helper, memory CD45RO+ subset of CD4+ cells than in CD45RA+ cells (Fig. 3C).

3.6. Upregulation of KIAA0603 in T cell activation

We have previously identified the ETEA gene, which is upregulated in T cells of AD patients and possibly involved in apoptosis[16]. To examine the involvement of KIAA0603 in T cell activation and activation induced cell death (AICD), the

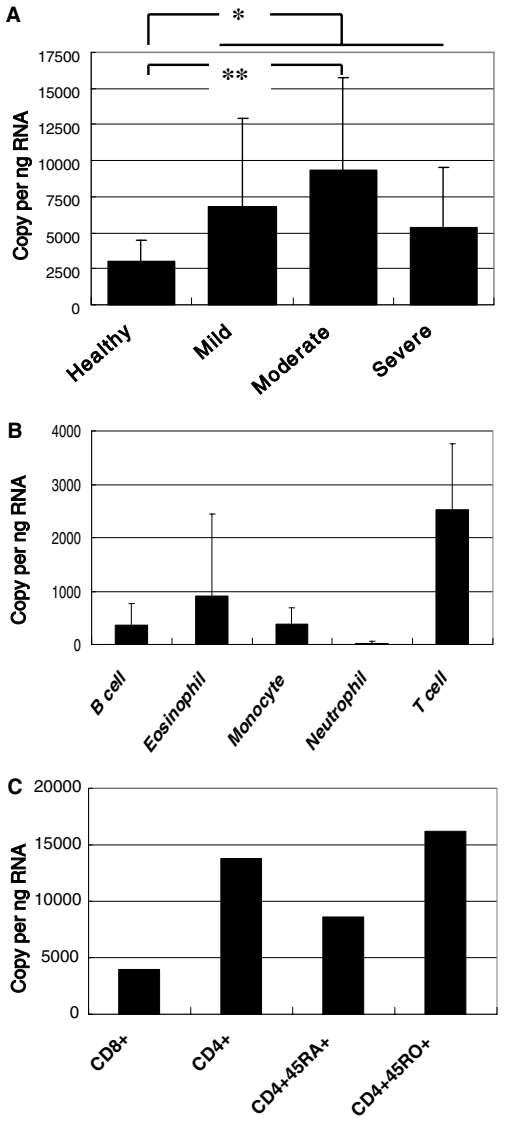

Fig. 3. Transcription levels of *KIAA0603* in peripheral blood leukocytes. (A) *KIAA0603* transcription levels in CD3+ cells from healthy controls and AD patients with either mild, moderate or severe disease diagnosis. *, $P < 0.05$; **, $P < 0.01$. Results are shown as means ± S.D. (B) Transcription levels of *KIAA0603* were examined in subsets of PBMCs from five healthy individuals. Results are shown as means ± S.D. (C) Transcription levels of *KIAA0603* were examined in subsets of T cells from a healthy individual. Results shown are representatives of 2 independent experiments.

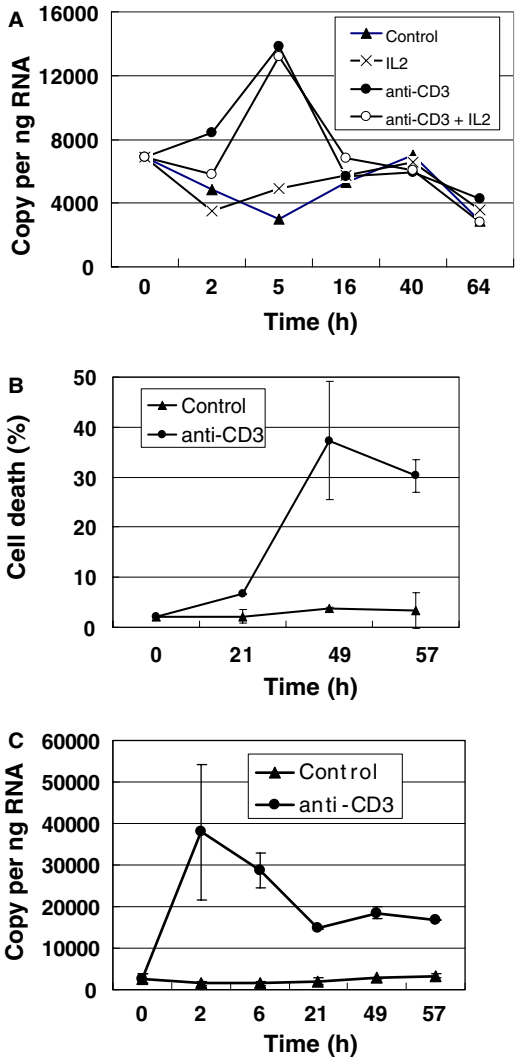

Fig. 4. Upregulation of *KIAA0603* transcription levels during stimulation of primary CD3+ cells. (A) First activation. Freshly prepared CD3+ cells were stimulated with anti-CD3 (closed circles), anti-CD3+IL-2 (open circles), or 100 U/ml of IL2 (crosses). Controls are shown as closed triangles. Total RNA was extracted from each sample. The transcription levels of *KIAA0603* were measured by real time RT-PCR and normalized to ribosomal RNA. Results shown are representatives of 2 independent experiments. (B) Induction of apoptosis. In the second activation of T cells, viable cells were counted by a trypan blue exclusion method. Cells treated with anti-CD3 (closed circles) and controls (closed triangles) are shown. Each value represents mean ± S.D. Results shown are representatives of 3 independent experiments. (C) Second activation. CD3 cells expanded by anti-CD3 stimulation were re-stimulated with anti-CD3 (closed circles). Closed triangles are controls. Total RNA was extracted from each sample and the transcription levels of *KIAA0603* were measured by real time RT-PCR. Each value represents mean ± S.D. Results shown are representatives of 3 independent experiments.

kinetics of transcription levels of *KIAA0603* were examined during the course of activation of T cells freshly isolated from peripheral blood. The transcription level of *KIAA0603* was transiently upregulated at 5 h after the first in vitro stimulation with mAb to CD3 (Fig. 4A). A 37% AICD was induced 49 h after the second stimulation of primary peripheral blood T cells with CD3 mAb (Fig. 4B). The transcription level of *KIAA0603* was markedly upregulated at 2 h after the stimulation. The elevated levels gradually declined but at 21 h were still higher than the unstimulated control (Fig. 4C). In similar experiments, the expression of *KIAA0603* was shown to be markedly induced by stimulation with 1 μg/ml of ionomycin (data not shown).

4. Discussion

Using differential gene expression analysis of CD3+ cells, we found that the expression levels of *KIAA0603* were higher in AD patients than in healthy individuals. The expression level of the intron 164 bp DNA fragment in asthmatic patients was not significantly higher than in the healthy controls. This suggests that upregulation of KIAA0603 may be associated

with AD, but not allergic asthma. To clarify this point, it is important to understand the KIAA0603 mRNA levels in asthmatic patients.

The expression level of KIAA0603 was higher in patients with moderate pathology as compared to healthy controls. In our previous study, we have identified several genes, including MAR/SAR DNA-binding protein and heat shock protein 40, that were also expressed at significantly higher levels in moderate AD patients than in healthy controls [3]. The relevance of these genes to severity of AD is uncertain. The upregulation of KIAA0603 might reflect accelerated AICD in T cells of patients with moderate AD.

Analysis of secondary and tertiary structure of KIAA0603 gave results that were compatible with an assignment to a family of Rab GAPs. A database surveillance for genes predicting proteins related to GAP identified KIAA0603 as a RabGAP that contained both a phosphotyrosine-binding domain(s) and a TBC domain and was most closely related to the Drosophila ortholog, pollux [17]. Rab GTPases are the largest group of the Ras superfamily of small monomeric G proteins. The Rab proteins have been implicated in the regulation of various transport steps in both the endocytic and exocytic pathway. They are potential regulators of transport vesicle budding and delivery to their correct target compartments [18]. The GTPase proteins cycle between biologically active GTP-bound and inactive GDP-bound conformations. GAPs inactivate the GTP-binding proteins by enhancing their intrinsically low GTPase activity. Rab proteins are thought to contribute to polarized secretion during the course of T cell activation. Upon encountering a B cell, Th2 cells rearrange the cytoskeleton and release IL-4 in a polarized fashion over the area of receptor crosslinking [19]. In cell-mediated cytotoxicity, conjugate formation between CD8+ and target cells is accompanied by capping of the adhesion molecule LFA-1 in the contact area. Simultaneously, the Golgi apparatus and the microtubule organizing center polarize toward the point of contact, resulting in a directed release of cytotoxic effector molecules [20].

When primary T cells were stimulated through the T cell receptor in our experiments, the transcriptional levels of *KIAA0603* were upregulated. In particular, marked upregulation and sustained expression of *KIAA0603* was observed in the course of AICD. It is generally assumed that GAPs are specific for GTPases that are within their own branch of the Ras superfamily. In addition, several GAPs are specific for a subset of GTAases within the branch [17]. The upregulation of *KIAA0603* may be involved in the modulation of the activity of a KIAA0603-related Rab GTPase protein.

Recently, Kane et al. [21] identified KIAA0603 (gi7662198: TBC1 domain family, member 4), which they designated AS160, as a substrate for Akt protein kinase in adipocytes. They demonstrated that insulin-stimulated phosphorylation of AS160 is required for GLUT4 translocation and hypothesized that insulin-stimulated translocation of GLUT4 requires a Rab in its active GTP form. Phosphorylation of AS160 inhibits its GAP activity toward the Rab. Consequently, the GTP form of the Rab increases and the Rab-dependent steps in GLUT4 translocation can proceed [22].

There is considerable evidence that Akt is activated with the co-stimulation through CD28 and functions as an important mediator of T cell survival. Functions of Akt are transcriptional upregulation of the anti-apoptotic molecule Bcl-XL through activation of NFkB, phosphorylation resulting in inhibition of the pro-apoptotic molecule BAD, and inhibition of Fas-mediated apoptosis. When T cells are repeatedly stimulated through a T cell receptor without co-stimulation by CD28, AICD is induced. AICD is thought to be a feedback mechanism for terminating an ongoing immune response [23], as well as means of inducing peripheral tolerance.

In our previous study of expression kinetics of apoptosis-related genes during AICD induced by anti-CD3 stimulation, pro-apoptic genes such as Fas, Bax and FAF1 were upregulated and the elevated levels were maintained until 48h. In contrast, the transient upregulation of anti-apoptic genes (Bcl2 and BclXL) was followed by downregulation at 48 h [16]. During AICD, the transcription kinetics of *KIAA0603* were similar to those of pro-apoptic genes. Since co-stimulation through CD28, the predominant activator of PI3K/PKB pathway [24], was not present in this experiment, the PKB was considered to be inactive. Therefore, the PKB substrate KIAA0603/AS160 was in an activated, unphosphorylated form, which led to inactivation of Rab proteins. The corresponding Rab protein may have a role in the activation and survival of T cells. Therefore, the upregulation and accumulation of the activated form of KIAA0603 may be involved in the induction of AICD by inactivating Rab proteins.

Very recently, Akdis et al. [25] reported that circulating cutaneous lymphocyte associated antigen-bearing (CLA+) CD45RO+ T cells undergo AICD in patients with AD. In particular, the Th1 subset of CLA+ CD45RO+ T cells selectively undergoes AICD, skewing the immune response toward surviving Th2 cells in AD. Because mRNA levels of KIAA0603 were strongly upregulated in the course of AICD of primary T cells, the higher expression of KIAA0603 in CD4+ CD45RO+ T cells that we observed probably reflects the AICD of Th1 subset of CLA+ CD45RO+ T cells.

The apoptosis of CLA+ CD45RO+ T cells was confined to atopic individuals. Non-atopic patient, such as psoriasis and healthy controls, showed no evidence of enhanced T cell apoptosis in vivo [25]. Psoriasis is a dermatological disorder in which Th1 cells are dominant. Based on the reported results, we can speculate that the expression level of KIAA0603 is not upregulated in T cells of patients with psoriasis. Further studies of the expression level of KIAA0603 in psoriasis should confirm this hypothesis.

In summary, we have found that the transcription levels of *KIAA0603* were significantly higher in AD patients than in normal individuals. KIAA0603, a Rab GAP, probably participates in modulation of activation and AICD of T cells, especially memory helper T cells, by interacting with a corresponding Rab protein(s). Our data point to the possible involvement of KIAA0603 in the pathogenesis of AD.

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