Novel Epitopic Region of Glucosyltransferase B from *Streptococcus mutans*

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Abstract

In the development of a component vaccine against caries, the catalytic region (CAT) and glucan-binding domain (GBD) of glucosyltransferase B (GtfB) from *Streptococcus mutans* have been employed as target antigens. These regions were adopted as primary targets because they theoretically include epitopes associated with enzyme function. However, their antigenicity has not been fully evaluated. Although there are many reports about successful vaccination using these components, the principle has not yet been put to practical use. For these reasons, we came to doubt the effectiveness of the epitopes in vaccine production and reevaluated the antigenic region of GtfB using *in silico* analyses combined with *in vitro* and *in vivo* experiments. The results suggest that the ca. 360-amino-acid variable region (VR) in the N-terminus of GtfB would be more reactive than CAT and GBD. This region is *S. mutans*- and/or GtfB-specific, non-conserved among other streptococcal Gtfs, and of unknown function. Immunization using an adenovirus vector-borne DNA vaccine confirmed that VR is an epitope that shows promise for the development of a caries vaccine.

Introduction

In spite of some success with preventive measures, dental caries continue to be prevalent

and costly in many populations, and the development of a vaccine to prevent dental caries is

still a desirable goal. Attempts to develop a vaccine against dental caries began with

immunization using whole cells of the primary etiologic agent *Streptococcus mutans* in rhesus

monkeys (23, 24). Although dental caries were successfully prevented in these studies, it was

reported that the induced antibodies showed cross-reactivity with the human heart muscle (10,

48). Thereafter, components derived from *S. mutans* were investigated to avoid induction of

antibodies that cross-reacted with human tissue. The molecules primarily focused as target

antigens are glucosyltransferase B (GtfB) (46) and antigen I/II (Ag I/II) (34).

GtfB was further truncated and the catalytic region (CAT) (37) and the glucan-binding

domain (GBD) (20) were employed as effective antigenic components. These components

were combined with the cholera toxin B subunit (22), the saliva-binding region derived from

Ag I/II (50), or both (30) to further enhance immunogenicity.

Despite these successful results reported more than 10 years ago, a GtfB-based vaccine

against caries has not yet been put to practical use. One reason may be that induction of

mucosal immunity is needed to produce the effective secretory IgA (S-IgA) antibodies that

can prevent dental caries. Although encouraging results have been reported (3-6, 30, 38, 39),

salivary S-IgA responses of are often variable, transient, and of low magnitude compared with

serum IgG responses. Another cause for the delay of the anti-caries vaccine may be the

antigenicity of the target antigens CAT and GBD, which were selected due to their association

with enzyme function (40, 41). No study has addressed the immunogenicity of CAT and GBD

by immunological, biochemical, or bioinformatic methods. It was recently suggested that

effective screening for candidate vaccine antigens should include *in silico* analysis followed

by *in vitro* and *in vivo* evaluation of antigenicity to reduce time and cost (29).

In this study, we reevaluated the immunogenicity of GtfB using *in silico* approaches

followed by *in vitro* and *in vivo* experiments to identify antigenic regions that may be

employed in an effective anti-caries vaccine. On the basis of our findings, we constructed a

DNA vaccine using an adenovirus vector and confirmed its ability to induce specific

antibodies contributing to immunity in mice.

MATERALS AND METHODS

Bacterial strains and growth media. *S. mutans* MT8148 (serotype *c*) was routinely

cultured in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) or on

Mitis-Salivarius (MS) agar (Difco) at 37 °C in air. *Escherichia coli* XL-2 (Stratagene Ltd.,

Cambridge, UK) was cultured aerobically in Luria-Bertani (LB) medium. When appropriate,

erythromycin, kanamycin, and ampicillin (Wako Pure Chemicals, Osaka, Japan) were added

to the LB medium to final concentrations of 500, 30, and 100 µg/mL, respectively.

Preparation of GtfB. *S. mutans* MT8148 was cultured in 5 L of TTY medium (composed

of Trypticase, tryptone, yeast extract, salts, and 1% glucose) instead of BHI broth, because of

increased yield of bacterial cells (16) at 37 °C, to an optical density of 0.8 at 550 nm. The

bacteria were collected by centrifugation, and cell-associated Gtf (CA-Gtf) was extracted by

treatment with 50 mL of 8 M urea at 25 °C for 1 h. The extract was dialyzed against 10 mM

sodium phosphate buffer (NaPB, pH 6.5) and adjusted to 60% saturation with ammonium

sulfate. After centrifugation, the precipitate was dissolved in 10 mM NaPB (pH 7.5) and then

dialyzed against the same buffer. The crude CA-Gtf sample was applied to a

diethylaminoethyl (DEAE) Sepharose FF (GE Healthcare UK Ltd., Buckinghamshire, UK)

column (bed vol., 10 mL) and eluted with a linear gradient of 0–1.0 M NaCl in the same

buffer. Active fractions measured as described below were pooled, concentrated by

ammonium sulfate precipitation, dialyzed against 10 mM NaPB (pH 7.5), applied to a

Bio-Scale CHT10-I column (bed vol., 10 mL; BioRad Laboratories, Hercules, CA, USA), and

then eluted with a linear gradient of 10–500 mM potassium phosphate buffer (KPB) (15). To

select the GtfB fractions, we performed a glucan synthesis assay (15), an enzyme-linked

immunosorbent assay (ELISA) using anti-CA-Gtf antibody, and a western blot using

anti-Gtf-I (GtfB) and anti-Gtf-SI (GtfC) monoclonal antibodies (47).

Glucan synthesis assay. Glucan-synthesizing activity was determined using

[^{14}C-glucose]-sucrose as described previously (15). In brief, reaction mixtures composed of

Gtf and 10 mM [^{14}C-glucose] sucrose (11.47 GBq/mmol) in 20 μL of 50 mM KPB (pH 6.0)

were incubated for 1 h at 37 °C, spotted on a filter paper (1.0 × 1.5 cm), and air-dried. The

filters were washed with methanol to remove non-incorporated [^{14}C-glucose]-sucrose and

then immersed in scintillation fluid to estimate the amount of *de novo*-synthesized

[^{14}C]-glucan.

Generation of anti-CA-Gtf antiserum. Antisera were prepared by repeatedly injecting

rabbits intramuscularly with the emulsion made by mixing Gtf-active fractions that were

purified using a DEAE Sepharose FF column and Freund's complete adjuvant (Difco) once,

followed by Freund's incomplete adjuvant (Difco) twice. The antibodies were purified from

rabbit antiserum by 3× precipitation with 33% ammonium sulfate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western

blotting. Conventional SDS-PAGE and western blot analyses were carried out as described

previously (12). In brief, Gtf samples and *E. coli* cells carrying the recombinant plasmid were

suspended in SDS gel-loading buffer and boiled for 5 min. Proteins separated by SDS-PAGE

were transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon; Millipore,

Billerica, MA, USA). After blocking with 5% bovine serum albumin (BSA), the membrane

was reacted with a primary antibody at 37 °C for 1 h, and the bound antibody was

subsequently detected using a solid-phase immunoassay employing goat anti-rabbit IgG

antibody conjugated with alkaline phosphatase (AP), or goat anti-mouse IgG antibody

conjugated with horseradish peroxidase (HRP).

When NuPAGE® 4–12% Bis-Tris gels (Invitrogen by Life Technologies, Carlsbad, CA,

USA) were used, the samples were suspended in NuPAGE® LDS sample buffer and

NuPAGE® Sample Reducing Agent (Invitrogen) and incubated at 70 °C for 10 min.

Coomassie brilliant blue (CBB) staining of the gels was performed using SimplyBlue

SafeStain™ (Invitrogen). For immunoblotting, the proteins were transferred onto 0.2-μm

PVDF membranes (Invitrogen) for 30 min at a constant voltage of 200 V. After blocking with 5% nonfat dry milk and 0.2% Tween 20 in Tris borate saline (TBS) at 4 °C overnight, the membranes were incubated with primary antibody in TBS containing 1% BSA at room temperature for 1 h. The membranes were washed 5 times with TBS containing 0.2% Tween and then incubated with secondary antibodies conjugated with HRP at a dilution of 1:3000 in TBS with 1% BSA at room temperature for 1 h. The membranes were then washed 5 times with TBS, and the signals were detected using a conventional solid-phase immunoassay.

ELISA. The ELISA was used to measure the amounts of GtfB or titers of antibody. The GtfB samples were applied to a 96-well plate, incubated at 37 °C for 1 h, and washed 5 times with phosphate buffered saline (PBS) containing 0.2% Tween 20 (PBST). The primary antibody at a dilution of 1:1000 in PBST was applied onto each well, and the plates were incubated at 37 °C for 1 h and then washed 5 times. The appropriate secondary antibody conjugated with AP at a dilution of 1:1000 was applied to each well and allowed to react at 37 °C for 1 h, and the plates were then washed 5 times. Subsequently, the GtfB amount or antibody titer was assessed using an Alkaline Phosphatase Substrate Kit according to the manufacturer's instructions (BioRad).

Protein assay. The amounts of GtfB or protein in antiserum were estimated using a Pierce[®]

BCA Protein Assay Kit according to the supplier's manual (Takara Bio Inc., Shiga, Japan).

Polymerase chain reaction (PCR). All PCR analyses in this study were performed using

TaKaRa EX Taq[®] Hot Start version (Takara) according to the manufacturer's instructions as

described previously (18). In brief, PCR was performed using 30 cycles of a denaturing step

at 98 °C for 10 s and a primer-annealing and extension step at 60 °C for 30 s or 1 min

according to the amplicon size.

Prediction of antigenic regions of GtfB *in silico***.** The antigenic regions of GtfB were

predicted using Kolaskar's method (21) (available at http://imed.med.ucm.es/Tools/

antigenic.pl) or Parker's method (33) and Welling's method (49) using ANTHEPROT 2000

version 6.0 software (7) (http://antheprot-pbil.ibcp.fr/index.php).

Preparation of recombinant CAT and GBD and generation of anti-CAT and anti-GBD

antisera. Based on the result of Kolaskar's method, PCR primers to amplify DNA fragments

encoding CAT and GBD were designed (Table 1). The pSK6 plasmid that harbors the *gtfB*

gene from *S. mutan*s MT8148 (13) was used as a PCR template. Each amplified fragment was

digested using the restriction enzymes that were added to the PCR primers and cloned into

pGEX-6P-1 (GE Healthcare). The recombinant CAT and GBD were expressed as glutathione

S-transferase (GST) fusion proteins. These recombinant proteins were digested by passing

through PreScission Protease (GE Healthcare), and their GST regions were removed using a

GSTrap FF column (GE Healthcare). Recombinant proteins and the adjuvant were used to

immunize the rabbits, and anti-CAT and anti-GBD antisera were obtained as described above.

Construction of DNA vaccine plasmid and variable region (VR) adenovirus. The VR

region containing the antigenic regions predicted by Parker's method was amplified by PCR

using the pSK6 plasmid as a template and the primers shown in Table 1. The amplicon

digested by *Kpn*I and *Pst*I was cloned into pSecTag2B (Invitrogen), and pSecTag2B-VRGB

was constructed as the DNA vaccine plasmid for VR (Fig. 1 A). The expression cassette was

cleaved by *Nru*I and *Sph*I from pSecTag2B-VRGB, blunted, and used to construct the

recombined adenovirus according to a previous report (44). In brief, the blunted expression

cassette was ligated with *Swa*I-digested pALC3 vector and introduced into *E. coli* DH10B

using an *in vitro* λ phage packaging kit (Nippon Gene). The structure of the resulting cosmid,

pALC3-VRGB, is illustrated in Figure 1B. To generate infectious recombinant adenoviral

vectors, the pALC3-VRGB cosmid and pOG44 plasmid (Invitrogen) for the expression of

FLP recombinase were cotransfected into HEK 293 cells (293 cell) seeded on a gelatin-coated

well using Lipofectamine (GIBCO-BRL) according to the supplier's instructions, and the

recombinant adenovirus AdV-VRGB was harvested after 15 days from wells in which

bacteriolysis complement was observed (Figs. 1C and D). The titer of the harvested

adenoviral suspension was measured using an Adeno-X^{TM} Rapid Titer Kit (Takara).

Detecting the expression of AdV-VRGB-mediated gene and protein in 293 cells. Total

RNA was extracted using TRIZOL$^®$ reagent (Invitrogen) from the 293 cells 3 days after they

were infected with AdV-VRGB, and reverse transcription was performed using SuperScript

III (Invitrogen) and Oligo (dT)$_{20}$ primer (Invitrogen). To detect the mRNA of

AdV-VRGB-mediated genes, we used primers to amplify the VR region (Table 1) and β-actin

primers (Invitrogen) as an internal control. In addition, total protein was extracted from the

293 cells 3 days after AdV-VRGB infection and was assessed by western blotting and

enhanced chemiluminescence.

Immunization with DNA vaccine for VR and generation of anti-VR antiserum. The

DNA vaccine for VR was used to immunize 6 week old female mice (BALB/c AnNcrlCrlj;

Charles River Laboratory Japan, Inc.) 4 times every night using retrograde intra-common

ductal injections (43). The mice were injected with AdV-VRGB suspension (1×10^9 pfu in

200 μL of lactated Ringer's solution) the first time according to a previous report (43), and

with 50 μg of pSecTag2B-VRGB plasmid in lactated Ringer's solution the other 3 times. The

antiserum titer was measured by ELISA using 10 ng of the purified GtfB as an antigen, and

serum was harvested from ascites 2 weeks after the last immunization.

Inhibition assay of GtfB activity by anti-VR antiserum. The effect of anti-VR antiserum

on the GtfB was analyzed according to a previous method (11). A solution of purified GtfB

(conc., 1.5 μg/μL) was incubated with an equal volume of anti-VR antiserum, anti-CA-Gtf

antiserum, or sham serum undiluted or diluted 1:3 in PBS. The reaction mixtures of GtfB and

serum were then incubated with [^{14}C-glucose]-sucrose at 37 °C for 1 h, and the amount of

[^{14}C]-glucan was measured as described above. Triplicate samples were analyzed in this

experiment. Significant differences were determined using Student's *t*-test ($p < 0.05$).

RESULTS

   Purification of GtfB. The cell-associated GtfB and GtfC enzymes were extracted from *S.*

mutans MT8148 cells using 8 M urea, and were purified by DEAE-anion exchange

chromatography and the following hydroxyapatite column chromatography (Fig. 2A and B).

In DEAE-anion exchange chromatography, Gtf activity was observed in fractions 6–17,

which were recovered as active fractions. During hydroxyapatite column chromatography,

GtfB and GtfC were eluted in fractions 29 and 24, respectively. SDS-PAGE of GtfB and GtfC

gave a single protein band with molecular masses of approximately 165 and 163 kDa,

respectively (Fig. 2C). Anti-CA-Gtf antiserum reacted with both GtfB and GtfC (Fig. 2D),

and anti-Gtf-I and anti-Gtf-SI antisera reacted with GtfB and GtfC, respectively (Figs. 2E and

F). The anti-Gtf-I antiserum cross-reacted slightly with GtfC. The purified GtfB was used in

further experiments.

   Predicting antigenicity using Kolaskar's method and the practical antigenicity of

CAT and GBD derived from GtfB. As shown in Figure 3, primary catalytic and

glucan-binding sites were included in the group of highly antigenic candidates. On the basis

of this prediction, we designed PCR primers for CAT, such that the primer could amplify the

region including primary and secondary catalytic sites. Likewise, the PCR primers for GBD

that could amplify the region including the 6 repeating units of GBD were designed. The

CAT and GBD region fragments amplified by these PCR primers were ligated into

pGEX-6P-1 to form pGEX-6P-1-CAT and pGEX-6P-1-GBD, respectively. The recombinant

CAT and GBD were expressed as GST fusion protein, and the GST regions were removed

from them by PreScission protease (Invitrogen). The recombinant CAT and GBD proteins

were then used to immunize rabbits from which anti-CAT and anti-GBD antisera were

obtained. The lysates from *E. coli* recombined with pGEX-6P-1, GEX-6P-1-CAT, and

pGEX-6P-1-GBD were electrophoresed by SDS-PAGE and observed as 26, 38, and 72 kDa

of GST fusion protein, respectively (Fig. 4A). In the western blot analyses used to evaluate

the antigenicity of CAT and GBD, it was observed that anti-CA-Gtf antibody did not react

with the CAT or GBD proteins (Fig. 4B) while anti-CAT and anti-GBD antisera reacted with

the corresponding recombinant proteins, and the native GtfB served as a positive control (Figs.

4C and D). In the inhibition assays of these antibodies (Fig. 5), anti-CAT and anti-GBD

antibody were less able to inhibit GtfB activity than was anti-CA-Gtf antibody, although there

was significant difference between the glucan synthesis inhibited by the anti-GBD antibody

and the sham serum. Thus, it was suggested that CAT and GBD regions were not

immunodominant compared with the other antigenic regions that reacted with anti-CA-Gtf

antibodies, although they were antigenic in rabbits.

Reevaluation of the antigenic region in GtfB using ANTHEPROT software. As it was suggested that CAT and GBD were non-immunodominant, we reevaluated the antigenic region of GtfB by the other *in silico* predictions identified by the Parker and Welling methods using the ANTHEPROT software (Fig. 6).

According to these analyses, the antigenicity of the primary catalytic site was predicted to be low by both methods as this region was highly hydrophobic, lowly hydrophilic, and lowly solvent-accessible. On the other hand, the GBD region was also predicted to have relatively low antigenicity by both methods, although the 6 periodic antigenic peaks corresponding to the 6 repeating structures were identified as antigenic epitopes in Parker's prediction.

When GtfB was roughly divided into 3 parts, VR, CAT, and GBD, Parker's prediction indicated that the N-terminal VR had more antigenic peaks and was more solvent-accessible than the other 2 parts. No report has evaluated the antigenicity of VR from GtfB. Thus, this region was adopted in the following experiments.

Expression of AdV-VRGB-mediated gene and protein in 293 cells. In the RT-PCR analysis used to confirm AdV-VRGB-mediated mRNA expression, appropriately 1.1-kbp fragment was amplified in the sample from the Adv-VRGB-infected 293 cells (Fig. 7A).

Western blot analysis was then used to confirm that the protein derived from AdV-VRGB was produced; an appropriate protein band with a molecular mass of approximately 46 kDa, including myc epitope and histidine tag derived from pSecTag2B was observed in the AdV-VRGB-infected 293 cells (Fig. 7C).

Immunization of mice with AdV-VRGB and pSecTag2B-VRGB. To induce the development of antibodies against VR, AdV-VRGB and pSecTag2B-VRGB were injected at the back of the hind legs of mice once and thrice, respectively. The reason why AdV-VRGB was not used for all 4 immunizations was to prevent the mice from dying due to the injection of adenovirus. To measure the titer of anti-VR IgG, ELISA using the purified GtfB as the antigen and goat anti-mouse IgG antibody as the secondary antibody was performed. It was observed that the titer of anti-VR IgG increased in 2 of 5 mice (Fig. 8). Thus, it was revealed that immunization with AdV-VRGB and pSecTag2B-VRGB could induce the development of antibody against VR**.** The antiserum against VR recovered from these 2 mice was pooled and used in further experiments.

Evaluation of the antiserum induced by DNA vaccine against VR. Reactivity of the antiserum induced by DNA vaccines against VR was evaluated by western blot analysis using crude CA-Gtf extracted from *S. mutans* cells using urea as an antigen (Fig. 9). The results

showed that the antiserum induced by DNA vaccines against VR reacted with natural GtfB.

Thus, it was suggested that the VR of GtfB is the antigenic region and is more reactive than

CAT and GBD.

Inhibition of GtfB activity by anti-VR antiserum. We carried out an inhibition assay to

prove that the anti-VR antiserum from mice contains antibodies reacted with a native protein

such as anti-CA-Gtf, and to evaluate the effect of anti-VR antiserum on the glucan synthesis

of GtfB (Fig. 10). In this experiment, the protein concentration of each non-diluted anti-VR

antiserum, anti-CA-Gtf antiserum, and sham serum was 60 µg/µL. When the ELISA using

equal volumes of GtfB (conc., 1.5 µg/µL) was performed, the absorbance at 405 nm of the

non-diluted anti-VR antiserum, anti-CA-Gtf antiserum, and sham serum was 1.2, 1.6, and 0,

respectively. These results revealed that the anti-VR antiserum significantly inhibited GtfB

activity compared with the sham serum. The anti-VR antiserum inhibited GtfB activity almost

to the same degree, as did the anti-CA-GtfB antiserum when 60 µg protein of the antiserum

was used. Thus, these results suggested that the anti-VR antiserum induced by AdV-VRGB

and pSecTag2B-VRGB effectively inhibited GtfB activity.

DISCUSSION

In the present study, we reevaluated the effectiveness of the CAT and GBD as epitopes in

vaccine production and also found that the ca. 360-amino-acid VR, which exists at the

N-terminus of GtfB, is a more reactive vaccine target.

The large Gtf molecules hamper complete functional domain mapping. However, studies

comparing the amino acid sequences of Gtf from various oral streptococci (Figure 6) have

revealed that Gtf consists of 4 regions: an ca. 40-amino-acid conserved signal sequence; an ca.

360-amino-acid VR with unknown function that is species-specific and not conserved among

the other Gtfs; an ca. 500-amino-acid catalytic domain, which contains conserved amino acids

that are necessary for sucrose hydrolysis (14, 25, 27); and a series of 6 direct repeats that

function in glucan binding (1, 9, 28). The latter 2 functional domains were employed as

potential vaccine target regions in previous attempts to develop a component vaccine against

GtfB, owing to their association with enzyme function and the high degree of sequence

conservation among streptococci. However, the antigenicity of those 2 regions was confirmed

only by western blot analyses using antibodies induced by synthetic peptides corresponding to

the CAT or GBD regions derived from GtfI of *S. downei* (40, 41). Thereafter, to make up for

antigenicity, the diepitopic antigen consisting of both the CAT and GBD (45), the chimera of

CAT and the B subunit of cholera toxin (22), and the chimera of GBD and the saliva-binding

region of Ag I/II (50) were applied. On the basis of these successful reports, we also tried to

develop a DNA vaccine against CAT and GBD, but did not obtain the expected results. One

result was that the recombinant CAT and GBD did not react with the antiserum induced by

native Gtf from *S. mutans* (Fig. 4), although these recombinant proteins did induce antibodies

that reacted with native GtfB and the corresponding recombinant proteins. In addition, the

DNA vaccine plasmid for CAT and GBD using pcDNA3 did not mediate sufficient amounts

of the recombinant proteins to induce specific antibodies, although expression of the objective

mRNA was confirmed by RT-PCR in an *in vitro* experiment (data not shown). Thus, we

began to doubt the antigenicity of these regions as a way to express antigenic protein and

decided to focus on alternative domains.

Secondary structure predictions suggest that the Gtfs are members of the α-amylase

superfamily and contain a circularly permuted $(\alpha/\beta)_8$-barrel motif (8, 19, 26). Our results in

Figure 6 revealed that the primary CAT site (FDSIRVDAVDN) of GtfB was highly

hydrophobic and its solvent accessibility was very low. Since these observations suggested

that the primary CAT site would exist on the inside of the $(\alpha/\beta)_8$-barrel structure, it was

thought that this region may be difficult to recognize as an antigen for the immune system,

although low-molecular-weight sucrose is able to pass through this structure as an enzyme

substrate. Thus, it was suggested that the antigenicity of CAT would be low as shown by

Parker's prediction.

In our previous study of glucosyltransferase from *S. oralis* (GtfR; 11), the incomplete

recombinant GtfR without GBD reacted with the antiserum induced by native GTFR from *S.*

oralis. An antiserum preliminarily prepared by immunization with the recombinant GtfR

without GBD not only reacted with native GtfR in western blot analysis but also inhibited

glucan synthesis by GtfR (data not shown). On the other hand, GbpA and GBD are

homologous, and it was reported that immunization with the entire GbpA protein did not elicit

a protective immune response (2, 36). Taken together, these observations indirectly suggested

that GBD antigenicity would be lower than that of the other Gtf regions. Practically, our

results suggested that GBD would not be immunodominant because few antigenic peaks were

seen compared with the other GtfB regions according to analysis using Parker's method

(Figure 6).

In this study, we reevaluated the antigenic GtfB region and tried to find a more potent

antigenic component. Results of the *in silico* analysis using Parker's method suggested that

the VR derived from GtfB was immunodominant, as evidenced by its many antigenic peaks

compared with those of CAT and GBD (Fig. 6). As it was reported that the VR is specific to

the Gtf of individual *Streptococcus* species (17, 18), our DNA vaccines would induce

GtfB-specific antibodies that would have an effect only on *S. mutans* glucan synthesis and

colonization, and would not interfere with Gtf-expressing members of the oral commensal

microbiota.

It was recently reported that non-immunodominant regions are effective as building blocks

in a streptococcal fusion protein vaccine (42). Although CAT and GBD were suggested to be

non-immunodominant in this study, they nevertheless may be attractive vaccine targets. Thus,

fusion protein of VR and CAT or GBD may induce more effective antibodies to inhibit

glucan synthesis of GtfB.

We adopted the adenovirus vector that possesses an intensive promoter and a high

infectious ability as a way to deliver recombinant antigen protein sufficient for mucosal

immunization as well. Since it was demonstrated that AdV-VRGB expressed sufficient

recombinant antigen protein to induce the development of antibodies that could inhibit GtfB

activity in murine *in vivo* experiments, use of AdV-VRGB may also be effective in mucosal

route immunization. As a natural consequence, AdV-VRGB ought to be applied to oral

experiments in animals to test whether dental caries can be prevented. We realize that there is

a safety problem when it comes to application of this DNA vaccine virus. Even if it were safe,

its use would not be permitted in the human oral cavity for dental caries prevention. Because

of safety issues, we hesitated to use only DNA vaccine virus in all immunization steps;

instead, we used DNA vaccine plasmid in the last 3 immunizations to reduce stress in the

mice. We plan to use AdV-VRGB to make a hybridoma that produces anti-VR antibody to

clone the gene encoding anti-VR antibody by phage display (35), and to transform this gene

into a rice plant using *Agrobacterium*-mediated transformation (31, 32). Rice harvested in this

planned study may be evaluated for passive immunization to prevent dental caries.

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FIGURE LEGENDS

FIG. 1. Construction of DNA vaccine plasmid and adenovirus. The gene coding the variable

region of *gtfB* was cloned into pSecTag2B and pSecTag2B-VRGB was constructed as a DNA

vaccine plasmid for VRGB (A). The expression cassette of VRGB elicited from

pSecTag2B-VRGB and pALC3 cosmid vector was ligated, packaged in λ phage, and

introduced into *Escherichia coli*. The cosmid obtained was pALC3-VRGB (B).

pALC3-VRGB and pOG44, FLP recombinase expression plasmid, were cotransfected into

293 cells and the infectious recombined adenovirus AdV-VRGB was harvested (C).

Non-cotransfected 293 cells (NC) and cotransfected 293 cells 8 and 15 days after

pALC3-VRGB and pOG44 cotransfection are shown (D).

FIG. 2. Glucosyltransferase B (GtfB) purification from *Streptococcus mutans* MT8148. The 8

M urea extraction from *S. mutans* cells was precipitated with 60% saturated ammonium

sulfate and was applied onto a DEAE Sepharose FF column. Fractions 6–17 were collected as

Gtf-active fractions (A). Gtf-active fractions were concentrated with ammonium sulfate

precipitation and applied to a Bio-Scale CHT10-I column. The purified GtfB and GtfC were

eluted in fractions 29 and 24, respectively (B). Samples at each purification step were

separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (C) and assessed

with western blotting analyses using anti-CA-Gtf (D), anti-Gtf-I (E), and anti-Gtf-SI (F)

antisera. Lane M, molecular weight marker; 1, 8 M urea extraction; 2, precipitant of 1 by

ammonium sulfate; 3, Gtf-active fraction eluted using a DEAE Sepharose column; 4, fraction

29 eluted using a CHT10-I column; and 5, fraction 24 eluted using a CHT10-I column.

FIG. 3. Glucosyltransferase B (GtfB) antigenicity prediction using Kolaskar's method.

Average antigenic propensity of the deduced GtfB amino acid sequence (1475 amino acid

residues) was estimated (A), and the antigenic region sequences using Kolaskar's methods are

listed (B). The asterisks and brackets indicate the primary GtfB catalytic site and

glucan-binding domain region, respectively.

FIG. 4. Antigenicity evaluation of the recombinant catalytic region (CAT) and the

glucan-binding domain (GBD). The recombinant CAT and GBD proteins were separated by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A), and their antigenicity was

evaluated by western blotting analyses using anti-CA-Gtf (B), anti-CAT (C), and anti-GBD

(D) antisera. Lane M, molecular weight marker; 1, *Escherichia coli* lysate from

pGEX-6P-1-CAT; 2, *E. coli* lysate from pGEX-6P-1-GBD; 3, recombinant glutathione

S-transferase protein; and 4, Gtf-active fraction eluted using a DEAE Sepharose column.

FIG. 5. Effect of anti-catalytic region (CAT) and anti-glucan binding domain (GBD) antisera

on glucosyltransferase B (GtfB) activity. Purified GtfB (1.5 µg protein) reacted with 0, 20, or

60 µg of protein of anti-CAT, anti-GBD, or anti-CA-Gtf antisera or sham serum at 37 °C for 1

h was incubated with [^{14}C-glucose]-sucrose at 37 °C for 1 h, and the amount of [^{14}C]-glucan

was measured. GtfB activity in non-serum was defined as 100%. Triplicate samples were

analyzed in this experiment, and the asterisks indicate statistical significance by Student's

t-test (*, $p < 0.01$).

FIG. 6. Glucosyltransferase B (GtfB) antigenicity prediction using ANTHEPROT software.

The deduced GtfB amino acid sequence was evaluated for antigenicity using Parker's method

(A), hydrophobicity (B), Welling's method (C), hydrophilicity (D), helical membranous

regions (E), and solvent accessibility (F).

FIG. 7. Adv-VRGB-mediated mRNA and recombinant protein expression. Total RNA from AdV-VRGB-infected 293 cells was analyzed by reverse transcription polymerase chain reaction using the variable region (VR) primers shown in TABLE 1 (A) and β-actin primer (B). Objective recombinant protein expression by the Adv-VRGB-infected 293 cells was assessed using western blotting analysis using anti-CA-Gtf antiserum (C). Lane M, molecular weight or DNA size marker; B, purified GtfB in Figure 2; 1, sample of 293 cells; 2, sample of 293 cells transfected with pSecTag2B; 3, sample of 293 cells transfected with pSecTag2B-VRGB; 4, sample of 293 cells infected with non-combined adenovirus; and 5, sample of 293 cells infected with AdV-VRGB. The arrowheads indicate the band that reacted with anti-CA-Gtf antiserum.

FIG. 8. Immunization of DNA vaccine for the variable region (VR). The DNA vaccine for VR, AdV-VRGB, and pSecTag2B-VRGB was intramuscularly injected into 5 mice according to the immunization schedule in the Materials and Methods section. Serum was obtained before 4 immunizations and every 2 weeks after the last immunization. The serum titer was evaluated by measuring the absorbance at 405 nm in an enzyme-linked immunosorbent assay using purified glucosyltransferase B as an antigen.

FIG. 9. DNA vaccine-induced antiserum reactivity against the variable region (VR). The

crude CA-Gtf that contained glucosyltransferase B (GtfB) was separated by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (A), and western blotting analyses with

anti-CA-Gtf antiserum (B) and DNA vaccine-induced VR antiserum (C). Lane M, molecular

weight marker and G, Gtf-active fraction eluted using a DEAE Sepharose column.

FIG. 10. Effect of anti-variable region (VR) antiserum on glucosyltransferase B (GtfB)

activity. Purified GtfB (1.5 μg protein) reacted with 0, 20, or 60 μg protein of anti-VR

antiserum, anti-CA-Gtf antiserum, or sham serum at 37 °C for 1 h was incubated with

$[^{14}C\text{-glucose}]$-sucrose at 37 °C for 1 h, and the amount of $[^{14}C]$-glucan was measured. The

GtfB activity in the non-serum was defined as 100%. Triplicate samples were analyzed in this

experiment, and the asterisks indicated statistical significance by Student's t-test (*, $p < 0.01$;

**, $p < 0.05$).

TABLE 1. The polymerase chain reaction primers used in this study.

Amplified region Primer name	Primer sequence	Restriction enzyme site
CAT (1223–1521)		
GtfB/CAT-F	5′-TTAG**GAATTC**CTTATGGCTAACGATGTGG-3′	*Eco*RI
GtfB/CAT-R	5′-CCG**CTCGAG**ATCATATTGTCGCCATCATC-3′	*Xho*I
GBD (3238–4431)		
GtfB/GBD-F	5′-GGTCGC**GGATCC**GTTTATTATCAACGAGTGG-3′	*Bam*HI
GtfB/GBD-R	5′-AGCGG**GAATTC**CATTAGTTAATCCGAAC-3′	*Eco*RI
VR (115–1216)		
GtfB/VR-F	5′-GTT**GGTACC**GATTCTAATGAATCGAAATC-3′	*Kpn*I
GtfB/VR-R	5′-AA**CTGCAG**TTCGTAACCGCCGATAG-3′	*Pst*I

The numbers in parentheses indicate the amplified nucleic acid position from the 5′ start of *gtfB*. The bold characters in the primer sequences indicate the added restriction enzyme site. CAT, catalytic region; GtfB, glucosyltransferase B; GBD, glucan-binding domain; and VR, variable region.
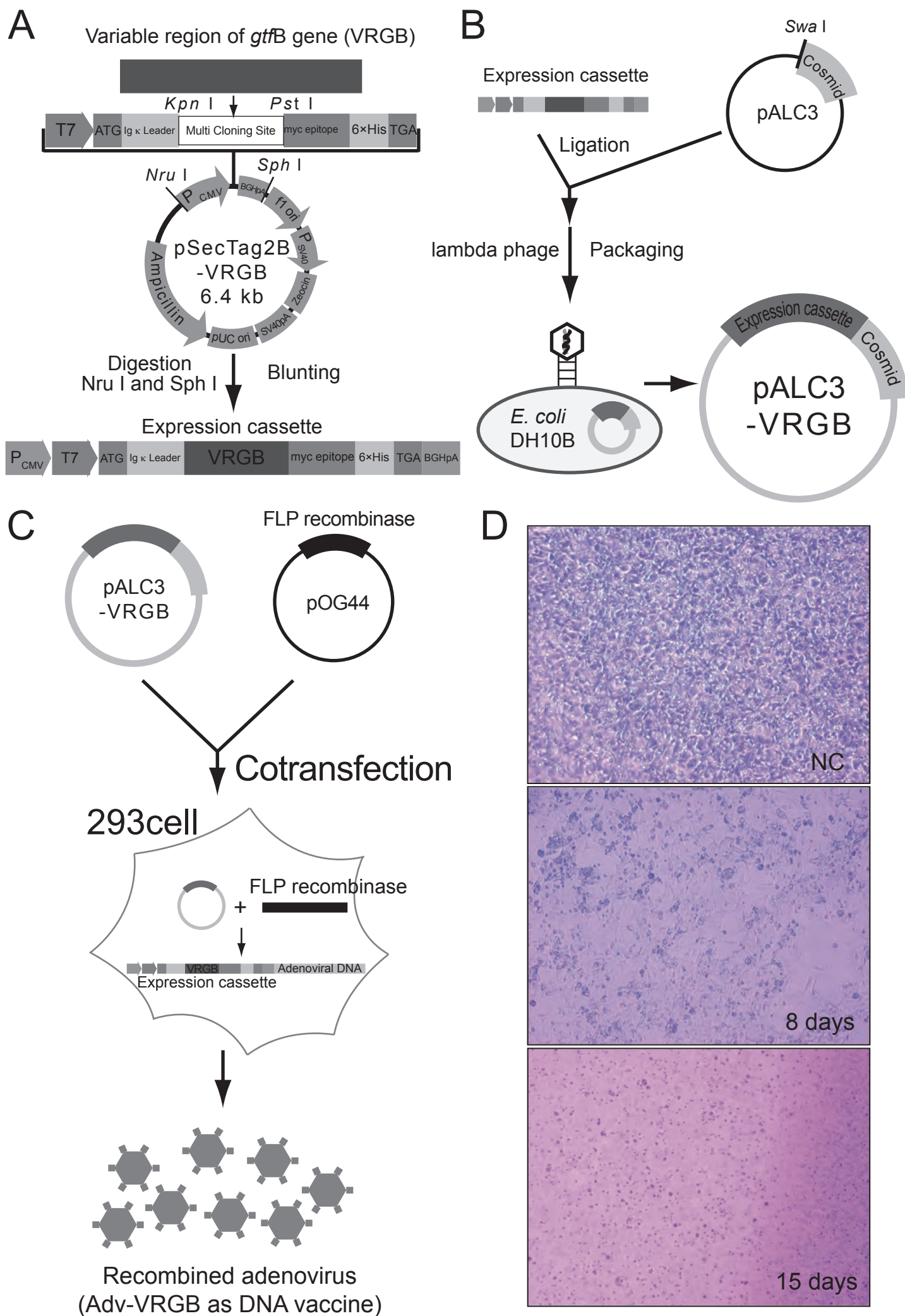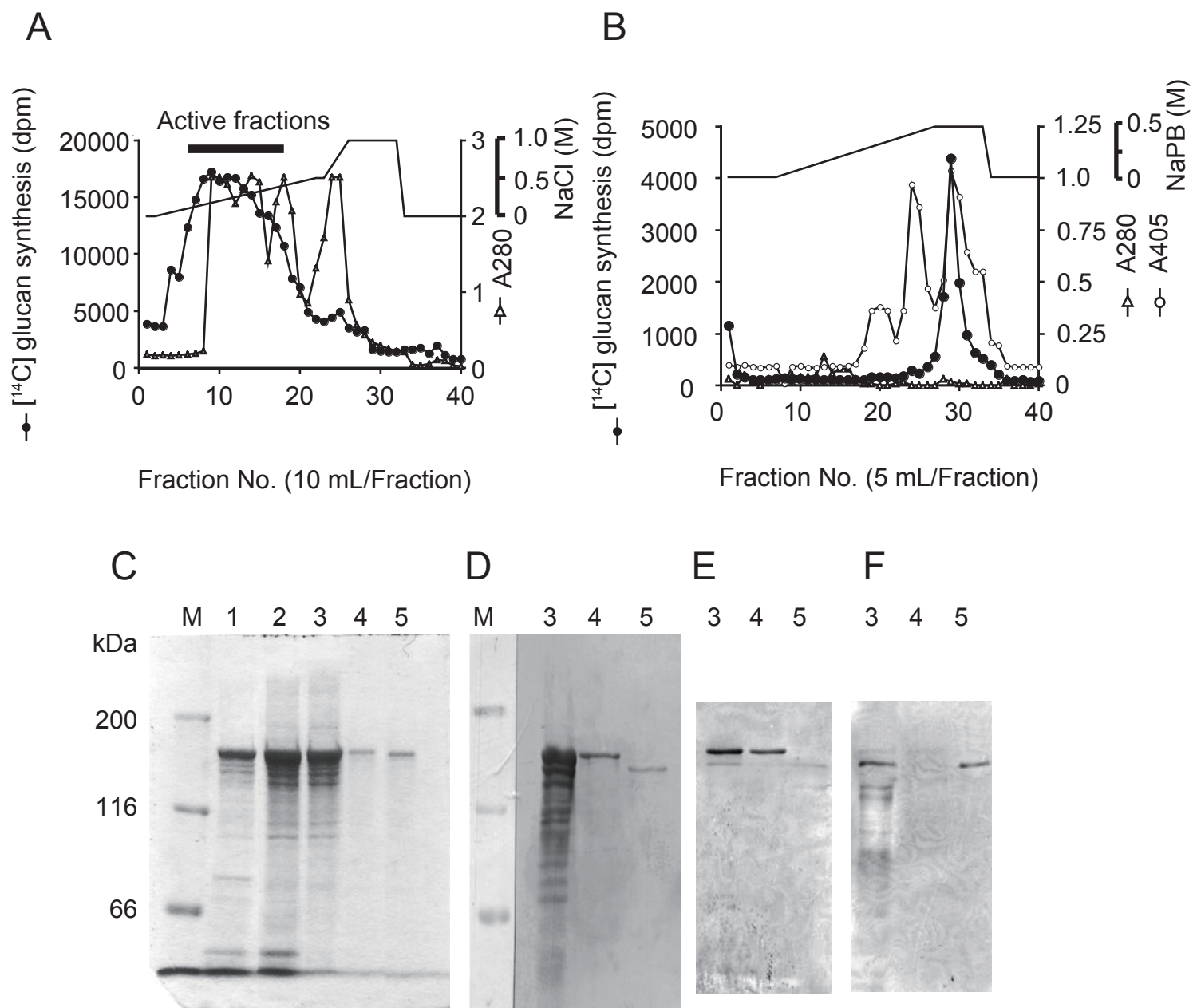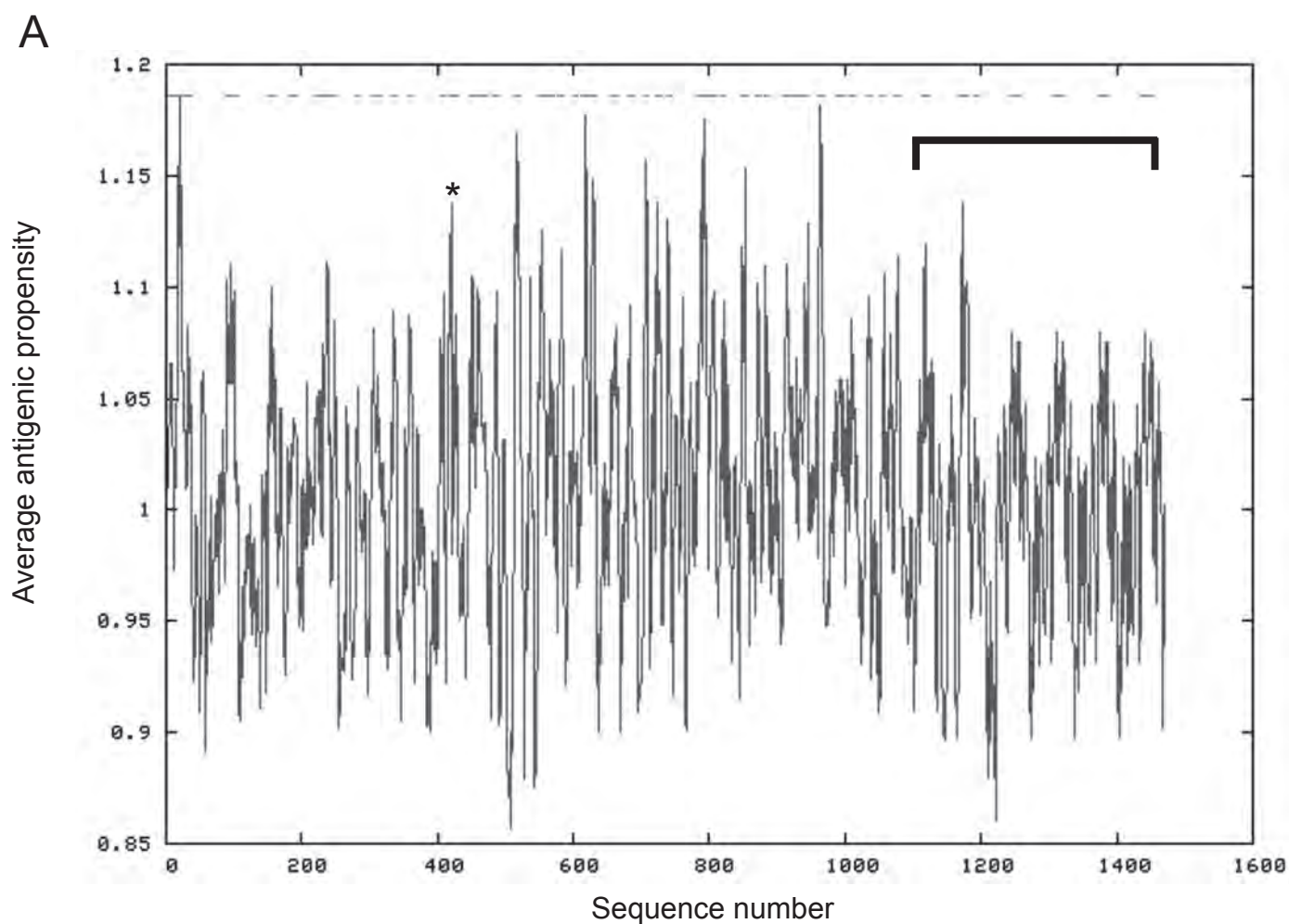
Figure 1. Hoshino et al.

Figure 2. Hoshino et al.

A

Average antigenic propensity

1.2
1.15
1.1
1.05
1
0.95
0.9
0.85

*

0   200   400   600   800   1000 1200 1400 1600

Sequence number

B

n	Start Position	Sequence	End Position
1	4	KVRYKLRK	11
2	13	KKRWVTVSVASAVMTLTTLSGGLVK	37
3	88	SSTSVVNPKEVVSNPYTVG	106
4	151	ANLQILEKLPNV	162
5	182	FTLIADGKILH	192
6	220	KKYNQVYDR	228
7	231	QSFEHVDHYLTA	242
8	246	YRPKYIL	252
9	280	QRQYVNY	286
10	302	NQLQLNIAAATIQAKI	317
11	331	RQTISAFVKT	340
12	357	QNGAVLY	363
13	403	GYEFLLA	409
14	414	NSNPVVQA	421
15	423	QLNWLHFL	430
16	446	DSIRVDAVDNVDADLLQIAGDYLKAAK	472
17	481	ANDHLSILE	489
18	513	LRLSLLYSLAK	523
19	548	ETAAVPSYSFIR	559
20	561	HDSEVQDLIRD	571
21	577	INPNVVGY	584
22	599	NKDLLAT	605
23	611	HYNTALSYALLLTNKSSVPRVYY	633
24	653	AIETLLKARIKYVSG	667

| n	Start Position	Sequence	End Position
25	681	IITSVRYGK	689
26	703	RTSGVVVI	710
27	721	ASDRVVVNM	729
28	737	AYRPLLL	743
29	782	ANPQVSGYLGVWVPVGA	798
30	800	ADQDVRVAAST	810
31	816	GKSVHQNA	823
32	847	YTNVVIAKNV	856
33	869	MAPQYVSS	876
34	881	FLDSVIQN	888
35	909	ADDLVKAIKALHSKGIKVMA	928
36	932	PDQMYALPEKEVVTA	946
37	960	QIKSVLYVAD	969
38	985	FLEELQAKYPELFAR	999
39	1007	PMDPSVKI	1014
40	1031	GAGYVLK	1037
41	1053	INFLPKT	1059
42	1073	GKGYVYYSTS	1082
43	1114	NGVNYYFLSNG	1124
44	1169	MSVGLTVIDGQVQC	1182
45	1190	AKGKFVT	1196
46	1242	NGQHLYFRANGVQVKG	1257
47	1307	NGQHLYFRANGVQVKG	1322
48	1372	NGQHLYFRANGVQVKG	1387
49	1437	NGQHLYFRANGVQVKG	1452

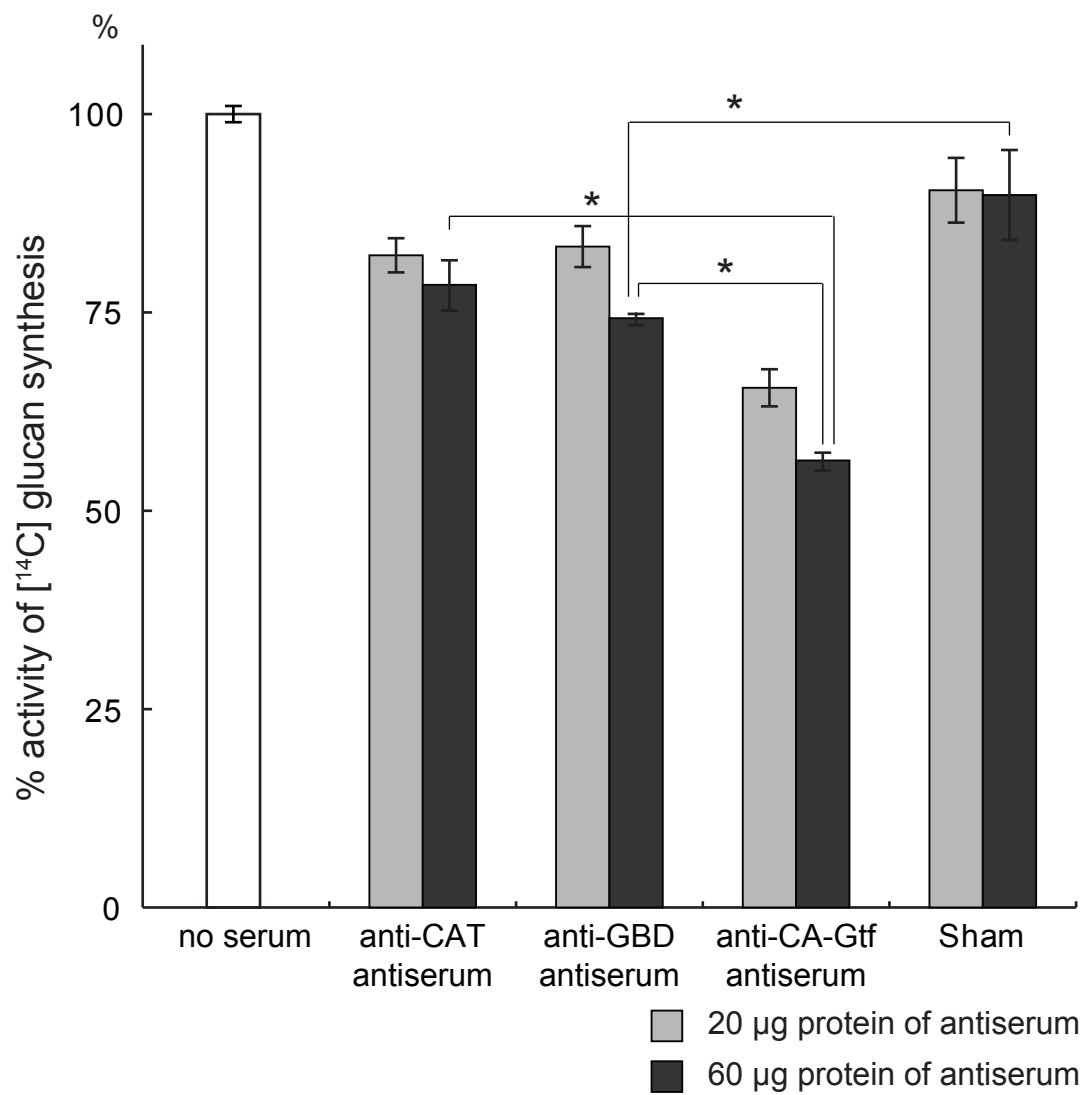Figure 3. Hoshino et al.
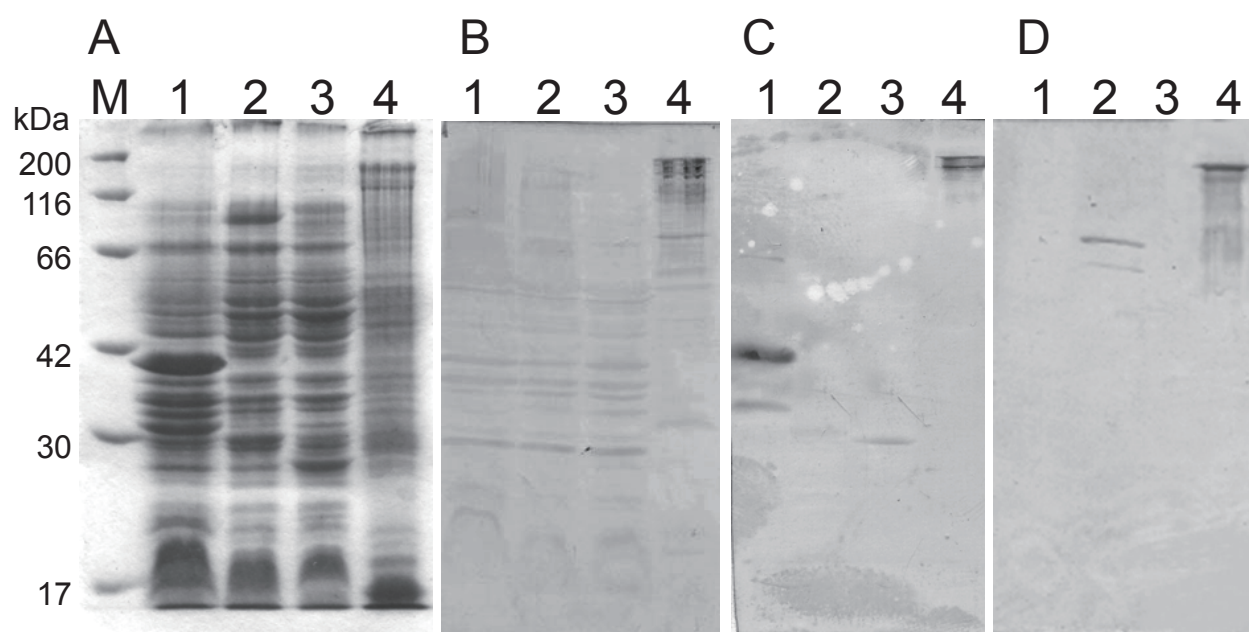
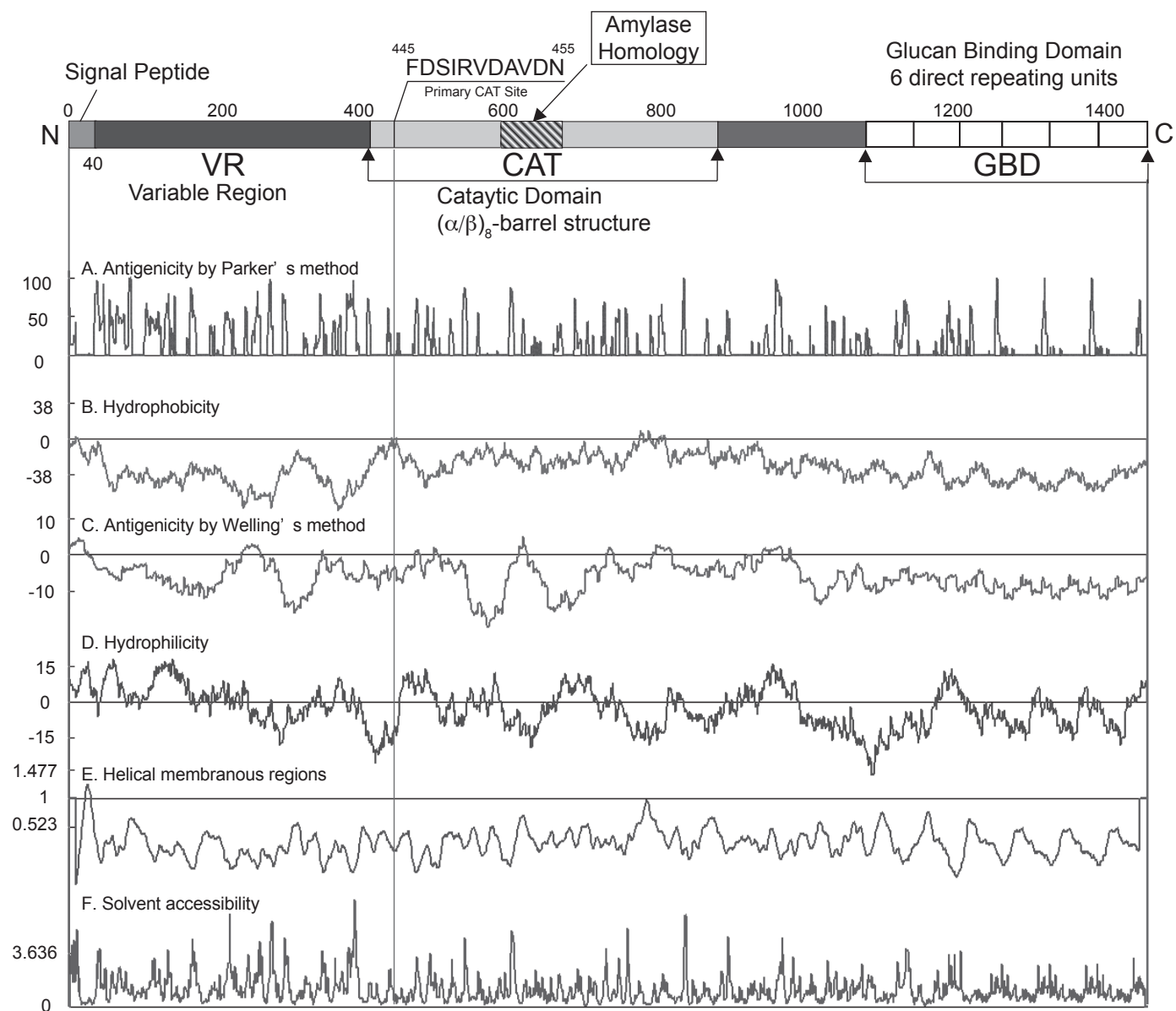Figure 4. Hoshino et al.

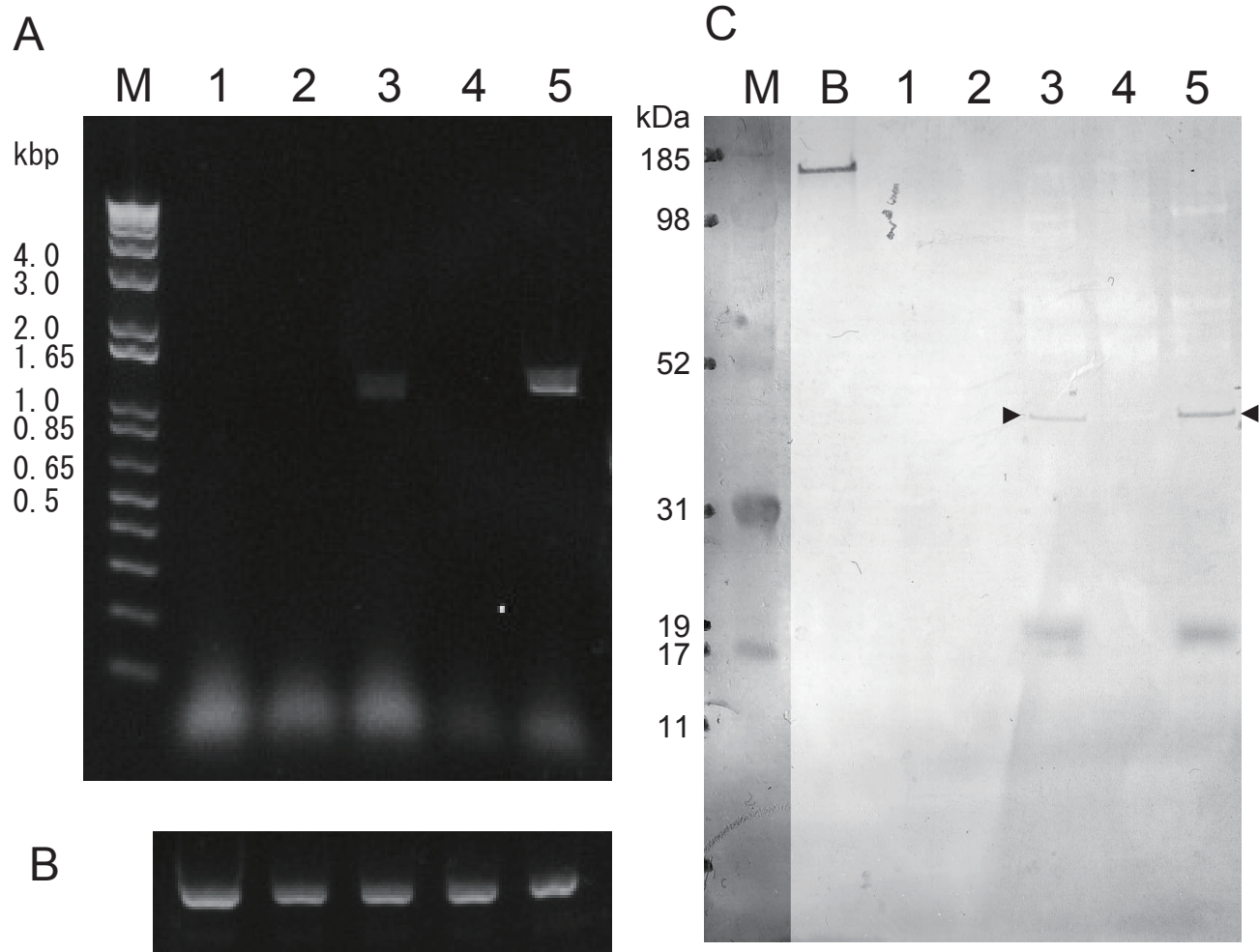Figure 5. Hoshino et al.

Figure 6. Hoshino et al.
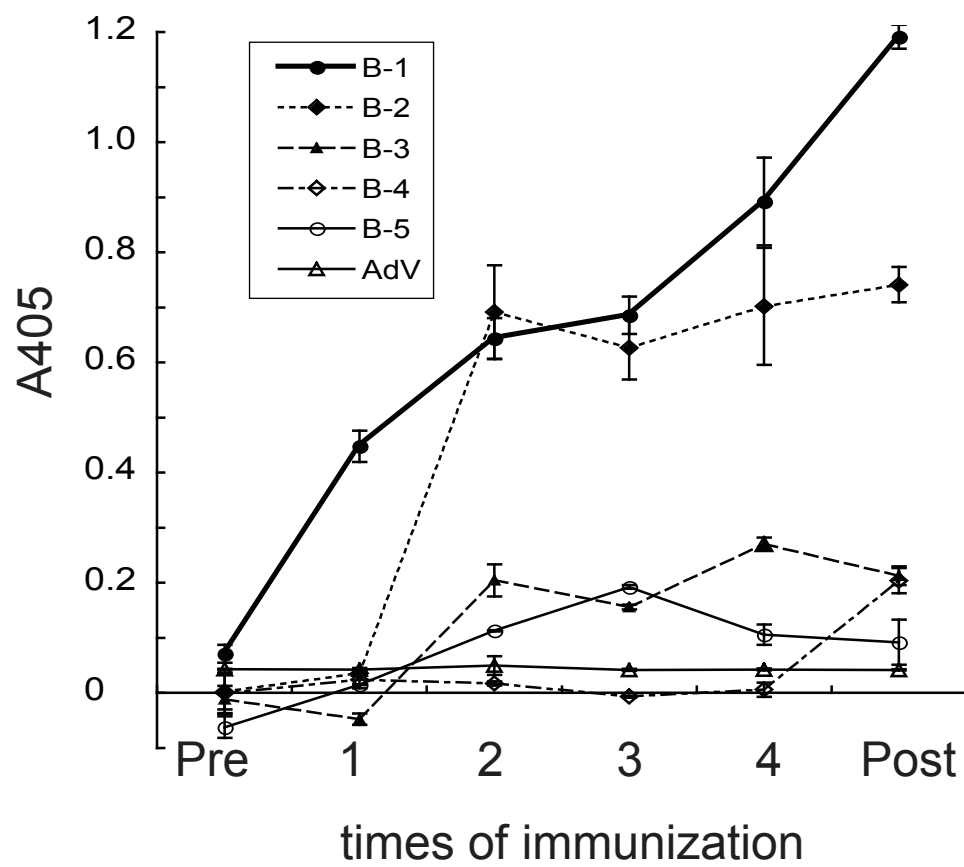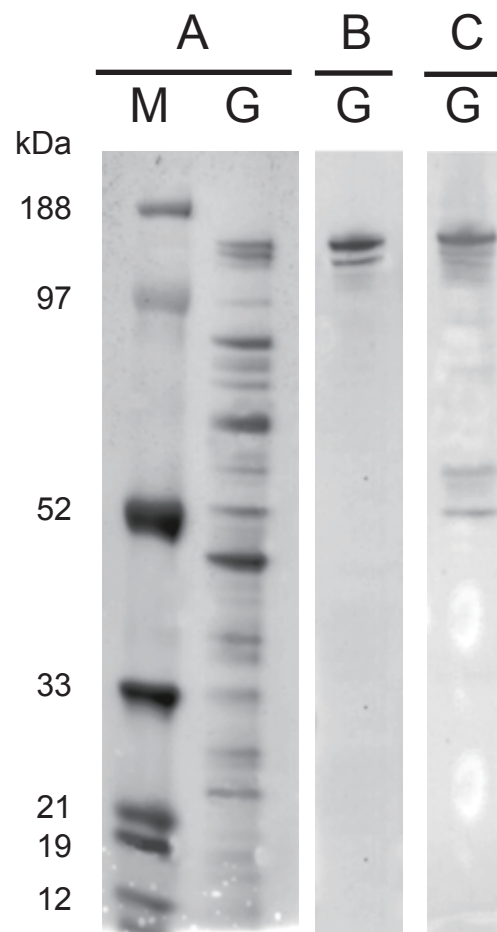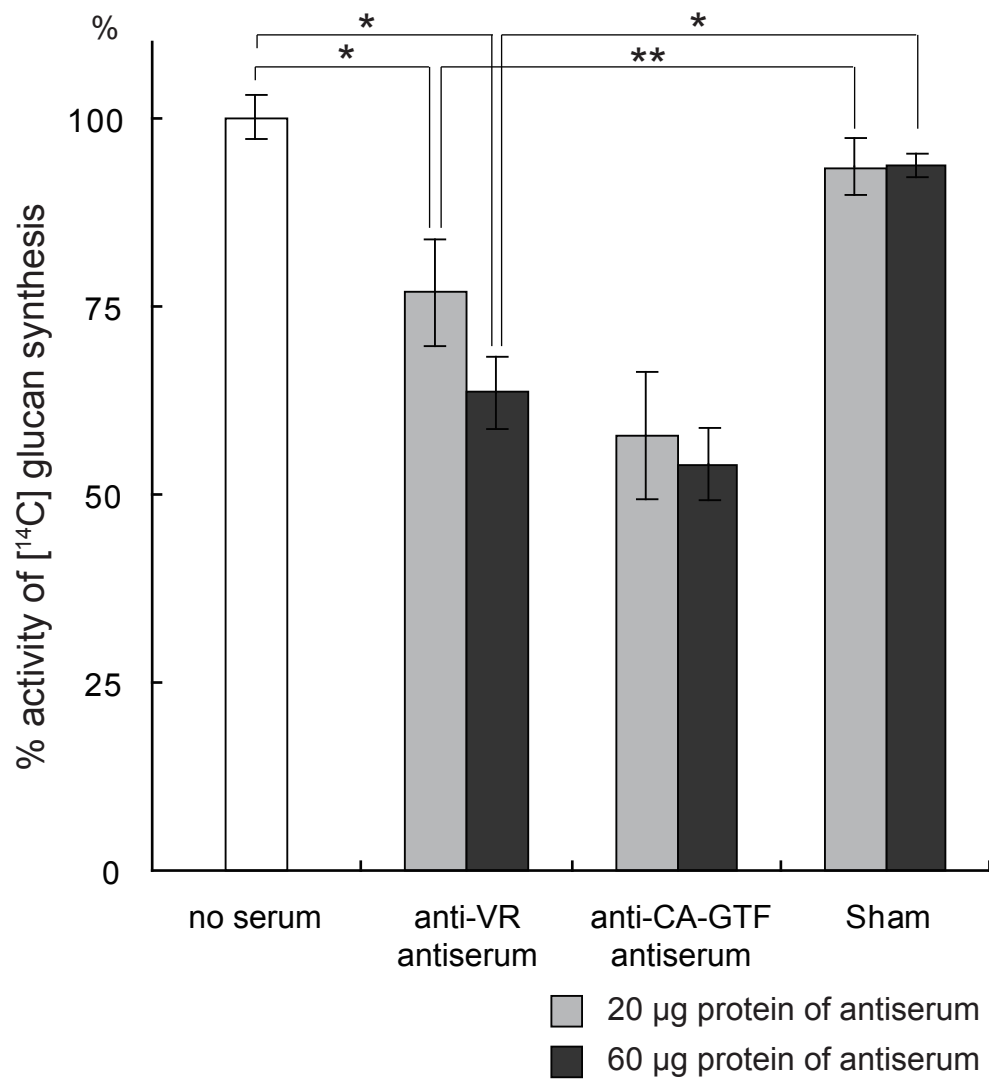
Figure 7. Hoshino et al.

Figure 8. Hoshino et al.

Figure 9. Hoshino et al.

Figure 10. Hoshino et al.