Caveolin 3-mediated integrin β1 signaling is required for the proliferation of folliculostellate cells in rat anterior pituitary gland under the influence of extracellular matrix

Kotaro Horiguchi, Ken Fujiwara, Cimi Ilmiawati, Motoshi Kikuchi, Takehiro Tsukada, Tom Kouki and Takashi Yashiro
Division of Histology and Cell Biology, Department of Anatomy, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
(Correspondence should be addressed to K Horiguchi; Email: kota@jichi.ac.jp; T Yashiro; Email: tyashiro@jichi.ac.jp)

Abstract

Folliculostellate (FS) cells in the anterior pituitary gland are believed to have multifunctional properties. Using transgenic rats that express green fluorescent protein (GFP) specifically in FS cells in the anterior pituitary gland (S100b-GFP rats), we recently revealed that FS cells in primary culture exhibited marked proliferation in the presence of laminin, an extracellular matrix (ECM) component of the basement membrane. In a process referred to as matricrine action, FS cells receive ECM as a signal through their receptors, which results in morphological and functional changes. In this study, we investigated matricrine signaling in FS cells and observed that the proliferation of FS cells is mediated by integrin β1, which is involved in various signaling pathways for cell migration and proliferation in response to ECM. Then, we analyzed downstream events of the integrin β1 signaling pathway in the proliferation of FS cells and identified caveolin 3 as a potential candidate molecule. Caveolin 3 is a membrane protein that binds cholesterol and a number of signaling molecules that interact with integrin β1. Using specific small interfering RNA of caveolin 3, the proliferation of FS cells was inhibited. Furthermore, caveolin 3 drove activation of the mitogen-activated protein kinase (MAPK) signaling cascades, which resulted in upregulation of cyclin D1 in FS cells. These findings suggest that matricrine signaling in the proliferation of FS cells was transduced by a caveolin 3-mediated integrin β1 signaling pathway and subsequent activation of the MAPK pathway.

Introduction

The anterior pituitary gland is composed of five types of hormone-producing cells and folliculostellate (FS) cells that do not produce classical anterior pituitary hormones. These cell aggregations form cell cords or clusters surrounded by different types of extracellular matrices (ECM) that provide the mechanical integrity, rigidity, and elasticity that are essential for these cells to perform their respective roles (Soji & Herbert 1989, Kaidzu et al. 2000, Paez-Pereda et al. 2005). FS cells have a star-like appearance and form a pseudolumen at the central portion of FS cell clusters (Soji & Herbert 1989). Physiologically, it has been speculated that FS cells may be stem cells, phagocytes, or regulating cells for hormone release (Allaerts & Vankelecom 2005) and that they accomplish cell-to-cell communication via the gap junction in the anterior pituitary gland (Fauquier et al. 2001, Sato et al. 2005).

Generally, cells can receive ECM as a signal that causes changes such as migration, proliferation, and differentiation—a process referred to as ‘matricrine’ action (Miyamoto et al. 2007). In our recent attempt to investigate matricrine action in anterior pituitary cells, FS cells markedly extended their cytoplasmic processes and promoted gap junction formation between them under the influence of laminin that is an ECM component of the basement membrane (Horiguchi et al. 2010, 2011). In addition, we showed that FS cells exhibited marked proliferation in the presence of ECM (Horiguchi et al. 2010). However, it is unclear at present how FS cells receive laminin as a signal and exhibit these phenomena, which is crucial for understanding matricrine action in FS cells. In this study, we, therefore, attempted to determine the matricrine signaling pathway in the proliferation of FS cells. First, we examined whether proliferation of FS cells in the presence of laminin is mediated by integrin β1 as a laminin receptor. Then, we analyzed the downstream events of the integrin β1 signaling pathway with mediation by caveolin (Echarri et al. 2007), which is a membrane protein that binds cholesterol and a number of signaling molecules potentially linked with integrin function.
Materials and Methods

Animals

Transgenic S100b-green fluorescent protein (GFP) rats express GFP under control of the promoter of the S100b protein gene, a marker of FS cells. The rats were donated by Prof. K Inoue of Saitama University and were bred in our laboratory (Itakura et al. 2007). Male rats aged 8–10 weeks weighing 250–300 g were given access to food and water and housed under conditions of 12 h light:12 h darkness. The rats were killed by exsanguination from the right atrium under deep Nembutal anesthesia and were then perfused with Ca2+- and Mg2+-free Hank’s solution for primary culture. All animals were treated in accordance with the Guidelines for Animal Experimentation of Jichi Medical University, which are based on the NIH Guidelines for the Care and Use of Laboratory Animals.

Cell culture

Anterior pituitary cells of male S100b-GFP rats were dispersed as described previously (Horiguchi et al. 2008). Dispersed cells were separated into GFP-positive and -negative cells by a cell sorter (MoFlo XDP; Beckman Coulter, Inc., Fullerton, CA, USA). GFP-positive cells were plated onto 8-well glass chamber slides (1 cm2/well; Nalge Nunc International, Rochester, NY, USA), with or without a coating of 10 μg/cm2 of laminin (Millipore, Bedford, MA, USA), at a density of 1×105 cells/cm2 in 400 μl of Medium 199 with Earle’s salts (Invitrogen) supplemented with 10% FBS (Sigma–Aldrich Corp.), 0·5 U/ml penicillin, and 0·5 μg/ml streptomycin (Invitrogen). In the other experimental group, laminin-coated slides were treated in medium containing 5 μg/ml monoclonal anti-integrin β1 antibody (clone Ha2/5, BD Biosciences, San Jose, CA, USA), which was shown to block signaling mediated by integrin β1 (Schultz & Armanit 1995); hamster IgM served as control. Cells were then cultured for 72 h at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

Proliferation assay

To visualize the proliferative activities of FS cells, the nucleotide analog 5-bromo-2′-deoxyuridine (BrdU; Sigma–Aldrich Corp.) was added to primary culture for 24 h at a concentration of 3 μg/ml. Cells were fixed in 4% paraformaldehyde in 25 mM phosphate buffer (pH 7·4) for 20 min at room temperature and were then treated with 4 M HCl in TBST, specific immunoreactivity was visualized with Can Get Signal solution (Toyobo, Osaka, Japan), followed by TBST washes, and incubated for 1 h with HRP-labeled secondary antibodies (Envision+ System–HRP, Dako, Glostrup, Denmark). After washing with TBST, specific immunoreactivity was visualized using Chemiluminescence ECL Plus Systems (GE Healthcare, Mississauga, ON, Canada) with lumi-shot film (Fujifilm, Tokyo, Japan). The film was scanned and densitometric analysis was performed with ImageJ software (NIH, Bethesda, MD, USA). The results were normalized with β-actin. Each analysis was performed in triplicate.

Quantification of mRNA levels by real-time reverse transcription-PCR

Total RNA fractions were prepared with Trizol (Invitrogen) from cultured cells and incubated with RNase-free DNase I (1 U/tube; Promega Corp.). After inactivation of DNase I by heating for 10 min at 65 °C, cDNA was synthesized using the PrimeScript RT reagent kit (Takara) with oligo(dT)20 primer (Invitrogen). Quantitative real-time PCR (ABI PRISM 7900HT; Applied Biosystems, Carlsbad, CA, USA) was performed using gene-specific primers and SYBR Premix Ex Taq (Takara) containing SYBR Green I. The following primers were used to amplify cDNA fragments of cyclin D1 (GenBank accession no. NM_171992): forward 5′-TGCAAATGGAACCTGGCTCTG3′ and reverse 5′-GC-GGATGATCTGCTTTGTTCT-3′ (125 bp); caveolin 1 (NM_031556): forward 5′-GGGAACGGGCAACA-TCTAC-3′ and reverse 5′-GCGGTTGACCAGATCA-ATT-3′ (107 bp); caveolin 2 (NM_133194): forward

Journal of Endocrinology (2011) 210, 29–36
Induction of caveolae depletion

The GFP-positive cells were cultured for 72 h on the laminin-coated surface as described above. The culture medium was changed to 400 µl Medium 199 with Earle’s salts (Invitrogen) supplemented with 10% FBS (Sigma–Aldrich Corp.), 0.5 U/ml penicillin, 0.5 µg/ml streptomycin (Invitrogen), and 10 mM methyl-β-cyclodextrin (MβCD; WAKO, Osaka, Japan; Bellott et al. 2005, Chung et al. 2009) and cultured for 8 h. MβCD was used at a concentration of 10 mM because this concentration has been shown to markedly disrupt caveolae structure in cultured cells, without affecting cell viability, by removing cholesterol from the plasma membrane (Ushio-Fukai et al. 2001). After washing with Medium 199, the culture medium was changed without MβCD for 40 h. The culture medium without MβCD was used as a negative control. We time-lapse recorded the cells as described previously (Horiguchi et al. 2010, 2011) using a digital camera (ORCA-ER; Hamamatsu Photonics, Shizuoka, Japan) and MetaMorph software (Molecular Devices Corp., Downingtown, PA, USA). Each observation was performed in triplicate.

Small interfering RNA for caveolin 1 and 3

For small interfering RNA (siRNA) transfection, the culture medium was replaced by 400 µl Medium 199 with Earle’s salts (Invitrogen) supplemented with 10% FBS (Sigma–Aldrich Corp.), 0.5 U/ml penicillin, 0.5 µg/ml streptomycin (Invitrogen), siRNA against caveolin 1 or 3 mRNA (0.2 µM, Qiagen), and INTERFERin (1:100 v/v, PolyPlus Transfections, Inc., New York, NY, USA) transfection reagent at 24 h. Nonsilencing siRNA (Qiagen) with no homology to any known mammalian gene was used as a negative control. After that, the GFP-positive cells were cultured for 48 h. Each analysis was performed at least three times.

Immunocytochemistry

Cultured cells fixed with 4% paraformaldehyde in 50 mM phosphate buffer for 20 min at room temperature were first immersed in PBS containing 2% normal goat serum for 20 min at 30 °C and then incubated overnight with anti-rat caveolin 3 mouse monoclonal antibody (12-5 ng/ml; BD Biosciences) at room temperature. After washing with PBS, cells were incubated in PBS with Alexa Fluor 568-conjugated goat anti-mouse IgG diluted to 1:200. Absence of an observable nonspecific reaction was confirmed using normal mouse serum. Cells were scanned using a confocal laser microscope (FV1000, Olympus Corp., Tokyo, Japan).

Statistical analysis

All results were presented as mean ± S.E.M. Statistical analysis was performed using ANOVA with Fisher’s protected least significant difference test (StatView 5.0 software, SAS Institute, Inc., Cary, NC, USA) and Dunnett’s test. Differences between groups were considered to be statistically significant at a P value of <0.05.

Results

Role of integrin β1 in laminin-induced FS cell proliferation

A cell sorter was used to isolate FS cells from male S100b-GFP rat anterior pituitary for primary culture. The histological features of FS cells in primary culture were the same as in our previous study (Horiguchi et al. 2011). At 72 h, FS cells on the uncoated surface and laminin-coated surface with integrin β1 antibody formed clusters of ~10–20 cells (Fig. 1A and C). However, almost all FS cells on laminin with or without hamster IgM were flattened and had interconnected cell bodies (Fig. 1B and D). The percentage of BrdU-positive cells among all FS cells was 14.9 ± 0.7 and 13.9 ± 0.5% on the laminin-coated surface with and without hamster IgM respectively (Figs 1J, L and 2A). In contrast, BrdU-positive FS cells were not observed on laminin-coated surfaces with integrin β1 antibody or on uncoated surface (Figs 1I, K, and 2A). Then, we examined whether ERK activation and cyclin D1 expression level were regulated by integrin β1. Using western blot analysis, we detected pERK, ERK, and cyclin D1 immunoreactivity,
at their expected masses, in FS cells (Fig. 2B). When normalized with β-actin, the phosphorylation of ERK and the production of cyclin D1 were greater in FS cells on laminin-coated surfaces with or without hamster IgM than on the uncoated and laminin-coated surfaces with integrin β1 antibody (Fig. 2C and D).

Change in caveolin mRNA and protein expression level in FS cells on laminin

To determine whether laminin affects gene expression of caveolins in FS cells, we performed quantitative real-time RT-PCR. As compared with the uncoated surface, caveolin 1 and 3 expression was higher in FS cells on the laminin-coated surface (Fig. 3A and C). However, their expression levels on the laminin-coated surface with anti-integrin β1 antibody were lower than those on the laminin-coated surface with hamster IgM. Caveolin 2 expression was not higher in cells on the laminin-coated surface compared with the uncoated surface (Fig. 3B). On western blot analysis, caveolin 1 and 3 immunoreactivity was greater in FS cells on the laminin-coated surface compared with the uncoated surface (Fig. 3D–F). In contrast, treatment with anti-integrin β1 antibody completely blocked the increases in caveolin 1 and 3 immunoreactivity in FS cells on the laminin-coated surface with hamster IgM (Fig. 3D–F).

Time-lapse imaging of FS cells in primary culture with MβCD

To examine the importance of caveolin protein in the proliferation of FS cells under the influence of laminin, FS cells were exposed to MβCD, a cholesterol-depleting agent that disassembles caveolae (Bellott et al. 2005, Chung et al. 2009), from 72 to 80 h in primary culture. MβCD treatment inhibited proliferation of FS cells (Fig. 4A, B and Supplementary Movie 1, see section on supplementary data given at the end of this article). After MβCD was removed from medium in primary culture at 80 h, FS cells resumed proliferation (Fig. 4C and Supplementary Movie 2, see

Figure 1 Proliferation of FS cells in primary culture. (A–D) GFP images superimposed on phase-contrast images on the uncoated surface, laminin-coated surface, laminin-coated surface with integrin β1 antibody, and laminin-coated surface with hamster IgM respectively. Scale bars, 100 μm. (E–H) Phase-contrast image of the same field shown in I–L on the uncoated surface, laminin-coated surface, laminin-coated surface with integrin β1 antibody, and laminin-coated surface with hamster IgM respectively. (L–L) Confocal image of BrdU incorporation (green, FS cells and red, BrdU).

Figure 2 Analysis of proliferation of FS cells in primary culture. (A) Percentage of BrdU-positive cells among FS cells on the uncoated surface (uncoated), laminin-coated surface (laminin), laminin-coated surface with integrin β1 antibody (laminin Itgb-1 AB), and laminin-coated surface with hamster IgM (laminin Ham IgM). ND, not determined. (B) The amount of protein was determined by western blotting. The four panels show pERK, ERK, cyclin D1, and β-actin. (C and D) pERK and cyclin D1 protein levels in FS cells on the uncoated surface (uncoated), laminin-coated surface (laminin), laminin-coated surface with integrin β1 antibody (laminin Itgb-1 AB), and laminin-coated surface with hamster IgM (laminin Ham IgM) respectively. Western blotting data from three experiments (mean ± S.E.M., n = 3) were quantified by densitometry and normalized with β-actin. pERK and cyclin D1 protein levels were calculated as ratios of uncoated values respectively. *P<0.05.
respectively. Caveolin 1, 2, and 3 protein levels were calculated as ratios of uncoated values quantified by densitometry and normalized with blotting data from three experiments (mean S.E.M., n = 5). Caveolin 1, 2, and 3 mRNA levels were calculated as ratios of uncoated values respectively. Caveolin 1 and 3 mRNA expression using siRNA in FS cells of primary culture (Fig. 5F). We examined the localization of caveolin 3 by immunocytochemistry at 72 h in primary culture on both the uncoated and the laminin-coated surfaces. Immunoreactive caveolin 3 was located on cell bodies and cytoplasmic processes at FS cells on both the uncoated and the laminin-coated surfaces (Fig. 6A–D). Caveolin 3 immunoreactivity of FS cells was clearly greater on the laminin-coated surface than on the uncoated surface (Fig. 6B and D). We also detected the localization of caveolin 3 by immunohistochemistry in rat anterior pituitary gland. Immunoreactive caveolin 3 was located on FS cells in the anterior pituitary gland (Fig. 6E).

**Discussion**

In this study, we revealed that matricrine signaling of the proliferation in FS cells was transduced by the association of caveolin 3 with integrin β1, which promotes mitogen-activated protein kinase (MAPK).

By using transgenic rats (S100b-GFP rats) that express GFP specifically in FS cells in the anterior pituitary gland (Itakura et al. 2007), we were able to use a cell sorter to obtain pure GFP-positive FS cells, which allowed us to examine the direct involvement of ECM on FS cells. As shown in Fig. 1, isolated FS cells directly receive laminin as a signal and thus proliferate in the absence of GFP-negative cells, which are primarily hormone-producing cells.

ECM generally transduces signals to cells through a family of transmembrane receptors called integrins (Hynes 2002). Many reports suggest that integrin signaling induces proliferation of cells, including mesangial cells, mammary proliferection.

**Knockdown of caveolin 1 and 3 by siRNA**

To examine the functional role of caveolin 1 and 3 in the proliferation of FS cells, we downregulated caveolin 1 and 3 gene expression using siRNA in FS cells of primary culture on laminin-coated surfaces. When caveolin 1 and 3 were downregulated with a specific siRNA, their respective expression levels were also downregulated (Fig. 5A–D). Quantitative analysis revealed that cyclin D1 mRNA level in caveolin 3 siRNA-treated FS cells was lower than that in caveolin 1 siRNA-treated and control FS cells (Fig. 5E). The percentage of BrdU-positive cells among caveolin 3 siRNA-treated FS cells was also lower than that among caveolin 1 siRNA-treated and control FS cells (Fig. 5F).
epithelial cell, and mesenchymal cells, in the presence of ECM (Schöcklmann et al. 2000, Li et al. 2005, Fernandes et al. 2006). Integrins comprise an α-subunit and a β-subunit that form a heterodimer. In mammals, 18 types of α-subunits and eight types of β-subunits are known; their various combinations give rise to 24 integrin heterodimers that differ in ligand specificity (Hynes 2002). With respect to these ligand specificities, we recently reported that FS cells bind laminin through integrin-α3β1 and/or integrin-α6β1 in the anterior pituitary gland (Horiguchi et al. 2010). As shown in Fig. 2, an antibody that inhibits the integrin β1 cascade blocked the increase of ERK phosphorylation and cyclin D1 production, which suggests that integrin β1 receives laminin as a signal on FS cells and that its signaling activates MAPK signaling cascades, leading to cyclin D1 transcription and contributing to cell cycle progression. However, there are several signal transduction molecules between integrin β1 and MAPK, including focal adhesion kinase (Li et al. 2001, Ding et al. 2009), protein kinase C (Han et al. 2004, Kudirka et al. 2007), and caveolin (Echarri et al. 2007). Caveolin is a cholesterol-binding membrane protein component of caveolae that are small (50–100 nm) cell invaginations that play a role in numerous cell functions (Parton 2001). Caveolin isoforms 1 and 3 are involved in the formation of…

**Figure 5** Downregulation of caveolin 1 and 3 by siRNA. (A and B) The expression of caveolin 1 and 3 mRNA determined by real-time PCR was normalized with an internal control (GAPDH) for 48 h incubation after FS cells were transfected by siRNA respectively (mean ± S.E.M., n = 4). Caveolin 1 and 3 mRNA levels (Cav 1 siRNA and Cav 3 siRNA) were calculated as ratios of control values (control). Caveolin 1 and 3 mRNA expression of experimental group was lower than that of control. (C and D) The protein levels of caveolin 1 and 3 were determined by western blotting. The upper panel shows caveolin 1 (Cav 1) or 3 (Cav 3) and the lower panel shows β-actin as the loading control. Western blotting data from three experiments (mean ± S.E.M., n = 3) were quantified by densitometry and normalized with β-actin. The protein level of caveolin 1 and 3 in FS cells of siRNA treatment (Cav 1 siRNA and Cav 3 siRNA) was significantly lower than that of control (control). (E) Expression of cyclin D1 mRNA determined by real-time PCR was normalized with an internal control (GAPDH) for 48 h incubation after FS cells were transfected by siRNA. Cyclin D1 mRNA levels in FS cells of caveolin 1 and 3 siRNA treatment (Cav 1 siRNA and Cav 3 siRNA) were calculated as ratios of control values (control) respectively (mean ± S.E.M., n = 4). (F) The percentage of BrdU-positive cells among FS cells on the laminin-coated surface with siRNA of caveolin 1 and 3 (Cav 1 siRNA and Cav 3 siRNA) respectively. The number of BrdU-positive cells with siRNA of caveolin 3 was significantly lower than that of control (control). *P < 0.05.

**Figure 6** Caveolin 3 expression in anterior pituitary gland. (A–D) Immunocytochemistry for caveolin 3 in primary culture of FS cells; (A and B) 72 h culture of FS cells on the uncoated surface; (C and D) 72 h culture of FS cells on the laminin-coated surface; (A and C) phase-contrast images; (B and D) fluorescent images. (E) Immunohistochemistry of caveolin 3 in the anterior pituitary gland. Caveolin 3 (red) immunoreactivity was observed on FS cells (green). Scale bar, 10 μm.
caveolae and interact with integrin β1 signaling molecules via a specific domain (Echarri et al. 2007). Caveolin 3 is expressed in smooth and skeletal muscle cells, cardiac myocytes, and astrocytes (Song et al. 1996, Ikezu et al. 1998). As shown in Figs 3 and 6, FS cells in anterior pituitary also expressed caveolin 3, and its expression level increased in the presence of laminin. Furthermore, the proliferation of FS cells was inhibited by MβCD, which abolishes the distribution of caveolin protein, and by siRNA specific for caveolin 3 (Figs 4 and 5). These results suggest that integrin β1 signaling in the proliferation of FS cells was mediated by caveolin 3. We recently reported that hormone-producing cells also expressed integrin β1 and showed almost no proliferation in the presence of laminin (Horiguchi et al. 2010). Thus, the difference in response to laminin must be due to the expression of caveolin 3. Our findings in this study showed that the caveolin 3-mediated integrin β1 signaling pathway was specific to FS cells in anterior pituitary. Integrin β1 signaling in the presence of laminin also triggered an increase in gap junction formation between FS cells in our previous report (Horiguchi et al. 2011). There are some reports that caveolin 3 upregulated the expression of connexin 43 in gap junction channel protein (Chung et al. 2009, Liao et al. 2010). This leads us to speculate that caveolin 3 plays a role in gap junction formation between FS cells. In anterior pituitary gland, it has been shown that the lobular structures surrounded by basement membrane are responsible for organizing the functional unit and that the cytoplasmic processes of FS cells attach to the basement membrane (Soji & Herbert 1989, Shirasawa et al. 2004). In addition to these histological features, we hypothesize that FS cells exert matricrine action via caveolin 3-mediated signaling for their functional roles.

In this study, we observed increased expression of not only caveolin 3 but also caveolin 1, under the influence of laminin in FS cells (Fig. 3). However, caveolin 1 has no effect on the proliferation of FS cells (Fig. 5). Kurzchalia et al. (1992) showed that caveolin 1 is localized to plasma membrane caveolae, the Golgi apparatus, and trans-Golgi-derived transport vesicles. In a previous study, we confirmed by electron microscopy that, in the presence but not in the absence of laminin, FS cells displayed well-developed Golgi apparatus that consisted of several layers of Golgi lamellae and many vesicles and vacuoles in the Golgi area (Horiguchi et al. 2011). These results suggest that the increase in caveolin 1 is due to the hyperfunctional status of FS cells under the influence of laminin.

In a series of studies investigating matricrine action in FS cells (Horiguchi et al. 2010, 2011), we have shown that FS cells in the presence of laminin may play important functional roles in the anterior pituitary gland. In this study, we have succeeded in elucidating the specific mechanism of matricrine action in FS cells. Our findings suggest that proliferation of FS cells is induced by a caveolin 3-mediated integrin β1 signaling pathway, leading to activation of the MAPK pathway in the anterior pituitary gland.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-11-0103.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was partly supported by a Grant-in-Aid for Scientific Research (C) (21570067 and 22590192), and a Grants-in-Aid for Young Scientists (B) (22790190), from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by promotional funds for the Keirin Race of the Japan Keirin Association, and by the Jichi Medical University Young Investigator Award from Jichi Medical University, School of Medicine.

Acknowledgements
We thank Prof. K Inoue (Saitama University, Japan) for supplying the transgenic rats. We are grateful to M Yatabe for her excellent technical assistance and to Prof. Y Hanazono and Y Furukawa for their support in fluorescence-activated cell sorting. We also thank David Kipler, ELS, of Supernatant Communications for revising the language of the manuscript.

References

www.endocrinology-journals.org


Received in final form 7 April 2011

Accepted 20 April 2011

Made available online as an Accepted Preprint 20 April 2011