ORIGINAL ARTICLE

Endocrine Research

The Role of the Aryl Hydrocarbon Receptor-Interacting Protein Gene in Familial and Sporadic Pituitary Adenomas

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Context: Mutations have been identified in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene in familial isolated pituitary adenomas (FIPA). It is not clear, however, how this molecular chaperone is involved in tumorigenesis.

Objective: AIP sequence changes and expression were studied in FIPA and sporadic adenomas. The function of normal and mutated AIP molecules was studied on cell proliferation and protein-protein interaction. Cellular and ultrastructural AIP localization was determined in pituitary cells.

Patients: Twenty-six FIPA kindreds and 85 sporadic pituitary adenoma patients were included in the study.

Results: Nine families harbored *AIP* mutations. Overexpression of wild-type AIP in TIG3 and HEK293 human fibroblast and GH3 pituitary cell lines dramatically reduced cell proliferation, whereas mutant AIP lost this ability. All the mutations led to a disruption of the protein-protein interaction between AIP and phosphodiesterase-4A5. In normal pituitary, AIP colocalizes exclusively with GH and prolactin, and it is found in association with the secretory vesicle, as shown by double-immunofluorescence and electron microscopy staining. In sporadic pituitary adenomas, however, AIP is expressed in all tumor types. In addition, whereas AIP is expressed in the secretory vesicle in GH-secreting tumors, similar to normal GH-secreting cells, in lactotroph, corticotroph, and non-functioning adenomas, it is localized to the cytoplasm and not in the secretory vesicles.

Conclusions: Our functional evaluation of AIP mutations is consistent with a tumor-suppressor role for AIP and its involvement in familial acromegaly. The abnormal expression and subcellular localization of AIP in sporadic pituitary adenomas indicate deranged regulation of this protein during tumorigenesis. **(*J Clin Endocrinol Metab* 93: 2390–2401, 2008)**

Whereas pituitary adenomas are common, familial cases are relatively rare (1). Familial pituitary adenomas are associated with the classical syndromes of multiple endocrine neoplasia type 1 (MEN-1) and Carney complex, but an autosomal dominant disease with incomplete penetrance with isolated pituitary adenomas has been described as isolated familial somatotrophinoma (2, 3), familial isolated pituitary adenoma (FIPA) (4), or pituitary adenoma predisposition (5). Linkage and loss of heterozygosity (LOH) data suggested a candidate locus on chromosome 11q13 (3, 6, 7). Subsequently germline mutations were identified in a gene in this region encoding aryl-hydrocarbon receptor (AhR)-interacting protein [AIP; also known as XAP2 or ARA9 (8, 9)] in families with somatotroph adenomas and families with both somatotroph and lactotroph tumors or rarely in families with other types of pituitary tumors (5, 10–12). The 330 amino-acid AIP is a molecular co-chaperone protein involved in the functional maturation of AhR, an orphan nuclear receptor known to bind the environmental toxin dioxin (8, 9). Structurally, AIP contains tetratricopeptide repeat motifs, which mediate protein-protein interactions (13). AIP modulates the function of AhR by both protecting AhR from ubiquitination and therefore prolonging its half-life and retaining AhR in the cytoplasm and preventing AhR acting as a transcription factor (14–16). Interaction of AIP with phosphodiesterase (PDE) isoforms has recently been shown (17, 18) (and could be of relevance due to the known involvement of the cAMP pathway in somatotroph cell function), whereas other AIP partners have also been described (19–22). There are therefore several possible ways in which mutations in AIP could promote tumorigenesis, but the exact mechanism remains unknown.

In the current paper, we present new mutations and novel functional data on the effect of wild-type and mutant AIP protein on cell proliferation and protein-protein interaction and the expression and cellular location of *AIP* in both normal and adenomatous pituitary tissue.

Patients and Methods

FIPA patients

Twenty-six families were identified on the basis of at least one member having a somatotroph adenoma and at least one other member having a somatotroph or lactotroph adenoma (Table 1). All subjects provided written informed consent, and institutional review board approval was obtained. MEN-1 and Carney complex was considered highly improbable on the basis of the absence of MEN-1- or Carney complex-associated tumors in patients or their family members, demonstration of a normal serum calcium and PTH level in all cases and the lack of detectable *menin* mutations where assessed. AIP sequencing data were compared with random Caucasian controls (n = 96, European Collection of Cell Cultures, Health Protection Agency, Porton Down, UK) and Japanese normal controls (n = 78). Sequencing covered all the exons and exon-intron junctions and 1200 bp of the promoter area.

0021-972X/08/$15.00/0
Printed in U.S.A.
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doi: 10.1210/jc.2007-2611 Received November 27, 2007. Accepted March 26, 2008.
First Published Online April 17, 2008

Patients with sporadic adenomas

Patients with sporadic pituitary tumors had no family history of pituitary or other endocrine tumors. Leukocyte-origin genomic DNA from seven childhood-onset (Table 1) and 30 adult-onset acromegaly cases (male to female ratio 1:1, age at diagnosis 38.9 ± 13.1 yr, mean ± SD) were examined for possible genomic changes in the AIP gene. cDNA from 48 sporadic pituitary adenomas was also sequenced and was studied for AIP expression using real-time PCR. Details of reactions are available in the supplementary material.

Cell proliferation

Site-directed mutagenesis reactions were carried out using the template pCI-neoAIP-FLAG (kind gift from Professor Perdew, Director of the Center for Molecular Toxicology and Carcinogenesis Department of Veterinary and Biomedical Sciences, Penn State University, University Park, PA) for generation of point mutations (V49M, C238Y, R271W), nonsense mutations (R304X, R81X, Q217X) and insertion mutation Ins274 (Quikchange; Stratagene, La Jolla, CA). These were then subcloned into the pCI-neoAIP-myc plasmid and the pBABE-puro retroviral vector. HEK293, human diploid embryonic lung fibroblast cells (TIG3), and the rat somatomammotroph cell line GH3 were transfected with wild-type and mutant vectors. Details of cell cultures and proliferation assays are described in the supplementary material.

AIP-PDE4A5 interaction

Interactions between AIP and PDE4A5 were performed as described previously (17). PDE4A5 (GenBank no. L27057) was cloned into the *Not*I site of pLEXAN to generate a LexA DNA-binding-domain fusion. Wild-type AIP or various mutants thereof were cloned into the *Not*I site of pGADN to generate GAL4 activation-domain fusions. All mutations were created by the circular mutagenesis method and were verified by sequencing before use (17). Yeast two-hybrid filter β-galactosidase assays were performed in *Saccharomyces cerevisiae* strain L40.

Immunostaining

Details of the methods for immunostaining, immunofluorescent confocal microscopy, and immunogold electron microscopy of normal (n = 9) and pituitary adenoma samples (n = 47) are described in the supplementary material. Sparsely and densely granulated adenomas were separated based on the pattern of GH staining, cytokeratin staining, and electron microscopy in some cases (23).

Results

Genetic studies

We identified 67 patients with pituitary tumors in 26 families with familial pituitary adenomas, in which 21 families had two to seven members with a GH-secreting tumor, four families had members with somatotroph and lactotroph, and one family with somatotroph and nonfunctioning adenoma (Table 1). The mean (± SD) age at disease onset (or diagnosis) was 31.6 ± 15.1 yr. Forty-seven subjects from nine families were found to have a heterozygote germline *AIP* mutation, and 31 of these had clinical disease at the time of the study (66%). Affected family members with mutations had a mean age at diagnosis of 24.2 ± 10.8 yr.

Abbreviations: AhR, Aryl-hydrocarbon receptor; AIP, AhR-interacting protein; FIPA, familial isolated pituitary adenoma; LOH, loss of heterozygosity; MEN-1, multiple endocrine neoplasia type 1; NFPA, nonfunctioning pituitary adenoma; PDE, phosphodiesterase.

TABLE 1. Clinical data of familial pituitary adenoma families

Families	Country of origin	Clinical diagnosis	Sex, height, and age (yr) at onset/diagnosis of affected patients	Leukocyte gDNA- AIP mutation status	Leukocyte cDNA-AIP mutation status	LOH in 11q13 in tumor tissue	Histology diagnosis	AIP staining	Responsiveness to somatostatin analogs	Number of known or obligate carriers	Number of known manifest disease	Percentage of manifest disease in carriers
Family I[a]	**Mexico**	**Acromegaly**	**M, 173 cm/23 yr** **M, 165 cm/21 yr** **M, 175 cm/19 yr**	**C238Y**	**NA**	**Yes**	**GH spars.g.** **GH** **No surgery**		**Not done**	**4**	**3**	**75**
Family II[b]	**Brazil**	**Gigantism** **Acromegaly** **Gigantism** **Acromegaly** **Gigantism** **Gigantism** **Gigantism**	**M, 224 cm/17 yr** **F, 165 cm/24 yr** **F, 185 cm/15 yr** **M, 172 cm/17 yr** **F, 164 cm/13 yr** **F, NA/16 yr** **M, NA/18 yr**	**E24X**	**Confirmed**	**Yes**	**GH** **GH, PRL** **GH** **GH** **NA** **NA** **NA**		**Poor response**	**9**	**7**	**78**
Family III[b]	USA	Gigantism Acromegaly	M, 202 cm/23 yr F, 177.5 cm/21 yr	No mutation	No mutation	Yes	No surgery Acidophil tu.		Good response	3	2	67
Family IV[b]	Serbia	Gigantism	M, 211.5 cm/20 yr F, 176 cm/10 yr	No mutation	No mutation	Yes	GH spars.g. GH spars.g.	Focally strong Focally strong	Poor response	3	2	67
Family V[b]	**USA**	**Acromegaly**	**F, NA/32 yr** **M, NA/32 yr**	**R81X**	**NA**	**Yes**	**GH,PRL spars.g.** **GH,PRL spars.g.**		**Not done**	**5**	**2**	**40**
Family VI[b,c]	**Japan**	**Gigantism**	**F, 184 cm/14 yr** **F, 160 cm/10 yr (>99%ile)**	**c.-270–269CG>AA and c.-220G>A**	**NA**	**Yes**	**GH,PRL spars.g.** **GH spars.g.**		**Good response**	**3**	**2**	**67**
Family VII[b]	Japan	Acromegaly	M, NA/49 yr M, NA/24 yr	No mutation	No mutation	Yes	GH staining GH staining		Not done	2	2	100
Family VIII[b]	USA	Acromegaly	M, NA/39 yr M, NA/38 yr	No mutation	NA	Yes	GH spars.g. NA		NA	3	2	67
Family IX[b]	Sweden	Acromegaly	F, NA/71 yr F, NA/70 yr M, NA/48 yr	No mutation	No mutation	Yes	NA NA NA		Not done	5	3	60
Family X[b]	**UK**	**Gigantism acromegaly** **Gigantism**	**M, 203 cm/15 yr** **M, 170 cm/29 yr** **M, 208 cm/15 yr**	**Ins274**	**Confirmed**	**Yes**	**GH spars.g.** **GH spars.g.** **GH spars.g.**	**Diffuse med** **Diffuse stron** **NA**	**Good response**	**10**	**3**	**30**
Family XI	UK	Acromegaly prolactinoma	F, 157 cm/30 yr F, NA/29 yr	No mutation	No mutation	NA	GH spars.g. No surgery	Diffuse weak	Not done	3	2	67
Family XII	**UK**	**Acromegaly** **Gigantism** **Acromegaly** **Acromegaly** **Prolactinoma** **Acromegaly** **Acromegaly** **Acromegaly**	**F, 175 cm/42 yr** **F, 200 cm/24 yr** **F, NA/24 yr** **F, NA/27 yr** **M, NA/25 yr** **M, NA/NA** **M, NA/NA** **M, NA/NA**	**R304X**	**Confirmed**	**NA**	**GH spars.g.** **GH spars.g.** **NA** **GH,PRL** **PRL**	 **Focally strong** **Focally strong**	**Poor response**	8	7	88
Family XIII	UK	Acromegaly	F, 165 cm/68 yr F, 163 cm/45 yr	No mutation	No mutation	NA	No surgery GH spars.g.		Good response	6	2	33
Family XIV	Brazil	Acromegaly prolactinoma	M, 165 cm/41 yr F, 160 cm/25 yr	No mutation	No mutation	NA	GH spars.g. No surgery	Diffuse med	Good response	3	2	67
Family XV	Finland	Acromegaly	F, 165 cm/54 yr F, NA/34 yr	No mutation	No mutation	NA	NA		Poor response	4	2	50
Family XVI[d]	**UK**	**Acromegaly**	**F, 160 cm/47 yr** **M, NA/58 yr**	**F269F splicing**	**NA**	**NA**	**No surgery** **NA**		**Not done**	**3**	**2**	**67**
Family XVII[d]	UK	Acromegaly	F, NA/52 yr M, NA/45 yr	No mutation	NA	NA	NA GH spars.g.		Not done	2	2	100
Family XVIII	UK	Acromegaly	M, NA/43 yr M, NA/46 yr	No mutation	No mutation	NA	GH staining No surgery		Poor response	3	2	67

(*Table continues*)

TABLE 1. Continued

Families	Country of origin	Clinical diagnosis	Sex, height, and age (yr) at onset/ diagnosis of affected patients	Leukocyte gDNA- AIP mutation status	Leukocyte cDNA-AIP mutation status	LOH in 11q13 in tumor tissue	Histology diagnosis	AIP staining	Responsiveness to somatostatin analogs	Number of known or obligate carriers	Number of known manifest disease	Percentage of manifest disease in carriers
Family XIX	Serbia	Acromegaly	M, 191 cm/36 yr M, NA/50 yr	No mutation	No mutation	NA	GH spars.g. NA	Diffuse strong	Poor response	4	2	50
Family XX	UK	Acromegaly	M, 188 cm/35 yr F, NA/40 yr	No mutation	NA	NA	GH densely g. NA	Diffuse med	Poor response	5	2	40
Family XXI	UK	Acromegaly	M, 198 cm/35 yr M, NA/19 yr	No mutation	NA	NA NA	GH spars.g.		Good response	3	2	67
Family XXII[e]	Malta	Acromegaly	F, 178 cm/53 yr F, NA cm/54 yr M, NA/58 yr	No mutation	NA	NA	GH staining GH staining GH staining		NA	2	2	100
Family XXIII	Serbia	Acromegaly	F, 170/35 yr F, 170/36 yr	No mutation	NA	NA	GH staining GH staining		NA	7	2	29
Family XXIV	**Romania**	**Acromegaly prolactinoma/ acromegaly**	**F, 172 cm/17 yr** **F, 169 cm/26 yr**	**R304X**	**NA**	**NA**	**No surgery (↑ serum GH, PRL)** **No surgery (↑ serum PRL and GH not suppressible)**		**Not done**	**3**	**2**	**67**
Family XXV	**Romania**	**Acromegaly**	**F, 168 cm/30 yr** **F, 165 cm/17 yr**	**R304Q**	**NA**	**NA**	**No surgery** **GH staining**		**Not done**	**2**	**2**	**100**
Family XXVI	UK	Acromegaly NFPA	M, 189 cm/30 yr M, 172 cm/42 yr	No mutation	NA	NA	GH staining Negative hormone staining		Not done	3	2	67
Giants												
G1	Australia	Gigantism	F, >97 percentile/18 months	No mutation	NA		GH staining		Not done			
G2	UK	Gigantism	M, 229 cm/24 yr	No mutation	NA		No surgery		Not done			
G3	Brazil	Gigantism	M, 195 cm/23 yr	No mutation	No mutation		GH, PRL spars.g.	Focally strong	Not done			
G4	UK	Gigantism	M, 194 cm/18 yr	No mutation	NA		GH spars.g.	Diffuse medium	Good response			
G5	USA	Gigantism	M, 186.5 cm/14 yr	No mutation	NA		GH, PRL staining		Not done			
G6	Brazil	Gigantism	F, 180 cm/25 yr	No mutation	No mutation		GH staining		Not done			
G7	UK	Gigantism	M, 198 cm/25 yr	No mutation	NA		No surgery		Poor response			

Families with mutation are marked with bold.

C, Cysteine; T, threonine; R, arginine; X, stop codon; Ins, insertion; F, phenylalanine; SS, somatostatin; spars.g., sparsely granulated; med., medium; NA, not available.

[a] Family previously reported (2, 6).

[b] Family previously reported (7).

[c] Family previously reported (41).

[d] Family previously reported (42).

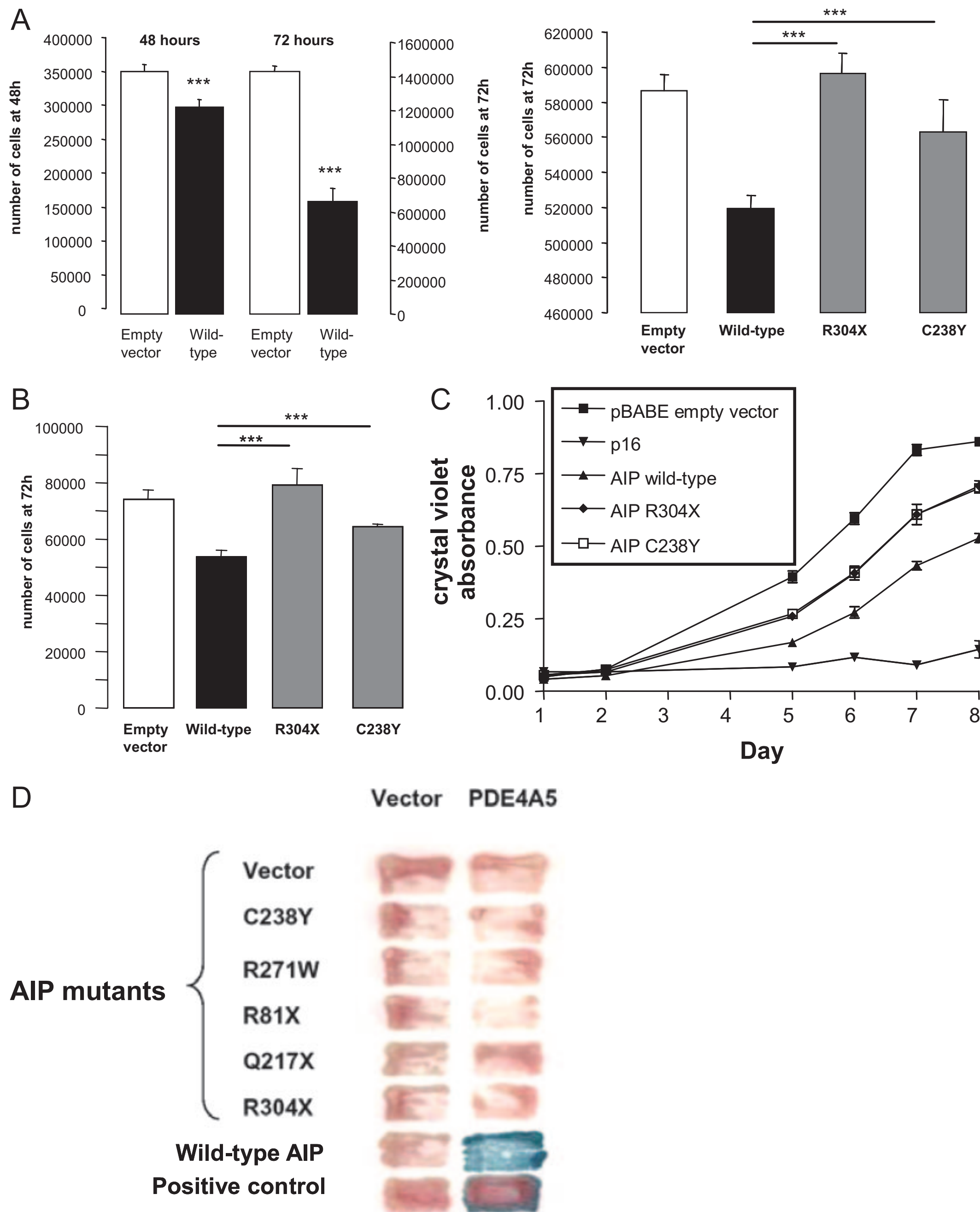

FIG. 1. Changes in cell proliferation as assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay of HEK293 cells 48 and 72 h after transfection with empty plasmid or pCI-neo-wild-type-AIP plasmid (mean ± SEM, $P < 0.001$). A, Changes in cell proliferation as assessed by MTS assay of GH3 cells transfected with wild-type and mutant AIP plasmids at 72 h (mean ± SEM, ***, $P < 0.001$). B, Cell proliferation assays in TIG-3 human fibroblasts expressing wild-type and mutant forms of AIP. The empty vector and $p16^{INK4a}$ serve as negative and positive controls, respectively (mean ± SEM, $P < 0.001$). C, A β-galactosidase filter two-hybrid assay showing interactions between PDE4A5 and wild-type and mutant AIP proteins. There is an interaction of wild-type AIP with PDE4A5 (*blue interactions* in the *right column*), whereas none of the studied mutants showed interactions with PDE4A5 (*pink interactions* in the *right column*). The incorporated positive controls were the human oncoproteins RAS and RAF1. Empty yeast vector pLEXAN served as negative control (*left columns*).

We identified six novel and two previously described heterozygous mutations in nine families (supplementary Fig. 1, A and B, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org): three stop mutations (E24X, c.70G>T; R81X, c.241C>T; R304X, c.910C>T), two amino acid changes (C238Y, c.713G>A; R304Q, c.911G>A), one large in-frame insertion (c.794_823dup), one splicing mutation (c.807C>T, F269F leading to loss of a splice acceptor site and therefore loss of exon 6), and a double promoter mutation (c.-270–269CG>AA and c.-220G>A). Further details are described in the supplementary material.

Patients (n = 36) from the 17 families with no detectable *AIP* mutation had a mean age at diagnosis of 38.2 ± 15.1 yr ($P < 0.0001$ *vs.* patients with a mutation). To detect possible intronic mutations leading to abnormal splicing of the exons, we se-

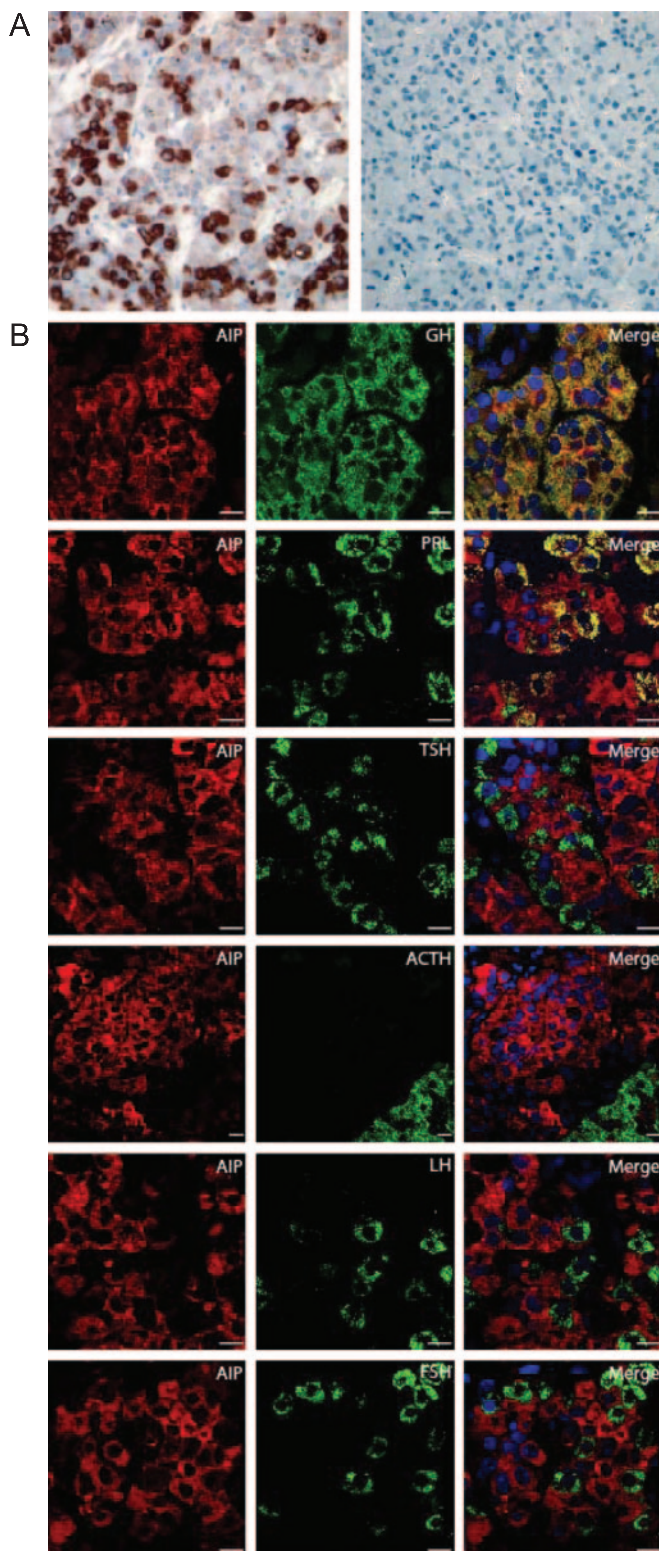

FIG. 2. A, Immunostaining in normal human pituitary using monoclonal AIP antibody (1:1000) in the *left panel*; *right panel* is negative control (×400). B, Double immunofluorescent staining using monoclonal AIP (*red staining*) and polyclonal GH, prolactin (PRL), TSH, ACTH, FSH, and LH (*green staining*) antibodies. Colocalization is shown by *yellow color* (*scale bar*, 10 μm).

quenced leukocyte-derived cDNA from one affected member of the 13 families in which an appropriate sample was available (Table 1). No further mutation was detected, whereas common single-nucleotide polymorphisms were detected as expected.

Similar numbers of affected male and female patients were identified. Previously studied pituitary adenoma samples (from 10 families) showed LOH at the 11q13 region (6, 7, 24), and five of these families harbored an *AIP* mutation (Table 1). Sparsely granulated GH-secreting adenomas were diagnosed in 19 subjects from 13 families of the 14 families in which an appropriate report was available, whereas one family (no *AIP* mutation identified) showed a densely granulated tumor (Table 1). Poor responses to somatostatin analogs (less than 50% reduction of GH/IGF-I levels) were observed in seven of the 13 treated families; one patient (family XX) is currently on GH antagonist treatment (pegvisomant) with IGF-I levels within the normal range. In addition to the pituitary adenomas, subjects with acromegaly or obligate *AIP* mutation carriers from four families developed other tumors: in families with *AIP* mutations adrenal carcinoma (family XXII) and lipomas (family XVI) were observed, whereas breast (family XV), thyroid (family XIX), and testicular cancer (family III) were diagnosed in three families without detectable *AIP* mutations. In the patient from family XXII with somatotroph adenoma and adrenal carcinoma, LOH was observed in both the pituitary and adrenal tumor tissues, although LOH in the 11q13 region is common in adrenal cancers (24).

In sporadic pituitary adenomas, we failed to find mutations in the gDNA sequence of the seven sporadic childhood- and 30 consecutive adult-onset cases of acromegaly and in the cDNA sequence of the AIP gene in any of 48 consecutive samples of sporadic pituitary adenoma tissue.

Effect of AIP on cell proliferation

We generated pCI-neoAIP-FLAG and pcDNA3-AIP-myc plasmids and retrovirus-based vectors encoding *AIP* mutations identified in familial pituitary adenoma families [in the current study and in previously published patients (10, 12)]. We studied the effect of the AIP variants on cell proliferation in three different types of cell lines: in GH3 cells (a rat somatomammotroph cell line) as the most relevant available cell line model for familial acromegaly, in HEK293 cells (an adenovirally transformed cell line with disrupted G1 regulation), and in a primary human fibroblast cell line (TIG3) with intact cell cycle regulation. Transient transfection of wild-type *AIP* caused reduced cell proliferation, compared with the empty vector control in HEK293 and GH3 cells at 48 and 72 h after transfection (Fig. 1, A and B, and supplementary Fig. 2, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org). Similar results were seen with cell counting (data not shown). Two AIP variants identified in our patients (R304X and C238Y) had no or reduced ability to block cell proliferation in both cell lines (Fig. 1, A and B).

Infection of TIG3 human fibroblasts with a recombinant retrovirus encoding wild-type *AIP* resulted in a reduced rate of cell proliferation (crystal violet-assay: 0.37 ± 0.01 arbitrary absorbance units at d 8 relative to empty vector control 0.69 ± 0.02, $P < 0.001$) (supplementary Fig. 3, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org). The known tumor suppressor p16^{INK4a} was used as a positive control, and this caused a complete block of cell proliferation under similar conditions (0.08 ±

0.01). Two AIP mutants (R304X and C238Y) showed a reduced ability to inhibit cell proliferation, compared with wild-type AIP (Fig. 1C).

AIP-PDE4A5 interaction

To study the effect of mutations on the AIP–PDE4A5 interaction, five *AIP* mutations (C238Y, R271W, R81X, Q217X, and R304X) were introduced into the AIP cDNA that we previously cloned into two-hybrid vectors (17). A β-galactosidase filter, two-hybrid assay showed an interaction of wild-type AIP with PDE4A5 (Fig. 1D), whereas none of the studied mutants showed interactions.

AIP expression

mRNA. We detected AIP mRNA expression in normal pituitary tissue and sporadic somatotroph, lactotroph and, surprisingly, corticotroph and nonfunctioning pituitary adenomas (NFPAs) (supplementary Fig. 4, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org).

Immunostaining. Immunostaining of normal pituitary with a well-characterized commercially available antihuman monoclonal antibody (25, 26) showed strong or moderate cytoplasmic AIP staining in some cells, whereas no staining was seen in others (Fig. 2A). Using double-immunofluorescence staining of normal pituitary, AIP could be detected in normal GH and prolactin-positive cells but not normal TSH-, ACTH-, LH-, or FSH-containing cells (Fig. 2B).

Immunostaining for AIP in familial pituitary adenomas from two families with an AIP mutation (Families X and XII) and in 5 families with no detectable AIP mutation (Families IV, XI, XIV, XIX and XX) revealed positive AIP staining in all cases (Fig. 3). On double-immunofluorescence staining colocalization of AIP with GH was seen (Fig. 3).

In sporadic pituitary adenomas, AIP protein immunostaining was seen in all types of adenomas (Fig. 4, A and B). Immunoblotting confirmed AIP protein expression (supplementary Fig. 5, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org). On double-immunofluorescence staining, colocalization of AIP with GH was seen in somatotroph adenomas (Fig. 4B). However, in prolactinomas, corticotrophinomas, and FSH-positive NFPAs, AIP and the hormone staining were seen in the same cells (both red and green signal), but no significant subcellular colocalization (yellow signal) was seen (Fig. 4B). This is in contrast with the findings of normal pituitary in which only GH and prolactin cells show AIP positivity (Fig. 2B). We therefore carried out electron microscopy studies to investigate the subcellular localization of AIP.

Electron microscopy. In the normal pituitary, immunogold AIP staining was observed only in the GH- and prolactin-secreting cells in which it was associated with the secretory granules (Figs. 5, A and B, and 6, A and B). No detectable AIP staining was seen in normal ACTH, TSH, and LH/FSH cells or folliculostellate cells in normal pituitary tissue (Fig. 6E). In sporadic somatotrophinomas, AIP staining was detected in the secretory vesicles (Fig. 5C), similar to normal GH cells. GH3 cells also showed AIP in secretory granules (data not shown). However, in sporadic prolactinomas, subcellular distribution of AIP immunogold was different from that observed in GH-positive cells: AIP was not detected in association with the secretory granules but was instead distributed within the cytoplasm of the endocrine cells (Figs. 5D and 6C). AIP immunogold staining was also detected in corticotroph and nonfunctioning adenomas in the cytoplasm and not in the secretory vesicles (Figs. 5, E and F, and 6D). These data correspond to the lack of colocalization of AIP and prolactin and ACTH and FSH staining in the double-immunofluorescence pictures of sporadic pituitary adenomas (Fig. 4B).

Discussion

We describe three major novel findings in this paper. First, we show that overexpression of wild-type AIP slows down cell proliferation in three different cell types including a pituitary cell

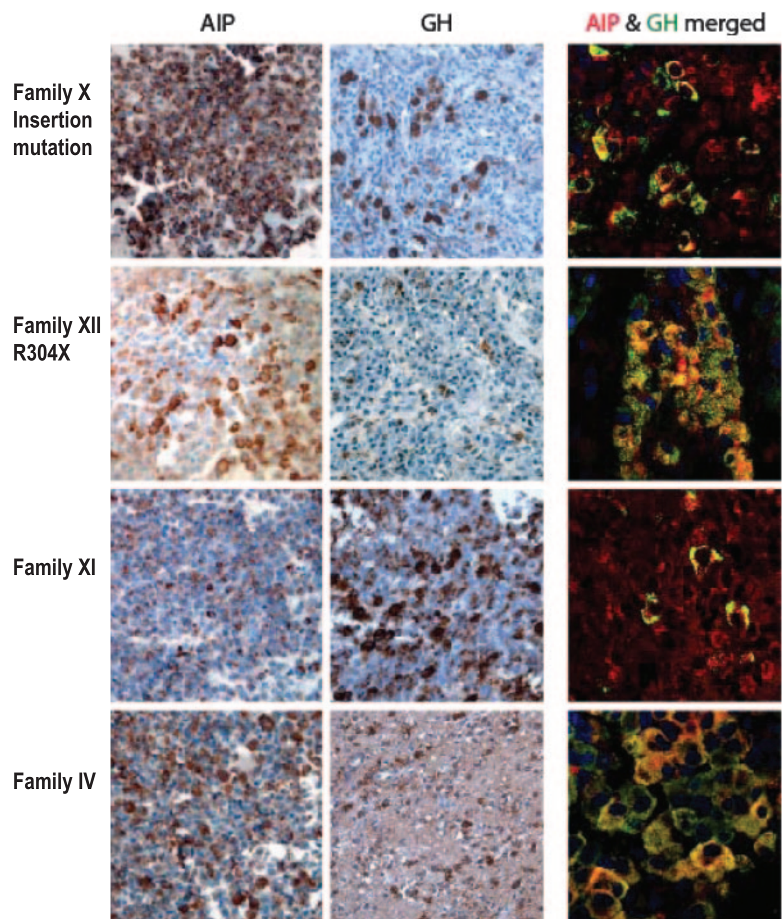

FIG. 3. Immunostaining of somatotroph tumors from patients with familial pituitary adenoma with AIP antibody (*left column*), GH antibody (*middle column*), and double-immunofluorescent staining with AIP (*red*) and GH (*green*) antibodies (*right column*). Colocalization is shown by *yellow color*; nuclei were stained with DAPI blue.

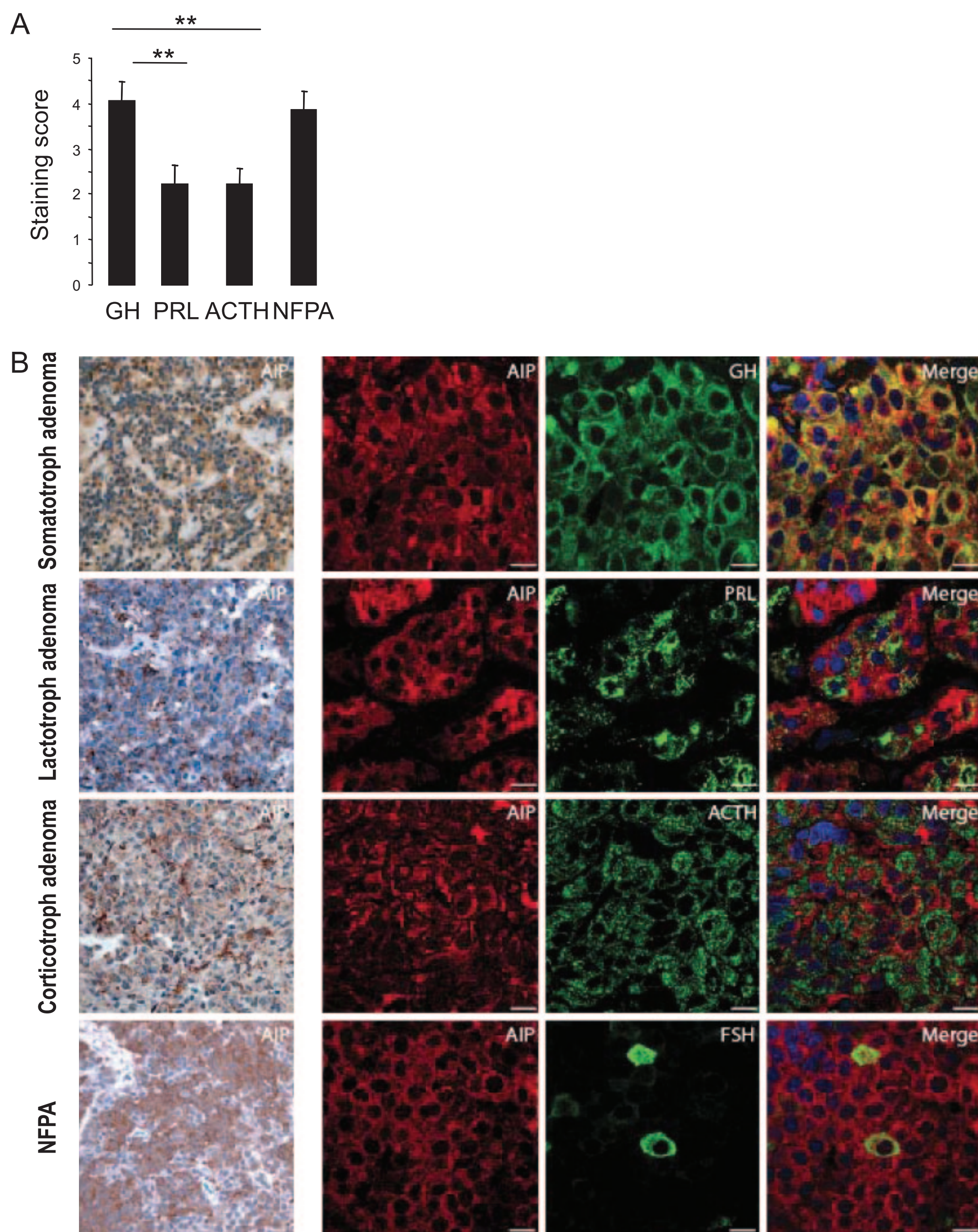

FIG. 4. A, AIP immunostaining in sporadic pituitary adenomas (mean ± SEM, **, $P < 0.01$). GH, somatotroph (n = 14), prolactin (PRL), lactotroph (n = 10), ACTH, corticotroph adenoma (n = 9), and NFPA (n = 14). B, AIP immunostaining in sporadic pituitary adenomas is shown in the *left column*. Double-immunofluorescent staining of sporadic pituitary adenomas using monoclonal AIP (*red*) and polyclonal GH, PRL, TSH, ACTH, and FSH (*green*) antibodies. Colocalization is shown by *yellow color*.

line. We also show that the mutations disrupt this function and also disrupt protein-protein interaction between AIP and its known interacting partner, PDE4A5. Second, we describe the cellular distribution of AIP in normal pituitary cells and show its exclusive association of GH and prolactin secretory vesicles and present data in pituitary adenomas with the surprising finding that non-GH-secreting sporadic adenomas express AIP, which is abnormally localized. Third, we present 26 familial pituitary adenoma families in which 10 kindreds were found to have AIP mutations; six of these mutations were not previously described, including the first identified promoter mutation. Although the *in silico* analysis is very convincing, further studies are necessary to prove that our splicing and double-promoter mutations indeed disrupt the AIP gene.

To assess the functional consequences of the AIP mutations proliferation studies and protein-protein interaction assays were carried out. Our data show that AIP has properties consistent with a tumor suppressor gene, as previously hypothesized (2, 5). Wild-type AIP attenuated cell proliferation in three different cell lines, one with disrupted G1 regulation due to adenovirus trans-

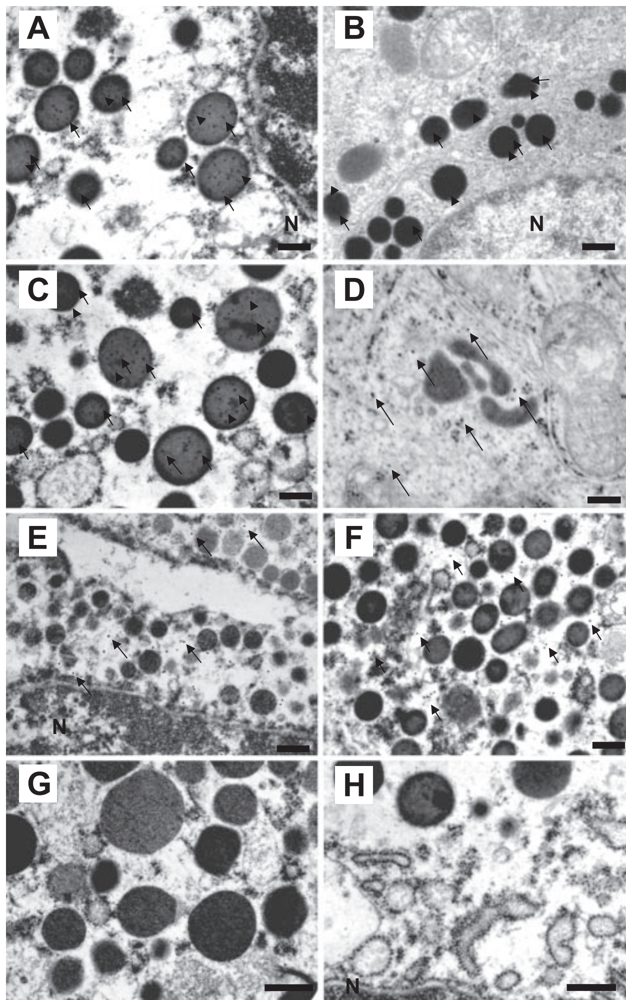

FIG. 5. Typical electron micrographs demonstrating immunogold labeling of AIP in a GH (A) and a prolactin cell (B) in normal pituitary tissue obtained postmortem, a GH-secreting adenoma (C), a prolactin-secreting adenoma with classical type 1 lactotroph cells distinguished by irregular shape of granules (D), an ACTH-secreting adenoma (E), and a nonfunctioning adenoma (F). In normal GH and prolactin cells and GH-secreting adenoma sections, AIP was evident in secretory granules, whereas in the nonfunctioning, ACTH- and prolactin-secreting adenomas, AIP was distributed within the cytoplasm of the endocrine cells and not associated with the secretory granules. There is a lack of immunogold AIP labeling in normal pituitary (G) and the ACTH-secreting adenoma (H) sections incubated with nonimmune serum in place of primary antibody. N, Nucleus. *Scale bar*, 200 nm. *Arrowheads* indicate 5 nm GH (A) or PRL (B) immunogold and *arrows*, 15 nm AIP immunogold.

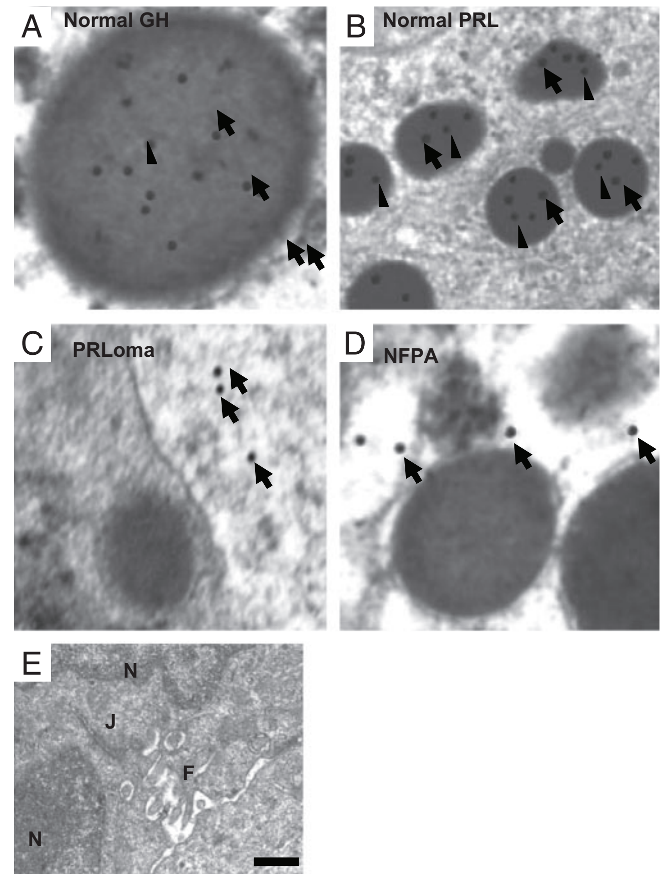

FIG. 6. Enlargement of electron microscopy images of secretory vesicles of normal GH cell (A), normal prolactin cell (B), prolactinoma (C), and NFPA (D). E, Follicle formed between three adjacent folliculostellate cells in normal human pituitary. Folliculostellate cells did not contain AIP granules. *Arrowheads* indicate 5 nm PRL immunogold and *arrows*, 15 nm AIP immunogold. J, Cell junction; F, follicle, N, nucleus. *Scale bar*, 200 nm.

formation (HEK293), one with no functional p27 protein (GH3), and one with intact cell cycle regulation (TIG3). We have shown that mutant AIP protein completely or partially loses this ability. Our data suggest that the mutations observed in the AIP gene are causally associated with the pituitary tumors seen in the familial cases because: 1) they were not seen in any of the normal subjects screened; 2) in each case they are predicted to cause either a disruption of the translated protein sequence specially affecting the last two tetratricopeptide repeat motif domains or a splicing mutation leading to loss of the last exon or possibly interfering with the regulation of the expression of the gene; and finally 3) our functional data suggest that these mutations disrupt the normal function of the AIP protein. Our data also show that the effect of AIP is not specific to pituitary cells despite the fact that lack of functional AIP primarily causes pituitary adenomas in the familial cases.

We have shown that mutant human AIPs lose the ability to bind to the known interacting partner PDE4A5. We have shown previously that AIP mutations block the interaction of AIP with PDE4A5 and its effect to modulate cAMP (17). Another PDE isoform, PDE2A, has also been recently reported to bind to the C-terminal region of AIP (18). In general, somatotroph secretory function is positively linked with the activity of cAMP and its downstream pathway as it is seen in McCune-Albright syndrome and Carney complex, but at this point it is not clear how PDEs, AhR, or any other partner of AIP is involved in its tumor suppressor effect. Recent data suggest that a repressor of AhR is down-regulated in various sporadic carcinomas, but pituitary tumors were not studied (27). Clearly, further studies are needed to clarify the mechanism of action of AIP.

Together with the six novel mutations identified in this study there are now 33 different mutations reported in the *AIP* gene (5, 10–12, 28–31). In our families, subjects with a mutation showed an age of onset of disease earlier than subjects with no detectable AIP mutation, in accordance with earlier data (4). The penetrance of the disease originally was suggested to be very low (5),

but a recent study from a large single family suggested it to be considerably larger (33%) (32). Our data of a mean penetrance of 66% may be skewed due to limited genealogical data in some of the families. We observed sparsely granulated tumors in the vast majority of the cases of familial somatotroph adenomas, a higher proportion of cases than reported in sporadic tumors (23). Sparsely granulated tumors are known to be more invasive and respond less well to somatostatin analog therapy (23, 33). Indeed, seven of the 13 families for which data were available showed a poor response to somatostatin analogs. In one of the families described earlier with the R304X mutation (5, 34), somatostatin analogs were also noted to be ineffective in reducing serum GH levels into the safe range. In contrast, more than 75% of acromegalic patients with sporadic tumors show responsivity to somatostatin analog therapy (1, 35). However, the numbers are currently too small to come to any definitive conclusions, and further clinical and experimental data are necessary to establish this point (23).

The families studied here were originally identified as having FIPAs. However, careful history taking revealed tumors at other sites in both acromegalic patients and unaffected carriers including breast, thyroid, and testicular cancer as well as lipomas. Lipomas were also reported earlier in an isolated familial somatotrophinoma kindred (36), and thyroid carcinomas were observed by Raitila *et al.* (37) in familial pituitary adenoma patients with *AIP* mutations. Because these are relatively common tumors, a larger number of families is needed to clarify whether AIP is influencing predisposition to neoplasms in tissues other than the pituitary.

We did not identify germline or somatic *AIP* mutations in leukocyte gDNA or tumor tissue cDNA in any of the patients with sporadic acromegaly, including those with childhood-onset disease. Some studies described mutations in sporadic patient cohorts, specially in early-onset cases, whereas others did not (5, 12, 28, 29, 31, 37–39), although some of these studies did not do comprehensive sequencing (38, 39).

Immunostaining of normal pituitary revealed AIP staining in the cytoplasm of somatotroph and lactotroph cells but not in other cell types. AIP was associated with the secretory vesicles in these cells as shown by electron microscopy. According to the classical Knudson hypothesis of tumor suppressor genes, the normal allele of *AIP* should be lost from the adenoma tissue of patients with an *AIP* mutation, so that the AIP immunostaining in these cases indicates the mutant AIP protein. In our cases with Ins274 and R304X mutations, AIP staining was observed in the adenomas from all four subjects studied (Fig. 3). Georgitsi *et al.* (29) observed no AIP staining in nine of 12 adenomas from patients with AIP mutations. However, nine of their 12 cases had an early stop mutation (Q14X), and it seems most likely that the severely truncated AIP protein in these cases is either not present due to degradation or that the epitope required for the antibody binding is missing. Our AIP mutation-negative familial somatotroph adenomas also stained positive for AIP.

In the sporadic adenomas, we were surprised to see significant *AIP* expression at both the mRNA and protein level, not just in GH- and prolactin-secreting tumors but also in corticotroph adenomas and NFPAs, the latter being predominantly of gonadotroph origin (40). This suggests an increase in *AIP* expression during tumorigenesis in these cell types as AIP protein could not be detected with immunostaining in normal corticotroph and gonadotroph cells. In addition, whereas AIP colocalizes with GH in the secretory vesicles of somatotroph tumor cells (Figs. 3, 4B, and 5C), similar to normal somatotrophs (Figs. 2B and 5A), lactotroph, corticotroph, and nonfunctioning adenomas do not show actual intracellular spatial colocalization of AIP and prolactin, ACTH, or FSH within the adenoma cells. Using electron microscopy, in sporadic lactotroph, corticotroph, and nonfunctioning adenomas, AIP was detected in the adenoma cells but was not associated with the secretory vesicles; rather, AIP was found to be distributed throughout the cytoplasm (Figs. 5, D, E, and F; and 6, C and D). The electron microscopy data therefore confirmed the double-immunofluorescence staining results: the close colocalization of AIP with GH and prolactin (both in the secretory vesicles) in normal cells and GH adenoma cells and the lack of close colocalization of the hormone and AIP in sporadic lactotroph, corticotroph, and nonfunctioning adenoma cells. These findings raise several questions that need to be addressed in further studies. What is the role of AIP in association with the secretory vesicles in normal somatotroph and lactotroph cells? Why can AIP retain secretory vesicle targeting in GH cells when adenomatous and go astray in other adenoma types including prolactinomas? Is this abnormal expression and localization of AIP linked with tumorigenesis?

The question remains as to the genetic cause for familial pituitary adenomas in almost two thirds of our familial cases in whom we failed to find an exonic or promoter mutation of *AIP*. In a recent study, Daly *et al.* (10) showed that some 50% of familial pure acromegalic cases had no mutation in the coding region of the AIP. From our cohort of 17 AIP mutation-negative families, five had been previously studied for LOH (7), and all of them were shown to have LOH at 11q13. Whereas both additional intronic and promoter changes are possible, the fact that only a minority of patients show an exonic mutation of *AIP* leads us to speculate that there is yet another gene involved in familial pituitary adenomas in the 11q13 region that remains to be identified. The possibility of genetic heterogeneity in familial pituitary adenomas is supported by the fact that age of onset is much higher in the AIP-negative families than the AIP-positive families, suggesting that the other tumor suppressor gene(s) possibly involved do not initiate tumorigenesis at such an early age as AIP.

We suggest that in families in which multiple pituitary adenoma cases are recognized and there is no clinical suspicion of MEN-1 or Carney complex, subjects at risk should be screened biochemically, whereas genetic screening probably will remain a tool of research-oriented units. In the series by Daly *et al.* (10), 50% of pure acromegaly families showed AIP mutations, whereas in our cohort seven of the 21 pure acromegaly families and two of the four mixed acromegaly/prolactinoma families showed AIP mutations. The question of AIP screening of childhood-onset sporadic acromegaly patients is controversial. Several sporadic giants have been shown to harbor germline mutations (12, 28, 31) and 10 of 33

(30%) of our familial cases with an AIP mutation had gigantism, but in our series no mutations were detected in any of our seven sporadic giants.

In summary, we have identified several new AIP mutations, and our functional data suggest that wild-type AIP is indeed a tumor-suppressor gene and the mutations disrupt the effects of the AIP protein. AIP is associated with secretory vesicles in normal GH and prolactin cells but shows abnormal expression and localization in non-GH sporadic pituitary adenomas.

Acknowledgments

We are grateful for the families providing samples and to the referring doctors including Mr. M. Powell, Drs. S. Ball, A. James, and A. Levy (United Kingdom); Drs. H. Widell and J.-O. Johansson (Sweden); Dr. P. Gallego (Australia); and Dr. K. Choudhry, E. Dimaraki, and Professor A. Barkan (United States). We are grateful to Professor G. Perdew (United States) for the provision of *AIP* plasmids and Drs. P. Smith, P. King, and M. Rodrigez-Niedenführ (United Kingdom) for technical advice and to the Cancer Research Committee of St. Bartholomew's Hospital for funding.

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Disclosure Statement: The authors have nothing to disclose.

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