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N-linked glycosylation selectively regulates autonomous precursor BCR function

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Developing B cells express distinct classes of B cell antigen receptors (BCRs) that differ in their heavy chain (HC). Although only μHC is expressed in early stages, δHC-containing BCRs dominate on the surface of mature B cells. The reason for the tightly regulated expression of these receptors is poorly understood. Here we show that μHC was specifically required for precursor BCR (pre-BCR) function and that δHC was unable to form a functional pre-BCR. A conserved asparagine (N)-linked glycosylation site at position 46 (N46) in the first conserved domain of μHC was absolutely required for pre-BCR function, and swapping that domain with δHC resulted in a functional δHC-containing pre-BCR. When tested in the context of the BCR, μHC with a mutant N46 showed normal function, which indicated that N46-glycosylation is specifically required for pre-BCR function. Our results suggest an unexpected mode of pre-BCR function, in which binding of the surrogate light chain to N46 mediates autonomous crosslinking and, concomitantly, receptor formation.

The development, survival and activation of B cells are critically dependent on the expression of functional immunoglobulin receptors. During early B cell development, productive recombination of the immunoglobulin heavy-chain (HC) locus (*Igh*) leads to expression of a μHC protein that assembles with the surrogate light chain (SLC) and the signal-transducing heterodimer immunoglobulin-α–immunoglobulin-β (CD79a (A000586) and CD79b (A000587)) to form a precursor B cell antigen receptor (pre-BCR)[1]. The SLC consists of two invariant polypeptides: λ5 (IGLL (A001380)), which resembles the constant (C) region of conventional λ-light chains, and VpreB, of which there are two isoforms, Vpreb1 (A002369) and Vpreb2 (A002370). Membrane assembly of the pre-BCR is essential for developmental progression, as mutations that prevent transmembrane anchoring of μHC as well as experimentally introduced defects in SLC genes are associated with a block in B cell development at the pro-B cell stage[2,3]. Signaling events initiated by the pre-BCR then induce several rounds of cell division, immunoglobulin light-chain (LC) recombination and development into immature B cells expressing BCRs of the immunoglobulin M (IgM) isotype[4,5]. However, it is still not clear whether initiation of pre-BCR signal transduction requires a specific external ligand. For example, it has been suggested that galectin-1 in humans and heparan sulfate in mice act as pre-BCR ligands[6,7]. However, it has also been shown that signaling is initiated in an autonomous, ligand-independent manner[8,9]. Biochemical analysis has shown that recombinant antigen-binding Fab-like pre-BCR molecules consisting of HC variable region–μ-chain constant region 1 (V_H-C_μ1) and SLC occur mainly as heterodimers, which have also been visualized by electron microscopy[8]. In agreement with that finding, published studies have shown that activating the function of the signaling molecule SLP-65 (BLNK (A000381)) in pre-B cells is sufficient to induce sustained intracellular calcium flux in the absence of bone marrow stromal cells or additional receptor stimulation via crosslinking antibodies or ligands[4]. Notably, both the amino-terminal λ5 tail and the complementarity-determining region 3 loops of autoreactive BCRs contain positively charged amino acids[10], and replacement of the λ5 tail by complementarity-determining region 3 loops of autoreactive BCRs restores pre-BCR function[11]. This result emphasizes the role of the arginine residues in the λ5 tail for the initiation of signal transduction, whereas the exact interaction sites that lead to receptor aggregation have not yet been characterized.

Immunoglobulin molecules are glycoproteins, but the effect of immunoglobulin glycosylation in immunoregulatory processes is only partially understood. Modulating effects caused by altered glycosylation patterns on immunoreceptor function have been shown for CD8 (ref. 12) and CD22 (refs. 13,14). For IgG1, IgG2a and IgG2b, it has been shown that differences in sialylation of the carbohydrate attached to Asn297 on the γHC controls inflammatory responses by mediating interactions with either activating or inhibitory Fcγ receptors expressed on innate immune effector cells[15,16]. Asparagine-linked glycosylation (N-glycosylation) of IgM is more complex than that of IgG. Five sites have been described in IgM, in which the C_H1 domain has the highest diversity[17]. IgM glycosylation has been linked to serum half-life, complement activation and IgM oligomerization[18,19]. However, the role of μHC carbohydrates in BCR

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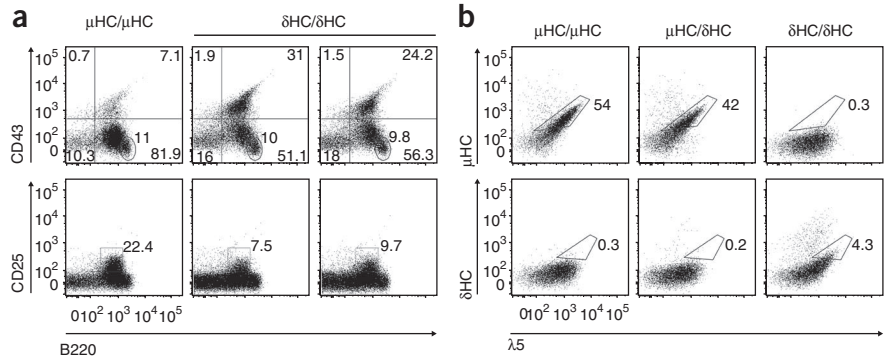

Figure 1 B cells deficient in μHC and expressing δHC show an early developmental block. (**a**) Flow cytometry of bone marrow cells from HC wild-type (μHC/μHC) mice and μHC-deficient (δHC/δHC) mice ($n > 3$ per genotype), stained with anti-CD43 or anti-CD25, plus anti-B220. Numbers in quadrants indicate percent cells in each; numbers adjacent to outlined areas indicate percent cells in the lymphocyte gate. (**b**) Flow cytometry of bone marrow cells from HC wild-type, HC-heterozygous (μHC/δHC) and μHC-deficient mice after 10 d in culture, stained with anti-μHC or anti-δHC, plus anti-λ5, assessing expression of μHC and δHC on the cell surface. Numbers adjacent to outlined areas indicate percent cells in the lymphocyte gate. Data are representative of more than three independent experiments.

function has not yet been addressed. At later stages of development, B cells coexpress BCRs of the IgD and IgM classes on the cell surface. A potential role for N-glycosylation in IgD function has not been addressed yet. Moreover, as the mechanism of pre-BCR function is not understood, it is unclear how the selection of pre-B cells is achieved and whether a specific HC is required. Here we show that differences between μHC and δHC in their glycosylation were important for the function of the respective HC and that the formation of a functional pre-BCR was strictly dependent on a specific N-glycosylation site in the C_H1 domain of μHC. Our results suggest an unexpected model of pre-BCR function in which intrinsic carbohydrates initiate pre-BCR formation, thereby allowing autonomous pre-BCR crosslinking.

RESULTS

Impaired B cell development in μHC-deficient mice

Mice deficient in μHC carry a deletion of the C_μ and μ-δ intron and therefore express δHC instead of μHC[20]. As differences in signal transduction from IgM and IgD have been described[21–23], we compared the B cell development in μHC-deficient and wild-type mice and found that the population of $B220^+CD43^+$ pro-B cells–pre-B cells was larger in the bone marrow of μHC-deficient mice (**Fig. 1a**). Furthermore, the compartment of $B220^+CD25^+$ cells (late pre-B cells–early immature B cells) was smaller in μHC-deficient mice (**Fig. 1a**). Together, the enlarged $CD43^+CD25^-$ pro-B cell–pre-B cell population and the fewer $B220^+CD25^+$ B cells suggested that μHC-deficient pre-B cells are blocked in differentiation at the pre-B cell stage.

Inefficient pre-B cell enrichment

To test whether the observed block at the pre-B cell stage in μHC-deficient mice was caused by defective pre-BCR function, we crossed μHC-deficient mice on the SLP-65-deficient background. Pre-B cells from SLP-65-deficient mice[24] show enhanced pre-BCR expression on the cell surface and more pre-BCR dependent proliferation *in vitro*[25]. In agreement with those observations, we detected conventional pre-BCRs (μHC and λ5) on the surface of SLP-65-deficient pre-B cells that were wild-type for μHC, as well as SLP-65-deficient pre-B cells heterozygous for μHC (μHC/δHC; **Fig. 1b**). In contrast, we detected only a minor population of cells expressing pre-BCRδ (δHC and λ5) among the pre-B cells doubly deficient in SLP-65 and μHC. The SLP-65-deficient μHC/δHC culture, which contained a substantial population of cells expressing the conventional pre-BCR, seemed to lack pre-BCRδ expression. We obtained similar results with intracellular flow cytometry staining for μHC and δHC (data not shown). As the number of cells expressing pre-BCRδ was substantially less than the number of cells expressing the conventional pre-BCR (**Fig. 1b**), we concluded that δHC is either not able to form a pre-BCR or is expressed as a signaling-incompetent pre-BCRδ.

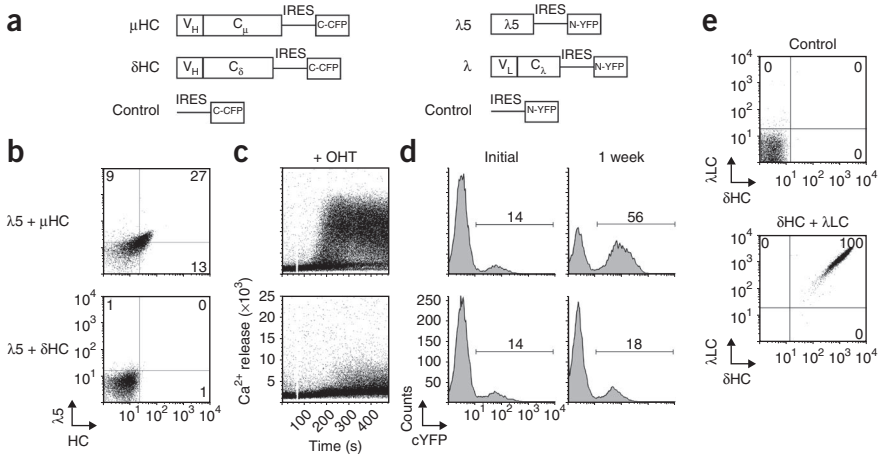

Figure 2 The δHC is not able to form a pre-BCR. (**a**) Bimolecular fluorescence-complementation vectors for the expression of NIP-specific μHC or δHC together with λ5 or λLC. Control, empty vector; IRES, internal ribosomal entry site; C-CFP, carboxy-terminal fragment of cyan fluorescent protein; N-YFP, amino-terminal fragment of YFP. (**b**) Flow cytometry of TKO cells reconstituted with either μHC or δHC, each together with λ5, stained with anti-λ5 and either anti-μHC (top) or anti-δHC (bottom) and sorted for yellow fluorescence. Numbers in quadrants indicate percent cells in each. (**c**) Ca^{2+} mobilization in ERT2–SLP-65$^+$ TKO cells reconstituted with λ5 plus either μHC or δHC and treated with 2 μM OHT. (**d**) Pre-B cell enrichment of TKO cells reconstituted with λ5 plus either μHC or δHC, assessed by flow cytometry at day 1 (Initial) and 1 week after transduction. Numbers above bracketed lines indicate percent cYFP$^+$ cells. The empty vector pair in **a** served as a control for **b**–**d** (data not shown; **Fig. 3b**–**d**). (**e**) Flow cytometry of TKO cells reconstituted with δHC together with λLC, stained with anti-λLC and anti-δHC and sorted for yellow fluorescence. Control, untransduced cells. Numbers in quadrants indicate percent cells in each. Data are representative of more than three independent experiments.

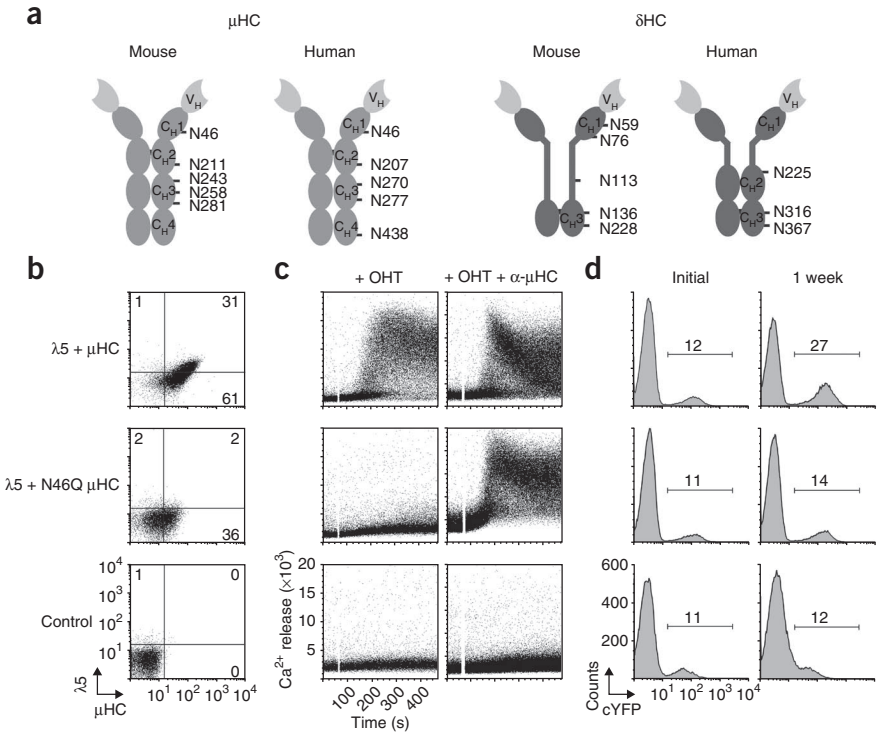

Figure 3 N46-glycosylation in μHC is essential for normal pre-BCR formation. (**a**) Mouse and human μHC and δHC structures. Right margins, N-linked glycosylation sites. (**b**) Flow cytometry of TKO cells reconstituted with λ5 and either μHC or N46Q μHC, stained with anti-λ5 and anti-μHC and sorted for yellow fluorescence. Control, empty vector pair (**Fig. 2a**). Numbers in quadrants indicate percent of total cells plotted. (**c**) Ca^{2+} mobilization in ERT2–SLP-65^{+} TKO cells reconstituted as in **b**, after treatment with 2 μM OHT with (right) or without (left) the addition of anti-μHC (α-μHC; 10 μg/ml). (**d**) Pre-B cell enrichment of TKO cells reconstituted as in **b**, assessed by flow cytometry at day 1 and 1 week after transduction. Numbers above bracketed lines indicate percent cYFP^{+} cells. Data are representative of more than three independent experiments.

Pre-BCR expression requires μHC

To characterize the molecular reason for the developmental block in μHC/δHC mice, we examined the expression and signaling properties of pre-BCRδ with those of the conventional pre-BCR by using a cell line triply deficient in SLP-65, λ5 and the recombinase component RAG-2 (TKO cells) as a reconstitution system[4,11]. Because of their deficiencies in RAG-2 and λ5, TKO cells do not express endogenous HC, LC or SLC and thus allow the exogenous reconstitution of various immunoglobulin receptors. We cotransduced TKO cells with a retroviral vector encoding λ5 and vectors encoding either μHC or δHC, each containing the B1-8 V_H region, which is specific for the hapten 4-hydroxy-5-iodo-3-nitrophenyl acetyl (NIP), and differing only in the C_H region (**Fig. 2**). To easily monitor cells expressing the receptor, we used bimolecular fluorescence-complementation expression vectors[11,26], which enabled complementation of yellow fluorescent protein (cYFP) only in cells containing both constructs (**Fig. 2a**). Flow cytometry of cells sorted for cYFP fluorescence showed that only the conventional pre-BCR, not pre-BCRδ, was expressed at the cell surface (**Fig. 2b**). An important advantage of our TKO reconstitution system was the inducible activation of SLP-65 function by fusion to a mutant

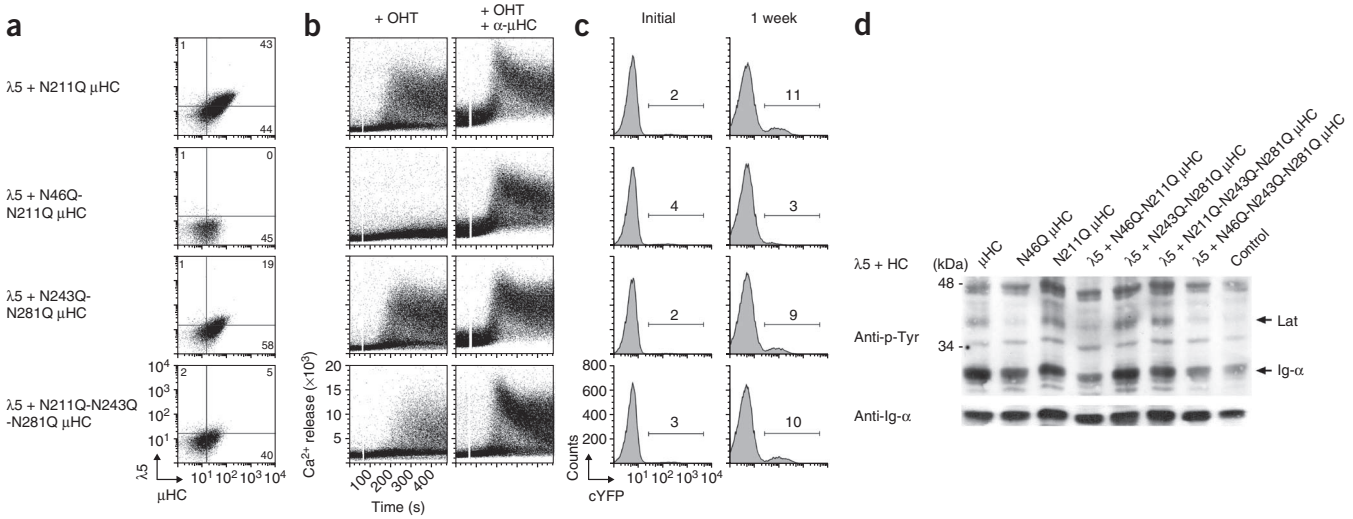

Figure 4 Glycans at positions N211, N243 and N281 in μHC are not required for the formation of a functional pre-BCR. (**a**) Pre-BCR surface expression by TKO cells reconstituted with λ5 together with μHC glycosylation mutants (left margin), stained with anti-λ5 and anti-μHC and sorted for yellow fluorescence. Numbers in quadrants indicate percent of total cells plotted. (**b**) Autonomous Ca^{2+} signaling ERT2–SLP-65^{+} TKO cells reconstituted as in **a**, after treatment with 2 μM OHT without or with the addition of anti-μHC (10 μg/ml). (**c**) Pre-B cell enrichment by TKO cells reconstituted as in **a**, assessed by flow cytometry at day 1 and 1 week after transduction. Numbers above bracketed lines indicate percent cYFP^{+} cells. (**d**) Immunoblot analysis of tyrosine phosphorylation in lysates of TKO cells reconstituted with λ5 and μHC glycosylation mutants (above lanes), assessed with 4G10 (antibody to tyrosine phosphorylation (Anti-p-Tyr)). Control, empty vector pair (**Fig. 2a**). Bottom, immunoblot analysis with antibody to immunoglobulin-α (Anti-Ig-α; loading control). Data are representative of more than three independent experiments (**a**–**c**) or are from one of three independent experiments (**d**).

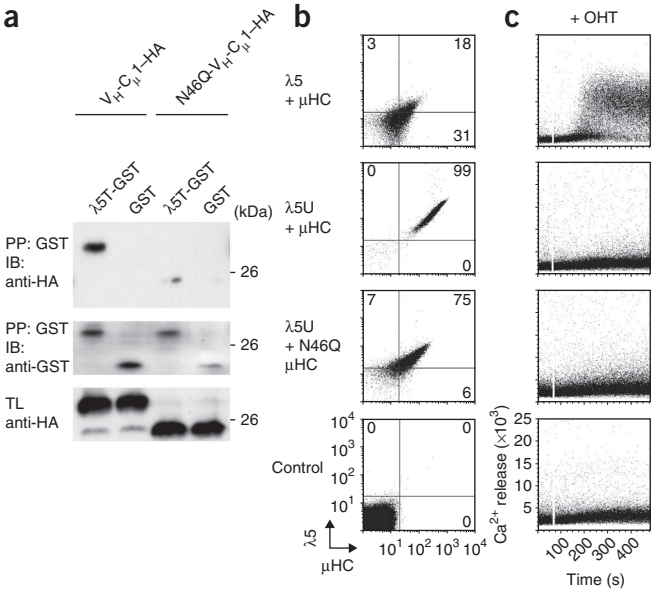

Figure 5 N46Q μHC associates with a λ5 mutant lacking the arginine-rich amino terminus. (**a**) Precipitation (PP) of the GST-fused λ5 tail (λ5T-GST) and GST from total lysates of Phoenix cells cotransfected with λ5T-GST or GST plus V_H-$C_\mu1$ or N46Q V_H-$C_\mu1$, tagged with hemagglutinin at the carboxyl terminus (–HA), followed by immunoblot analysis (IB) with anti-GST or anti-hemagglutinin (anti-HA). Bottom, immunoblot analysis of total lysates (TL) with anti-hemagglutinin. Right margin, molecular size in kilodaltons (kDa). (**b**) Flow cytometry of TKO cells reconstituted with μHC or N46Q μHC together with a λ5 mutant lacking the arginine-rich tail (λ5U) or λ5, stained with anti-λ5 and anti-μHC and sorted for yellow fluorescence. Control, untransduced cells. Numbers in quadrants indicate percent cells in each. (**c**) Ca^{2+} mobilization in ERT2–SLP-65⁺ TKO cells reconstituted as in **b**, assessed after treatment with 2 μM OHT. Data are representative of more than three independent experiments (**a**,**b**) or three independent experiments (**c**).

version of the estrogen receptor ligand-binding domain (ERT2) that is barely responsive to estradiol but is very sensitive to the synthetic ligand 4-hydroxytamoxifen (OHT). Activation of SLP-65 function in this ERT2–SLP-65 fusion by the administration of OHT leads to ligand-independent calcium mobilization when a functional pre-BCR is coexpressed[4,11]. In agreement with the inability of δHC to express a pre-BCR, induction of SLP-65 function did not lead to calcium mobilization when SLC was coexpressed with δHC (**Fig. 2c**). Moreover, and in contrast to results obtained for μHC, combining the δHC with SLC did not induce enrichment of pre-B cells (**Fig. 2d**). Notably, δHC was expressed as IgD when a conventional LC was cotransduced (**Fig. 2e**), which suggested that the inability to express pre-BCRδ was related to the SLC. Together these results indicate that δHC cannot form a pre-BCR and is therefore unable to induce autonomous signaling and pre-B cell proliferation.

Pre-BCR expression requires μHC glycosylation

To investigate why δHC is unable to form a pre-BCRδ, we compared the C_H1 domain of δHC with that of μHC, as the latter associates with the SLC. We found that mouse and human C_H1 of μHC both contained the N-linked glycosylation site N46, whereas C_H1 of mouse or human δHC contained two or no glycosylation sites, respectively (**Fig. 3a**). We addressed the question of whether the conserved N46-glycosylation site is important for pre-BCR function by site-specific mutation. We then expressed μHC with substitution of glutamine for the asparagine at position 46 (N46Q μHC) together with λ5 and measured expression and autonomous pre-BCR signaling. We did not detect SLC on the surface when it was coexpressed with N46Q μHC (**Fig. 3b**). Moreover, combining N46Q μHC with SLC failed to induce autonomous calcium flux after activation of SLP-65 (**Fig. 3c**) and did not result in pre-B cell proliferation (**Fig. 3d**). Although we did not detect the SLC, we did detect small amounts of N46Q μHC on the cell surface, and treatment with antibody to μHC (anti-μHC) induced calcium flux in cells expressing N46Q μHC (**Fig. 3b**,**c**).

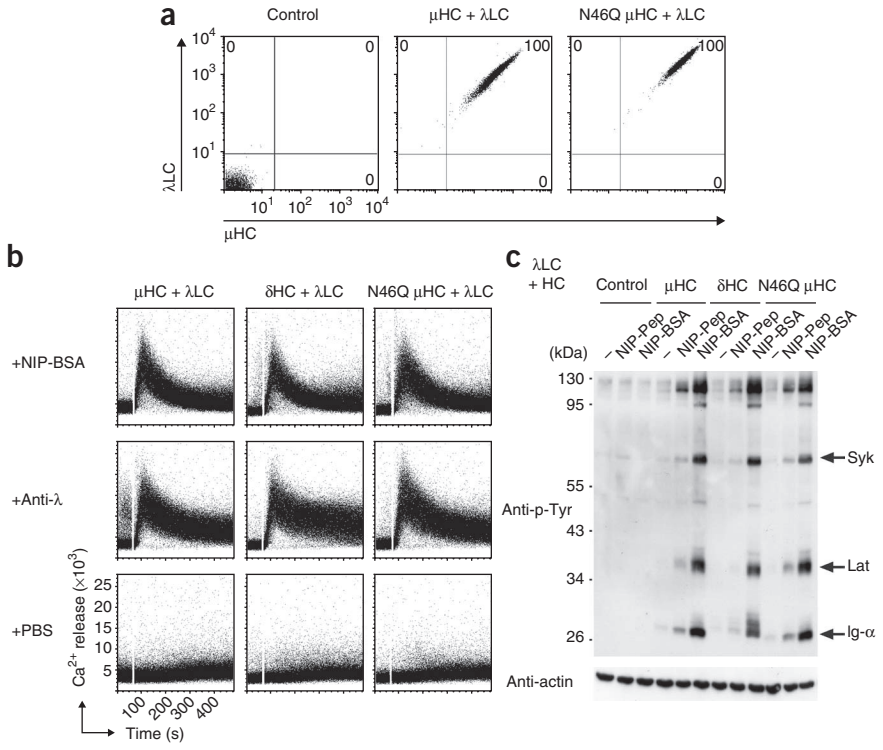

Figure 6 BCR formation is independent of N46-glycosylation in the μHC. (**a**) Flow cytometry of TKO cells reconstituted with μHC or N46Q μHC, each together with λLC, stained with anti-λLC and anti-μHC and sorted for yellow fluorescence. Control, empty vector pair (**Fig. 2a**). Numbers in quadrants indicate percent cells in each. Data are representative of more than three independent experiments. (**b**) Ca^{2+} mobilization in ERT2–SLP-65⁺ TKO cells reconstituted with λLC in combination with μHC, δHC or N46Q μHC, after stimulation with NIP-BSA (1 μg/ml) or anti-λLC (10 μg/ml). PBS serves as a control. Data are representative of three independent experiments. (**c**) Immunoblot analysis of tyrosine phosphorylation in total lysates of the cells in **a**, incubated with PBS (–; control), bivalent NIP2,3-peptide (in which NIP molecules are conjugated to the lysine residues (K) at positions 3 and 7 of peptide KSKGESKG; NIP-Pep) or multivalent NIP-BSA, assessed with antibody 4G10. Bottom, immunoblot analysis with anti-actin (loading control). Results are from one of three independent experiments.

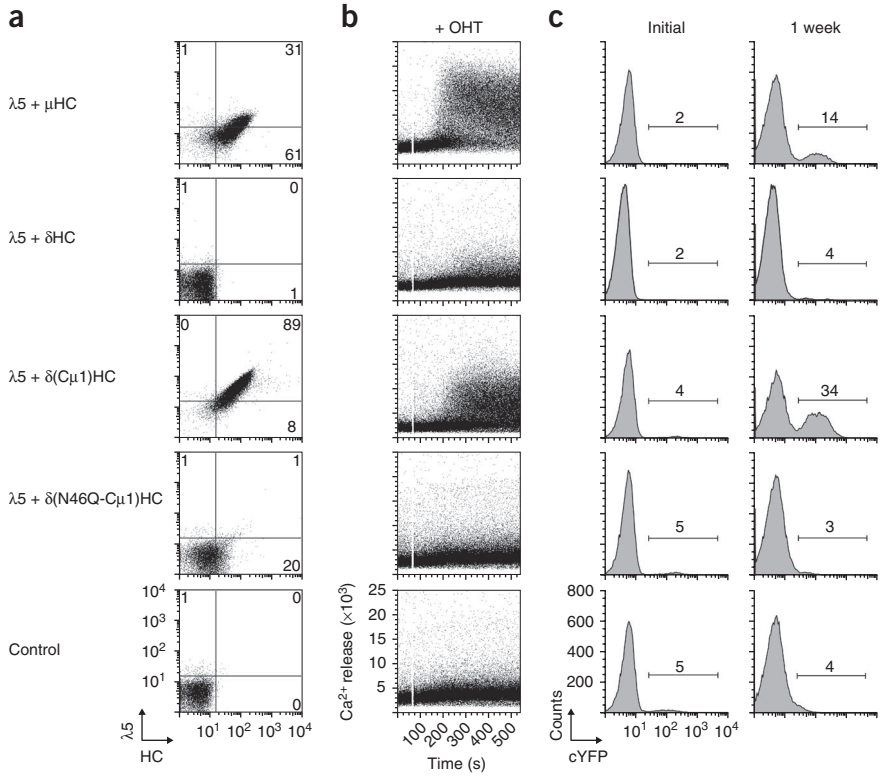

Figure 7 Transferring the $C_\mu1$ domain to δHC facilitates the formation of a functional pre-BCR. (**a**) Flow cytometry of TKO cells reconstituted with λ5 together with various HCs (left margin), stained with anti-λ5 and either anti-μHC or anti-δHC and sorted for yellow fluorescence. Control, empty vector pair (**Fig. 2a**). Numbers in quadrants indicate percent cells in each. (**b**) Ca^{2+} mobilization in ERT2–SLP-65$^+$ TKO cells reconstituted as in **a**, assessed after treatment with 2 μM OHT. (**c**) Pre-B cell enrichment of TKO cells reconstituted as in **a**, assessed by flow cytometry at day 1 and 1 week after transduction. Numbers above bracketed lines indicate percent cYFP$^+$ cells. Data are representative of more than three independent experiments.

To show that the low expression of N46Q μHC was independent of coexpressed SLC and was similar to that of wild-type μHC, which can be expressed without the LC[27–29], we expressed the HCs in TKO cells in the absence of any cotransduced LC. Similar amounts of N46Q μHC or wild-type μHC were expressed on the cell surface in the absence of SLC, and treatment with anti-μHC induced similar calcium flux in both cases (**Supplementary Fig. 1**). In summary, our data suggest that apart from pre-BCR formation, N46Q μHC acted like wild-type μHC, which suggests that N46-glycosylation is specifically required for pre-BCR formation.

To confirm that specific role of N46, we substituted other N-linked glycosylation sites in μHC and tested the ability of the resulting HCs to form a functional pre-BCR. The results showed that single or combined substitutions of N211, N243 and N281 with glutamine had little effect on the expression and autonomous signaling capacity of the pre-BCR (**Fig. 4a–c** and **Supplementary Fig. 2**). Notably, autonomous calcium flux correlated with the SLC surface expression, as shown for the glycosylation triple mutant by both lower SLC surface expression and weaker autonomous calcium signaling (**Fig. 4a**,**b**). In addition to the autonomous calcium flux and pre-B cell proliferation, tyrosine phosphorylation of signaling proteins such as immunoglobulin-α or Lat[30] was also lower after substitution of N46 with glutamine (**Fig. 4d**). Thus, the results suggest that N46 is specifically required for expression and proper function of the pre-BCR.

Enhanced binding of the λ5 tail to glycosylated $C_\mu1$

The arginine-rich tail of λ5 has an important role in pre-BCR function and is suggested to be responsible for the ability of the pre-BCR to bind many structures with moderate affinity[4,11]. We hypothesized that glycosylation of $C_\mu1$ might be needed to enhance the association between λ5 and μHC. Immunoprecipitation analysis of TKO cells expressing the SLC with either wild-type or N46Q hemagglutinin-tagged V_H-$C_\mu1$ constructs[31] showed that only glycosylated V_H-$C_\mu1$ precipitated with anti-λ5 (data not shown). This observation suggests that the glycan attached to N46 is important for the association of μHC and SLC. We next examined whether the arginine-rich tail is the structural element of λ5 responsible for binding to glycosylated V_H-$C_\mu1$. Therefore, we generated a fusion protein of recombinant glutathione *S*-transferase (GST) and the λ5 tail and coexpressed it with either wild-type or N46-mutant hemagglutinin-tagged V_H-$C_\mu1$ constructs. More glycosylated V_H-$C_\mu1$ than N46Q V_H-$C_\mu1$ purified together with the GST-fused λ5 tail when coexpressed *in vivo* (**Fig. 5a**). In summary, the data indicate that N46-glycosylation is required for better binding of $C_\mu1$ to the λ5 tail. However, deleting the λ5 tail results in more pre-BCR expression on the surface of pre-B cells[4,11,32], which suggests that binding of the λ5 tail to glycosylated N46 is not required for simple association of the SLC with μHC. We hypothesized that if binding of the λ5 tail to glycosylated N46 is required for a crucial step of pre-BCR function but not for simple association of SLC with μHC, then deleting the λ5 tail might enable the association of SLC with N46Q μHC. To test our hypothesis, we coexpressed N46Q μHC with a λ5 mutant lacking the arginine-rich tail. Indeed, SLC lacking the λ5 tail associated with N46Q μHC on the cell surface (**Fig. 5b**). Yet, as with wild-type μHC, coexpression of N46Q μHC with the λ5 mutant resulted in a pre-BCR that failed to induce autonomous signaling (**Fig. 5c**). Together our experiments suggest that binding of the λ5 tail to glycosylated N46, although not required for the association of SLC with μHC, is required for an initial step of pre-BCR function and that preventing this step inhibits the formation of a normal pre-BCR. Therefore, we propose that the λ5 tail–dependent interaction between SLC and the N46 carbohydrate puts the SLC into position for *trans* association with adjacent HCs (**Supplementary Fig. 3**).

BCR expression is independent of N46

The finding that N46Q μHC was expressed on the surface with tailless λ5 suggested that the folding and export of N46Q μHC were similar to those of wild-type μHC. To confirm that idea, we coexpressed N46Q μHC with its cognate λLC derived from the NIP-specific B1-8 BCR[22]. We found that λLC was expressed together with N46Q μHC as efficiently as it was with wild-type μHC on the cell surface (**Fig. 6a**). Moreover, BCRs containing N46Q μHC, wild-type μHC or wild-type δHC led to similar calcium flux and tyrosine phosphorylation after stimulation with NIP–bovine serum albumin (BSA) or anti-λ (**Fig. 6b**,**c**). These data suggest that the N46Q

substitution did not affect the association of μHC with LCs and allowed normal BCR-dependent activation of signaling processes.

Functional pre-BCR formation by δHC containing N46

Our findings so far suggested that N46-glycosylation of μHC is specifically required for pre-BCR expression and that δHC cannot form a pre-BCR because it lacks N46-glycosylation. If that were true, then swapping the N46-glycosylation from μHC to δHC should result in a functional pre-BCRδ chimera. Replacing the C_H1 domain of δHC with that of μHC resulted in pre-BCR expression, as detected by SLC expression on the cell surface (**Fig. 7a**). Notably, this synthetic pre-BCRδ was able to induce autonomous calcium fluxes and activated pre-B cell proliferation (**Fig. 7b**,**c**). Substituting N46 in the chimeric δ($C_μ1$)HC (in which C_H1 of δHC was replaced by that of μHC) abrogated expression and autonomous pre-BCR signaling (**Fig. 7a–c**). In summary, our results have shown that providing δHC with the ability to associate with the SLC leads to pre-BCR formation and autonomous signaling. Furthermore, N46-glycosylation is crucial for pre-BCR formation and autonomous signaling.

DISCUSSION

Here we have shown that replacing μHC with δHC resulted in partial blockade of early B cell development caused by the inability of δHC to form a functional pre-BCR. We found that a conserved N-linked glycosylation site in the C_H1 domain of μHC was the crucial element that regulated pre-BCR formation. In agreement with that finding, swapping the C_H1 domains of μHC with those of δHC enabled the latter to form a functional pre-BCR. In contrast to wild-type δHC, the chimeric δ($C_μ1$)HC led to surface expression and autonomous pre-BCR signaling that were dependent on N46-glycosylation. Our results suggest that N46-glycosylation is not required for C_H1 folding because N46Q μHC was as efficient as wild-type μHC in BCR expression when N46Q μHC was combined with a conventional LC. In addition, human δHC and all γHCs do not contain glycosylation sites in C_H1, which indicates that N-linked glycosylation is dispensable for HC and LC association. Thus, N46-glycosylation is instead specifically required for pre-BCR formation. It could be argued that the C domain of λ5 specifically requires N46-glycosylation to associate with the C_H1 of μHC. However, deleting the arginine-rich tail of λ5 resulted in an SLC that was able to associate with N46Q μHC. This result suggests that N46Q μHC contains all the structural requirements for association with λ5 and that the arginine-rich tail inhibits this association either by binding to unknown protein(s), and thus sequestering the SLC, or by affecting SLC folding and thereby inhibiting its association with HCs. Thus, removing the inhibitory role of the λ5 tail is essential for the association of SLC with HCs and can be achieved either by deletion or by involvement of the λ5 tail in specific interactions. In this scenario, glycosylation of N46 is probably involved in neutralizing the inhibitory role of the arginine-rich λ5 tail. Notably, the λ5 tail has been shown to inhibit the folding of λ5 until it binds to VpreB, the second component of the SLC[31]. Similar to the association of the SLC with N46Q μHC, removal of the λ5 tail allows λ5 folding in the absence of VpreB[31]. Given that the arginine residues are positively charged and that glycans might contain negatively charged groups, it is conceivable that the λ5 tail interacts directly with the glycan attached to N46 in μHC. HCs and LCs are able to associate efficiently without arginine-rich regions or N46-glycosylation. Deletion of the λ5 tail allowed association of the SLC with the HC. This result suggests that similar to conventional LCs, λ5 contains all necessary structural elements for association with HCs. Thus, it is unlikely that association of the λ5 tail with glycosylated N46 serves as a recruitment mechanism for *cis* association of HCs and the SLC. More likely, we propose that an interaction between the λ5 tail and N46 removes the inhibitory function of the λ5 tail and renders the SLC available for *trans* association with adjacent HCs. This model suggests a previously unknown and unexpected mechanism for pre-BCR function in which binding of the λ5 tail to N46-glycosylation allows receptor formation, thereby connecting autonomous crosslinking with receptor assembly. The conventional view that the pre-BCR is formed and transported to the cell surface, where it seeks interaction partners, may not be entirely true. A single pre-BCR complex with the highly polyreactive λ5 tail might undergo separate heterotypic interactions that could interfere with autonomous pre-BCR signaling. In contrast, the glycosylation-induced assembly of the pre-BCR identifies glycosylated N46 as a crucial element and suggests that pre-BCR formation is initiated by engagement of the λ5 tail in a specific interaction with glycosylated N46, which allows autonomous crosslinking and concomitant pre-BCR assembly. Subsequent transport to the cell surface might then allow association of the pre-BCR with essential signaling molecules if pre-BCR signaling is not already initiated in the cell[33].

It has been suggested that the main role of the pre-BCR is to test newly generated HCs for their ability to pair with conventional LCs that appear later in development[34]. However, none of the various HCs that were supposed to be unable to pair with SLC have been tested for pairing with conventional LCs *in vitro*, nor have they been used in transgenic models to show their inability to support B cell development. In contrast, our results have shown that the molecular requirements for the pairing of HC with SLC are totally different from those for the pairing of HC with conventional LC. It is therefore questionable that the SLC has any role in testing HC-LC pairing. We propose that the arginine-rich tail of the SLC is required for a previously unknown type of interaction that involves a specific N-linked glycosylation. It should be noted, however, that the importance of N46-glycosylation for association of the SLC with HCs does not exclude the possibility of effects on association that might be influenced by the composition of the V_H domains. That caveat might explain the low abundance of cells expressing pre-BCRδ observed in bone marrow–derived pre-B cell cultures from μHC-deficient mice. That suggestion, in addition to prematurely recombined conventional LCs[35], might explain why B cell development is not completely blocked in μHC-deficient mice. Notably, μHC-deficient mice that express γHC instead of μHC also show impaired pre-B cell development[36], which is most probably caused by the inability of γHC to form a pre-BCR, as C_H1 of γHC lacks the essential N46-glycosylation site. Altogether, our study has identified N-linked glycosylation as a critical post-translational modification that confers selective functional characteristics to the μHC in developing B cells. Notably, μHC is expressed mainly in pre-B cells and immature B cells, which often express polyreactive BCRs able to recognize various autoantigens[10]. Given that polyreactive BCRs mimic pre-BCR function and induce autonomous signaling[11], one question is whether N46-glycosylation is required for the function of polyreactive BCRs, thereby controlling the selection of polyreactive B cells into the mature B cell repertoire.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A002369, A002370, A001380, A000586, A000587 and A000381.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

R.Ü. and M.P.B. did experiments, analyzed data and contributed to the writing of the manuscript; C.E. did experiments and provided advice; T.W. analyzed the μHC-deficient mice; M.R. provided suggestions for experimental design; and H.J. supervised the work, designed experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Mice deficient in μHC[20], SLP-65-deficient mice[24] and mice doubly deficient μHC and SLP-65 were bred at the animal facility of the Max-Planck Institute of Immunobiology. All mice were on the BALB/c background and were crossed to the SLP-65-deficient background. Animal experiments were done in compliance with the guidelines of German law.

Cell culture and enrichment studies. TKO cells[4] and primary bone marrow cells from mice expressing or deficient in μHC were grown in Iscove's medium (Biochrom) containing 5% (vol/vol) heat-inactivated FCS (PAN-Biotech), L-glutamine (10 mM; Gibco), penicillin (100 U/ml), streptomycin (100 U/ml; Gibco), 2-mercaptoethanol (50 mM) and 9 ml culture supernatant of J558L mouse plasmacytoma cells stably transfected with mouse IL-7 expression vector. J558L cells were grown in RPMI-1640 medium (PAA Laboratories) complemented with 10% (vol/vol) heat-inactivated FCS, HEPES-buffered solution (10 mM; Gibco), pH 7.2–7.5, L-glutamine (10 mM; Gibco), penicillin (100 U/ml), streptomycin (100 U/ml; Gibco) and 2-mercaptoethanol (50 mM).

For enrichment studies, transduced cells were cultivated in Iscove's medium supplemented with IL-7-containing supernatant, and the percentage of $cYFP^+$ cells was determined by flow cytometry at day 1 and between days 8 and 10 after transduction (called '1 week' here).

Plasmids and retroviral transduction. The ERT2–SLP-65 fusion construct[4] was expressed from a retroviral vector encoding tdTomato[37] (provided by R.Y. Tsien) as an expression marker. HCs were expressed from retroviral vectors containing an internal ribosomal entry site sequence followed by an open reading frame encoding a fusion of GCN4 (a basic leucine zipper transcriptional activator from the yeast *Saccharomyces cerevisiae*) and a carboxy-terminal fragment of cyan fluorescent protein (pMIZCC)[11]. LCs were expressed from constructs with that same retroviral vector backbone with an amino-terminal fragment of YFP (pMIZYN) in place of the carboxy-terminal fragment of cyan fluorescent protein. Vectors pMIZYN-λB1-8, pMIZYN-λ5, pMIZYN-λ5U and pMIZCC-μB1-8, which encodes the NIP-specific B1-8 μHC, have been described[11]. The vector pMIZCC-δB1-8 was generated by PCR amplification of V_HB1-8 and C_δ with partially overlapping primers. PCR fragments were then used as templates to produce fragments that contain the complete δB1-8 open reading frame for cloning into pMIZCC. The asparagine codon (AAC), which represents the attachment sites for N-linked glycans on μHC, was mutated to a glutamine codon (CAG) through the use of overlapping primers containing the mutated codon. Retroviral transduction was done as described[38]. Transduced cells were sorted with a MoFlo (Dako Cytomation).

Flow cytometry. For flow cytometry, cells were stained with the appropriate antibodies, including phycoerythrin–anti-B220 (anti-CD45R; RA3-6B2; Becton Dickinson), phycoerythrin-indodicarbocyanine–anti-B220 (RA3-6B2; eBioscience), fluorescein isothiocyanate–anti-CD43 (S7; Becton Dickinson), allophycocyanin–anti-CD25 (PC61; Becton Dickinson), indodicarbocyanine–anti-IgM (polyclonal; 115-175-075; Jackson Immunoresearch), Alexa Fluor 647–anti-IgD (11-26; eBioscience), biotin–anti-IgD (11-26; Southern Biotech), phycoerythrin-biotin–anti-λ5 (LM34; a gift from A.G. Rolink), phycoerythrin-biotin–anti-λ (polyclonal; 1060-09-08; Southern Biotech) and streptavidin–peridinin chlorophyll protein (340130; Becton Dickinson). Coexpression of the bimolecular fluorescence-complementation vector pairs was detected by yellow fluorescence, and ERT2–SLP-65 was detected directly via tdTomato[11,37]. A FACSCalibur (Becton Dickinson) was used for flow cytometry.

Ca^{2+} measurement. Ca^{2+} was measured as described[39]. Ca^{2+} responses were induced by the addition of goat anti–mouse μHC (10 μg/ml; 1021-01; Southern Biotech), goat anti–mouse λLC (10 μg/ml; 1060-01; Southern Biotech), a conjugate containing BSA and seven molecules of NIP (NIP_7-BSA (called 'NIP-BSA' here; 1 μg/ml; Biosearch Technologies), NIP2,3-peptide (1 μg/ml; IRIS Biotech)[40] or 2 μM OHT. As a negative control, cells were treated with equivalent volumes of the solvents PBS or ethanol. Ca^{2+} flux was measured with an LSR II (Becton Dickinson).

Immunoblot analysis. Cells (3×10^6) in Iscove's medium were stimulated for 5 min at 37 °C with NIP-BSA (1 μg/ml), NIP-peptide (1 μg/ml)[40] or the equivalent volume of PBS. Stimulated and unstimulated cells were lysed in 45 μl lysis buffer containing 50 mM Tris-HCl, pH 8, 1% (vol/vol) Triton (Sigma-Aldrich), 137.5 mM NaCl, 1% (vol/vol) glycerol, 1 mM sodium orthovanadate, 0.5 mM EDTA, pH 8, and protease inhibitor 'cocktail' (Sigma-Aldrich). Samples were separated by 10% SDS-PAGE and were transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 90 min at 25 °C in 5% (wt/vol) milk powder in PBS with 0.1% (vol/vol) Tween-20. Primary antibodies were diluted in PBS with 0.1% (vol/vol) Tween-20 supplemented with 2% (wt/vol) BSA fraction V (Biomol) and 0.1% sodium azide (Sigma-Aldrich). Secondary antibodies were diluted in 5% (wt/vol) milk powder in PBS with 0.1% (vol/vol) Tween-20. Immunoreactive proteins were detected with the ECL chemoluminescence detection system. Antibody to phosphorylated tyrosine (4G10; Santa Cruz), anti-immunoglobulin-α (a gift from J.C. Cambier), anti-hemagglutinin (3F10; Roche Diagnostics), polyclonal anti-GST (A190-121A; Bethyl Laboratories) and anti-actin (l-19; Santa Cruz) were used for immunoblot analysis.

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