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Generation of High-Affinity Antibody against T Cell-Dependent Antigen in the Ganp Gene-Transgenic Mouse

Nobuo Sakaguchi,*,† Tetsuya Kimura,‡ Shuzo Matsushita,† Satoru Fujimura,* Junji Shibata,† Masatake Araki,‡ Tamami Sakamoto,§ Chiemi Minoda,§ and Kazuhiko Kuwahara*†||

Generation of high-affinity Ab is impaired in mice lacking germinal center-associated DNA primase (GANP) in B cells. In this study, we examined the effect of its overexpression in ganp transgenic C57BL/6 mice (GanpTg). GanpTg displayed normal phenotype in B cell development, serum Ig levels, and responses against T cell-independent Ag; however, it generated the Ab with much higher affinity against nitrophenyl-chicken gammaglobulin in comparison with C57BL/6. To further examine the affinity increase, we established hybridomas producing high-affinity mAbs and compared their affinities using BIAcore. C57BL/6 generated much higher affinity against nitrophenyl-chicken gammaglobulin in comparison with C57BL/6. To further examine the affinity mutation. GanpTg-generated high-affinity anti-nitrophenyl mAbs (Kd ~ 2.50 × 10^−7 M) of IgG1/A1 and contained the VH186.2 region with W33L mutation. GanpTg generated much higher affinity (Kd > 1.57 × 10^−7 M) by usage of VH186.2 as well as noncanonical VH1783 regions. GanpTg also generated exceptionally high-affinity anti-HIV-1 (V3 peptide) mAbs (Kd > 9.90 × 10^−11 M) with neutralizing activity. These results demonstrated that Ganp is involved in V region alteration generating high-affinity Ab. The Journal of Immunology, 2005, 174: 4485–4494.

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The expression construct of mouse ganp cDNA under the mouse Ig promoter and human Ig enhancer (6) was used for establishing the transgenic mouse. GanpTg mice express high-affinity Ab in response to TD-Ag in vivo. However, there remained several possibilities to account for the molecular mechanism in generation of high-affinity V regions by the expression of Ganp in GC B cells. Ganp might augment the induction of SHM in the V region, resulting in the affinity maturation of V regions during the proliferation and differentiation of Ag-driven B cells in GCs. Furthermore, Ganp might be involved in the survival of the high-affinity BCR B cells for the positive selection through the interaction of Ags captured on the follicular dendritic cell network. The GCs of the B-ganpTg−/− mouse displayed an increase of apoptotic cells upon immunization with TD-Ag SRBC, which suggested a partial involvement of Ganp in the survival of GC B cells. However, the ganpTg−/− B cells do not show marked abnormalities in the levels of apoptotic and proapoptotic molecules after BCR cross-linkage (5). To study the function of Ganp in generation of high-affinity Ab response, it is necessary to examine whether the affinity maturation of BCR on the GC B cell is generated by the genetic alteration in the V region gene.

We speculated that it would be possible to generate a high-affinity Ab if we used mice with higher level Ganp expression in B cells. We studied whether the transgenic mouse with increased expression of ganp gene could generate high-affinity Ab against TD-Ag using a model epitope of 4-hydroxy-3-nitrophenyl acetyl (NP)-hapten in the C57BL/6 background. To demonstrate the increased affinity of the Ab in detail, we established the hybridomas secreting anti-NP mAbs after immunization with NP-chicken gammaglobulin (CG) in GanpTg mice. After selecting the high-affinity mAbs against NP-hapten by differential ELISA method and the BIAcore system, we examined the V region gene usage of the hybridomas and compared the sequences with those from wild-type C57BL/6 mice. The results suggest that the affinity maturation of BCR on GC B cells is generated by the altered V region usage with increased SHM in GanpTg mice.

Materials and Methods

GanpTg−/− mouse

The expression construct of mouse ganp cDNA under the mouse Ig promoter and human Ig enhancer (6) was used for establishing the transgenic mouse.

*Department of Immunology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan. E-mail address: nobusaka@kaiju.medic.kumamoto-u.ac.jp
†Division of Clinical Retrovirology and Infectious Diseases, Center for AIDS Research, Division of Bioinformatics, Institute of Resource Development and Analysis, Kumamoto University, and\(^2\) Trans Genic, Kumamoto Japan; \(^6\) Core Research for Evolutional Science and Technology Program, Saitama Japan; and \(^7\) PRESTO, Japan Science and Technology Agency, Saitama, Japan
\(^1\) Address correspondence and reprint requests to Dr. Nobuo Sakaguchi, Department of Immunology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan. E-mail address: nobusaka@kaiju.medic.kumamoto-u.ac.jp
\(^2\) Abbreviations used in this paper: GC, germinal center; GANP, germinal center-associated DNA primase; NP, 4-hydroxy-3-nitrophenyl acetyl; SHM, somatic hypermutation; TD-Ag, T cell-dependent Ag; MCM, minichromosome maintenance; CG, chicken gammaglobulin; KLH, keyhole limpet hemocyanin; TNP, 2,4,6-trinitrophenyl; LTR, long terminal repeat.

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mouse by the standard procedure. Mice were screened for the transgene by PCR using gagp 1–5’ primer (5’-TCCCCGCTTCCAGCTTGAC-3’) and gagp 1–3’ primer (5’-GTGCTGCTGTGTTAT GTCC-3’) and Southern blot analysis using gagp probe A (1143–1939 nt) of tail genomic DNAs. The G apoptosis that express 1.5- to 2.0-fold increase of gagp gene grew normally under specific pathogen-free condition and were immunized with Ags. G apoptosis were detected by two primers (gagp 1–5’ and gagp 1–3’) in comparison with B-actin control (5). All mice were maintained in the Center for Animal Resources and Development (Kumamoto University, Kumamoto, Japan).

Flow cytometric analysis

Single-cell suspensions from lymphoid organs were stained with each biotin-labeled mAb in combination with FITC-conjugated streptavidin (Amersham Biosciences) and PE-conjugated mAbs. Lymphoid cells were analyzed by FACSCalibur (BD Biosciences) using CellQuest software.

**FIGURE 1.** Generation of transgenic mice that overexpress the gagp gene in B cells. A, A schematic diagram of construct for G apoptosis under the human Ig enhancer, mouse Ig promoter, and followed by rabbit β-globin 3’-untranslated region (UTR). The construct contains restriction enzyme sites: Xb, Xhol; H, HindIII; E, EcoRI; and S, SalI. The probe for Southern blot analysis (probe A) is indicated. B, Detection of the gagp transgene by Southern blot analysis. Southern blot analysis with EcoRI-digested genomic DNAs of G apoptosis displayed a 5.3-kb band hybridized with probe A. C, Up-regulation of gagp transcripts in B cells from G apoptosis. Semiquantitative PCR was performed using the primers gagp 1–5’ and gagp 1–3’, in comparison with B-actin transcripts. From densitometer analysis, gagp transcripts in B cells from G apoptosis showed an 80% increase in comparison with C57BL/6 mice. D, Flow cytometric analysis. Bone marrow, spleen, and lymph node cells from 8-wk-old C57BL/6 and G apoptosis were analyzed with indicated markers. E, In vitro proliferation assay of purified B cells from G apoptosis. [3H]Thymidine incorporation was measured in the presence or absence of B cell mitogenic stimulants in C57BL/6 mice (■) and G apoptosis mice (□). The representative data are shown from four independent experiments. * p < 0.05. F, Kinetics of GC formation after TD-Ag in G apoptosis. C57BL/6 and G apoptosis mice were immunized by SRBC. At day 10 or day 14, the sections were doubly immunostained with peanut agglutinin (brown) and IgD (blue). Arrows indicate GCs. G, T cell-independent Ag (type II)-specific or TD-Ag-specific immune responses in G apoptosis. Sera from mice immunized with TNP-Ficoll or TNP-KLH were collected at day 14. TNP-specific Ab titers were measured by ELISA. C57BL/6 mice (■) and G apoptosis mice (□) are indicated. H, Relative affinity of serum Abs in G apoptosis. Sera from C57BL/6 and G apoptosis mice immunized with NP-CG were collected at days 14 and 28. The NP1 to NP17 ratios of anti-NP IgG1 were measured by ELISA. I, W33L mutation of V-region transcripts from C57BL/6 and G apoptosis mice. Mice were i.p. immunized by 20 μg of alum-precipitated NP-CG. V-region transcripts of γ1-isotype were amplified by RT-PCR and cloned into pBluescript vector for sequencing. The calculated percentage from sequence data was shown in C57BL/6 (■) and G apoptosis (□) mice. * p < 0.05.

**In vitro proliferation assay**

Purified B cells were cultured for 48 h at a density of 2 × 10⁶ cells/well in 96-well microtiter plates in RPMI 1640 medium containing 10% heat-inactivated FCS (JRH Biosciences), 2 mM L-glutamine, and 5 × 10⁻⁵ M 2-ME. The cells stimulated with or without various mitogenic stimulants were pulsed with 0.2 μCi/well of [3H]thymidine (ICN Pharmaceuticals) for 16 h before harvesting, and the incorporated radioactivity was measured by scintillation counter. Stimulatory reagents were affinity-purified goat anti-mouse μ-chain-specific Ab (F(ab’)2, 10 μg/ml; ICN Pharmaceuticals), rat anti-mouse CD40 mAb (LB429, 10 μg/ml) (4), and LPS (10 μg/ml; Sigma-Aldrich).

**Immunohistochemistry**

The 8-μm sections of spleen from SRBC-immunized mice were lightly fixed with acetone. Slides were blocked with 3% BSA in PBS-Tween 20.
and incubated with anti-IgD mAb in combination with alkaline phosphatase-conjugated anti-rat IgG (ICN Pharmaceuticals). The first development step was conducted with Vector Blue kit (Vector Laboratories). For second staining, slides were incubated with biotin-conjugated peanut agglutinin (Vector Laboratories) in combination with HRP-conjugated streptavidin (Kirkegaard & Perry Laboratories), followed by 3,3'-diaminobenzidine tetrahydrochloride (Dojindo). After fixation with 1% glutaraldehyde in PBS, mounting was done by Aquatex (Merck).

Ag and immunization

2,4,6-Trinitrophenyl (TNP)-keyhole limpet hemocyanin (KLH), TNP-Ficoll, and NP28-CG were purchased from Biosearch Technologies. From 20 to 100 μg of TNP-KLH and NP-CG precipitated by alum (Pierce), or 25 μg of TNP-Ficoll dissolved in PBS was injected i.p. into C57BL/6 and Ganpβ2 mice.

Measurement of Ag-specific Ab production

Five micrograms per well of TNP-BSA (Biosearch Technologies) were coated on ELISA plate, blocked with 3% BSA in PBS, and incubated with the serial-diluted sera obtained at day 14 after Ag immunization. After washing with PBS-0.1% Tween 20, the wells were incubated with biotin-conjugated isotype-specific mAb in combination with alkaline phosphatase-conjugated streptavidin (Southern Biotechnology Associates). The development was performed in the presence of substrate.

Sequence analysis of V_{H}186.2 gene

The Ganpβ2 mice were immunized with alum-precipitated NP-CG once as described (5). After 28 days, the spleen B cells were purified, and the total
RNA was used for RT-PCR analysis with the sequence primers for IgG1-VH/IgG2a and the sequences were compared with those of C57BL/6.

Establishment of mAbs
Ag immunization was conducted with CFA as a primary immunization and then followed by boosting with IFA (4). For anti-NP-specific mAbs, NP28-CG emulsified in CFA was injected i.p. and boosted after 2 wk with IFA. The mice with higher serum Ab titers were further immunized, and 3 days later, the spleen cells were obtained for cell fusion by polyethylene glycol method with mouse myeloma cell line X63 under the standard procedure (4). The fused cells were selected with hypoxanthine/aminopterin/thymidine medium on the microculture plates at the concentration of 2 × 10^5 cells/well with IL-6 (5 U/ml). The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure. The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure. The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure.

ELISA screening
For anti-NP mAbs, supernatants of individual wells were divided into two aliquots (each 50 μl) and measured by the differential ELISA method with two different Ag-coating as NP2-BSA and NP2g-BSA (Biosearch Technologies) under the standard procedure. The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure. The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure. The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure.

ELISA assay
For anti-NP mAbs, supernatants of individual wells were divided into two aliquots (each 50 μl) and measured by the differential ELISA method with two different Ag-coating as NP2-BSA and NP2g-BSA (Biosearch Technologies) under the standard procedure. The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure. The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure. The mAbs binding to the Ags were captured with protein A-peroxidase (Amersham Biosciences) with the subunit specificities under the standard procedure.

BIAcore assay
Affinity of the mAbs was determined by the BIAcore assay (7). The on and off rate constants (k_a and k_o) for binding of the mAbs to NP or HIV-1 V3 loop peptide were determined by BIAcore system (Biacore Technologies). The carboxyl-methylated dextran surface of the sensor chip was activated with EDC (N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide) and NHS (N′-hydroxysuccinimide) (8). V3 loop peptide was immobilized through the carboxyl group of a cysteine residue that was deliberately placed at the N terminus, by injection of 35 μl of a 20 μg/ml solution in 10 mM MES buffer (pH 6) to the EDC-NHS-activated surface that had been reacted with 2-(2-pyridylidyldithio)ethanesulfonic acid. The excess disulfide groups were deactivated by the addition of cysteine. The mAbs were diluted in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.05% (v/v) BIAcore surfactant P20 and injected over the immobilized Ag at a flow rate of 5 μl/min. The association was monitored by the increase of the refractive index of the sensor chip surface per unit time. The dissociations of the mAbs were monitored after the end of the association phase with a flow rate of 50 μl/min. Kinetic rate constants were calculated from the collected data using the Pharmacia Kinetics Evaluation software (9). The K_D was determined by measuring the rate of binding to the Ag at different protein concentrations.

DNA assay
The DNA fragments corresponding to the rearranged VH regions were amplified using Pfu-Turbo (Stratagene) from the genomic DNA. The oligonucleotide primers are as follows (10, 11): VH186.2 forward, 5′-CTGAC CCGCATCCGCTTTCCACGGCAGG-3′; VH186.2 reverse, 5′-GCA GCTGGTGAGGACTTGCG-3′; JH16.4-3, 5′-CTTCAGCGCCCTCTCTCAGGG-3′; Vα1 forward, 5′-TGCTGACAAATATTTGAAAA-3′; Jα1 reverse, 5′-AGCACCTGAACTGTGAGAGG-3′. For rearranged V κ chain genes, the cDNA fragments were amplified using the primers designed as follows (12): V κ-1.1 forward, 5′-ATGGATATCTAGGGAATATTTTCA-3′; Vκ-21B forward, 5′-AGGTATTGCAAGAACACTCTGTGCA-3′; and Cκ reverse, 5′-TGGAAGATGAGGATACGTGTTGGTCAG-3′. Amplification of Cμ region was conducted with the primers: Cμ-Ex1 forward, 5′-AGCTAGCTTCTCCCAATGTTCTTCCC-3′ and Cμ-Ex3 reverse, 5′-TGAAGGTAGATGACTGTGAGGG-3′. The amplified DNA fragments cloned into blunt-ended pBluescript were sequenced.

In vitro binding assay into NL4-3 envelope
293T cells were transfected with pLP1-RES2 enhanced GFP (BD Clontech) or pLP-NL4-3 envelope enhanced GFP using Effectene Transfection Reagent (Qiagen). After 36 h, cells were harvested, incubated with each anti-HIV-1 mAb in combination with allophycocyanin-conjugated goat anti-mouse IgG Ab (BD Pharmingen), and analyzed in comparison with GFP expression by FACSCalibur. The anti-CD19 mAb was purchased from BD Biosciences.

Neutralization activity assay
HIV-1 strain NL4-3 (prototype X4; T cell tropic) was propagated in PM1 cells in RPMI 1640 medium with 10% (v/v) heat-inactivated FCS, and the cell-free supernatant was collected and stored as virus stocks at −80°C. The chemiluminescent assay (Galacto-Star; Applied Biosystems) for β-galactosidase released from the HeLa-Cd4/long terminal repeat (LTR) β-galactosidase/CCR5 (MAGI/CCR5) cells were conducted as previously described (13). Tissue culture-effective dose (TCID)50 of virus stock was predetermined with MAGI/CCR5 cells by the method of Reed and Muench (14). For the assay of neutralizing activity against HIV-1 infection, MAGI/CCR5 cells were plated in 96-well microtiter plates at a density of 1 × 10^4 cells/well, and on the next day, the cells were incubated with 50 μl of each mAb and 50 μl of HIV-1 solution (500 TCID50) for 30 min at 37°C in combination with 10 μg/ml DEAE-dextran (Amersham Biosciences) in a pre-incubation assay. After 48 h, the foci-galactosidase activity for 1 s using the Galacto-Star system according to the manufacturer’s protocol and showed results as percentages of the negative control.

Results
Establishment of GanpT8 mice
GanpT8 mice were established under control by human Ig enhancer and mouse Ig promoter in C57BL/6 background (Fig. 1, A and B), and the adult mice showed an increase of ganp transcripts (~2-fold) in B cells (Fig. 1C). GanpT8 mice had normal B lineage differentiation by surface marker studies of B220, IgM, and IgD on lymphoid cells in the bone marrow, spleen, and lymph nodes (Fig. 1D). B cell numbers and the levels of serum IgMs were also normal in GanpT8 mice (data not shown). These results demonstrated that B cell differentiation undergoes normally in GanpT8 mice compared with wild-type littermates.

In vitro B cell proliferation and GC formation of GanpT8 mice
Next, we examined the potential of B cell proliferation of GanpT8 mice in vitro. GanpT8 mice showed comparable proliferation activities to wild-type littermates in response to anti-μ Ab, anti-μ Ab plus anti-CD40 mAbs, or LPS (Fig. 1E). Interestingly, GanpT8 B cells showed augmented responses to anti-CD40 stimulation in comparison to wild-type B cells. This was only observed in the response to anti-CD40 stimulation but not in the response to anti-μ Ab or LPS stimulation, suggesting that GanpT8 mice augment CD40-stimulated response in vivo.
We examined whether GanpTg mice showed the alteration of GC formation in vivo. GanpTg mice did not show any difference in the size and number of GCs at day 10 after SRBC immunization; however, in contrast to the findings observed in B-ganp−/− mice (4), GanpTg mice showed the accelerated resolution of GC formation in vivo (Fig. 1F). This response could be due to the efficient production of high-affinity Ab in GanpTg mice.

Responses of GanpTg mice against T cell-independent Ag and TD-Ag

Because GANP expression is selectively up-regulated in GC B cells, we studied the Ab responses of GanpTg mice. After Ag immunization, the responses were measured for T cell-independent type II Ag and TD-Ag at various time points and the results of day 14 were shown. The serum titers of Ag-specific Abs against TNP-Ficoll as T cell-independent Ag and TNP-KLH as TD-Ag were normal with similar distributions of various Ig isotypes in comparison with wild-type littermates (Fig. 1G).

Enhanced affinity maturation of GanpTg mice against TD-Ag

However, after immunization with NP-CG, GanpTg showed high affinity by the differential ELISA with the pauci NP2-BSA conjugate that yielded 42% of the response to the multihapten NP17-BSA conjugate in comparison with C57BL/6 (Fig. 1H). Jacob et al. (15, 16) showed that, in (NP-CG)-immunized C57BL/6 mice, the Abs in the secondary response against NP were exclusively IgG1/A1 and had a single Vh region (Vh186.2) carrying a peculiar pattern of mutation for high affinity. We investigated whether the affinity increase of anti-NP Ab generated in GanpTg mice accompanied with the similar mutation pattern in the Vh186.2 locus. The Vh186.2 sequence was studied by RT-PCR using the spleen B cells from (NP-CG)-immunized mice. GanpTg showed striking increases in mutation at W33L to the Vh186.2 locus in splenic B cells (W33L, Fig. 1I). These results demonstrated that GanpTg induced a higher frequency of the high-affinity mutation during the immune response to TD-Ag.

Establishment of hybridomas secreting high-affinity anti-NP-hapten

Anti-NP hybridomas were established by immunization of GanpTg with NP-CG. Supernatants from >6000 clones were screened by the differential ELISA to identify wells with high-affinity mAbs, and the selected hybridoma cells were cloned. Affinities of those
FIGURE 2. Sequence analysis of the V region genes of the hybridomas secreting anti-NP mAbs. V<sub>H</sub> regions were determined using the primers commonly applicable to the major V<sub>H</sub> families and the J<sub>H4</sub> primer (12). The individual V<sub>H</sub> region sequence was determined after cloning of the genomic PCR products. Each sequence was determined by three sequencing reactions. A, The sequences of the clones using the V<sub>H</sub>186.2 region are aligned and the site of W33L mutation is boxed. B, The sequences of the VDJ recombination regions are aligned. The part of the V<sub>H</sub>186.2 region was boxed. C, The V<sub>L</sub> region sequences were determined similarly to the method used for V<sub>H</sub> regions. D, The sequences of the V<sub>H</sub> and V<sub>L</sub> regions of the two anti-NP mAbs that used noncanonical V<sub>H</sub> regions are shown. The mutations shared between the two mAbs are boxed.
The usage and mutation of V region genes in the anti-NP hybridomas

The high-affinity mAbs obtained from C57BL/6 mice used the canonical VH186.2 gene with the W33L mutation that is responsible for high affinity. This change increased the affinity from $K_D = 2 \times 10^{-6} \text{M}$ to $2 \times 10^{-7} \text{M}$ (17, 18). The other mutations in the VH186.2 gene segment would not have contributed to increased affinity against NP-hapten (19). Therefore, we sequenced the VH regions of the hybridomas to examine whether there were similar mutation profiles of the V region. The mAbs from C57BL/6 mice generated typical high affinity against NP-hapten by using the VH186.2 region with W33L mutation in combination with DFL16.1 and JH2 gene segments. 

The high-affinity mAbs from GanpTg (NP-G2-15 and NP-G2-25) also used noncanonical VH186.2 in combination with both $\kappa$ and $\lambda$ yielding affinities from $K_D = 1.10 \times 10^{-7} \text{M}$ to $1.57 \times 10^{-9} \text{M}$. Interestingly, the mAbs from GanpTg also used noncanonical VH region of VH7183 family in combination $\kappa$-chain but showed similarly high affinities.

The usage and mutation of V region genes in the anti-NP hybridomas

The sequences of the VH region, the Ag-binding activities, and the neutralizing activities of the mAbs against the V3 epitope of HIV-1 gp120. A. The VH region sequence (VHSm7) used for the high-affinity mAbs from GanpTg is shown. The mutation site commonly observed in the two regions is boxed. B. Binding activity of the mAbs against HIV-1 envelope. Specific binding of the mAbs were shown as mean fluorescence intensity (MFI) examined with allopurinol-conjugated goat anti-mouse IgG Ab in combination with the NL4-3 envelope-expressing GFP+ transfectants by using flow cytometry. Negative controls were measured with GFP+ mock transfectants. Another negative control for mAb binding was shown by CD19 mAb. C. Neutralizing activities were measured using a CD4-LTR/B-galactosidase-induced HeLa cell line. After TCID$_{50}$ of virus stock was predetermined with MAGI/CCR5 cells, the virus infection assay was conducted in vitro, to which anti-V3 epitope mAbs were added. Higher neutralization activities were significant (*) for the mAbs produced by GanpTg (V3-G2-10 and V3-G2-25) as compared with those of C57BL/6 mice at the concentration of 0.5 $\mu$g/ml. Negative control is shown with anti-CD19.
Moreover, two anti-NP mAbs from GanpTg yielding similarly high affinities \( (K_D = 2.34 \times 7.05 \times 10^{-8} \text{ M} \) used noncanonical V_H region sequences, both of which probably originated from the same genomic V_H7183 family. The best match in the Celera Discovery System was to the C1F221MH9 (V_H7183 family), but the two clones showed variations with 12 at differences from the genomic C1F221MH9 sequence (Fig. 2D). Mutations of W47C, N52C, S59T, G66D, and A97T were commonly observed in the V_H region (C1F221MH9), suggesting their contributions to raise the affinity of the mAb against NP-hapten. However, mutations in the V_H regions were not apparently increased in the comparison of the anti-NP mAbs from GanpTg and C57BL/6 mice. The generation of high-affinity BCR without the exchange at position 33 is a rare event, which suggested that the combination of particular D-JH sequences and/or many SHMs do not result in high affinity (18). A recent report only showed the case of mutation, Y99G in V_H186.2, which generated similar high-affinity mAb against NP-hapten comparable to W33L (10). Extensive earlier studies of anti-NP mAbs found that repeated immunization of C57BL/6 mice with NP-CG increased usage of noncanonical V_H regions and different L chain combinations (16). However, as far as we know, no study with conventional animals has demonstrated comparable high-affinity mAbs to those reported in this study. Although crystallographic studies are needed for definitive conclusions, we speculate that hypermutated C1F221MH9 V_H region (V_H7183 family) in association with other L chain combinations creates an effective tertiary structure for Ag-binding, yielding closer interactions of hypermutated C1F221MH9 V_H Region and NP-hapten, and might be as effective as mAbs with the V_H186.2 region.

Mutations induced in the noncanonical V_H region of the spleen B cells after immunization with NP-CG

Usually wild-type C57BL/6 mice do not induce such a frequent mutation in the noncanonical V_H region in GC B cells before and after immunization with NP-CG. We examined the mutation frequency of the V_H7183 family gene (C1F221MH9) under a non-immunization condition in spleen B cells of GanpTg mice but found no alteration of the V_H region (data not shown). To study whether such hypermutation could be observed in GanpTg spleen B cells after immunization, we investigated mutations in the V_H7183 family gene (C1F221MH9) by examining genomic DNA of NP-binding GC B cells purified by cell sorting. These DNAs showed 16-fold higher mutation frequencies (4.5 mutations/V_H region of GanpTg mAb vs 0.28 mutations/V_H region of C57BL/6 mAb) in the V_H7183 family (Fig. 3, left panel). In contrast, such higher mutation frequencies were not observed in the C_H region (Fig. 3, right panel). This is in agreement with the suggestion that GanpTg has a high frequency of SHM that contributes to the production of high-affinity BCR in vivo. Alternatively, GanpTg might effectively rescue and maintain B cells with high-affinity BCR during the immune response.

Establishment of high-affinity mAbs against HIV-1 by use of GanpTg mice

To apply this system for generating high-affinity Ab using GanpTg mice, we studied whether high-affinity anti-HIV-1 mAbs with significant neutralization activity against virus infection could be generated by immunization with the V3 loop peptide (NL4-3) of HIV-1 gp120. Differential ELISA using plates coated with high and low doses of the V3 peptide initially identified hybridoma cells with relatively high-affinity mAbs from >6000 wells of (V3 peptide)-immunized GanpTg and C57BL/6 mice. High-affinity clones were selected from each mouse strain. After further cloning, individual mAbs were purified and their affinities were measured using the BIACore system. The mAbs from both mouse systems showed higher affinities in a range from \( K_D = 2.81 \times 10^{-5} \text{ M} \) to \( 5.67 \times 10^{-9} \text{ M} \). However, we could obtain extraordinarily higher affinity mAbs (V3-G2-10 and V3-G2-25; \( K_D = 9.90 \times 10^{-13} \text{ M} \) from GanpTg over the level that is generally not attainable by conventional methods of mAb preparation (20). The highest affinity mAbs (V3-W1-2 and V3-W1-8) from C57BL/6 mice were up to \( K_D = 9.81 \times 10^{-9} \text{ M} \) and \( 7.58 \times 10^{-8} \text{ M} \). The high-affinity mAbs from GanpTg used the same V_H region (V_H7183 family) with the common mutation at T97I, suggesting that the T97I mutation contributed to an affinity increase against the V3 epitope (Fig. 4A). A binding assay involving HIV-1 envelope (NL4-3) gene-transfected cells was used to determine whether the mAb recognized the viral epitope. The binding activities to the transfectants were studied by flow cytometry as mean fluorescence intensity in comparison with the GFP-positivity as indicators of gene transfection. The mAbs (V3-G2-10, V3-G2-25, V3-W1-2, and V3-W1-8) showed higher binding activities to the virus epitope-expressing cells (Fig. 4B).

The neutralization activities of these anti-HIV-1 mAbs were examined using a CD4-LTR/β-galactosidase-transduced HeLa cell line that expresses high levels of human CD4 and contains a single integrated copy of a β-galactosidase gene under the control of a truncated HIV-1 LTR (13, 21). Neutralization activities of the two high-affinity mAbs (V3-G2-10 and V3-G2-25) were clearly detected at 0.5 μg/ml, which were more effective than those of mAbs (V3-W1-2 and V3-W1-8) from C57BL/6 (Fig. 4C). The simple comparison might indicate 50–100 times increase of affinity in the mAbs from GanpTg mice. These mAbs with high-affinity Ag-binding and neutralization activities should be useful for clinical diagnostic purposes and analogous human mAbs might have therapeutic possibilities (22).

Discussion

Expression of GANP is required for generation of high-affinity Ab response in vivo, which was demonstrated by conditional targeting of ganp gene in B cells that caused apparent decrease in production of high-affinity Ab against NP-hapten, accompanied with the decreased frequency of high-affinity type mutation of W33L at the V_H186.2 in NP-binding IgG1+ B cells (5). Several possibilities might be considered to explain the mechanism of GANP in generation of high-affinity BCR+ B cells in vivo. GANP might be directly linked in genetic alteration, including V region dsDNA breaks occurring in B cell proliferation (23), SHM events in association with activation-induced cytidine deaminase (24), uracil DNA glycosylase (25), and error-prone DNA polymerases up-regulated in GC B cells (26), DNA recombination and repair mechanisms or rather involved in the selection of high-affinity BCR+ B cells in the follicular dendritic cell network (27), and survival and maintenance of B cells with high-affinity type mutations throughout the immune response.

There are several possible mechanisms regarding the GANP function. Firstly, GANP might directly regulate generation of mutation frequency of the V_H region in GC B cells. The structure and expression of GANP indicated that GANP has two nuclear localizations, which probably originated from the same genomic RNA-primase region and the RNA-binding activity might cooperate during the transcription at G1 phase and introduce the alteration or the damage of the V_H region sequences during rapid cell proliferation in GCs. More interestingly, altered expression of

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mouse SHD1 that has a homology to the central part of GANP (630–950 aa) caused an apparent cell cycle abnormality involving with centrosome duplication and M phase transition (29), which was also in accordance with the information of the association of Saccharomyces Sac3 with Cdc31/centrin (30). Loss of SHD1 caused an impairment of centrosome duplication, deregulated nuclear division, with disappearance of Mad2 expression in the pro-metaphase. Because mouse GANP is considered as a homologue of Saccharomyces Sac3 (31), GANP might be also involved in the centrosome duplication or the chromatin segregation during cell division. These observations suggested the involvement of GANP in either one or several mechanisms of gene transcription, DNA replication, and chromatin separation and cell division. Loss of GANP caused the increased apoptotic cells in GCs after immunization with TD-Ags (4), whereas the gain of function did not show obvious difference (data not shown). Second, these functions of GANP regions might be involved in the repair of DNA injuries occurring under a transcription-coupled mechanism or in the DNA replication phase. If this is the case, existence of GANP is critical for maintenance of DNA stability during the GC B cell stage that undergoes genetic alteration with frequent SHM of the VH region and class switch recombination. Expression of GANP is necessary for the rescue of damaged GC B cells that potentially gain the high-affinity BCR. Third, additional function of GANP might be involved in generation or selection of high-affinity BCR+ B cells in GCs. GANP Tg mice showed accelerated kinetics of GC formation (Fig. 1F), whereas B-ganp−/− mice showed retarded GC formation (4). Recently, Mumics et al. (32) described that GANP is involved in downstream event(s) of Lyn. As Lyn is involved in CD40-mediated signal transduction (33) and Lyn-deficient mice showed lack of GCs (34), there might be functional interaction of CD40-mediated signaling with the GANP function involved in regulation of high-affinity B cells. The augmented anti-CD40 response of GANP Tg mice might support this notion, in which GANP is necessary for the rescue of high-affinity B cells during the selection in GCs. As a potential role of GANP in the selection process, GANP associates with a protein phosphatase component G5PR that associates with protein phosphatase 5 and protein phosphatase 2A (35). The complex of GANP with G5PR may regulate the other signaling pathways involved in cell survival mechanism or in regulation of BCR-mediated cell proliferation during maturation and selection of GC B cells. We have no definitive evidence to conclude the molecular mechanism at present but GANP is most likely a key molecule to elucidate the molecular mechanism in generation of high-affinity Ab in vivo.

To confirm the effect of GANP in generation of high-affinity Ab, we used a system to compare the affinity of the Abs at the monoclonal level by establishing the mAb-producing hybridomas. Affinity measurement with NP-hapten clearly demonstrated the high affinity of the mAbs generated from the GANP Tg mice. Sequence analyses of the V regions of individual mAb-producing hybridomas demonstrated that the high affinity was generated not only with increased SHM frequency in the VH1 86.2 region but also with the noncanonical VH region usage that was not seen in the control hybridomas.

The results of both the loss and gain of GANP expression caused adverse effects in generation of high affinity response, which confirmed that the GANP function is involved in generation of high-affinity Ab in vivo. Additionally, the high-affinity is generated with the genetic alteration of V region genes as increased SHM and the different V region usage. GANP function might be directly involved in the formation of high affinity V region of the GC B cells. GANP is not up-regulated in the nonimmunized condition and is not expressed in normal T cells at the similar level detected with anti-GANP mAb (3). We speculate that up-regulation of GANP is selective in the cells with frequent genetic alterations such as V region SHM and class switch recombination during rapid proliferation phase.

In summary, we have demonstrated that GANP Tg mice induces higher affinity Ab against TD-Ag in vivo, which was confirmed by BIA-core system with the purified mAbs against two model Ags of NP-hapten and the gp120 V3 peptide of HIV-1 by immunizing as TD-Ag. More importantly, the usage and the mutations of the V regions demonstrated that increased expression of GANP caused the genetic alteration of the V regions with increased mutations generating high affinity against TD-Ag in vivo. The results suggest that the GANP Tg mouse has an advantage in preparation of mAbs against various epitopes, for which conventional mice hardly generate high-affinity mAbs by the standard procedures. High-affinity mAbs generated this way show greater epitope binding constants and this binding is long-lasting as measured in vitro. It would be useful to generate high-affinity mAbs against various molecules, which can be applicable widely in the diagnostic and therapeutic purposes.

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Disclosures
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