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Identification of the novel autoantigen candidate Rab GDP dissociation inhibitor alpha in isolated adrenocorticotropin deficiency

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Abstract. Isolated adrenocorticotropin deficiency (IAD) is characterized by low or absent adrenocorticotropic hormone (ACTH) production. IAD is presumed to be caused in part by an autoimmune mechanism, and several lines of evidence have suggested the presence of anti-pituitary antibodies in IAD. However, the exact autoantigens remain unknown. The present study was designed to identify the autoantigen(s) in IAD using chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Rat anterior pituitary lysate was subjected to SDS-PAGE, and immunoblotting was performed using the sera from two patients with IAD and from a healthy subject. The bands detected by the patient serum samples, but not by the healthy subject sample, were excised, in-gel digested using trypsin, and subjected to LC-MS/MS analysis. On immunoblots, a 51-kDa band in the insoluble pellet was detected by the sera from the IAD patients but not from the healthy subject. Mass spectrometric analysis revealed the 51-kDa band contained Rab guanine nucleotide dissociation inhibitor (GDI) alpha. Consistent with the mass spectrometric analysis, a recombinant full-length human Rab GDI alpha was recognized by the two IAD patient samples but not by the healthy subject sample using immunoblotting. In total, anti-Rab GDI alpha antibodies were detected in serum samples from three of five patients with IAD (60%) but were absent in 5 healthy subjects. In addition, Rab GDI alpha was expressed in the anterior pituitary. In conclusion, it appears that Rab GDI alpha is a candidate autoantigen involved in IAD, and that anti-Rab GDI alpha antibodies are present predominantly in patients with IAD.

Key words: Isolated adrenocorticotropin deficiency, Autoantigen, Rab GDI alpha, Autoantibody

ISOLATED adrenocorticotropin deficiency (IAD) is characterized by insufficient production of adrenocorticotropic hormone (ACTH). IAD seems to be of pituitary origin, as indicated by the absence of an ACTH response to corticotropin-releasing factor. Although its pathogenesis remains to be elucidated, IAD is thought to be caused in part by an autoimmune mechanism. IAD is associated with other autoimmune disorders, for example, chronic thyroiditis [1]. In addition, IAD is also accompanied by idiopathic hypoparathyroidism and type 1 diabetes, suggesting polyglandular failure autoimmune process is involved in some, though not all, forms of IAD [2-4]. These findings strongly suggest the involvement of autoimmune processes in the pathogenesis of IAD.

Several lines of evidence suggest the presence of anti-pituitary antibodies in IAD [5-9]. Furthermore, it has been reported that the sera from IAD patients selectively target pituitary ACTH-secreting cells [8, 10, 11]. Sauter *et al.* tested sera from IAD patients for the presence of an anti-pituitary antibody using indirect immunofluorescence in rat pituitary tissue [8]. Positive immunostaining was observed in the anterior and intermediate lobes, and the immunopositive cells were revealed by immunoelectron microscopy to have ultrastructural characteristics of corticotrophs [8]. In a study by De Bellis *et al.,* it was reported that anti-pituitary antibodies in IAD selectively targeted pituitary ACTH-secreting cells when using young baboon

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pituitary gland samples as the substrate, and that antibodies targeting corticotrophs may be considered reliable markers of autoimmune processes involving the pituitary and impaired ACTH secretion in patients with IAD [10, 11]. Thus, identification of autoantigens would provide important insights into the pathology of IAD, and identification of the targets of anti-pituitary antibodies might lead to the development of more reliable diagnostic markers of IAD. However, autoantigens present in IAD are unknown. Proteomic analysis enables high-throughput, exhaustive analyses of candidate autoantigens in autoimmune diseases [12, 13]. In the present study, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify autoantigen(s) in IAD patients, and the results were confirmed using recombinant proteins of autoantigen candidates and immunoblotting. We further accessed the specificity of the autoantibodies in IAD.

Materials and Methods

Patients

In all IAD patients, morning basal serum levels of ACTH and cortisol, measured from 8:00 to 10:00, were low, and the response of ACTH to corticotropin-releasing hormone (CRH) was absent. In a CRH test, single doses (100 μg) of human CRH were injected intravenously. Blood samples were then collected before and 30, 60, 90, and 120 min after injection. Serum levels of other pituitary hormones, including thyroid stimulating hormone (TSH), growth hormone (GH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and the respective target organ hormones (free T4, free T3, insulin-like growth factor I, testosterone, and estradiol) were also within normal basal level ranges. In addition, TSH, GH, LH, and FSH responded appropriately to stimulation tests including those for thyrotropin-releasing hormone, growth hormone releasing peptide-2, and luteinizing hormone-releasing hormone. Serum prolactin basal levels were normal. Diabetes insipidus was not observed in any patient. No abnormal findings were observed by magnetic resonance imaging. Neither pituitary surgery nor radiation therapy was performed in any patient. The causes of IAD in all of the patients were unknown. Hydrocortisone (15-20 mg/day) was administered to all IAD patients. The profiles of the IAD patients were as follows: #1, male, 77 years old; #2, male, 67 years old; #3, female, 70 years old; #4, male, 62 years old; #5, female, 33 years old. The durations from IAD onset to blood sample collection were approximately 1, 1, 1, 13, and 2 years, respectively.

As control, we randomly chose five samples of healthy subjects from stocked samples. The profiles of the healthy subjects were as follows: male, 30 years old; male, 31 years old; male, 33 years old; male, 51 years old; female, 53 years old. The samples were obtained from Nagoya University and another facility in Japan, following approval of the protocol from the institutional review boards of each facility.

Western blot analysis to detect the pituitary autoantigens that react with IAD patient sera and to detect the expression of Rab GDI alpha in the pituitary

Male Sprague-Dawley rats (body weight 250–300 g; Chubu Science Materials, Nagoya, Japan) were housed in pathogen-free facility with a 12-h/12-h light/dark cycle, free access to food and water, and a room temperature of 22°C; and used when 7-8 weeks old. Upon sacrifice, rat pituitaries from Sprague-Dawley rats were collected and homogenized in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The soluble supernatant and insoluble pellet fractions were separated by ultracentrifugation as reported previously [14] and stored at -80°C. The supernatants and pellets were separated and subjected to SDS-PAGE, followed by immunoblot analysis. Proteins were transferred to PVDF membranes (Millipore, USA). Membranes were then blocked in 4% skim milk and incubated with sera (1:100) from patients and healthy subjects overnight at 4°C.

To evaluate the expression of Rab GDI alpha, anterior and posterior lysates were separated by SDS–PAGE and transferred to PVDF membranes. The membranes were then incubated overnight at 4°C with goat Rab GDI alpha antibody (1:200, sc-20447, Santa Cruz Biotechnology, Dallas, TX, USA).

After three washes in phosphate-buffered saline containing Tween 20 (PBST), membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP) conjugated human IgG or anti-goat IgG-HRP antibody (1:200, Dako, USA) for 60 min, as reported previously [15]. After washing with PBST for 10 min three times, the membranes were incubated with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA) and exposed to X-ray film (Kodak, Tokyo, Japan).

In-gel digestion and mass spectrometric analysis

The fractionated proteins from the anterior pituitary were separated by SDS-PAGE and subjected to silver staining using a Wako Silver Stain Kit (Wako, Tokyo, Japan). The gel bands corresponding to IAD-specific proteins were then excised and destained using acetonitrile (ACN). After reducing the proteins using 10 mM DTT, they were alkylated using iodoacetamide and digested using trypsin (Promega). The resulting peptides were extracted sequentially from the gel using 0.1% trifluoroacetic acid (TFA) in ACN. The extracts were evaporated and dissolved using 0.1% TFA in 5% ACN. Desalting was performed using a solid phase extraction tip (C-tip). The samples were then applied to a liquid chromatography (LC) system (Paradigm MS4; AMR) and analyzed by tandem MS/MS (LTQ Orbitrap XL; ThermoFisher Scientific) directly coupled to the LC system, as reported previously [16, 17]. Samples were injected onto the Paradigm MS4 HPLC System equipped with a Magic C18AQ column 0.1 mm in diameter and 50 or 150 mm in length (Michrom BioResources). Reverse-phase chromatography was performed using a linear gradient (0 min, 5% B; 100 min, 50% B) of solvent A (2% ACN with 0.1% formic acid) and solvent B (90% ACN with 0.1% formic acid) at an estimated flow rate of 1 μl/min. After ionization, a precursor ion scan was performed using a mass to charge ratio (m/z) of 400–2,000 prior to MS/MS analysis. Data were analyzed using Mascot software (Matrix Science Inc., Boston, MA, USA) to search the Swiss-Prot database. Protein identifications comprising at least two identified peptides with >95.0% probability were accepted [17].

Plasmids and recombinant proteins

Vectors containing the full-length cDNAs of human GDP dissociation inhibitor (GDI) 1 (protein name, Rab GDI alpha) and serotransferrin were purchased from Open Biosystems, Inc. Each open reading frame was amplified by PCR, inserted into the expression vector pcDNA 3.1D/V5-His-TOPO (Invitrogen, Carlsbad, CA, USA), and fully sequenced using the Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA). HEK293FT cells cultured in 10-cm dishes were transfected with each vector or an empty vector control using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions, as described elsewhere [14]. After 48 h, transfected HEK293FT cells were collected. Expression of each recombinant protein was confirmed by Western blotting using an anti-V5 antibody (Invitrogen).

Detection of the anti-Rab GDI alpha antibodies in patient sera

To evaluate the presence of antibodies in patient serum samples, recombinant full-length human proteins of Rab GDI alpha or serotransferrin and the control lysate were electrophoresed on a 7.5% polyacrylamide gel and transferred to a PVDF membrane, which was subjected to immunoblotting using each serum sample (1:50 dilution) or an anti-V5 antibody (1:1,000) as a positive control.

Immunohistochemistry

Cryostat sections (7 μm) of the pituitary gland from rats sacrificed under deep ether anesthesia administered by transcardial perfusion were generated using 4% paraformaldehyde in phosphate-buffered saline. Immunostaining was performed as reported previously [18]. All fluorescently stained sections were examined using a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan). The following antibodies were used: mouse monoclonal anti-ACTH antibody (1:50, ab8615; Abcam, Cambridge, MA, USA), goat anti-Rab GDI alpha antibody (1:50, sc-20447, Santa Cruz Biotechnology), donkey anti-mouse IgG-AlexaFluor 488 (Invitrogen), and donkey anti-goat IgG-AlexaFluor 594 (Invitrogen).

All procedures were performed in accordance with the institutional guidelines for animal care at Nagoya University, which conform to the National Institutes of Health animal care guidelines.

Results

Sera from IAD patients recognized autoantigens in the anterior pituitary gland

On immunoblots, an ~51-kDa band in the insoluble pellet fraction and an ~75-kDa band in the soluble supernatant fraction from anterior pituitary extracts were detected by serum samples from two IAD patients (patients 1 and 2) but not by that from a healthy subject (Fig. 1).

Autoantigen candidates were identified by mass spectrometry analysis

To identify autoantigens, we excited the ~51- and ~75-kDa bands, and performed LC-MS/MS analysis. Several proteins were detected in the excised bands.

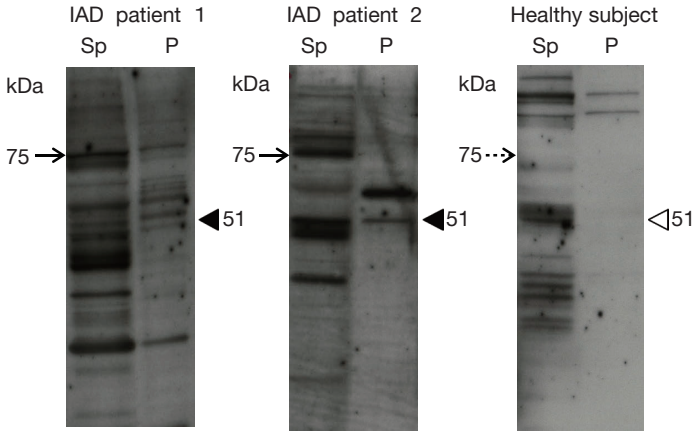

Fig. 1 Recognition of anterior pituitary proteins by sera from IAD patients
Anterior pituitary lysates were resolved by SDS-PAGE, followed by immunoblotting using serum samples. An ~51-kDa band (arrow) in the insoluble pellet fraction and an ~75-kDa band (arrowhead) in the soluble supernatant fraction were detected by serum samples from two IAD patients (patients 1 and 2) but not by that from a healthy subject. Sp, soluble supernatant fraction; P, insoluble pellet fraction.

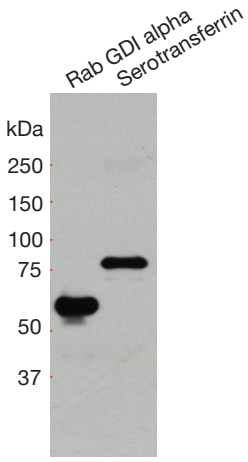

Fig. 2 Expression of recombinant full-length of human Rab GDI alpha and serotransferrin proteins
Recombinant full-length human Rab GDI alpha and serotransferrin proteins were expressed in HEK293FT cells, after which they were resolved by SDS-PAGE and subjected to immunoblotting using an anti-V5 antibody.

We retrieved proteins whose molecular weights corresponded to the size of the band from a search against the Swiss-Prot database. Rab GDI alpha (mascot score, 173.0; sequence coverage, 10.0%) and serotransferrin (mascot score, 3,412.2; sequence coverage, 51.2%) were detected with high scores in the 51-kDa and 75-kDa bands, respectively. Rab GDI alpha (UniProtKB/Swiss-Prot: P50398) contains 447 amino acid residues and has a molecular weight of 50,537 Da. Serotransferrin (UniProtKB/Swiss-Prot: P12346) contains 698 amino acid residues and has a molecular weight of 76,395 Da.

Sera from IAD patients recognized recombinant full-length human Rab GDI alpha

To confirm recognition of Rab GDI alpha and serotransferrin by the sera from the IAD patients, we created recombinant full-length human proteins and expressed them in HEK293FT cells (Fig. 2). The number amino acid residues of human Rab GDI alpha is the same as that of rat Rab GDI alpha, and the amino acid sequence homology between human and rat is high (98.7%). Consistent with the mass spectrometric analysis, sera from two IAD patients (patients 1 and 2) recognized the recombinant Rab GDI alpha, while that from a healthy subject did not (Fig. 3). The recombinant serotransferrin was not recognized by the sera from IAD patients or from a healthy subject (data not shown).

Prevalence of anti-rab GDI alpha antibodies

We performed additional immunoblotting to detect anti-Rab GDI alpha antibodies using three serum samples that were all we had from IAD patients and four serum samples from healthy subjects. Anti-Rab GDI alpha autoantibodies were detected in one (patient 3) of the three patients with IAD (Fig. 3) but were absent in the four healthy subjects (data not shown). These data are summarized in Table 1. In total, the sensitivity for detection of anti-Rab GDI alpha antibodies was 60% (3 of 5) in IAD patients (Table 1). Anti-Rab GDI alpha autoantibodies were absent in 5 healthy subjects (Table 1). It should be noted that the duration from IAD onset to blood sample collection in the samples from patients (patients 1, 2, and 3) was within one year.

Rab GDI alpha is expressed in the anterior pituitary

We next examined the expression of Rab GDI alpha in the anterior pituitary. Immunoblotting analysis showed Rab GDI alpha was expressed in the anterior pituitary (Fig. 4A). Consistent with results of the LC-MS/MS analysis and immunoblotting, immunohistochemical analysis showed Rab GDI alpha immunoreactivity was mainly observed in the anterior pituitary (Fig. 4B-I). In addition, the expression of Rab GDI alpha was partially

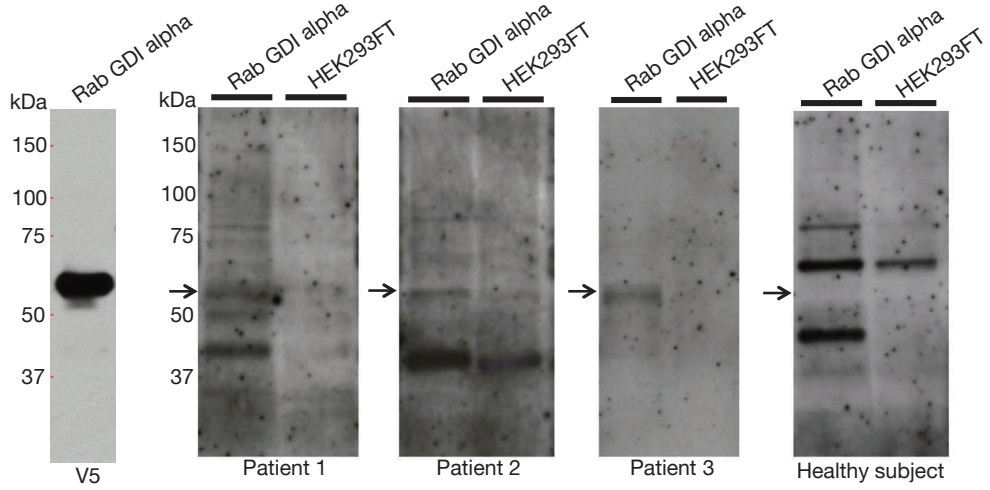

Fig. 3 Detection of anti-Rab GDI alpha antibodies in IAD patient serum by immunoblotting
Recombinant full-length human Rab GDI alpha protein (Rab GDI alpha,left lane) expressed in HEK293FT cells as well as total cell lysates from HEK293FT cells transfected with the empty vector (HEK293FT, right lane) were subjected to immunoblotting using sera samples from IAD patients 1, 2, and 3 and a healthy control. Recombinant full-length human Rab GDI alpha was detected using an anti-V5 antibody as a positive control. Sera from IAD patients recognized the recombinant Rab GDI alpha (arrows).

Table 1

Number	Sex	Age of IAD onset	Duration from IAD onset to blood sample collection	Anti-Rab GDI alpha antibody
1	Male	77	1 year	Positive
2	Male	67	1 year	Positive
3	Female	70	1 year	Positive
4	Male	62	13 years	Negative
5	Male	33	2 years	Negative

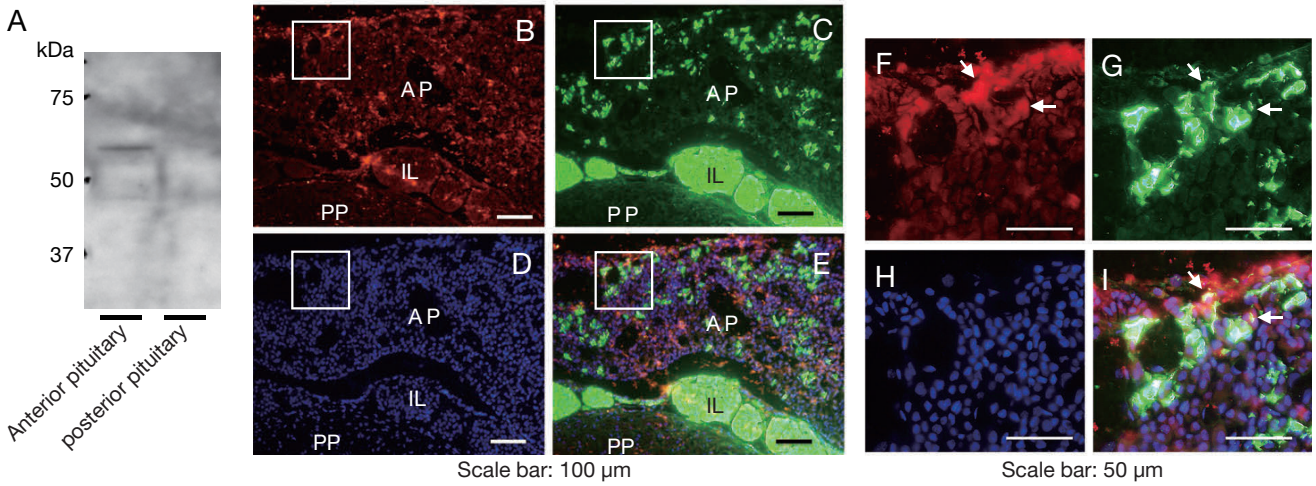

Fig. 4 Expression of Rab GDI alpha in the anterior pituitary
Protein extracts from pituitary were analyzed by Western blotting with an antibody directed against Rab GDI alpha (A). Rat pituitaries were immunostained with Rab GDI alpha (B, F), ACTH (C, G), and DAPI (D, H). Merged image (E, I). The regions in white boxes (B, C, D, E) are shown magnified in the right panels (F, G, H, I, respectively). Arrows indicate the colocalization of Rab GDI alpha and ACTH. Scale bar shows 50 μm (B-E). Scale bar shows 100 μm (F-I).
AP, anterior pituitary; PP, posterior pituitary; IL, intermediate lobe.

colocalized with that of ACTH (Fig. 4B-I).

Discussion

In the present study, we identified a novel anterior pituitary autoantigen candidate, Rab GDI alpha, recognized predominantly by the sera of patients with IAD. To our knowledge, this is the first report of an autoantigen candidate in IAD. With regard to studies on autoantigen candidates in IAD, Sauter *et al*. reported that the immunostaining patterns of IAD patient's sera colocalized with that of ACTH-secreting cells, and that they searched for autoantigens in the sera using immunoabsorption with proopiomelanocortin (POMC)-derived peptides. The immunolabeling intensities of the proteins in the pituitary were not reduced by the use of ACTH-(1-24), ACTH-(1-39), gamma MSH, corticotropin-like intermediate lobe peptide, beta-endorphin, or beta-lipotropin, leading the authors to conclude that these molecules were not autoantigens in IAD [8]. They speculated that one candidate autoantigen could be a cell-specific granular factor involved in the posttranslational processing of POMC or secretion of ACTH.

GDIs regulate GDP/GTP exchange among members of the Rab family. Rab GDI was identified originally as a factor that prevents the release of GDP from Rab, thereby stabilizing the inactive form [19, 20]. In addition, it has been reported that Rab GDI functions as a chaperone Rab GTPase in the cytosol, mediating the delivery of Rab proteins to membranes and recycling them back to the cytosol [21, 22]. Rab GDI alpha mRNA is expressed abundantly in the brain of rats [23] and plays a specialized role in Rab3A recycling, thereby regulating the sorting of neurosecretory vesicles in the brain [24]. In the present study, Rab GDI alpha was expressed in the anterior pituitary and partially colocalized with corticotrophs. Thus far, the physiological or pathophysiological role of Rab GDI alpha antibodies has not been reported. However, we speculate that Rab GDI alpha antibodies may suppress ACTH vesicle trafficking or that antibody-dependent, cell-mediated cytotoxicity causes the selective destruction of corticotrophs. In addition, the result of the expression of Rab GDI alpha in cells other than ACTH secreting cells in the anterior pituitary suggests the involvement of Rab GDI alpha in other anterior hormone deficiencies.

IAD is thought to be associated with lymphocytic adenohypophysitis (LAH) [25-27], a form of lymphocytic hypophysitis that is considered to be an autoimmune disease of the pituitary gland [27]. In LAH, several autoantigen candidates, such as GH [28], alpha enolase [29, 30], pituitary gland-specific factors 1a and 2 [31], secretogran II [32], chromosome 14 open reading frame 166 (C14orf166) and chorionic somatomammotrophin [13], have been reported. Furthermore, in isolated anterior pituitary hormone deficiencies including IAD, anti-pituitary antibodies have been detected [33]. Bensing *et al.* reported that sera from patients with isolated GH deficiency that was associated with autoimmune polyendocrine syndrome type 1 (APS1) recognized anterior pituitary cells [34]. Recently, Iwama *et al*. reported that isolated prolactin deficiency was associated with serum autoantibodies, and that circulating autoantibodies recognize some antigens in PRL-secreting cells [35]. Rab GDI alpha has not been reported previously as a candidate autoantigen of LAH and other isolated anterior pituitary hormone deficiencies. The involvement of anti-Rab GDI alpha antibodies in LAH and isolated anterior pituitary hormone deficiencies would be of interest in that they may help clarify the similarities and differences in the molecular mechanisms between IAD and LAH or between IAD and isolated anterior pituitary hormone deficiencies other than IAD.

Although we need to collect more samples from IAD patients and controls, including those with other pituitary diseases that cause hypopituitarism, such as tumors, and those with other autoimmune disorders, such as chronic thyroiditis and collagen diseases, to validate the sensitivity and specificity of anti Rab GDI alpha antibodies in IAD, in this study, we detected Rab GDI alpha autoantibodies in 60% of patients. Several explanations exist for the lack of detection in the remaining 40% of IAD patients. The Rab GDI alpha autoantibody titers may decrease over time in patients after the onset of IAD. In fact, Rab GDI alpha autoantibodies were not detected in patient 4, who had developed the disease more than 10 years ago. Although our results suggest that Rab GDI alpha is the major pituitary autoantigen in patients with IAD, it is possible that other autoantigens exist. In the present study, we could not detect antibodies against recombinant serotransferrin. However, the improvements in biochemical methods, such as recombinant protein design or immunoblotting, may reveal autoantigen candidates other than Rab GDI alpha. In addition, using the pitu-

itaries from other species, including humans, would help us to complete our study more thoroughly. It is well known that genetic mutations can cause IAD. Examples include gene mutations in POMC [36] or the T box transcription factor, TPIT, which is important for terminal differentiation of pituitary POMC-expressing cells [37]. However, the onset of IAD due to these genetic mutations typically occurs during the neonatal or young childhood stage. Therefore, in the present study, there was a low possibility of involvement of POMC and TPIT gene mutations in IAD.

We anticipate identification of the novel autoantigen candidate Rab GDI alpha would provide important insights into the pathology of IAD. When IAD develops, steroid replacement therapy is needed over the course of one's life. Understanding the molecular mechanisms of IAD may contribute to the development of therapies targeting the molecules involved in the pathogenesis of IAD.

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Disclosures

All authors report they have nothing to declare.

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