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Core Fucosylation of IgG B Cell Receptor Is Required for Antigen Recognition and Antibody Production

Wenzhe Li,* Rui Yu,* Biao Ma,* Yan Yang,* Xinyan Jiao,* Yang Liu,† Hongyu Cao,‡ Weijie Dong,* Linhua Liu,* Keli Ma,* Tomohiko Fukuda,§ Qingping Liu,‡ Tonghui Ma,* Zhongfu Wang,‡ Jianguo Gu,§ Jianing Zhang,§ and Naoyuki Taniguchi‖

Ag recognition and Ab production in B cells are major components of the humoral immune response. In the current study, we found that the core fucosylation catalyzed by α1,6-fucosyltransferase (Fut8) was required for the Ag recognition of BCR and the subsequent signal transduction. Moreover, compared with the 3-83 B cells, the coalasing of lipid rafts and Ag-BCR endocytosis were substantially reduced in Fut8-knockdown (3-83-KD) cells with p31 stimulation and then completely restored by reintroduction of the Fut8 gene to the 3-83-KD cells. Indeed, Fut8-null (Fut8−/−) mice evoked a low immune response following OVA immunization. Also, the frequency of IgG-producing cells was significantly reduced in the Fut8−/− spleen following OVA immunization. Our results clearly suggest an unexpected mode of BCR function, in which the core fucosylation of IgG-BCR mediates Ag recognition and, concomitantly, cell signal transduction via BCR and Ab production. The Journal of Immunology, 2015, 194: 000–000.

Core fucosyltransferase (Fut8) catalyzes the transfer of a fucose residue from GDP-fucose to the innermost N-acetylgalactosamine (GlcNAc) residue of N-glycans via α1,6-linkage (1) as shown in Fig. 1A. FUT8 is a typical type II membrane protein and a Golgi apparatus–resident glycosyltransferase. Core fucosylation catalyzed by FUT8 has an important role in various biological events (2). In our previous studies, we found that lack of core fucosylation in TGF-β1R and/or epidermal growth factor receptor results in a marked dysregulation of their activation that is due to a decreased ligand affinity for the receptor (1, 3, 4).

The recognition of Ag by the BCRs displayed on the surface of B cells triggers signals that ultimately lead to B cell activation (5). The IgG-BCR molecule consists of two Fab arms that are involved in Ag binding. These arms are connected via a flexible hinge region to an Fc region. The BCRs form a complex that contains a membrane Ig with a short cytoplasmic domain that is non-covalently associated with a disulfide-linked heterodimer of Igα–Igβ (CD79a–CD79b); in their cytoplasmic domains, these contain ITAMs that couple the BCR to the signaling apparatus (5). Upon Ag engagement, the ITAMs become phosphorylated by the Src family kinase Lyn and provide a binding site for the SH2 domain-containing kinase Syk, which triggers the signaling cascades (6, 7). Previous biochemical studies provided evidence that one of the earliest events that accompanies the phosphorylation of the BCR by Lyn is the association of the BCR with detergent-insoluble, sphingolipid- and cholesterol-rich membrane microdomains that were defined as lipid rafts (8, 9). Subsequently, the BCR rapidly delivers Ag to intracellular compartments where it is proteolytically cleaved, which results in clonal expansion and differentiation of Ag-specific B cells into plasma cells or memory B cells (5).

The human IgM molecule contains two N-glycans in C3g2 at Asn297 of Fc regions (10). For IgG1, the core oligosaccharide normally consists of Asn297GlcNAc (core fucose)-GlcNAc-mannose-(mannose-GlcNAc)2. Microheterogeneity of N-glycans on IgG includes the attachment of galactose and/or galactose–sialic acid at one or both of the terminal GlcNAc and/or attachment of a third GlcNAc arm. The existence of attached fucose, galactose, or sialic acid residues is known to contribute to the folding, stability, and function of IgG molecules (11, 12). Kaneko et al. (11) showed that, for IgG1, IgG2a, and IgG2b, differences in sialylation of the carbohydrates control inflammatory responses by mediating interactions with FcγRs expressed on innate immune effector cells. Radaev et al. (13) also demonstrated that the carbohydrate attached to the Asn297 of IgG has a significant impact on the receptor-Fc recognition. Multiple carbohydrate moieties were visible in the electron density extending from the Asn297 of both chains of Fc toward each other into the interchain region, and they could support the structural framework of the Fc regions. IgG is a highly core fucosylated glycoprotein, and the lack of core fucosylation of IgG could increase the binding affinity of Fc regions to FcγRs and enhance the Ab-dependent cell-mediated cytotoxicity that is mediated by NK cells (14). Also, Mori et al. (15) showed that the highly defucosylated (~60%) human IgG1 exhibited >100-fold higher Ab-dependent cell-mediated cytotoxicity compared with high fucosylated (~10% defucosylated) IgG1, without any change in Ag binding.
In our previous studies, the loss of Fut8 led to a decrease in the generation of pre-B cells with a low response to stromal cells in the bone marrow (16, 17). Given the important biological functions of Fut8, the function of the core fucosylation of IgG-BCR deserves a more detailed investigation. In the current study, we showed an unexpected mode of IgG-BCR function, in which core fucosylation mediates the Ag recognition of IgG-BCR and lipid raft association, thereby allowing cell signal transduction via BCR and Ab production. Our results suggested that core fucosylation could regulate the BCR-mediated humoral immune response.

Materials and Methods

Mice

Fut8+/− mice on the ICR background (18) were maintained in a room illuminated for 12 h (08:00 to 20:00) and kept at 24 ± 1°C with free access to food and water in the specific pathogen–free laboratory animal facility of Dalian Medical University. Homozygous wild-type (Fut8+/+) and knockout (Fut8−/−) mice were obtained by crossing heterozygous Fut8+/− mice. The total and differential blood leukocyte counts were analyzed by the Clinical Laboratory of Dalian Municipal Central Hospital.

Abs

FITC-labeled anti-IgM (II/41), PE-labeled anti-IgD (11-26), PE-Cy5-labeled anti-CD19 (MB19-1), anti-CD5 (Ly-1, 5-3,7,3), alkalinephosphycin-labeled anti-CD45 (RA3-6B2), and anti-CD11b (Mac-1, M1/70) were obtained from eBioscience; anti-phosphotyrosine Ab (PY20) and anti–caveolin-1 mAb (2234) were from BD Transduction Laboratories; a mouse anti-FUT8 mAb (15C6) was obtained from Fujirebio (Tokyo, Japan); an HRP-conjugated goat anti-mouse IgG and an HRP-conjugated goat anti-rabbit IgG were from ICN Biomedicals; Alexa Fluor 647 labeled goat anti-mouse IgG were from Sigma-Aldrich; anti-Syk and phospho-Syk Abs were purchased from Cell Signaling; and Alexa Fluor 488 labeled anti-CD45R (RA3-6B2), and anti-CD11b (Mac-1, M1/70) were obtained from BD Pharmingen.

Preparation of lipid rafts

Lipid rafts were isolated using modified lysis conditions and flotation on sucrose gradients with frosted slides and then passing them through nylon mesh. RBCs were lysed by incubation with 0.14 M NH4Cl and 2 mM Tris (pH 7.4) for 3 min at RT. The splenic lymphocytes (SPLs) were incubated with an anti-CD16/CD32 (2.4G2) mAb to block FcRs, and the cells were stained with 1:200 diluted FITC-labeled anti-CD16/CD32 (2.4G2) mAb to block FeRs and then stained on ice for 15 min with several combinations of mAbs.

Preparation of lipid rafts

Lipid rafts were isolated using modified lysis conditions and flotation on discontinuous sucrose gradients (6).

Oligomerization assay

The cells were washed with HBSS (20 mM HEPES [pH 7.5]) and incubated or not with 0.1 μg/ml bivalent p31 at 37°C. The medium was replaced rapidly with HBSS containing freshly prepared 5 mM bis(sulphosuccinimidyl) suberate (BS3, Pierce), and the incubation continued with gentle shaking for 30 min at 37°C. The reaction was quenched by the addition of 20 mM (final) Tris-HCl (pH 7.5). The cells were washed with PBS, the membrane proteins were labeled with sulfo-NHS-biotin, and the proteins (~1 mg) were immunoprecipitated with Protein G Sepharose (50% slurry) (Amersham Biosciences) overnight at 4°C. After washing three times in lysis buffer, the bound protein were released by boiling for 5 min in a sample buffer without 2-ME.

Flow cytometric analysis

Single-cell suspensions of spleens were prepared by gently grinding the tissues with frosted slides and then passing them through nylon mesh. RBCs were lysed by incubation with 0.14 M NH4Cl and 20 mM Tris (pH 7.4) for 3 min at RT. The splenic lymphocytes (SPLs) were incubated with an anti-CD16/CD32 (2.4G2) mAb to block FeRs and then stained on ice for 15 min with several combinations of mAbs.

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Analysis of BCR endocytosis by confocal microscopy

To analyze BCR endocytosis, B cells were incubated with 0.1 μg/ml biotinylated p31 on ice for 0, 5, 10, or 15 min to allow endocytosis of cell surface Ag–BCR complexes and then washed to remove unbound Ag. The cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100. Anti-CD16/CD32 (2.4G2) mAb was used to block FeRs, and the cells were stained with 1:200 diluted FITC-labeled.
streptavidin (BD Pharmingen, San Diego, CA) on ice for 30 min, washed, and mounted in anti-fade medium (PermaFluor Mountant Medium; Immunon, Pittsburgh, PA). The slides were examined under a confocal microscope at 200× magnification using the appropriate filter.

Fluorescence and confocal microscopy

For the triple-staining procedure, cells were fixed with 4% PFA for 10 min at RT and then incubated with goat anti-mouse IgG2a mAb for 30 min at RT. After washing, cells were incubated with FITC-conjugated donkey anti-goat IgG secondary mAb, followed by incubation with Alexa Fluor 594 CT-B (Invitrogen) for 30 min at RT. After several washes, the cells were incubated with mouse anti–caveolin-1 mAb and Alexa Fluor 647 goat anti-mouse IgG1 secondary mAb. The cells were examined by confocal microscopy using a Bio-Rad Radiance 2000 confocal system fitted on a Nikon microscope.

Electrospray ionization mass spectrometry

Mass spectrometry analysis was carried out using an LTQ-XL linear ion trap electrospray ionization mass spectrometer coupled with a HPLC system (Thermo Scientific, Bremen, Germany), as described previously (20).

Animal immunization

Mice (12 wk old) were immunized by i.p. injection with 200 μg OVA, mixed with an equal volume of CFA. Two weeks later, mice were immunized with 200 μg OVA by i.p. injection. Sera were collected at 0, 14, and 28 d postimmunization.

ELISA

Five micrograms of OVA was coated on 96-well plates and incubated with antisera diluted 1:100–1:204,800 at 0, 14, and 28 d postimmunization. After washing, the wells were incubated with HRP-conjugated goat anti-mouse IgG diluted 1:8,000. HRP activity on the immunoplate was detected using O-phenylenediamine and H2O2 as enzyme substrates. The absorbance was measured at 492 nm using a computer-interfaced microplate reader (Bio-Rad, Houston, TX).

ELISPOT assay

Production of OVA-specific Abs by SPLs was analyzed by ELISPOT assay. In brief, 96-well microplates were coated with 50 μg/50 μl OVA. After blocking with 3% skim milk, SPL suspensions from OVA-immunized mice were added to each well (5 × 10^5 cells) and incubated at 37˚C for 16 h in air with 5% CO2 at 95% humidity. After incubation, 1:5000 diluted HRP-labeled goat anti-mouse IgG was added to each well. The spots were developed with the AEC coloring system and analyzed by Dakewe Biotech (Shenzhen, China).

PCR array

Total RNA was extracted from cells with TRizol reagent (Invitrogen, Carlsbad, CA). A Mouse B Cell Activation PCR Array was used to simultaneously examine the mRNA levels of 96 genes, including 6 “housekeeping genes,” in 96-well plates, according to the protocol of the manufacturer (SA Biosciences). The mean number of cycles to the threshold of fluorescence detection was calculated for each sample as the difference in gene expression between 3-83 and 3-83-KD cells.

Real-time PCR

Total RNA was prepared from sorted cells using TRIzol reagent (Invitrogen) following the protocol recommended by the manufacturer. Real-time PCR analyses were performed using a SmartCycler II System (Cepheid, Sunnyvale, CA). The thermal cycling conditions for real-time PCR were 10 s at 95˚C to active SYBR Ex Taq, followed by 40 cycles of denaturation for 5 s at 95˚C and annealing/extension for 20 s at 60˚C. The primer pairs used for PCR were as follows: 5’-AAC AGT TGA AGG CCA AAG-3’ and 5’-GCC TGT CTT TGA AGT TCA TTT C-3’ for Fut8 (NM_016893); 5’-GCA AGA GCA ACA GCA TGA GAG-3’ and 5’-TGC CGC CAG CGA AGA GAC GCT-3’ for Fut8 (NM_012042); 5’-TCA AGC CAT CAA CAT CAA C-3’ and 5’-GTT GGG GAC GAT GTA CTC GAA GG-3’ for Gor III (NM_011795); 5’-CAG CGC CAG CAA CTC GAC TA-3’ and 5’-TCG ATT GTA CAT GTC TCC AG-3’ for B4GalT-I (NM_022305); and 5’-AAA TGG TGA AGG TCG GTG TG-3’ and 5’-TGA AGG GGT CTT TGA GCG TGG-3’ for GAPDH (NM_001001303).

Statistical analysis

The results are expressed as mean ± SD. Statistical analyses were carried out using the Student t test. A p value < 0.05 was considered statistically significant.

Results

Lack of core fucosylation results in impaired B cell generation in the spleen after OVA immunization

To determine the effects of targeting Fut8 on the immune response, we immunized 12-wk-old Fut8+/+ mice (body weight, 34 ± 2.3 g) and Fut8−/− mice (body weight, 29 ± 3.5 g) with OVA. Leukocyte numbers in the peripheral blood of Fut8+/+ mice and Fut8−/− mice immunized with OVA were 6040 ± 381/μl and 4730 ± 213/μl, respectively (Supplemental Fig. 1), whereas they were 4930 ± 227/μl and 4400 ± 178/μl, respectively, without immunization. Fut8−/− mice exhibited a significant decrease in lymphocyte counts and an increase in eosinophil and monocyte counts. The end product catalyzed by FUT8, a core fucosylated N-glycans, was ubiquitously expressed in Fut8+/+ spleen but not in Fut8−/− spleen, as evidenced by AOL blot (Fig. 1B), which preferentially recognizes core fucose structure. Moreover, the phenotypes of SPLs from Fut8+/+ and Fut8−/− mice immunized with OVA were compared using flow cytometry. The results revealed that CD45R+ CD19+ cells and IgM+/IgD- cells were markedly reduced in Fut8−/− spleen, and CD11b+ myeloid cells were increased in Fut8−/− spleen, whereas the number of CD5+ cells was relatively normal in Fut8−/− spleen (Supplemental Fig. 2). The absolute numbers of SPLs in immunized Fut8+/+ and Fut8−/− spleen were 188 × 10^6 and 92 × 10^6, respectively, whereas they were 100 × 10^6 and 80 × 10^6, respectively, in unimmunized Fut8+/+ and Fut8−/− spleen. In our previous study, we also found that the numbers of CD5+ cells, NK cells, and NK T cells were relatively normal.
in Fut8<sup>-/-</sup> bone marrow (17). These results indicated that Fut8 plays important positive roles in B cell generation with OVA immunization in spleens.

Establishment of 3-83-KD cells and 3-83-KD-Re cells

To investigate the mechanism involved in Fut8 regulation of B cell generation, we inserted a Fut8 siRNA sequence (1386–1404) into a retrovirus expression vector and established Fut8-knockdown 3-83 B cells, which are referred to as 3-83-KD cells. The expression of targeted Fut8 mRNA was verified using RT-PCR. As expected, Fut8 mRNA was barely detected in 3-83-KD cells. Reintroduction of the Fut8 gene into 3-83-KD cells resulted in recovery of Fut8 mRNA expression in 3-83-KD-Re cells, and the levels were comparable to those in 3-83 cells (Fig. 2A). In a real-time PCR assay, Fut8 mRNA expression of 3-83-KD cells was reduced to <5% of that in 3-83 cells (Fig. 2B). No apparent changes were found in the expression of other glycosyltransferase genes, such as Fut4, Gnt III, and β4GalT-I. Although a complex coordination among glycosyltransferases in the medial Golgi was reported (21), the loss of Fut8 did not influence the expression of other glycosyltransferases, suggesting that there was no off-target effect in this system. The results of Fut8 gene expression were further supported by analysis of FUT8 protein expression (Fig. 2C). Using an HPLC assay, FUT8 enzymatic activity of 3-83-KD cells was 26.3 pmol/h/mg, whereas that of 3-83 cells and 3-83-KD-Re cells was 761.1 and 697.2 pmol/h/mg, respectively (Fig. 2D).

Flow cytometry analysis showed no significant differences in the IgG2a-BCR expression of all three types of 3-83 cells used in this study (Fig. 3A). Also, biotin-labeling analysis showed no differences in the expression of IgG2a-BCR for three type of cells, whereas the core fucosylation of IgG2a-BCR was abolished in 3-83-KD cells and was rescued by the reintroduction of Fut8, as evidenced by AOL blot (Fig. 3B), indicating that IgG2a-BCR is the target of Fut8. No significant differences were found in the protein expression patterns of the three type of cells (Fig. 3E). Furthermore, IgG2a-BCRs on the membranes from three type of cells were purified with a Protein G Agarose column (Fig. 3C). In our analysis, using the NetNGlyc program and DNA sequencing, we found that IgG2a-BCR had an N-linked oligosaccharide chain in C<sub>H2</sub> at Asn<sup>293</sup> of Fc regions, whereas no glycosylation site was identified in the V region of the H chain (V<sub>H</sub>) or the V region of the L chain (Supplemental Fig. 3). Indeed, the core fucosylated N-glycans bearing non-, mono-, or di-galactose were analyzed in IgG2a-BCR by electrospray ionization mass spectrometry. The core fucose of N-glycans in IgG2a-BCR was eliminated by disruption of Fut8 and restored by reintroduction of Fut8 (Fig. 3D).

Core fucosylation regulates the interaction between IgG2a-BCR and p31

The recognition of Ag by IgG-BCR is the most important event for initiating B cell activation. We sought to elucidate the role of core fucosylation during the binding of Ag to IgG-BCR by chemically synthesizing the monobiotinylated p31 peptide. Compared with 3-83 cells, purified IgG2a from 3-83-KD cells showed an impaired binding affinity to the p31 peptide by an optical biosensor; reintroduction of Fut8 restored the binding affinity of IgG2a to p31 (Fig. 4A). Moreover, using fluorescence emission spectra analysis, we found that, compared with the core fucosylated IgG2a-BCR, the fluorescence intensity was significantly decreased in the de-core fucosylated IgG2a-BCR to p31; the intensity returned to near-normal levels with the reintroduction of Fut8 (Fig. 4B). These results indicated that core fucosylation is required for the functional interaction between IgG2a-BCR and p31.
oligomerization of BCR, whereas the de-core fucosylated BCR failed to do so (Fig. 5). The inhibited oligomerization was partially rescued by reintroduction of the \( \text{Fut8} \) gene to 3-83-KD cells (Fig. 5). Although Yang and Reth (24) showed that the IgM-BCR and IgD-BCR are an oligomer on the surface of resting cells, based on our results, the IgG2a-BCR appears to be a monomer without the stimulation (Fig. 5). These results suggested that core fucosylation of BCR is essential for its efficient oligomerization.

Core fucosylation is required for signal transduction via IgG2a-BCR

Given that BCR-mediated cellular responses to Ag stimulation regulate several biological functions, including B cell proliferation (5), we next analyzed the changes in the patterns of the tyrosine-phosphorylated proteins of 3-83 cells after p31 or anti-IgG2a mAb stimulation. As expected, 3-83 cells treated with 0.1 \( \mu \text{g/ml} \) p31 (Fig. 6A) or 0.005 \( \mu \text{g/ml} \) anti-IgG2a mAb (Fig. 6B) showed a striking increase in the levels of many phosphorylated species compared with the pattern seen in unstimulated cells. Notably, although 50–110-kDa bands were clearly evident in 3-83 cell lysates, none were found in 3-83-KD cell lysates (Fig. 6A, 6B). Moreover, in contrast to 3-83 cells, p31-induced phosphorylation at 5 and 15 min was reduced significantly in 3-83-KD cells, and the reduced phosphorylation was completely restored by the reintroduction of \( \text{Fut8} \) in 3-83-KD-Re cells (Fig. 6C). However,
no significant difference was found in the phosphoinositide-specific phospholipase Cγ-mediated signaling of different cell lines when using H2O2 as a stimulus, which bypasses the Ag-driven BCR signal (Fig. 6D).

**FIGURE 4.** Core fucosylation is required for the binding affinity of p31 to BCR. (A) Molecular interaction of p31 with BCR using an optical biosensor. The monobiotinylated p31 peptide (0, 0.1, 0.5, 1 μg/ml) was immobilized on a biotinyl-cuvette via streptavidin. Then, 1 μg/ml IgG2a purified from the lysates of 3-83, 3-83-KD, and 3-83-KD-Re cells was placed in the cuvette. Data shown are the means of triplicate experiments. **p < 0.01. (B) Comparison of p31 binding of IgG2a-BCR among 3-83, 3-83-KD, and 3-83-KD-Re cells by fluorescence emission spectra. The p31 peptide (0.1 μg/ml) binds to 1 μg/ml IgG2a purified from 3-83, 3-83-KD, and 3-83-KD-Re cells for 5 min at RT.

To more precisely define the diminished BCR signaling in 3-83-KD cells, we analyzed the phosphorylation profiles of the intracellular signaling molecules. In comparison with 3-83 cells, the phosphorylation levels of CD79a and Syk, as induced by p31, were greatly attenuated in 3-83-KD cells, and the reduced phosphorylation was rescued in 3-83-KD-Re cells (Fig. 6E). Consistent with the change in the phosphorylation pattern, the recruitment of Lyn was decreased significantly in 3-83-KD cells compared with 3-83 cells, and it was restored in 3-83-KD-Re cells (Fig. 6F). However, it is noteworthy that the status of CD79a phosphorylation, as induced by anti-CD79b mAb (HM79-12), was not altered by the loss of core fucosylation (Fig. 6G), suggesting that core fucosylation is specifically required for Ag-induced IgG2a-BCR-mediated signal transduction.

Core fucosylation regulates the efficient coalescing of a lipid raft

Lipid raft domains are characterized by high concentrations of glycosphingolipid, cholesterol, GPI-anchored proteins, and several other molecules involved in signal transduction (8, 25). To test whether BCR is present in the lipid raft domains, we used confocal microscopy to examine the distribution of BCR on the cell surface after p31 and/or anti-IgG2a mAb stimulation. As shown in Fig. 7A, both p31 and anti-IgG2a mAb induced prominent colocalization of BCR, caveolin-1, and GM1 on 3-83 cells at 3 min after stimulation, whereas the colocalization did not appear in the absence of p31. In 3-83-KD cells, we found only modest colocalization of GM1, caveolin-1, and BCR after stimulation with p31 (Fig. 7B); this was completely restored in 3-83-KD-Re cells (Fig. 7C). Indeed, the recruitment of caveolin-1 to BCR was significantly suppressed in 3-83-KD cells (Fig. 7F). We further purified lipid rafts from resting and stimulated cell lines with discontinuous sucrose density gradient centrifugation and analyzed the fractions in the sucrose gradient. GM1, one of the components of lipid rafts, has been used to visualize the raft domain. The position of the GM1-enriched fraction in the sucrose gradient was determined by dot blot analysis. As shown in Fig. 7D, after p31 stimulation, GM1 was enriched at fractions 4–5, located at the top of the sucrose gradient in 3-83 cells; however, in 3-83-KD cells, it was enriched at fractions 5–6, and it returned to fractions 4–5 in 3-83-KD-Re cells. These results were similar to those described above. To determine where the BCR resided in the gradients, immunoblots were probed with Abs specific for mouse IgG2a-BCR and for Lyn. In untreated cells, IgG2a-BCR and Lyn were found in the soluble fractions of the sucrose gradient and not in the detergent-insoluble lipid raft region, indicating that the BCR was excluded from the lipid rafts in resting cells. After p31 stimulation, a significant portion of both IgG2a-BCR and Lyn is translocated into the fraction 4 of lipid raft in 3-83 and 3-83-KD-Re cells, whereas the reduced translocation of IgG2a-BCR and Lyn to the lipid raft domains was found in fraction 6 in 3-83-KD cells (Fig. 7D), indicating that core fucosylation was required for the efficient coalescing of lipid raft. The kinetics of BCR-mediated Ag internalization have been studied extensively by multiple laboratories (25, 26). As illustrated in Fig. 7E, rapid and extensive Ag-BCR internalization was found in 3-83 cells after p31 stimulation at 5, 10, and 15 min, whereas p31-BCR endocytosis was reduced significantly in 3-83-KD cells and then was restored in 3-83-KD-Re cells.

Core fucosylation regulates IgG production

We next used Western blotting to analyze IgG2a secretion of three type of cells after p31 stimulation. Compared with 3-83 cells and 3-83-KD-Re cells, the amount of IgG2a secreted in cell media was

**FIGURE 5.** Core fucosylation is required for the oligomerization of BCRs. BCR oligomerization by p31 stimulation (left panel) and monomeric BCR (right panel) in resting cells. Cells were treated or not with 0.1 μg/ml p31 peptide for 2 min at 37°C, followed by cross-linking with 5 mM BS3; the incubation continued with gentle shaking for 30 min at 37°C. The cell surfaces were labeled by sulfo-NHS-biotin, and the lysates were incubated with Protein G Sepharose for immunoprecipitation and probed by HRP-labeled streptavidin.

**FIGURE 6.** IMPORTANCE OF N-GLYCANS IN ADAPTIVE IMMUNE RESPONSE

- **A** Biodistribution of the synthetic glycosylated tetramer with the different types of glycan compared with a synthetic glycosylated tetramer with a GlcNAc core fucosylation site. The tetramers were incubated with B lymphocytes from a mouse expressing an IgG2a-BCR in the absence or presence of p31 stimulation. The amount of IgG2a binding to the BCR was measured by flow cytometry. The data presented are the means of triplicate experiments. **p < 0.01.
- **B** Binding of IgG2a-BCR to different types of glycosylated tetramers. The monobiotinylated tetramers were immobilized on a biotinyl-cuvette via streptavidin. Then, 1 μg/ml IgG2a purified from the lysates of 3-83, 3-83-KD, and 3-83-KD-Re cells was placed in the cuvette. Data shown are the means of triplicate experiments.

**FIGURE 7.** Core fucosylation regulates IgG production

- **A** Biodistribution of the synthetic glycosylated tetramer with the different types of glycan compared with a synthetic glycosylated tetramer with a GlcNAc core fucosylation site. The tetramers were incubated with B lymphocytes from a mouse expressing an IgG2a-BCR in the absence or presence of p31 stimulation. The amount of IgG2a binding to the BCR was measured by flow cytometry. The data presented are the means of triplicate experiments. **p < 0.01.
- **B** Binding of IgG2a-BCR to different types of glycosylated tetramers. The monobiotinylated tetramers were immobilized on a biotinyl-cuvette via streptavidin. Then, 1 μg/ml IgG2a purified from the lysates of 3-83, 3-83-KD, and 3-83-KD-Re cells was placed in the cuvette. Data shown are the means of triplicate experiments.
decreased >3.75-fold in 3-83-KD cells after p31 stimulation for 12 h (Fig. 8A). ELISPOT assay was performed to further investigate the frequency of IgG-producing cells in SPLs from Fut8+/+ and Fut8−/− mice after OVA immunization. Compared with Fut8+/+ SPLs, the frequency of IgG-producing cells was significantly reduced in Fut8−/− SPLs (Fig. 8B). Moreover, the spots of IgG-producing cells in Fut8−/− mice were smaller than those of Fut8+/+ mice (Fig. 8C). In the current study, we found that the amounts of IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM in 12-wk-old Fut8−/− mice (ICR background) were not different from those seen in Fut8+/+ mice (Fig. 8D). To investigate the role of core fucosylation in an adaptive humoral immune response, Fut8+/+

**FIGURE 6.** Core fucosylation is required for signal transduction via BCR upon several stimuli. (A) Tyrosine phosphorylation levels of 3-83-derivative cells upon 0, 0.1, and 1 μg/ml p31 stimulation. After serum starvation overnight, cells were treated with p31 at the indicated concentrations for 5 min and solubilized for 15 min at 4°C in lysis buffer. The blots were probed by PY20. (B) Tyrosine phosphorylation levels of 3-83-derivative cells upon stimulation with 0, 0.005, 0.05, and 0.5 μg/ml anti-IgG2a mAb. Cells were treated with anti-IgG2a mAb at the indicated concentrations for 5 min. (C) Tyrosine phosphorylation levels of 3-83-derivative cells upon stimulation with 0.1 μg/ml p31 for 0, 5, and 15 min. (D) Tyrosine phosphorylation levels of 3-83-derivative cells upon stimulation with 0, 0.005, and 0.05% H2O2. Cells were treated with H2O2 at the indicated concentrations for 5 min. (E) Phosphorylation levels of CD79a and Syk with p31 stimulation. After serum starvation overnight, cells were treated with p31 at the indicated concentrations for 5 min and then solubilized in lysis buffer. The blots were probed with PY20 and anti-CD79a Ab for CD79a phosphorylation and probed with anti–p-Syk and anti-Syk Ab for Syk phosphorylation. (F) Recruitment of Lyn and caveolin-1. The blots were probed with anti-Lyn Ab and anti-caveolin-1 Ab. The membrane was stripped and reprobed with anti-GAPDH Ab to confirm equal loading. (G) Phosphorylation levels of CD79a with anti-CD79b mAb stimulation. Whole-cell lysates were immunoprecipitated with anti-CD79a Ab. The samples were subjected to 7.5% SDS-PAGE. The blots were probed with PY20 and anti-CD79a Ab. IP, immunoprecipitation; WB, Western blots.
and Fut8−/− mice (12 wk old) were immunized with 200 μg OVA, and the immune sera were collected 0, 14, and 28 d later. ELISA analysis showed that the titers of anti-OVA IgG in the immune sera of Fut8−/− and Fut8+/+ mice were 1:1,600 and 1:12,800, respectively, after the first immunization (14 d); after the boost immunization (28 d), they reached 1:25,600 and 1:102,400, respectively (Fig. 8E). Taken together, these results strongly suggest that the disruption of Fut8 led to a reduction in Ab production.

Core fucosylation regulates the expression of genes related to B cell activation

To further elucidate the underlying mechanism of the impaired B cell activation caused by the lack of core fucosylation of BCR, mRNA expression in 3-83 cells and 3-83-KD cells was compared after p31 stimulation. By PCR array analysis, we found 14 genes with >2-fold difference in their expression levels. Among them, eight genes (Runx3, Runx2, Lmo2, Lmo1, Blnk, Mapk3, Prkcb, and JUN), which are involved in immune system development, hematopoiesis, and BCR signaling, were downregulated in 3-83-KD cells, whereas six genes (Ikzf3, Trp53, Ifna2, Smo, Ccl3, and Ctgf), which function in cell cycle regulation and lymphocyte differentiation, were upregulated (Table I). BLNK, a downstream molecule from BCR and Lyn, could bridge Syk to a multitude of signaling pathways, such as the MAPK and protein kinase C pathways, which play important roles in controlling B cell activation and final Ab production.

Discussion

Almost all of the immunological molecules are glycoproteins (29, 30), and certain N-glycans contribute to the folding, stability, and biological function of the molecules (2, 12). N-glycans of IgG molecules could contribute to their binding (29), activity (12), and...
structural integrity (30). Core fucosylation catalyzed by FUT8 is a relatively common posttranslational modification in mammalian N-glycans. Substantial evidence confirmed that the core fucosylation of membrane receptors is an important parameter defining the affinity to ligands, oligomerization, and the biological function of the molecules (1, 16, 17). In the current study, the effects of FUT8 expression and its product, core fucosylation, on B cell activation and Ab production were investigated. We found that the core fucosylation of N-glycans is a crucial element that regulates Ag recognition of BCR, B cell activation, and Ab production. Moreover, core fucosylation enabled B cells to form an effective lipid raft domain that regulates signal transduction via BCR. Our results provide clear evidence that core fucosylation may play an important role in the BCR-mediated humoral immune response.

The initial step of B cell activation is a strict Ag-sensing event, which relies on the affinity of BCR to its ligand. The affinity of human IgG2 Ab was linked to the axial rotation of the IgG2 Fab arms (31). Indeed, IgG Fc regions do not interact, but have two oligosaccharide chains interposed between them (13). These chains cover the hydrophobic faces that would normally lead to domain pairing. Differences in the sequences of the oligosaccharide chains determine the variance in the orientation of the chains on the protein surface (13). Shields et al. (14) showed that the lacto core fucosylation of N-glycans at Asn 297 of IgG1 could increase the binding affinity of Fc regions to FcgammaRs on NK cells. In addition to the N-glycans at Asn297 in C1q2 of Fc regions, it was reported that the carbohydrate in the V_H of dextran-specific Abs was required for the affinity of the Abs for Ag (32). In the current study, we found that there are no glycosylation sites in the V_H of the IgG2a-BCR, whereas there are glycosylation sites

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**FIGURE 8.** Core fucosylation is required for IgG production. (A) Lack of core fucosylation reduced IgG production. Cells were first cultured for 24 h under serum-starved conditions and then replated at a density of 2 × 10^6 cells/well. The media were harvested after p31 stimulation. (B) Frequency of Ab-secreting cells in ELISPOT assay. Single SPLs from immunized mice were isolated and plated on OVA-coated 96-well plates at a density of 5 × 10^5 cells/well. The averages of the spots were calculated and expressed as the number of anti-OVA IgG-secreting B cells. Data are representative of the mean ± SD of five mice/genotype. *p < 0.05. (C) Comparison of Ab-secreting abilities of Fut8^+/+ and Fut8^-/- SPLs. Note that although the IgG-producing spots of Fut8^+/+ SPLs had a dark center, those of Fut8^-/- SPLs showed small spots. (D) Comparison of the levels of serum Ig isotypes in 12-wk-old Fut8^+/+ and Fut8^-/- mice. The concentrations of Ig isotypes in the sera of mice (n = 5/group) were measured by ELISA using mouse mAb isotyping reagents (Sigma-Aldrich). (E) Comparison of Ab production between Fut8^+/+ and Fut8^-/- mice with OVA immunization. Ab titers were detected by ELISA. OD values were measured at 492 nm using a microplate reader. Data are representative of the mean ± SD of four mice/genotype. **p < 0.01.
at Asn\textsuperscript{293} of the Fc region. The structure of N-glycans at Asn\textsuperscript{293} was further characterized by a mannosyl-chitobiose core (Man3GlcNAc2-Asn) with core fucose. Lack of core fucosylation impaired the binding affinity of IgG2a-BCR to p31, and the impairment was improved by the reintroduction of Fut8. Moreover, in Fut8\textsuperscript{−/−} SPLs, the response of OVA-stimulated B lymphocytes to Ag was significantly reduced by the disruption of Fut8. Because the core fucosylation could modulate ligand affinity of the membrane receptors (4, 33), and because the core fucosylation could affect the conformation and flexibility of the antenna of N-glycans (34), it is reasonable to postulate that core fucosylation of BCR could influence the axial rotation of the IgG2 Fab arms and regulate the ability of BCR to discriminate Ag. Given the intimate relationship between Ag recognition and BCR signaling, the signaling via BCR is thought to be carefully coordinated in B cells. The stimulation with Ag or cross-linking Abs could induce BCR activation, leading to the phosphorylation and recruitment of signaling molecules. Under the conditions used in the current study, 0.1 \( \mu \text{g/mL} \) p31 or 0.005 \( \mu \text{g/mL} \) anti-IgG2a mAb was sufficient to elicit a tyrosine phosphorylation response in 3-83 cells. However, the lack of core fucosylation reduced low phosphorylation of CD79a and Syk phosphorylation response in 3-83 cells. However, the failure of Lyn to BCR on the lipid raft domains, triggering a signal cascade (35). Moreover, loss of core fucosylation reduced the recruitment of Lyn to BCR in the cell lysate and resulted in the combined downregulation of the genes Blnk, Mapk3, Prkcb, and JUN, which accounts for the attenuated B cell activation and low Ab production.

In addition to their role in concentrating appropriate signaling molecules, lipid rafts are thought to function in sorting and trafficking transmembrane proteins through the endocytic pathway (39). Upon Ag stimulation, core fucosylation regulated the translocation of Lyn to BCR on the lipid raft domains, triggering a signal cascade (35). Moreover, loss of core fucosylation reduced the recruitment of Lyn to BCR in the cell lysate and resulted in the combined downregulation of the genes Blnk, Mapk3, Prkcb, and JUN, which accounts for the attenuated B cell activation and low Ab production.

The physiological importance of the fucosylation was highlighted by leukocyte adhesion deficiency II, which leads to an immunodeficiency caused by the absence of fucosylation, specifically core fucosylation (40, 41). Given that core fucosylation is an important posttranslational process and could modulate the ligand affinity for membrane receptors, the observation that core fucosylation regulates the Ag binding of BCR is perhaps not surprising. Ag recognition and Ab production in B cells are major components of the adaptive humoral immune response. The most important finding of the current study was the identification of the regulatory mechanism of Fut8 in Ag recognition, which, in turn, regulates several parameters of B cell activation, such as BCR oligomerization, signal transduction via BCR, raft association, and signal transduction.

<table>
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Table I. Genes upregulated and downregulated by the lack of core fucosylation
Ab production. Because the ability of BCR to discern an Ag is critical to self/non-self discrimination, and because the concentration of Ag–BCR clusters in the center of the B cell contact area was a critical process during immune synapse formation (42, 43), our results may have far-reaching implications that can further our understanding of the adaptive humoral immune responses from glycoimmunological aspects in immunity.

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Disclosures

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References


Disclosures

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