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Megakaryocyte proliferation and ploidy regulated by the cytoplasmic tail of glycoprotein Ibα

Taisuke Kanaji, Susan Russell, Janet Cunningham, Kenji Izuhara, Joan E. B. Fox, and Jerry Ware

We have investigated the ability of glycoprotein (GP) Ibα, a megakaryocytic gene product, to sequester the signal transduction protein 14-3-3ξ and to influence megakaryocytopoiesis. Using a Gp1ba$^{-/-}$ mouse colony, we compared the rescued phenotypes produced by a wild-type human GP Ibα allele or a similar allele containing a 6-residue cytoplasmic tail truncation that abrogates binding to 14-3-3ξ. The observed phenotypes illustrate an involvement for GP Ibα in thrombopoietin-mediated events of megakaryocyte proliferation, polyploidization, and the expression of apoptotic markers in maturing megakaryocytes. We developed a hypothesis for the involvement of a GP Ibα/14-3-3ξ/PI-3 kinase complex in regulating thrombopoietin-mediated responses. An observed increase in thrombopoietin-mediated Akt phosphorylation in the truncated variant supported the hypothesis and led to the development of a model in which the GP Ibα cytoplasmic tail sequestered signaling proteins during megakaryocytopoiesis and, as such, became a critical regulator in the temporal sequence of events that led to normal megakaryocyte maturation. (Blood. 2004;104:3161-3168)

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Introduction

Megakaryocytopoiesis and thrombopoiesis are highly specialized forms of cell maturation in which bone marrow precursors develop from stem cells, proliferate, and are triggered to differentiate into mature polyploid megakaryocytes.[1,2] A hallmark feature of the process is the repeated endomitotic divisions producing mature megakaryocytes with a DNA content that can reach 128n (diploid = 2n). Subsequent to platelet release, the cytoplasm of the mature megakaryocyte partitions to form a demarcation membrane system, leading to the formation of the platelet precursor, the proplatelet.[3,4] Thrombopoietin (TPO) is the essential cytokine for megakaryocytopoiesis,[5-7] and it stimulates bone marrow precursor cells through a series of signaling pathways, including the Janus kinase/signal transducer and activator of transduction (Jak/STAT), Ras/mitogen-activated protein kinase (Ras/MAPK), and phosphoinositol-3-kinase/Akt (PI3K/Akt) pathways.[8-10] Although the evidence is strong for each of these pathways participating in megakaryocytopoiesis, it is unclear what molecular mechanisms switch a proliferating precursor cell to a path of repeated endomitotic divisions to produce the polyploid megakaryocyte.

A hereditary bleeding disorder, Bernard-Soulier syndrome, is associated with abnormal bone marrow megakaryocytes with a poorly developed demarcation membrane system.[11,12] These abnormal megakaryocytes mimic the presentation of macrothrombocytopenia, characterized by giant circulating platelets and reduced platelet count. The genetic basis of Bernard-Soulier syndrome is well established. It is caused by mutations that impair expression of a multiple subunit receptor, the glycoprotein (GP) Ib-IX complex.[11,13] The molecular basis of macrothrombocytopenia is linked to an absence of the cytoplasmic tail of the GP Ibα subunit of the GP Ib-IX complex.[14] However, a role for the extracytoplasmic domains of the complex cannot be excluded because antibodies to GP Ib-IX can alter proplatelet formation in vitro.[15,16] In addition, in vitro evidence links the expression of GP Ib-IX to cell proliferation, further suggesting the GP Ib-IX complex might regulate megakaryocyte growth.[17]

The mouse phenotype mimicking Bernard-Soulier syndrome in humans can be rescued by expression of a human GP Ibα transgene under the control of a megakaryocytic gene promoter.[11] We have extended this model, generating a comparable mouse colony expressing a variant human GP Ibα subunit lacking the 6 terminal residues (605-610) on the cytoplasmic tail of GP Ibα critical for binding to the signal transduction protein, 14-3-3ξ.[18-21] Using animal models, we tested the hypothesis that sequestering 14-3-3ξ by GP Ibα alters megakaryocytopoiesis and thrombopoiesis. The basis for this hypothesis is the extensive literature on members of the 14-3-3 protein family and their influence in various physiologic processes, including intracellular signaling (Raf, MLK, MEKK, PI3K, IRS-1), cell cycling (Cdc25, Wee1, CDK2, centrosome), apoptosis (BAD, ASK-1), and the regulation of transcription (FKHRL1, DAF-16, p53, TAZ, TLX- 2, histone deacetylase).[22]

Our results identify a link between the GP Ibα/14-3-3ξ interaction and the phosphorylation state of Akt. A model is proposed

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Submitted March 12, 2004; accepted June 27, 2004. Prepublished online as *Blood* First Edition Paper, July 22, 2004; DOI 10.1182/blood-2004-03-0893.

Supported by grants HL50545 and HL31950 (J.W.) and HL30657, HL56264, and HL00903 (J.E.B.F.) from the Heart, Lung and Blood Institute of the National Institutes of Health. The authors are grateful for the support of the Sam and Rose Stein DNA Core Facility within the Department of Molecular and Experimental Medicine at The Scripps Research Institute.

An Inside *Blood* analysis of this article appears in the front of this issue.

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wherein the PI3K/Akt axis of TPO stimulation is regulated by the cytoplasmic tail of GP Ibα during megakaryocyte differentiation. The model is based on results obtained in several different experimental settings, including an in vivo model of severe thrombocytopenia, in vivo administration of TPO, and in vitro analysis of megakaryocyte proliferation and ploidy.

Materials and methods

Generation of mouse models

In vivo use and characterization of a megakaryocyte-specific promoter has been previously described.[23,24] Animals expressing a wild-type human GP Ibα transgene in the absence of mouse GP Ibα [mGp1ba(−/−)-TgN(hGPIbαWT)] and designated, hTg^{WT}, have also been described.[11] Briefly, hTg^{WT} animals were generated by breeding a colony of mice expressing a wild-type human transgene into a GP Ibα knockout mouse colony to produce offspring with heterozygous mouse GP Ibα alleles [mGp1ba(+/−)] and a portion of the offspring expressing the transgene. Expression of the human transgene was established by flow cytometry using the fluorescein isothiocyanate (FITC)–labeled anti–human monoclonal antibody LJ-P3. These mice were again bred to mGp1ba(−/−) mice, and the offspring from this cross were characterized by Southern blot analysis identifying mice lacking 2 mouse GP Ibα alleles and by flow cytometry to identify those expressing the human transgene. These mice are referred to as hTg^{WT} throughout this article. Using a similar breeding strategy, mice expressing a truncated GP Ibα subunit were also generated [mGp1ba(−/−)-TgN(hGPIbαY605X)]. These mice express a human transgene with a stop codon in place of the mature subunit Tyr605 codon (Figure 1). After breeding into the mGp1ba(−/−) mouse colony and selecting animals lacking both mouse GP Ibα alleles, these animals were designated hTg^{Y605X}. Expression of the hTg^{Y605X} transgene is driven by the rat platelet factor 4 promoter, provided by Katya Ravid and Robert Rosenberg (Massachusetts Institute of Technology, Cambridge).[25] The hTg^{Y605X} transgenic construct contained in a 5′ → 3′ direction the rat platelet factor 4 promoter, the coding sequence of human GP Ibα, and the 3′ untranslated cDNA sequence, including the polyadenylation signal sequence. Subsequent expansion of the hTg^{WT} and hTg^{Y605X} colonies was accomplished by breeding into the GP $Ib\alpha^{null}$ colony, a colony that has been backcrossed into the C57/Bl6 strain since its inception. Transgenic animals carrying human GP Ibα alleles have been backcrossed for approximately 6 generations. All animal experiments were performed with approval from the institutional review board of The Scripps Research Institute, La Jolla, CA.

Immunologic reagents

Anti–human GP Ibα monoclonal antibodies LJ-P3 and LJ-Ibα1 were kindly provided by Dr Zaverio Ruggeri (The Scripps Research Institute, La Jolla, CA). Both antibodies recognize the extracytoplasmic domain of platelet GP Ibα.[26] Either denaturation or reduction of human GP Ibα abrogates the epitope of LJ-P3,[27] whereas the LJ-Ibα1 epitope is not sensitive to denaturation and is used for Western blot detection of human GP Ibα antigen. FITC-labeling of purified LJ-P3 was performed according to standard procedures.[28] An antifilamin-1 polyclonal antibody was provided by Drs Hoffmeister and Stossel (Brigham and Women's Hospital, Boston, MA). Other antibodies were purchased from commercial vendors: an FITC-labeled monoclonal antibody recognizing the mouse integrin α_{IIb} subunit was purchased from PharMingen (La Jolla, CA), an anti–14-3-3ζ (C-16) polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), an antiphospho-Akt (Ser473) antibody was obtained from New England Biolabs (Beverly, MA), an anti-PI3K polyclonal was purchased from Upstate (Charlottesville, VA), and antiphosphotyrosine polyclonal antibodies (py20 and 4G10) were purchased from Chemicon (Temecula, CA).

von Willebrand factor binding

Using ristocetin (1.5 mg/mL; Sigma, St Louis, MO) as a modulator of von Willebrand factor binding to GP Ib, washed platelets were mixed at a final count of 2×10^8/mL with increasing concentrations of sodium iodide I 125 (^{125}I)–labeled von Willebrand factor (VWF).[23] The mixtures were incubated at 22°C to 25°C for 15 minutes. At the end of the incubation period, platelet-bound VWF and free VWF were separated by centrifuging the

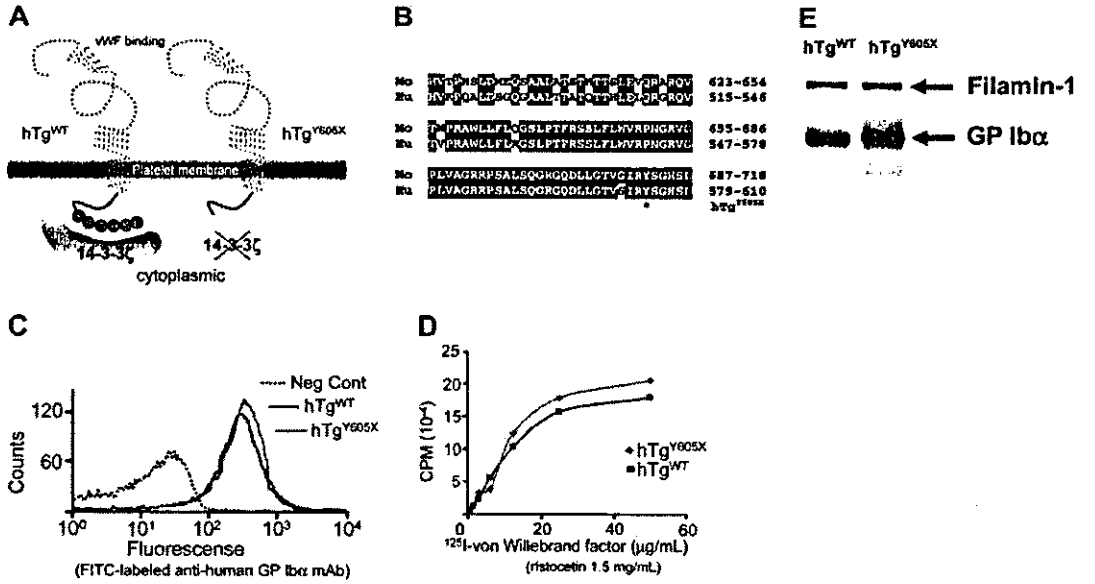

Figure 1. Generation and characterization of mouse models expressing wild-type human GP Ibα (hTg^{WT}) and a truncated form of GP Ibα (hTg^{Y605X}). (A) Animal models have been developed lacking the murine GP Ibα genes and expressing normal human GP Ibα (hTg^{WT}) and a truncated human GP Ibα variant (hTg^{Y605X}). Both expressed human transgenes were driven by megakaryocytic-specific promoters. (B) Shown are the primary sequence alignments for the cytoplasmic tails of mouse (Mo) and human (Hu) GP Ibα. *The amino acid position (605) where a Tyr605 codon was replaced with a stop codon to truncate the cytoplasmic tail of GP Ibα. (C) Flow cytometry profiles of platelets in whole blood are shown using blood from hTg^{WT} and hTg^{Y605X} animals. Fluorescence was produced using an FITC-labeled anti–human GP Ibα monoclonal antibody, LJ-P3. The negative control (dotted line) was fluorescence produced by nontransgenic or normal mice. (D) von Willebrand factor binding isotherms are shown for washed platelets from hTg^{WT} and hTg^{Y605X} blood. (E) Mouse platelet lysates were immunoprecipitated with LJ-P3 and immunoblotted with an antifilamin-1 polyclonal antibody and an anti–GP Ibα polyclonal antibody.

platelets through a sucrose layer, and the platelet-bound radioactivity was measured in a γ-scintillation spectrometer.

Western blot and immunoprecipitation analyses

Platelet pellets prepared from platelet-rich plasma (PRP) were lysed in solubilization buffer (2% Triton X-100, 0.1 M Tris [pH 7.4], 0.01 M EGTA [ethyleneglycotetraacetic acid], 0.15 M NaCl, 2 mM Pefabloc SC [Boehringer Mannheim, Indianapolis, IN]). For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the samples were mixed with an SDS sample buffer and were boiled (5 minutes) before electrophoresis. After transfer to nitrocellulose and reactivity with the designated antibodies,[29] the immunoreactive proteins were visualized using a chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ) and or an iodinated secondary antibody. Immunoreactive signals were identified using Kodak Biomax MR film (Kodak, Rochester, NY).

Immunoprecipitation experiments were performed with washed platelets (3.4×10^8 platelets) resuspended in 250 μL modified Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 2.8 mM dextrose, 0.4 mM NaH_2PO_4, 5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4]) and were lysed with an equal volume of solubilization buffer. The mixture was kept on ice for 45 minutes and was centrifuged (10 minutes, 13 000*g*) to remove the insoluble material. Lysates (500 μL) were mixed with 100 μL (50% vol/vol) protein A beads (IPA-300; Repligen, Cambridge, MA) and 10 μg of the indicated antibody for 90 minutes. The beads were then washed 4 times in an equal volume of modified Tyrode buffer and solubilization buffer. Bound proteins were eluted by boiling in SDS-PAGE sample buffer in the presence of 10 mM dithiothreitol.

In vivo model of thrombocytopenia and TPO administration

To induce thrombocytopenia in mice expressing human GP Ibα, 50 μg purified LJ-P3 immunoglobulin G (IgG) was injected into the tail vein of each mouse. Purified recombinant murine TPO was administered subcutaneously (1.5 μg per mouse) to 6- to 8-week-old animals. The TPO was a gift from Dr Hiroshi Miyazaki (Kirin Brewery, Tokyo, Japan). Circulating blood counts were determined using manual methods (Unopette; Becton Dickinson, Franklin Lakes, NJ) and an automated cell counter (Baker, Allentown, PA). The number of platelets and the DNA content of megakaryocytes were analyzed at the indicated time points.

Purification and analysis of mature megakaryocytes

After the induction of thrombocytopenia or the administration of TPO, mice were killed and whole marrow was flushed from both femurs and tibias using a 25-gauge needle until the bone appeared white. Marrow cells are collected in MK buffer (Ca^{2+}, Mg^{2+}-free phosphate-buffered saline [PBS] containing 3% bovine serum albumin [BSA], 5.5 mM D-glucose, 10.2 mM trisodium citrate, and 10 μM prostaglandin E1 [PGE1]) and were filtered through a sterile nylon mesh (70 μm) to remove small bone fragments. Megakaryocytes were harvested by centrifugation (5 minutes, 220*g*) and were overlaid on a discontinuous gradient (0%, 2%, 4%) of BSA. By this method, megakaryocytes made up more than 90% of the cells settling to the bottom within 40 minutes at 1*g*, as evidenced by reactivity with an anti–integrin α_{IIb} monoclonal antibody and acetyl cholinesterase staining.[9]

In vitro culture of murine bone marrow and megakaryocytes

Isolated cells from the bone marrow of mice were suspended at a concentration of 5×10^6 cells/mL in Iscove modified Dulbecco medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (HyClone, Logan, UT), 1 × Pen/Strep Fungizone Mix (BioWhittaker, Walkersville, MD), and 50 ng/mL murine TPO. Cells were cultured at 37°C in a humidified chamber with 5% CO_2.

Ploidy and TUNEL analysis

DNA ploidy was assessed by flow cytometry. Bone marrow–derived cells were centrifuged (5 minutes, 220*g*) washed, and fixed in 70% ethanol (4°C, overnight). Cells were then washed and resuspended in PBS, incubated for 30 minutes at room temperature with 10 μg/mL FITC-conjugated anti-α_{IIb}, 50 μg/mL propidium iodide (Sigma), and 10 U/mL RNase A (Sigma). Cells were analyzed by flow cytometry using a FACScan (Becton Dickinson). In case of antibody injection, megakaryocytes were enriched before staining using Percoll (Sigma) density centrifugation ($\rho = 1.07$ g/mL) according to the procedure of Tomer et al.[30]

To assay for DNA degradation (TdT-mediated dUTP nick-end labeling [TUNEL] assay), cells were fixed in 1% paraformaldehyde for 15 minutes on ice and were stored in 70% ethanol (−20°C, 30 minutes). This was followed by incubation with terminal deoxynucleotide transferase and fluorescein isothiocyanate-dUTP according to the manual in the APO-Direct Kit (PharMingen).

CFU-Meg assays

For megakaryocyte colony-forming unit (CFU-Meg) assays, bone marrow cells (2×10^5) were cultured with MegaCult-C media (StemCell Technologies, Vancouver, BC, Canada) in the presence of 10 ng/mL recombinant mouse interleukin-3 (IL-3), 20 ng/mL recombinant human IL-6, and 50 ng/mL recombinant mouse TPO (all cytokines courtesy of Kirin Brewery) according to the manufacturer's suggested conditions. After culturing for 7 days, the colonies were stained with acetylcholine esterase, and a CFU-Meg colony was defined as a colony with at least 3 acetylcholine esterase–positive cells.

Platelet preparation and TPO stimulation

Murine blood was withdrawn from the retro-orbital plexus using heparin-coated micro-hematocrit capillaries (Fisher Scientific, Pittsburgh, PA) and was transferred to tubes containing the anticoagulant heparin (Sigma), at a final concentration of 30 U/mL, or acid-citrate-dextrose at a ratio of 1:6. The blood was diluted (1:2) in modified Tyrode buffer, and PRP was prepared by 10-minute centrifugation at 220*g*. PGE1 [10 μM] and apyrase (5 U/mL; Sigma) were added to the PRP. Platelets were washed with 3 sequential centrifugations (2 minutes, 1000*g*) at room temperature. Platelet pellets were washed one additional time with modified Tyrode buffer followed by a final centrifugation (3 minutes, 1000*g*). Final platelet pellets were resuspended in a buffer appropriate for the next procedure, such as immunoprecipitation or Western blot analysis. To analyze the TPO-mediated phosphorylation of Akt, washed platelets were stimulated with TPO (200 ng/mL) at the indicated time points. Phosphorylation was terminated by the addition of an equal volume of 2% SDS, 50 mM Tris-HCl (pH 7.5), 10 mM EGTA, and 2 mM Na_3VO_4.

Results

Murine platelet model with an ablated GP Ibα/14-3-3ξ interaction

Animals devoid of mouse GP Ibα but expressing either of 2 human transgenes were generated to test the hypothesis that a GP Ibα/14-3-3ξ interaction controls aspects of megakaryocyte differentiation. Animals expressing the normal human GP Ibα subunit were designated hTg^{WT}, and those expressing a truncated human GP Ibα subunit were designated hTg^{Y605X} (Figure 1A). The validity of the model was based on a high degree of sequence similarity between human and mouse GP Ibα cytoplasmic domains (Figure 1B) and the ability of human GP Ibα to correct the mouse Bernard-Soulier syndrome.[11] Gross characterization of the hematologic parameters from hTg^{Y605X} animals, including platelet count and platelet size, gave results indistinguishable from those obtained using platelets from hTg^{WT} animals.[11] Tail bleeding time assays, crude measurements of mouse hemostasis and coagulation, were also comparable between the 2 colonies of mice and were indistinguishable from those of healthy mice (data not shown).

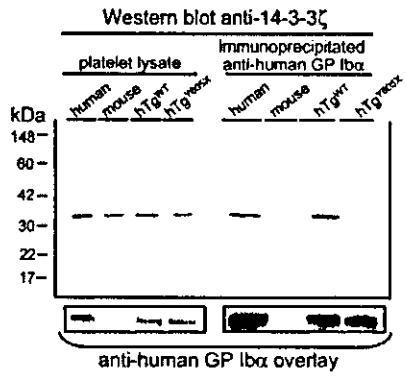

Figure 2. Truncation of platelet GP Ibα at Tyr605 ablates the interaction between GP Ibα and 14-3-3ξ. (Left) Western blot of platelet lysates detecting 14-3-3ξ protein at approximately 32 kDa in human platelets, mouse platelets, and mouse platelets expressing human GP Ibα subunits but devoid of mouse GP Ibα (hTg^{WT} and hTg^{Y605X}). The experiment demonstrated that total levels of 14-3-3ξ were similar in each of the samples. (Right) Results obtained after immunoprecipitation of human GP Ibα. Bound anti–14-3-3ξ antibody was detected using radiolabeled goat anti–rabbit IgG. Results demonstrate that the truncation of GP Ibα ablated the interaction with 14-3-3ξ, as evidenced by the lack of 14-3-3ξ antigen. Longer exposures of the autoradiograph failed to detect any 14-3-3ξ antigen in the hTg^{Y605X} sample. The same nitrocellulose filter was probed a second time with an anti–human GP Ibα monoclonal antibody, LJ-Ibα1, and demonstrated the presence of GP Ibα antigen in those samples in which the human subunit was present (bottom panel).

Critical for a comparison of the hTg^{WT} and hTg^{Y605X} transgenic megakaryocytes or platelets was the level of expressed human antigen. Using a monoclonal antibody that recognized a conformation-specific epitope within the amino terminus of GP Ibα, the expression levels produced by both transgenes were virtually indistinguishable (Figure 1C). Binding isotherms using the radiolabeled ligand, VWF, produced similar binding to hTg^{WT} and hTg^{Y605X} platelets (Figure 1D). The similar levels of GP Ibα antigen expressed by hTg^{WT} and hTg^{Y605X} reflected the rate-limiting requirement of GP Ibα and GP IX to assemble the multiple subunit GP Ib-IX complex.[31,32] Intracytoplasmic levels of GP Ibα antigen were higher in platelet lysates from hTg^{Y605X} animals (Figure 1E), even though the surface-expressed levels were similar. In addition, we observed similar levels of filamin-1 associated with each cytoplasmic tail, further validating structural integrity (Figure 1E). Thus, 2 mouse colonies were established expressing similar levels of human GP Ibα antigen and differing only in their cytoplasmic tail lengths.

Next we performed immunoprecipitation experiments using an anti–human GP Ibα monoclonal antibody to determine in vivo whether an absence of the 6-terminal residues of GP Ibα residues would lead to loss of interaction with 14-3-3ξ. Indeed, 14-3-3ξ was coimmunoprecipitated from mouse platelets expressing the wild-type human GP Ibα, whereas immunoprecipitation of the truncated GP Ibα molecule failed to copurify 14-3-3ξ, even though comparable levels of human GP Ibα antigen were immunoprecipitated (Figure 2).

Truncation of GP Ibα altered megakaryocyte ploidy in an acute model of severe thrombocytopenia

An anti–human GP Ibα mouse monoclonal antibody, LJ-P3, recognized an amino terminal extracytoplasmic domain of human GP Ibα and reacted with platelets from hTg^{WT} and hTg^{Y605X} animals (Figure 1C). When the intact IgG of LJ-P3 was injected into the tail veins of mice expressing human GP Ibα, it produced severe thrombocytopenia; circulating platelet counts dropped to 3% to 5% of normal level within 24 hours of injection (Figure 3A). The requirement of human GP Ibα and LJ-P3 to produce thrombocytopenia was evident by the failure of the antibody to produce any thrombocytopenia in nontransgenic animals (Figure 3A). After injections of LJ-P3 into hTg^{WT} animals, platelet counts started to increase after approximately 7 days (Figure 3A). In contrast, when LJ-P3 was injected into hTg^{Y605X} animals, platelet counts recovered more quickly, with a return to 50% of normal platelet count 4 days after antibody injection (Figure 3A).

To determine whether the platelet count recovery in the 2 animal models coincided with changes in bone marrow megakaryocytes, we examined megakaryocyte ploidy before and after antibody-induced thrombocytopenia. Before antibody injection, the megakaryocyte ploidy profiles were indistinguishable in hTg^{WT} and hTg^{Y605X} marrow (Figure 3B). In addition, the steady-state number of megakaryocytes was the same in the 2 colonies, as determined by the number of α_{IIb}-positive cells identified by flow cytometry of bone marrow aspirates (not shown). However, by day 4 after antibody-induced thrombocytopenia, the marrow from hTg^{Y605X} animals contained an increased percentage of megakaryocytes, with a ploidy of 32n or greater (Figure 3B). hTg^{WT} bone marrow by day 4 contained an increased percentage of 16n megakaryocytes. During the recovery from thrombocytopenia, the percentage of megakaryocytes in an 8n or less ploidy class was indistinguishable in the hTg^{WT} and the hTg^{Y605X} marrow (Figure 3B).

Truncation of GP Ibα alters the in vivo megakaryocytic response to TPO

Results from antibody-induced thrombocytopenia suggested a link between the cytoplasmic tail of GP Ibα and megakaryocytopoiesis. We further examined this possibility by testing the hypothesis that TPO-mediated proliferation of megakaryocytes differs in the 2 GP Ibα murine models. First, we injected hTg^{WT} mice and hTg^{Y605X}

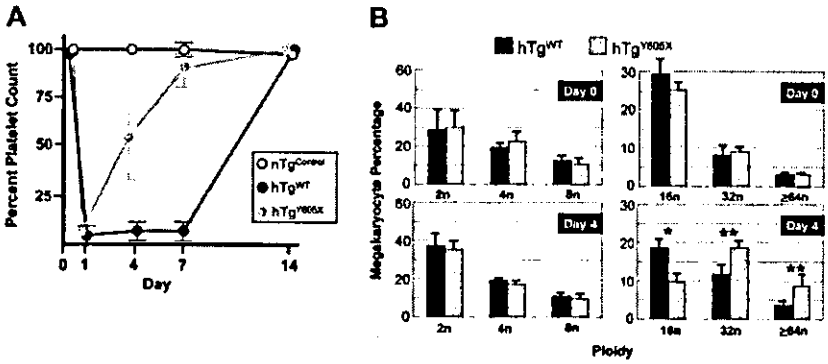

Figure 3. Acute model of severe thrombocytopenia. (A) The anti–human GP Ibα mouse monoclonal antibody, LJ-P3, injected into the tail veins of mice expressing human GP Ibα, produced severe thrombocytopenia. When injected into mice expressing the wild-type human GP Ibα subunit (hTg^{WT}), the platelet count started to return to a normal level after day 7. In mice expressing a truncated form of GP Ibα (hTg^{Y605X}), the platelet count began to recover by day 4, and by day 7 the platelet counts were near normal levels. A control LJ-P3 injection into nontransgenic mice ($nTg^{Control}$) is shown for comparison. (B) The ploidy profile of megakaryocytes is shown before injection of the anti–human monoclonal antibody, LJ-P3 (day 0) and 4 days after injection (day 4). Results were compiled from individual mice, and the mean and SEM are shown (n = 6). *P = .01; **P = .05.

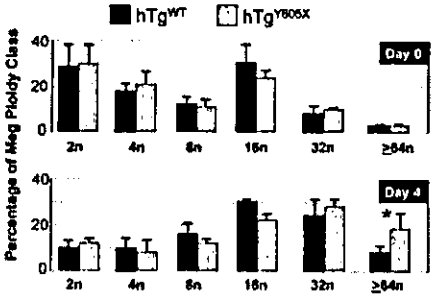

Figure 4. Ploidy analysis of bone marrow megakaryocytes after in vivo administration of TPO. After the injection of TPO, bone marrow megakaryocytes were harvested and analyzed for ploidy classes. Shown are the means and SEM from 6 mice of each genotype. *P = .05.

mice with equivalent amounts of TPO. A single subcutaneous injection of TPO (1.5 μg) caused a similar increase in platelet count in both models at days 2, 4, and 6, with a 3-fold increase over baseline as the maximum level of response (not shown). Next, we examined the effects of TPO on megakaryocyte ploidy. At 4 days after TPO injection, the extent of polyploidization was again different in the 2 models (Figure 4). Megakaryocytes of a high ploidy class, 64n or greater; were significantly increased in the marrow of hTg^{Y605X} animals, whereas the lower ploidy classes were indistinguishable (Figure 4).

Truncation of GP Ibα alters megakaryocytic proliferation and markers of apoptosis

Megakaryocytes from both animal models were tested in vitro for their proliferative potential and the ability to express terminal differentiation markers. CFU-Mk assays revealed no difference in the number of megakaryocytic progenitor cells for each model (hTg^{WT} = 13.8 ± 5.4; hTg^{Y605X} = 15.0 ± 3.9). However, after in vitro culturing for 5 days in the presence of TPO, hTg^{WT} cultures were enriched in megakaryocytes, as evidenced by a 2-fold increase in acetyl cholinesterase–positive cells when compared with similar cultures from hTg^{Y605X} marrow (Table 1; Figure 5A). Similar to the in vivo results presented in Figure 5, the culture of hTg^{Y605X} bone marrow produced an increase in the percentage of higher ploidy cells (not shown). Thus, the number of progenitor cells in each model was similar in the marrow, but the hTg^{WT} cells had a greater proliferative potential whereas the hTg^{Y605X} marrow produced megakaryocytes with higher ploidy content.

Next, we considered whether the hTg^{Y605X} cells containing an increased percentage of high ploidy cells might also express increased levels of terminal differentiation markers. TUNEL assays were performed to monitor apoptosis as a function of DNA degradation. After 5 days the in vitro culture of hTg^{Y605X} bone marrow produced megakaryocytes with a higher occurrence of apoptosis (Figure 5B), a difference no longer apparent by day 8 of culture (not shown). Further evidence for the changes in the hTg^{Y605X} megakaryocyte population was apparent by the increased accumulation of GP Ibα antigen (Figure 5C). An increase in GP Ibα expression also supported the more differentiated state of the hTg^{Y605X} megakaryocytes after stimulation with TPO. Thus, the truncation of the GP Ibα tail limited the proliferative potential of megakaryocytic precursors (Table 1) while increasing their intrinsic ability to progress through a sequence of events leading to the presence of late-stage megakaryocytes.

Table 1. In vitro bone marrow cultures

	Total no. cells, × 10^7		No. megakaryocytes, × 10^4	
Day	hTg^{WT}	hTg^{Y605X}	hTg^{WT}	hTg^{Y605X}
0	7.71 ± 0.65	7.57 ± 0.99	—	—
5	1.48 ± 0.17	1.44 ± 0.57	13.67 ± 1.58	7.54 ± 1.34

n = 4.
– indicates undetectable.

Truncation of GP Ibα leads to increased phosphorylation of Akt

To explain the results observed in in vivo and in vitro assays, we considered the possibility that the PI3K/Akt axis of TPO stimulation is altered in the hTg^{Y605X} model.[9] A physical link is reported among 14-3-3ξ, PI3K, and GP Ibα,[33] and the relevance of PI3K for TPO stimulation of megakaryocytes is well documented.[9,34] Our hypothesis suggested that ablation of the 14-3-3ξ interaction between GP Ibα and 14-3-3ξ has altered one of the major stimulatory pathways for megakaryocytopoiesis, the PI3K activation of Akt.[9]

To address this problem and to determine how much PI3K was associated with GP Ibα, we stimulated human platelets with TPO, immunoprecipitated with either an anti–GP Ibα antibody or an

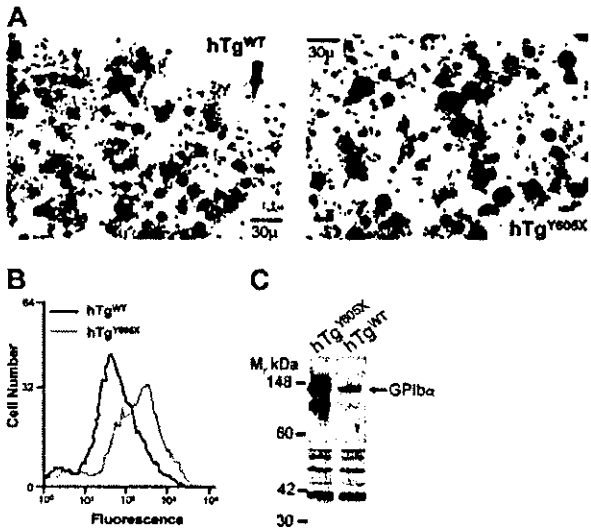

Figure 5. In vitro analysis of cultured bone marrow cells. (A) Shown are images from the cultured bone marrow preparations illustrating the increased proliferation of hTg^{WT} cells compared with hTg^{Y605X} cells (for comparison, see also Table 1). Images were captured with an Olympus IX71 inverted microscope equipped with a 40 ×/0.95 NA objective (Olympus, Tokyo, Japan). Images were photographed at 400 × original magnification with an Olympus DP70 CCD camera (Olympus) and acquired through Lumina Vision software (Mitani, Fukui, Japan). Cells were stained for acetylcholinesterase using 0.1 mol/L PBS containing 0.05% acetylthiocholine iodide, 0.1 mol/L sodium citrate, 30 mmol/L copper sulfate, and 5 mmol/L potasium ferricyanide (pH 6.0). (B) TUNEL assay on cultured bone marrow cells. Bone marrow cells were harvested and cultured for 5 days in the presence of TPO, and TUNEL assays were performed. Flow cytometry settings were used to gate and provide data for cells with greater than 4n ploidy. (C) Western blot from the same cultures depicted in the center panel was blotted for GP Ibα antigen, a marker of late-stage megakaryocytopoiesis. Ten micrograms protein (BCA assay) was applied to each lane, electrophoresed, transferred to nitrocellulose, and reacted with an anti–GP Ibα antibody. Shown is the resultant autoradiograph. Samples from hTg^{Y605X} show an increase in the amount of GP Ibα antigen compared with hTg^{WT}, consistent with an increase in high-ploidy cells seen after a 5-day culture in the presence of TPO. However, as seen in Figures 1 and 2, gene expression levels for both transgenes were similar, but coincident with an increase in the percentage of high-ploidy megakaryocytes was an increase in GP Ibα antigen. The blot was subsequently reprobed with a pool of antibodies to confirm a similar protein load and is shown below for comparison.

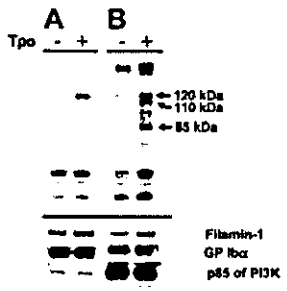

Figure 6. TPO-induced phosphorylation. Purified human platelets were stimulated with TPO (200 ng/mL, 10 minutes, 37°C) and were immunoprecipitated with an anti–GP Ibα monoclonal (LJ-P3) (A) or an anti-PI3K antibody (B). Immunoprecipitation products were electrophoresed and blotted with an antiphosphotyrosine antibody (4G10). Phosphorylated proteins in the absence (-) or presence (+) of TPO are shown. Subsequent control blots were performed on the same filter for filamin-1, GP Ibα, and the p85 subunit of PI3K.

anti-PI3K antibody. We blotted the immunoprecipitated material with an antiphosphotyrosine antibody (4G10) and looked for links among GP Ibα, PI3K, and c-Mpl. Figure 6A shows a protein (120 kDa) that became increasingly phosphorylated in the presence of TPO and immunoprecipitated by an anti–GP Ibα antibody. As shown in the accompanying control, PI3K was immunoprecipitated by the anti–GP Ibα monoclonal antibody, but a phosphorylated form of the protein was not detected. A protein of similar mobility (120 kDa) was also immunoprecipitated by an anti-PI3K antibody along with major phosphorylated proteins of 110 and 85 kDa (Figure 6B). Thus, a 120-kDa protein appeared to be immunoprecipitated by anti-PI3K and anti–GP Ibα antibodies after stimulation with TPO. The results also illustrate that only a minor portion of PI3K is associated with GP Ibα. These results suggest that a complex exists of GP Ibα, PI3K, and an unidentified 120-kDa protein and that several of these proteins become phosphorylated in the presence of TPO.

If the phosphorylation state of PI3K is relevant to the differences we observed between the hTg^{WT} and hTg^{Y605X} colonies, we would expect to observe further downstream consequences, such as the phosphorylation of a PI3K target, Akt. We examined the TPO-induced phosphorylation of Akt in platelets from both models and found a heightened Akt phosphorylation in hTg^{Y605X} platelets (Figure 7A). In contrast, the overall phosphorylation pattern observed with an antiphosphotyrosine antibody revealed no dramatic differences in the 2 colonies (Figure 7B). These results confirm a shift in the PI3K/Akt pathway and establish an involvement of the GP Ibα/PI3K/14-3-3ξ interaction in the regulation of this signaling pathway.

Discussion

The presented results are consistent with a model in which the cytoplasmic tail of GP Ibα influences the temporal sequence of events necessary for the proliferation and maturation of megakaryocyte progenitors. Our hypothesis began with 14-3-3ξ and its sequestering by GP Ibα as a regulator of megakaryocytopoiesis. The sequestration of 14-3-3ξ by GP Ibα has implications for platelet function by down-regulating the activation of Cdc42 and Rac.[35] Because our model with ablated interaction between GP Ibα and 14-3-3ξ displayed changes in megakaryocyte ploidy, we were led to consider the contributions of the major regulator of megakaryocyte development, TPO.[6,7] The relevance of TPO to our results was evident by a decreased in vitro proliferation potential of hTg^{Y605X} megakaryocytic stem cells (Table 1) and an increased percentage of high ploidy hTg^{Y605X} cells (Figure 4). Of the TPO activation pathways, the PI3K/Akt pathway seemed to be the most likely involved because both the TPO receptor and the GP Ibα/14-3-3ξ complex sequester PI3K.[34,36] Indeed, in the absence of a GP Ibα/14-3-3ξ interaction, an increased phosphorylation of Akt in response to TPO was observed (Figures 7, 8A). Thus, the TPO response in megakaryocytes can be influenced by membrane receptors other than c-mpl, just as platelet activation can be primed by TPO and by the activation of a PI3K-dependent pathway.[37]

Although the target of activated Akt could be any number of proteins, activated Akt increases the posttranslational stability of cyclins.[38] Cyclins are particularly relevant targets because they have been associated with promoting the endomitosis that leads to increased ploidy.[39] Cyclin D3 is active in promoting passage through the G_1 phase of mitosis,[40] and its importance as a megakaryocytic gene product has been established.[39,41] In addition, the overexpression of D1 or D3 cyclin in transgenic animals increases megakaryocyte ploidy.[42,43] Phenotypically, the overexpression of D3 impairs formation of the mature megakaryocyte demarcation membrane system,[43] similar to what occurs with a complete absence of a GP Ib-IX complex.[11,12] Thus, the abnormal megakaryocyte cytoplasm associated with Bernard-Soulier syndrome may reflect an unregulated activation of cyclins in the absence of GP Ib-IX or, in the current study, by a short truncation in the cytoplasmic tail. Together, these results illustrate the requirement for a highly coordinated temporal sequence of events leading to the formation of a developing platelet field in the cytoplasm of a normal megakaryocyte.

Our results demonstrate increases in Akt phosphorylation and in markers of apoptosis (Figure 5B). This seems to be in direct conflict with the dogma establishing the PI3K/Akt pathway as a cell survival or

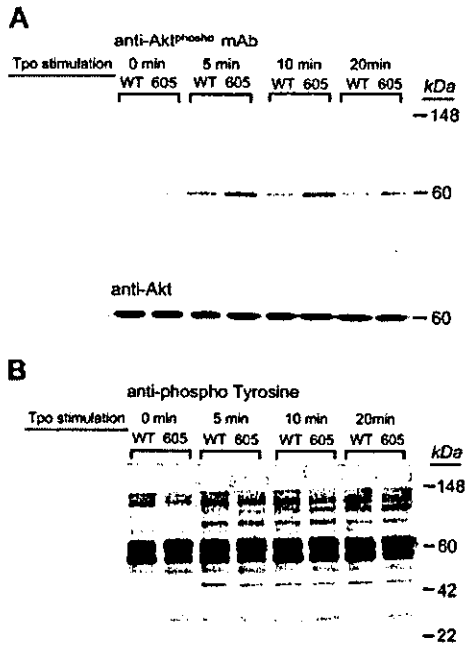

Figure 7. TPO-induced Akt phosphorylation. Purified platelets from hTg^{WT} and hTg^{Y605X} were stimulated with TPO (200 ng/mL) for the indicated times. Platelet lysates were subjected to Western blot analysis using either an antiphospho-Akt (Ser473) antibody (A) or an antiphosphotyrosine polyclonal antibody (B). The upper blot was reprobed using an anti-Akt polyclonal antibody to visualize protein loads and is shown for comparison (middle panel).

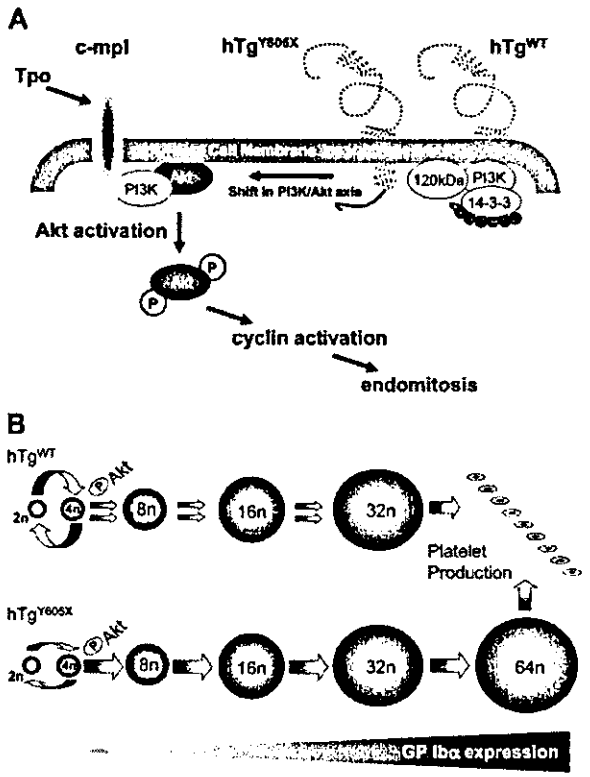

Figure 8. GP Ibα expression, megakaryocyte proliferation, and differentiation. (A) Results suggest a shift in the PI3K/Akt axis of TPO stimulation. A hypothesis is presented whereby the cytoplasmic tail of GP Ibα sequesters signaling proteins, such as 14-3-3ξ and PI3K, and down-regulates the Akt-dependent pathway. In the truncated GP Ibα variant, hTg^{Y605X}, a shift in the PI3K/Akt axis results in increased Akt activation and downstream consequences of increased endomitosis and accumulation of a greater percentage of high-ploidy megakaryocytes. (B) A schematic model is presented to illustrate how an increase in Akt phosphorylation results in a megakaryocyte population with increased ploidy. The precursor cell proliferation (2n → 4n → 2n) is more active in the hTg^{WT} model (Table 1) and is depicted by larger arrows compared with proliferation in the hTg^{Y605X} model. However, Akt phosphorylation in response to TPO is increased in the hTg^{Y605X} model (Figure 6A) and corresponds to an increase in the accumulation of cells of higher ploidy (Figures 3B, 4). Thus, repeated endomitotic divisions in the hTg^{Y605X} marrow are depicted by larger arrows compared with a similar process in the hTg^{WT} model. The increased proliferative potential of hTg^{WT} cells and the potential for hTg^{Y605X} cells to generate megakaryocytes of a higher ploidy class provide mechanisms whereby both models produce similar levels of platelet. Results highlight the independence of ploidy and platelet release in response to stimuli. GP Ibα expression increases during megakaryocyte maturation and, as such, becomes a critical timing factor for normal megakaryocyte maturation.

an antiapoptotic pathway.[44] Exceptions do exist, however, and they reflect the downstream targets of the activated Akt.[45] Worth considering are the unique properties of the megakaryocyte. Increased apoptotic markers are not a deleterious result of maturation but are part of the normal physiology of the megakaryocyte proceeding along the pathway to maturation. Clearly, the PI3K/Akt pathway is crucial to a wide range of processes in the multicellular organism, and our results underscore the inability to identify a single unifying theme for activation through a PI3K/Akt pathway.

Originally, we believed the faster platelet recovery observed in the hTg^{Y605X} model (Figure 3) could be a consequence of the increased percentage of megakaryocytes of higher ploidy. However, this conclusion did not fit when we observed the in vivo administration of TPO-altered megakaryocyte ploidy in the 2 models without altering the rate of increase in the circulating platelet count. Thus, by what mechanism does antibody-induced thrombocytopenia occur, and, more important, what explains the different platelet recovery rates in the 2 animal models? We present data showing that the expressed levels of transgene on the platelet surface and within platelet lysates were similar in both animal models (Figure 1C-E). The levels of megakaryocytic GP Ibα were also similar before the induction of thrombocytopenia (not shown). However, the levels of GP Ibα antigen changed dramatically when the megakaryocytic population changed, as seen in Figure 5C. The mechanism of antibody-induced thrombocytopenia could have existed at several points. First, it could have had a direct effect on proplatelet production in the marrow. This would be analogous to the in vitro inhibition described by others.[15,16] As such, proplatelet formation by megakaryocytes with increased GP Ibα antigen would be less inhibited, as would have occurred within the hTg^{Y605X} marrow after the induction of thrombocytopenia (Figure 3B). The net result would be an increased rate of circulating platelet recovery in the hTg^{Y605X} animal. Second, the antibody would have a direct effect on the circulating platelets. It is possible that by unlinking GP Ibα with 14-3-3ξ, we altered the clearance kinetics of the platelets in each model. In this case, the antibody produced a difference in platelet count that had nothing to do with the state of the megakaryocyte within the marrow.

In further support of the idea that the different platelet recovery rates observed in antibody-induced thrombocytopenia might be irrelevant to megakaryocyte proliferation and maturation, we observed that in vivo administration of TPO increased the hTg^{Y605X} ploidy (Figure 4) but did not increase the rate of platelet release. We suggest the similar increases in platelet count are explained by a level of compensation dependent on the increased cell proliferation in the hTg^{WT} model (Table 1) balanced by the higher ploidy in the hTg^{Y605X} marrow (Figure 8B). In support of this idea is the similar percentage of α_{IIb}-positive cells 4 days after TPO administration (2.50 ± 0.6 for hTg^{WT} vs 2.66 ± 0.08 for hTg^{Y605X}; $n = 6$). These results highlight an independence of ploidy and platelet release in the megakaryocyte population.[2,7,46]

One noticeable aspect of the presented results is the requirement for a stressed system to identify differences between the hTg^{WT} and the hTg^{Y605X} genotypes. The normal megakaryocyte ploidy profile and the circulating platelet counts are indistinguishable in the 2 models. However, changes are seen after induced severe thrombocytopenia or after in vivo administration of TPO. These results highlight the potential for compensation among signaling pathways in the unstressed situation. Indeed, the ability of hematopoietic cells to compensate in the absence of specific proteins or signaling pathways is not without precedence, and it highlights the complexity of normal hematopoiesis.[47-49]

In summary, we have found a mechanism by which the cytoplasmic tail of GP Ibα can modulate megakaryocyte ploidy and proliferation. The involved pathways most likely represent one defect contributing to the generation of the macrothrombocytopenic phenotype typical of Bernard-Soulier syndrome. Our results also identify factors capable of regulating the commitment of a normal megakaryocyte to become a polyploid cell. GP Ibα expression increases during megakaryocytic differentiation,[50] but the low levels of GP Ibα expression during early megakaryocytopoiesis may influence the level of Akt phosphorylation and balance the proliferative potential of a precursor cell compared with the cell's commitment to a pathway of repeated endomitotic cycles. Our results illustrate a temporal sequencing of events that control the commitment of the megakaryocyte to undergo endomitosis. Expressed levels of GP Ibα and sequestration of signaling proteins become relevant in controlling the mechanisms associated with megakaryocytopoiesis and thrombopoiesis.

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Recombinant Der p 1 and Der f 1 exhibit cysteine protease activity but no serine protease activity

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Received 5 January 2005
Available online 25 January 2005

Abstract

Although mite major group 1 allergens, Der p 1 and Der f 1, were first isolated as cysteine proteases, some studies reported that natural Der p 1 exhibits mixed cysteine and serine protease activity. Clarifying whether the serine protease activity originates from Der p 1 or is due to contamination is important for distinguishing between the pathogenic proteolytic activities of group 1 allergens and mite-derived serine proteases. Recombinant mite group 1 allergens would be useful tool for addressing this issue, because they are completely free from contamination by mite serine proteases. Recombinant Der p 1 and Der f 1, and highly purified natural forms exhibited only cysteine protease activity. However, commercially available natural forms exhibited both activities, but the two activities were eluted into different fractions in size-exclusion column chromatography. The substrate specificity associated with the serine protease activity was similar to that of Der f 3. These results indicate that the serine protease activity does not originate from group 1 allergens.
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Keywords: Recombinant major house dust mite group 1 allergens; Der p 1; Der f 1; Cysteine protease; Group 3 allergens; Serine protease

House dust mites of the *Dermatophagoides* species (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) are associated with various allergic diseases such as bronchial asthma, rhinitis, and atopic dermatitis, and are the most important causative factor of in-door allergens [1–3]. Group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2) allergens are considered to be the major allergens derived from house dust mites based on the frequency of patients sensitized by them, amount of specific IgE produced against them, and their content in mite extract [4]. Group 1 allergens exist in abundance in mite fecal pellets and account for over 50% of the IgE antibodies produced against total mite extract. Der p 1 of *D. pteronyssinus* and Der f 1 of *D. farinae* have 82% sequence homology in the amino acid sequences of their mature forms, which corresponds to their natural allergen state, and the sequences are homologous to those of papain-like cysteine proteases [5,6]. Recently, the cysteine protease activity of Der p 1 was suggested to be involved in the pathogenesis of allergy [7–15].

Although Der p 1 and Der f 1 were first isolated as cysteine proteases, some studies reported that natural Der p 1 exhibits mixed cysteine and serine protease activity [11,16,17]. The specificity for substrates and

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doi:10.1016/j.bbrc.2005.01.051

inhibitors associated with such serine protease activity within their preparations was different from that of the cysteine protease activity. However, house dust mites produce at least three groups of serine proteases, group 3, 6, and 9 allergens [18–21], and therefore, the possibility of contamination of cysteine protease Der p 1 preparations with these serine proteases cannot be excluded. Clarifying whether the serine protease activity originates from Der p 1 or is due to contamination is important for distinguishing between the pathogenic proteolytic activities of mite major group 1 allergens and mite-derived serine proteases. Recombinant mite group 1 allergens as recently reported [22–24] would be useful tool for addressing this issue, because they are completely free from contamination by mite serine proteases.

Using synthetic substrates and irreversible class-specific inhibitors, we compared the specificity of four types of preparation of group 1 allergens: recombinant Der p 1 and Der f 1 expressed in yeast *Pichia pastoris* with yeast-derived N-glycosylation, those without N-glycosylation, commercially available natural types, and natural types purified using a different method. Consequently, we demonstrate that the serine protease activity does not originate from group 1 allergens and that the substrate specificity of the contaminated serine protease is similar to that of a mite group 3 allergen Der f 3.

Materials and methods

Natural Der p 1 and Der f 1. Commercially available natural Der p 1 and Der f 1 were purchased from Indoor Biotechnologies (Charlottesville, VA, USA) and Asahi Breweries (Tokyo, Japan), respectively. Purification of natural Der p 1 and Der f 1 from whole culture extracts of house dust mites by the method of Yasueda et al. [25] was performed. Protein concentration was determined by the Bradford procedure using a protein assay kit (Bio-Rad, Richmond, CA, USA) with bovine IgG (Bio-Rad) as the standard.

Recombinant Der p 1 and Der f 1. Recombinant Der p 1 and Der f 1 were prepared as previously described with some modifications [22–24]. Briefly, proforms of four recombinant types, Der p 1-N52Q, Der p 1-WT, Der f 1-N53Q, and Der f 1-WT, were secreted into the culture supernatant of yeast *P. pastoris* and converted into their prosequence-removed mature forms. The mature forms were then purified by anion exchange column and size-exclusion column chromatography.

Measurement of proteolytic activity. Cysteine protease activity was measured as previously described with modifications [23,26]. Enzymes (400 nM) were incubated with or without 1 mM dithiothreitol (DTT) for 5 min at 37 °C in 25 μl. After further incubation with or without E-64 (50 μM) (Peptide Institute, Osaka, Japan) and/or 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (500 μM) (Sigma, St. Louis, MO, USA) for 15 min in 50 μl, assays were conducted in reaction buffer with substrates (0.1 mM) at 37 °C in 100 μl scale. The final concentrations of the enzymes were 100 nM, and those of the inhibitors were 25 μM for E-64 and 250 μM for AEBSF. The substrates used were: butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-QAR-MCA), succinyl-Leu-Leu-Val-Tyr-MCA (Suc-LLVY-MCA), succinyl-Ala-Ala-Ala-MCA (Suc-AAA-MCA), butyloxycarbonyl-Phe-Ser-Arg-MCA (Boc-FSR-MCA), butyloxycarbonyl-Gln-Gly-Arg-MCA (Boc-QGR-MCA), butyloxycarbonyl-Val-Leu-Lys-MCA (Boc-VLK-MCA), benzoyl-Arg-MCA (Bz-R-MCA), and succinyl-Ala-Ala-Pro-Phe-MCA (Suc-AAPF-MCA) (Peptide Institute). The fluorescence of aminomethylcoumarin released from the substrate was measured.

Protein sequencing. Samples of commercial natural Der p 1 and Der f 1 before and after SDS–PAGE and electroblotting onto membranes were subjected to N-terminal amino acid sequencing using protein sequencers [23,27].

Size-exclusion column chromatography. Recombinant and natural Der p 1 and Der f 1 were subjected to size-exclusion column chromatography on Protein-Pak 125 (Waters, Milford, MA, USA), and the absorbance at 280 nm was monitored. The eluted fractions were subjected to sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and silver stained (Silver stain II kit wako; Wako, Osaka, Japan).

Results

Substrate specificity of recombinant and commercial natural Der p 1 and Der f 1

Measurement of the proteolytic activity using eight synthetic substrates revealed that the activity of recombinant mite group 1 allergens with yeast-derived hyperglycosylation (Der p 1-WT and Der f 1-WT) and that of commercial natural types were different from each other in terms of substrate specificity and DTT-dependence (Fig. 1). The patterns of substrate specificity and DTT-dependence were similar between Der p 1-WT and Der f 1-WT, and also between commercial Der p 1 and Der f 1.

Der p 1-WT and Der f 1-WT showed DTT-dependent proteolytic activity against six synthetic substrates (Figs. 1A–F) and almost no activity against two (Figs. 1G and H). This DTT-dependency suggested that the activity of Der p 1-WT and Der f 1-WT was that corresponding to a cysteine protease. On the other hand, the commercial natural types showed proteolytic activity and high DTT-dependence against three substrates (Figs. 1A–C); that with low or no DTT-dependence against three substrates (Figs. 1D–F), and little or no activity against two substrates (Figs. 1G and H). This suggested that commercial natural Der p 1 and Der f 1 possessed the other class of proteases as well as that of cysteine protease activity.

Activity of recombinant and natural Der p 1 and Der f 1 treated with class-specific irreversible protease inhibitors

To identify the class of proteases exhibiting DTT-independent activity among the commercial natural allergens (Figs. 1D–F, right two panels) and to reveal whether the difference between recombinant and commercial natural allergens was caused by the hyperglycosylation of recombinant allergens and/or by the method used for purification, we analyzed recombinant allergens without glycosylation (Der p 1-N52Q and Der f 1-N53Q) and natural allergens purified by the method

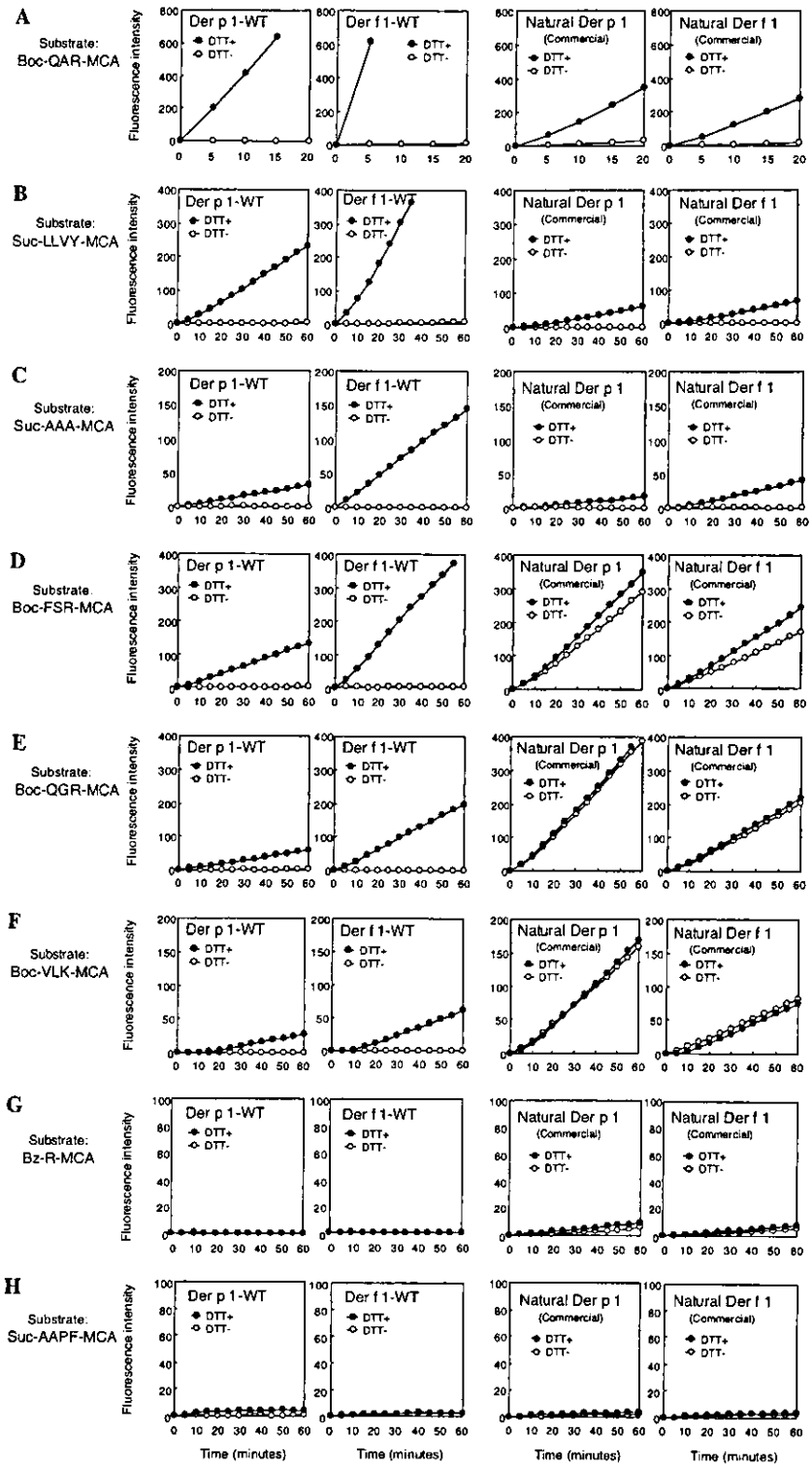


Fig. 1. Hydrolysis of synthetic substrates by recombinant and commercial natural Der p 1 and Der f 1. Eight substrates (A–H) were used. WT: recombinant forms with N-glycosylation. Commercial: commercial natural allergens. The reaction was performed in the presence (DTT+) or absence of DTT (DTT−).

by Yasueda et al. [25] and compared them with Der p 1-WT, Der f 1-WT, and commercial natural allergens using two irreversible class-specific protease inhibitors, cysteine protease-specific E-64 and serine protease-specific AEBSF (Figs. 2 and 3). We selected Boc-QAR-MCA and Boc-QGR-MCA for the substrates, because they are typical substrates suitable for measuring DTT-dependent and -independent activities of commer-

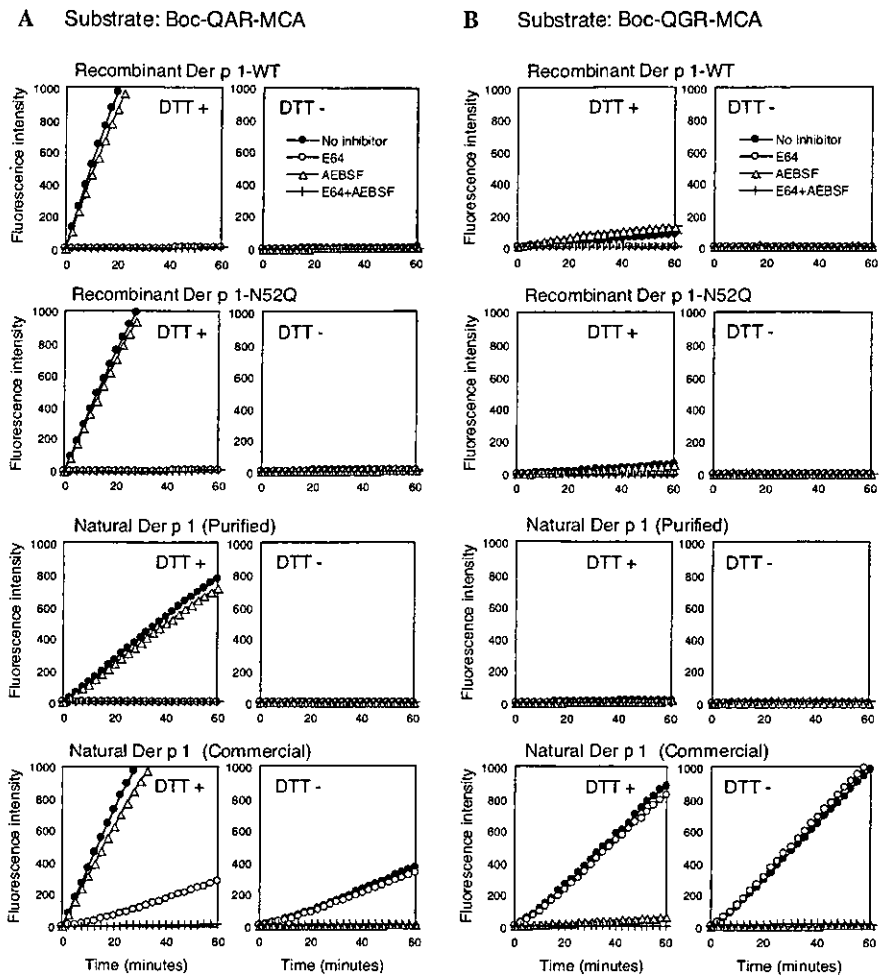


Fig. 2. Activity of recombinant and natural Der p 1 treated with irreversible class-specific protease inhibitors. Boc-QAR-MCA (A) and Boc-QGR-MCA (B) were used as substrates. Der p 1-WT: recombinant Der p 1 with yeast-derived N-glycosylation. Der p 1-N52Q: recombinant Der p 1 without N-glycosylation. Der p 1 (purified): natural Der p 1 purified by the method of Yasueda et al. [25]. Der p 1 (commercial): commercial natural Der p 1. The reaction was performed in the absence of inhibitors or in the presence of E-64, AEBSF or E-64 and AEBSF; and in the presence (DTT+) or absence of DTT (DTT−).

cial natural allergens, respectively (Figs. 1A and E, right two panels).

The activities of recombinant allergens and natural types purified by the method of Yasueda et al. against Boc-QAR-MCA (Figs. 2A and 3A, upper six panels) and Boc-QGR-MCA (Figs. 2B and 3B, upper six panels) were DTT-dependent, and E-64 completely inhibited the activities while AEBSF did not. This indicated that recombinant allergens and natural allergens purified by this method only possessed cysteine protease activity and that hyperglycosylation of recombinant allergens did not affect the activity.

On the other hand, the activities of commercial natural allergens against Boc-QAR-MCA consisted of major DTT-dependent and minor DTT-independent activities (Figs. 2A and 3A, panels at the bottom), and those against Boc-QGR-MCA possessed inverse major DTT-independent and minor DTT-dependent activities (Figs. 2B and 3B, panels at the bottom). The DTT-dependent and -independent activities of commercial natural allergens were inhibited by E-64 and AEBSF, respectively, and the addition of E-64 and AEBSF was necessary for complete inhibition in the presence of DTT. This indicated that the activity of commercial natural allergens consisted of cysteine protease and serine protease activity.

Substrate specificity of cysteine protease activity of recombinant and commercial natural Der p 1 and Der f 1

To compare the substrate specificity of the cysteine protease associated activities of recombinant and commercial natural allergens, DTT-dependent activities were calculated from the results shown in Fig. 1. The patterns of relative DTT-dependent activities were similar between recombinant and commercial natural allergens (Fig. 4A), although absolute DTT-dependent activity was higher for recombinant allergens than for commercial natural allergens (Fig. 1). This indicated

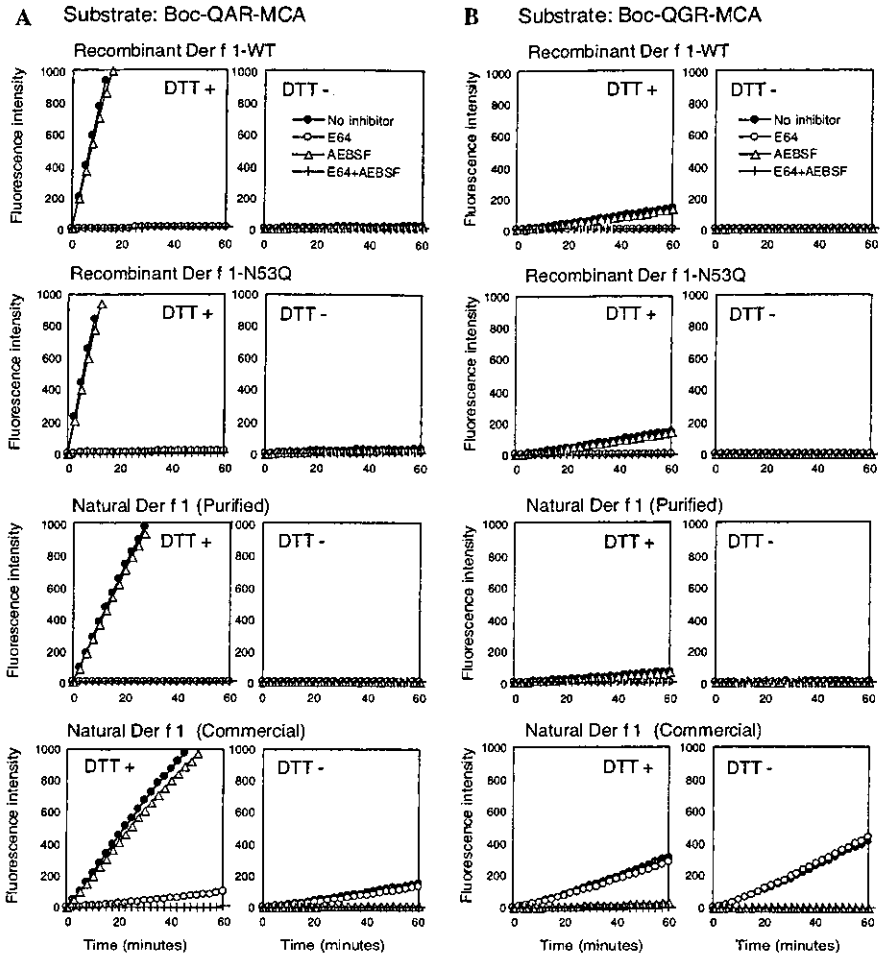


Fig. 3. Activity of recombinant and natural Der f 1 treated with class-specific irreversible protease inhibitors. The reaction was performed as that in Fig. 2, except that recombinant and natural Der f 1 were used.

that the substrate specificity of the cysteine protease associated activities was similar between recombinant and commercial natural allergens.

Substrate specificity of serine protease associated activity of commercial natural Der p 1 and Der f 1

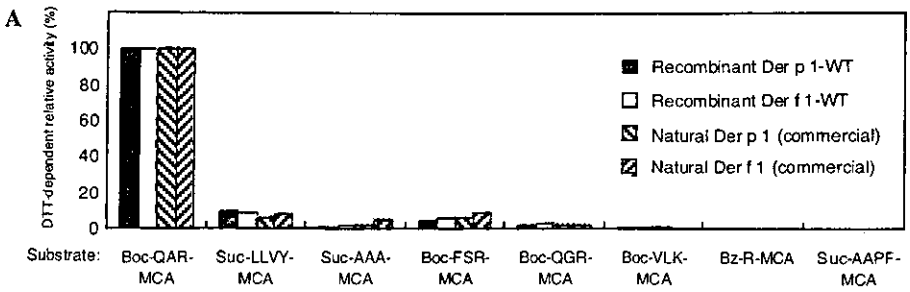
The substrate specificity of commercial natural allergens with DTT-independent activity (Fig. 1) was compared with the previously described specificity of mite serine proteases, except group 9 allergens, whose substrate specificity was not well characterized, and was found to be similar to the mite group 3 allergen Der f 3 but not to group 6 allergens (Fig. 4B, Boc-FSR-MCA, Boc-VLK-MCA, Suc-AAPF-MCA, and Suc-LLVY-MCA).

N-terminal sequencing of commercial natural Der p 1 and Der f 1

To determine the content of the serine protease within the commercial natural allergens, they were subjected to protein sequencing. N-terminal sequences for Der p 1 and Der f 1 were detected, but those for mite serine proteases that belong to three groups, group 3, 6, and 9 allergens, were not detectable. This indicated that contamination of mite serine proteases was minute if the serine protease activity was mite-derived. However, it was possible that isoforms of Der p 1 and Der f 1 exhibited the serine protease activity [16]. Therefore, to determine whether the cysteine and serine protease activities of the commercial natural allergens were from identical molecular species or not, we next analyzed the commercial natural allergens using size-exclusion column chromatography.

Size-exclusion column chromatography of recombinant and natural Der p 1 and Der f 1

The peak activity against Boc-QAR-MCA, which was used to detect cysteine protease activity in the presence of DTT, and that against Boc-QGR-MCA to detect serine protease activity in the absence of DTT were eluted into different fractions (Fig. 5, panels at



B

Substrate	DTT-independent relative activity (%)				
	Der p 1 (DTT -) (commercial)	Der f 1 (DTT -) (commercial)	Der f 3	Der p 6	Der f 6
Boc-QGR-MCA	135	120	n.d.	n.d.	n.d.
Boc-FSR-MCA	100	100	100	0.7	2
Boc-VLK-MCA	55	47	58	0.2	1.2
Suc-AAPF-MCA	0.9	1.8	0	100	100
Suc-LLVY-MCA	0.2	0.7	0	52	50
Suc-AAA-MCA	0	0.6	n.d.	n.d.	n.d.
Boc-QAR-MCA	46	45	n.d.	n.d.	n.d.
Bz-R-MCA	2.2	3.5	n.d.	n.d.	n.d.

n.d.: not determined

Fig. 4. Substrate specificity associated with the protease activity of recombinant and commercial natural Der p 1 and Der f 1. (A) Comparison of the specificity of cysteine protease activity. The activity for each substrate is shown as the relative DTT-dependent fluorescence intensity to that for the substrate Boc-QAR-MCA. The DTT-dependent fluorescence intensity used was calculated by subtracting the intensity in the absence of DTT from that in the presence of DTT. (B) Comparison of the specificity of serine protease activity. The activity for each substrate is shown as the relative DTT-independent fluorescence intensity to that for the substrate Boc-FSR-MCA in commercial natural Der p 1 and Der f 1, and natural Der f 3; and for Boc-AAPF-MCA in natural Der p 6 and Der f 6. The DTT-independent fluorescence intensity used was the intensity in the absence of DTT. The values for natural Der f 3, Der p 6, and Der f 6 were obtained from a previous report [20].

the bottom). The peaks for the cysteine protease activity of Der p 1-N52Q, Der f 1-N53Q, and the natural types purified by the method of Yasueda et al. were eluted into a fraction at an elution time equivalent to that associated with the cysteine protease activity of the commercial natural allergens (Fig. 5, middle four panels). Those for Der p 1-WT and Der f 1-WT were eluted earlier reflecting their larger molecular sizes (Fig. 5, panels at the top). The shapes of protein-elution curves and band density on SDS–PAGE of the eluted fractions were correlated well with the strength of the cysteine protease activity [22] but not with that of the serine protease activity (data not shown). These results indicated that the cysteine and serine protease activities associated with the commercial natural allergens originated from different molecular species and that the serine protease had an apparent molecular size different from those of Der p 1 and Der f 1.

Discussion

Several studies have found that cysteine and serine protease activities are associated with affinity-purified natural Der p 1 [11,16,17], and we obtained similar results using commercial natural Der p 1 and Der f 1. However, recombinant Der p 1 and Der f 1, and natural allergens purified by the method of Yasueda et al. only exhibited cysteine protease activity (Figs. 1–3). We showed that the substrate specificities of commercial natural Der p 1 and Der f 1 are similar to those of recombinant forms for DTT-dependent cysteine protease activity (Fig. 4A), and similar to that of a mite group 3 allergen Der f 3 for DTT-independent serine protease activity (Fig. 4B). The cysteine and serine protease activities eluted into different fractions during size-exclusion column chromatography (Fig. 5), indicating that these different types of protease activity are due to different molecular species. These results indicate that the major house dust mite group 1 allergens have only cysteine protease activity and that the serine protease activity detected within some preparations of natural group 1 allergens is due to contamination by serine protease with similar substrate specificity to Der f 3.

Hewitt et al. [11] reported that natural Der p 1, which was affinity-purified using a column coupled with murine anti-Der p 1 monoclonal antibody (mAb), exhibited cysteine and serine protease activities even after further active-site affinity purification using a thiol column. They showed that different substrate specificities associated with the cysteine and serine proteases are present using insulin B chain as a model peptide [16]. Recently, Brown et al. [17] reported similar results using two synthetic substrates, Boc-QAR-MCA and Tosyl-Gly-Pro-Arg-MCA;

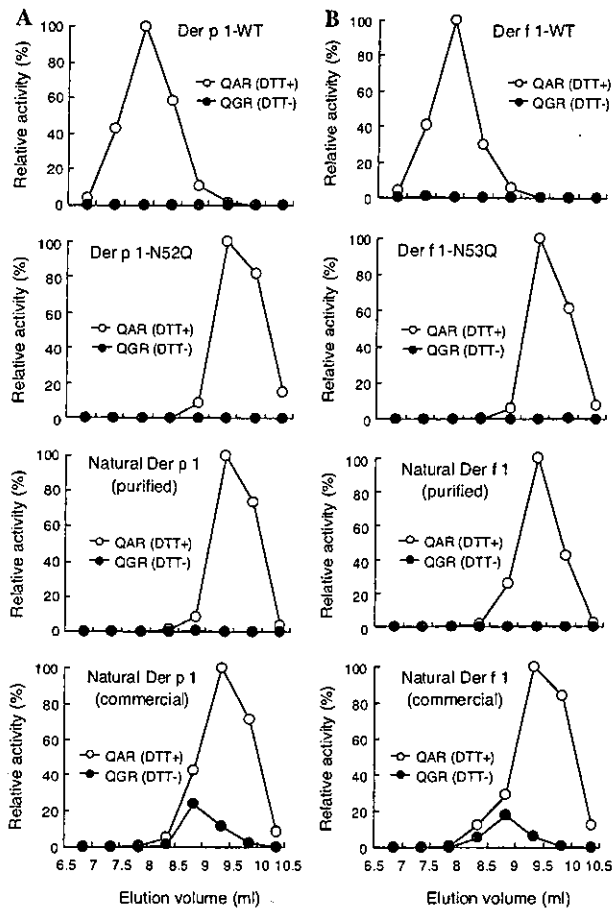


Fig. 5. Proteolytic activity associated with eluted fractions of recombinant and natural Der p 1 and Der f 1 separated by size-exclusion column chromatography. (A) Der p 1. (B) Der f 1. Eluted fractions were subjected to the measurement of proteolytic activity against the substrate Boc-QAR-MCA in the presence of DTT [QAR (DTT+)] and against Boc-QGR-MCA in the absence of DTT [QGR (DTT−)]. The relative fluorescence intensity to the peak intensity for Boc-QAR-MCA is shown.

and in the present study, we too observed similar behavior using several synthetic substrates of commercial natural Der p 1 and Der f 1, although the strength of the serine protease activity varied by lot and manufacturers (data not shown). Schulz et al. [26] and Brown et al. [17] reported that the serine protease found within the preparation of affinity-purified natural Der p 1 could be removed by passing it through a column coupled with soybean trypsin inhibitor (SBTI). However, whether such serine protease activity is associated with the group 1 allergens or contamination by mite-derived serine proteases [18–21] has not been clarified. We showed that recombinant Der p 1 and De f 1 purified by a simple method [22,23] and natural allergens purified by another method which used many purification steps without mAb and SBTI [25] exhibited only cysteine protease activity. Furthermore, we indicated that the serine proteases within commercial Der p 1 and Der f 1 have molecular sizes different from that of the cysteine protease and that their substrate specificities are similar to that of Der f 3 but not to those of Der p 6 and Der f 6 [20]. Therefore, the identity of the serine protease activity might be the group 3 allergens. The larger molecular sizes of the peaks associated with serine protease activity for commercial group 1 allergens compared to those of the peaks associated with cysteine protease activity for size-exclusion column chromatography do not conflict with the higher molecular weights of group 3 allergens Der p 3 and Der f 3 compared to those of the group 1 allergens estimated by SDS–PAGE [19,28].

Co-purification of serine protease activity with cysteine protease Der p 1 by both affinity purification using the mAb-coupled column and active-site affinity purification was reported by Hewitt et al. [11]. Possibilities that the residual serine protease activity is due to insufficient column washing after loading of the extract to the columns or that the serine protease have weak affinity for column materials or the murine mAb used are not to be excluded, as we have observed that some preparations of whole culture extracts of mites showed a much higher proteolytic activity for serine protease activity than cysteine protease activity. Der p 3 might bind to the thiol column via Cys12, which is predicted to be an unpaired cysteine residue based on its homology with trypsin proteins from various species [29].

The cysteine protease activity of Der p 1 is suggested to be involved in the pathogenesis of allergy [7–15]. Mite-derived serine protease activity also has biological activities including the ability to disrupt tight junctions [30], stimulate bronchial epithelial cells by cleaving the protease activated receptor-2 (PAR-2) [31], cleave an intrinsic protease inhibitor, elafin [17], and produce anaphylatoxins by cleaving complement components [32]. Although we considered that the amount of the serine proteases that contaminated the commercial group 1 allergens was small because no peaks associated with the group 3, 6, and 9 allergens were detected by protein sequencing analysis, their activities were not negligible. Therefore, recombinant Der p 1 and Der f 1, which can be easily prepared and exhibit only cysteine protease activity, will be useful for the accurate analysis of the pathogenicity of the cysteine protease activity of the major mite group 1 allergens, which are distinct from those of mite serine proteases. Additionally, further analysis of the contribution of serine protease activity including that by the group 3 allergens, which could have significant activities and different substrate specificities from the major group 1 allergens [17], to the pathogenesis of allergy may also be important.

Acknowledgments

We thank Dr. Reiko Mineki and Dr. Kimie Murayama (Central Laboratory of Medical Sciences, Juntendo

University School of Medicine) for protein sequencing. This work was supported in part by a Health and Labour Sciences Research Grant for Research on Allergic Disease and Immunology from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to T.T.).

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Clin Exp Allergy 2004; 34:1192–1201 doi:10.1111/j.1365-2222.2004.02008.x

Polymorphisms in *ADAM33* are associated with allergic rhinitis due to Japanese cedar pollen

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Summary

Background A recent report provided evidence that a disintegrin and metalloprotease domain 33 (*ADAM33*), a member of the ADAM family, is a novel susceptibility gene in asthma linked to bronchial hyper-responsiveness. However, there has been no investigation of the genetic role of *ADAM33* variants in nasal allergy.

Objective The purpose of this study was to test the association between *ADAM33* polymorphisms and Japanese cedar pollinosis (JCPsis), a most common seasonal allergic rhinitis in Japan.

Methods We conducted a case–control association study among a Japanese population, involving 95 adult individuals with JCPsis and 95 normal healthy controls. A total of 22 single-nucleotide polymorphisms (SNPs) in *ADAM33* were genotyped using PCR-based molecular methods.

Results Six SNPs of *ADAM33* gene, three in introns (7575G/A, 9073G/A and 12540C/T) and three in the coding region (10918G/C, 12433T/C and 12462C/T), were strongly associated with JCPsis ($P = 0.0002 - 0.022$ for absolute allele frequencies) and most of the SNPs were in linkage disequilibrium with each other. A higher frequency of the common alleles of these SNPs was noted for the subjects with JCPsis in comparison with healthy controls. We also identified a haplotype associated with the disease susceptibility. In addition, associations were found between *ADAM33* polymorphisms and various cedar pollinosis phenotypes including clinical severity, eosinophil counts in nasal secretion and allergen-specific IgE levels in sera, but not total serum IgE levels.

Conclusion These results indicate that polymorphisms in the *ADAM33* gene are associated with susceptibility to allergic rhinitis due to Japanese cedar pollen, but the functional relationship still needs clarification.

Keywords *ADAM33*, allergic rhinitis, Japanese cedar pollinosis, single-nucleotide polymorphism, susceptibility gene

Submitted 2 December 2003; revised 6 March 2004; accepted 26 April 2004

Introduction

Japanese cedar pollinosis (JCPsis) is an IgE-mediated type I allergy affecting the nasal mucosa caused by exposure to Japanese cedar (*Cryptomeria japonica*) pollen (JCP). It is one of the most common allergic diseases in Japan and represents a public health issue affecting 5–20% of the Japanese population, with increasing prevalence over the past two to three decades [1–3]. JCPsis is known to be a complex disease with genetic predisposition [4], although the underlying genes have not been pinpointed. Several previous studies, focused on the relationship between HLA class II molecules and JCPsis, showed antigen-specific T cell responsiveness to the major allergens of JCP was strikingly associated with certain specific *HLA-D* alleles [5–7]. Recently, a significant association has been found between a coding variant (Glu237Gly) of the β chain of the high-affinity IgE receptor (FcεRIβ) and JCPsis, as well as serum total and specific IgE levels [8], suggesting a strong genetic component to the onset and disease phenotype of JCPsis.

Allergic rhinitis (AR) and asthma may both be manifestations of the atopic syndrome, affecting different anatomical parts of the respiratory tract, which share a common genetic background [9], and are characterized by a similar inflammatory process [10, 11]. Significant progress has been made in

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the study of genetics of atopy during the past decade since Cookson et al. [12], in 1989, first reported a linkage between generalized IgE responsiveness and a variable number of tandem repeats polymorphism on chromosome 11q13 in British families. Nevertheless, intensive research on the complex genetics of allergic disease has focused primarily on asthma and related traits, with very little attention given to rhinitis [9, 13]. Recent information regarding asthma genetics comes from the work of Van Eerdewegh et al. [14]. These investigators performed a genetic study by combining linkage and association approaches in Caucasian affected sib-pair families from the UK and the US, and identified a disintegrin and metalloprotease domain 33 (*ADAM33*) as a major susceptibility gene for asthma linked to bronchial hyperresponsiveness (BHR). Most recently, the association of *ADAM33* polymorphisms with asthma and related phenotypes has been replicated in four unique asthma populations (African American, US white, US Hispanic and Dutch white) [15] and in the German samples [16], but not in the Latino populations (Puerto Rican and Mexican) [17].

We hypothesized that association of the *ADAM33* gene with asthma may not be specific for asthma *per se* but rather may reflect an overall genetic predisposition for allergic airway diseases. To clarify whether the *ADAM33* polymorphisms relate to JCPsis, a most important springtime AR in Japan, we genotyped and analysed a total of 22 single-nucleotide polymorphisms (SNPs) within the *ADAM33* locus in a case–control association study.

Materials and methods

Subjects

The study included 95 unrelated adult individuals with JCPsis and 95 age-matched unrelated healthy controls. All subjects were from the population of Kinki area, west Japan. The diagnosis of JCPsis was based on a positive history of rhinitis symptoms during the cedar pollen season, and positive allergen-specific IgE reactivity to JCP in serum. Exclusion criteria were a present or past history of asthma and asthma-like symptoms. Among a total of 95 patients with JCPsis, 45.3% were also allergic to house dust mites (HDM), and 18.9% of patients had perennial symptoms with seasonal exacerbations. None of the patients had received specific immunotherapy for allergic conditions. The control subjects were all symptom free, had no history of atopic disorders, and had negative allergen-specific IgE against JCP as well as HDM. All participants gave written informed consent to participate in the study, according to the process approved by the Ethical Committee in RIKEN Yokohama Institute.

Clinical parameters

Detailed characteristics of recruited patients are described in Table 1. Among a total of 95 patients with JCPsis, five cases (5.3%) were mild, 22 cases (23.2%) were moderate and 68 cases (71.6%) were diagnosed as severe according to the scores of three main nasal symptoms (sneezing, rhinorrhoea and nasal obstruction), on the basis of the clinical severity classification for AR (Okuda's method) described in detail elsewhere [18, 19].

Table 1. Phenotypic characteristics of recruited patients

Number of subjects	95
Clinical severity	
Mild	5 (5.3%)
Moderate	22 (23.2%)
Severe	68 (71.6%)
Total serum IgE (IU/mL)	
Geometric mean	162.5
Ranges	5.3–10 000
RAST scores for Japanese cedar pollen	
Score 2	9 (9.5%)
Score 3	42 (44.2%)
Score 4	25 (26.3%)
Score 5	16 (16.8%)
Score 6	3 (3.2%)
RAST scores for house dust mites	
Score ⩾2	43 (45.3%)
Eosinophil counts in nasal secretions	
None	13 (13.7%)
1+	36 (37.9%)
2+	16 (16.8%)
3+	30 (31.6%)

Serum total and allergen-specific IgE levels were measured for all participants with an AutoCAP System (Pharmacia Diagnostics AB, Uppsala, Sweden). The geometric mean (range) of total IgE levels in patients and control subjects was 162.5 (5.3–10 000) IU/mL and 30.0 (3.2–240) IU/mL, respectively. Subjects with JCPsis had higher total IgE levels than healthy controls (2.21 ± 0.51 (mean ± SD) log IU/mL vs. 1.48 ± 0.51, $P<0.001$, *t*-test). Allergen-specific IgE levels were determined by RAST. The grading of RAST scores 0–6 was made according to the manufacturer's instructions, with a specific IgE level of 0.7 UA/mL (RAST score 2 or more) being positive. In addition, to evaluate the nasal eosinophilia in patients with JCPsis, eosinophils in nasal secretion (nEOS) obtained from nose blowing were stained by Hansel staining and semi-quantitated ranging from 0 to 3+ (0: none; 1+: scattered; 2+: between 1+ and 3+; and 3+: clustered) [18, 19].

SNPs

We screened a total of 22 SNPs at the human *ADAM33* locus as the target polymorphisms for a case–control association study. The SNPs studied were based on the database of SNPs in Japanese population (JSNP, data are available through website at http://snp.ims.u-tokyo.ac.jp/) [20, 21], and our own data for SNP discovery (data not shown). The 22 SNPs are shown in Table 2, five SNPs (−3989T/C, −3982C/T, −3835C/T, −3705C/A and −85T/C) are in the 5′ genomic region, 10 SNPs (6716G/C, 7575G/A, 7667A/G, 9073G/A, 11188A/T, 11434C/A, 12540C/T, 12601T/G, 12946G/A and 12954C/T) are in introns, four SNPs (7441A/G, 10918G/C, 12433T/C and 12462C/T) are in the coding region and the remaining three SNPs (13236T/C, 13506C/G and 13527A/G) belong to the 3′ untranslated region (3′UTR) of exon 22.