Further investigation of the light chain shifting phenomenon: Light chain replacement through secondary rearrangement induced by lectin stimulation in the hybridoma cell line HB4C5

Chatchadaporn Krungkasem, Kyoko Ueda, Hirofumi Tachibana & Sanetaka Shirahata
Graduate School of Genetic Resources Technology, Kyushu University, 6-10-1, Hakozaki, Fukuoka, Japan

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Abstract

We found that when the hybridoma cell line HB4C5 was stimulated with wheat germ agglutinin (WGA), loss of production of the original λ light chain occurred, followed by production of new light chain, which mirrored the reaction when stimulated with concanavalin A (ConA). We previously reported that the RAG genes are expressed not only in HB4C5 and its ConA-treated variant subclones, but also in the parental Namalwa cells, which are known to be in the plasma state. However, the new λ light chains were expressed only in the HB4C5 cells and not in the parental Namalwa cells. Here we found that the RAG genes are expressed in HB4C5 cells after continuous stimulation with WGA. To further investigate the mechanism of this loss of original λ light chain production by stimulation with lectins in HB4C5 cells, which leads to a sIg-negative subpopulation, we analyzed the differences between HB4C5 and Namalwa cells. In this present study, we found that a 70 kDa phosphorylated protein in HB4C5 cells became undetectable after stimulation with lectins (WGA and ConA), and was not detected in Namalwa cells before or after lectin stimulation. It has been believed that the RAG genes and loss of original λ light chain production are required to induce expression of a new λ light chain in the HB4C5 cells. We suggested that the phosphorylated 70 kDa protein in HB4C5 cells play important roles in regulating the production of new λ light chains which is induced by lectins.

Abbreviations: WGA – wheat germ agglutinin; sIg – surface immunoglobulin; ConA – concanavalin A; CDR – complementary determining region; FITC – fluorescein isothiocyanate

Introduction

Immunoglobulin (Ig) gene rearrangement is performed during B cell development and is completed before reaching maturity. Only one heavy chain and one light chain, which combine to confer monospecificity on any particular single B cell, is produced by sequential rearrangement events (Alt *et al.*, 1986). Allelic exclusion of Ig gene expression is thought to be mediated by the production of one heavy chain which restricts the expression of the other. Expression of membrane bound μ heavy chain may signal allelic exclusion at the heavy chain locus and inhibits further rearrangement of the heavy chain gene (Nussenzweig *et al.*,

1987; Papavasiliou *et al.*, 1995). Light chain rearrangement is also thought to be inhibited by sIg expression, which results in the assembly of a light chain with a μ heavy chain. However, light chain rearrangement could still be detected in the pre-B and immature stage even though the Ig μ heavy chain is not expressed (Kitamura *et al.*, 1992). The mechanism of exclusion at the light chain locus has not been clearly elucidated. We previously isolated ConA-resistant variant clones from an antibody-secreting human B cell line HB4C5, which were shown to express a new light chain replacing the original light chain (Tachibana *et al.*, 1994). It was demonstrated that the loss of production of the original λ light chain was a result of a reduction of the level

of mRNA transcripts, and production of a new light chain was found to be a result of a new VJ rearrangement on a previously excluded allele. We have termed this process 'light chain shifting'. The loss of original λ light chain production and expression of the RAG genes seem to be necessary to induce new light chain expression in HB4C5 cells. ConA is thought to be important in inducing a reduction of the original light chain transcript level which leads to loss of the original light chain production (Tachibana *et al.*, 1996).

In this study, we used WGA instead of ConA to stimulate HB4C5 cells for further investigation of lectins which can induce loss of λ light chain production and induce rearrangement on a previous excluded allele in human plasma B cell lines. We show further that light chain shifting was induced not only by ConA but also by WGA in RAG genes expressing, human Ig-secreting B cell lines. In addition we found that a 70 kDa serine phosphorylated protein in HB4C5 cells will become undetectable by stimulation with lectins (WGA or ConA).

Materials and methods

Cells and cell culture

The human hybridoma HB4C5, which secretes an antibody reactive to lung carcinoma cells, was established by fusing a Namalwa derived NAT30 cells with IgM secreting B lymphocytes (Murakami *et al.*, 1985). The cells were maintained in ERDF medium (Kyokuto Seiyaku, Tokyo, Japan) containing 5% fetal bovine serum (FBS, Whittaker Bioproducts, MD, USA) and maintained at 37 °C under the humidified atmosphere of 5% CO₂.

Cell stimulation

HB4C5 variants, resistant to WGA (Seikagaku, Japan), were isolated and cells were maintained in serum-free ERDF medium (containing 5 μ g ml⁻¹ insulin, 20 μ g ml⁻¹ transferrin, 20 μ M ethanolamine, 1 nM sodium selenite; ITES) (Murakami *et al.*, 1982) and supplemented with 2 μ g ml⁻¹ WGA.

PCR amplification

Total RNA, which was isolated from 1×10^7 cells using TRIZOL (Gibco, BRL, MD, USA), was synthesized into cDNA using a kit (Life Science, Florida,

USA) following instructions provided by the manufacturer. Each cDNA served as a template for PCR amplification using specific primers. One PCR cycle consisted of 0.7 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C in a DNA thermo cycler (Thermolyne).

Primers

The primers pairs used to amplify the original λ light chain (λ C5) are Pv λ C5 (CDR1 region of λ C5) 5'-AACAGCTCCAACATTGGGAA-3' (sense) and Pv λ C5 (λ light chain constant region) 5'-CCAAGCTTGMGCTCCTCAGAGGAGGG-3' (antisense). Primers used to amplify the μ heavy chain transcript are PV μ NAT30 (CDR1 region) 5'-AGTAGTAACTGGTGGAGCTG-3' (sense) and PC μ (first exon) 5'-CCAAGCTTAG ACGAGGGGGGAA-AAGGGTT-3' (antisense) (Tachibana *et al.*, 1996). Primers for the RAG-1 genes: 5'-ACTACTCGAGGC-TrcTGGCTCAGTCTAC-3' (sense) and 5'-ACTAAAG-CTTGCCTGAGGGTTTCATGG-3' (antisense) (Schatz *et al.*, 1989; Oettinger *et al.*, 1990; Van Gent *et al.*, 1995), for the RAG-2 genes: 5'-ATACCTGGTTTACGCGCAAA-3' (sense) and 5'-CCAGCCTTTTTGTCCAAAGAA-3' (antisense) (Oettinger *et al.*, 1990; Abe *et al.*, 1994) and the internal standard β -actin genes: 5'-GAAATCGTGCGTGACATTAAG-3' (sense) and 5'-CTAGAAGCATTTGCGGTGGA CGATGGAGGGGCC-3' (antisense) (Abe *et al.*, 1994) were used.

Southern blot hybridization

Each cDNA was amplified as described above using the RAG or β -actin primer pairs. After a 30 cycle-amplification run, the PCR reaction products were electrophoresed on 1.5% agarose gels and transferred to nylon membrane (Hybond-N+, Amersham). Hybridization with ³²P-radiolabeled probes prepared by isolating the RAG genes or β -actin genes was performed. The membrane was then autoradiographed on X-ray film.

RNA analysis

Total RNA 10 μ g/lane was electrophoresed on 1.2% agarose gels under denaturing conditions, transferred to nylon membrane (Hybond-N+, Amersham), and hybridized with the appropriate probe. The probe was detected by autoradiography on a X-ray film. For detection of the μ chain, λ chain and β -actin transcripts,

the primers described above were used to construct the following probes: μ NAT-30-C μ 302 bp, V $\sim$ C5-J λ 251 bp, and β -actin 510 bp.

Probes

Probes to detect the λ C5 gene, μ NAT30 gene, RAG genes and β -actin gene were constructed as follows. Using the appropriate primer, the PCR products obtained from a 30 cycle amplification run were isolated on 1% agarose gel. The recovered DNA was then labeled using the Radi-Prime kit (Amersham) and [α^{32} P]-dCTP (ICN). These labeled DNA fragments were then used as probes.

Nucleotide sequence analysis

The V-region genes of the λ light chain from the WGA-resistant clones were amplified by PCR using the primers that were described by Larrick *et al.* (1989). The PCR product was then inserted into pGEM-T vector (Promega, USA) according to the instructions produced by manufacturer. The nucleotide sequences of V-region genes were determined, after subcloning and analyzed by a sequencer (LI-COR 4000L, ALOKA).

Immunofluorescence analysis

Cultured cells were prepared as previously described (Krungskasem *et al.*, 1995) and stained with either of the following monoclonal antibodies; goat anti-human μ chain conjugated to FITC (Biosource, CA, USA) or goat anti-rabbit IgG conjugated to FITC which was used as a negative control. The relative fluorescence intensity was detected by Flow cytometry (FACSCaliburTM, BECTON DICKINSON, USA).

Western blot analysis of serine phosphorylated proteins

The hybridoma cells, 2×10^5 cells ml $^{-1}$, were stimulated with WGA and incubated at 37 °C in culture medium without serum. The cells were pelleted and lysed in lysis buffer as described by Kato *et al.* (1994). A substance in the supernatant was immunoprecipitated with anti-phosphoserine covalently linked to proteinA-sepharose (Pharmacia, Sweden). This immunoprecipitated substance was then electrophoresed using a SDS-PAGE gel and immunoblotting was performed to detect the serine-phosphorylated proteins using 5 ng ml $^{-1}$ of the primary antibody, an

anti-phosphoserine mouse IgG1 (Sigma, USA). A peroxidase conjugated to anti-mouse IgG γ was used as a secondary antibody (Biosource, CA, USA).

Results

Isolation and characterization of WGA-resistant variant clones from HB4C5 cells

We analyzed the following antibody-secreting human plasma B cells; the parental Namalwa cells, HB4C5 cells, the WGA-resistant HB4C5 variant clones WO, W26, W29 and W42, which are shown to express variant new λ light chains (Figure 1A). Each clone was demonstrated to have the ability to secrete a new λ light chain, each different from the other clones. The new λ light chains were detected 7 weeks after continuous stimulation with WGA. The appearance of the clones producing a new light chain when stimulated with WGA occurred at an average rate of 16/96 cells. We selected 4 different clones among these clones. Three clones of them (W26, W29 and W42) were new λ light chain producers and the rest (WO) was a non- λ chain producer. To characterize these new λ light chains, we sequenced the variable region (V-region) of one WGA-resistant clone (Figure 1B). The λ C5 chain has been shown to belong to the λ light chain subgroup I, and has one N-glycosylation consensus sequence in its hypervariable region (Tachibana *et al.*, 1993). The V segment of the W29 λ light chain is shown to belong to subgroup VI, and no N-glycosylation consensus sequence was found in the V-region. This emphasizes the fact that W29 clone has the new λ light chain and is different from its parental cell line, HB4C5. To assess whether a new light chain producer can secrete yet another new light chain, we continued to culture these WGA-resistant clones (W29, W42 and WO) with WGA for 8 weeks. We found that these clones produced the same product over the entire time course (Figure 1C). This suggests that WGA-resistant clones of HB4C5 cannot be induced to further light chain shifting. HB4C5 cells and its variant clones, except WO cells express surface Ig (sIg) (see the data below). The clone WO does not contain the sIg-positive phenotype, suggesting that the WO cell line may be a derivation of the sIg-negative expression which may be due to the loss of production of the original light chain. This loss of sIg expression may be a signal for inducing the production of new light chain.

Figure 1. Western blot analysis of λ light chain expression by WGA-resistant cells. (A) Immunoblotting with an anti-human λ light chain/HPR conjugate was performed on the supernatant from Namalwa, HB4C5 and WGA-resistant clones of HB4C5 cells (W0, W26, W29 and W42). (B) The light chain V-region sequence of the W29 cells. The cDNA sequence of the light chain V-region of each clones are shown in a 5' to 3' direction. (C) The WGA-resistant clones of HB4C5 cells (W29, W42 and W0), which were maintained in culture in the presence of WGA for 1–8 weeks, were assayed for λ chain production. The molecular sizes shown by arrows.

Figure 2. Northern blot analysis of the expression of λ C5 light chain, μ C5 heavy chain and β -actin genes in Namalwa, HB4C5 and WGA-resistant clones of HB4C5 cells (W26, W29, W42 and W0). (A) Schematic for probes used to detect each transcript is shown. (B) Cells used as a source of mRNA were indicated above each lane. The arrows indicated the size for each transcript.

Figure 3. RAG genes expression analysis by PCR amplification. RAG-1, RAG-2 and β -actin gene transcripts were amplified by RT-PCR followed by Southern blot hybridization.

Reduction of the original λ light chain transcript and rearrangement of the new λ light chain on a previous excluded allele

We found that when we compared the transcript produced by HB4C5 cells to WGA-resistant cells, the loss

of the original light chain production was due to a significant reduction of the original light chain transcript. However, no difference was found in the level of heavy chain transcript produced (Figure 2). The significant reduction of the level of original λ light chain mRNA may be a reason why the original λ chain protein is not detected in these WGA-resistant clones. This suggests that the expression of a new λ light chain occurs by a process of continuing rearrangement on a previously excluded allele, not on the original allele.

RAG genes expression in the λ light chain variant clones

RAG-1 and RAG-2 are necessary for Ig rearrangement and are expressed only when B cells undergo rearrangement at the Ig heavy and light chain loci during the early stages of B cell differentiation and in mature cells but not in plasma cells (Oettinger *et al.*, 1990; Yancopoulos *et al.*, 1990; Ma *et al.*, 1992). To clarify whether RAG expression participates in a new λ light chain expression, we assessed the expression of the RAG genes in both the HB4C5 cells and WGA-treated HB4C5 cells. Although HB4C5 cells and the WGA-resistant cells exhibit a plasma cell phenotype, they express the RAG-1 and RAG-2 genes (Figure 3). After a 7-week culture in the presence of WGA, the

RAG-1 and RAG-2 genes were still expressed in these cells.

Immunological identification of cell surface

The induction of lectin-derived light chain shifting has led to the hypothesis that cross-linking of receptor molecules on the surface membrane may control the reduction of the original light chain transcript. WGA can induce light chain shifting in HB4C5 cells, but not in Namalwa cells. When the supernatants were assayed by SDS-PAGE and immunoblotted with anti-human light chain/horseradish peroxidase (HPR), Namalwa cells were shown to only produce the original 32 kDa λ light chain even after stimulation with WGA for 7 weeks. A new light chain variant was detected from WGA stimulated HB4C5 cells after 4 weeks, and after 7 weeks even more variants were found (Figure 4A). To determine the relationship between the loss of the C5 λ chain production and WGA stimulation, we tested for sIg expression on HB4C5 and Namalwa cells in the presence of WGA by immunofluorescent staining. The sIg-positive subpopulation was present in both cell lines. However, after 7 weeks in continuous culture in WGA, a sIg-negative subpopulation was detected in HB4C5 cells (Figure 4B).

Serine phosphorylated protein in HB4C5 cells

To assess the relationship between protein serine phosphorylation in HB4C5 or Namalwa cells and light chain shifting, serine-phosphorylated proteins from these cells were analyzed by Western blot after they were isolated by immunoprecipitation with anti-phosphoserine. A 70 kDa serine phosphorylated protein was consistently detected in HB4C5 cells, but we could not detect the serine-phosphorylated 70 kDa protein around 40 min after stimulation (Figure 5A). WGA stimulated HB4C5 cells which already completed light chain shifting or Namalwa cells which has been shown to be unable to undergo light chain shifting did not exhibit this serine-phosphorylated 70 kDa protein (Figure 5B). Under the same conditions, we stimulated HB4C5 or Namalwa cells with ConA instead of WGA, we detected the same serine-phosphorylated 70 kDa protein in ConA-untreated HB4C5 but did not detect it in ConA-treated HB4C5. The 70 kDa serine phosphorylated protein was not detected in Namalwa cells whether or not they were treated with either WGA or ConA (Figure 5B). Moreover, we did not detect the serine-phosphorylated 70 kDa protein in the WGA-

resistant clones, which are also believed to be already completed light chain shifting (Figure 5C).

Discussion

In this report, we show that WGA-resistant clones derived from HB4C5 cells produce new λ light chains which replaced the originally expressed λ light chain. The complete inactivation of Ig rearrangement could be signaled by the expression of the Ig complex on the cell surface which occurs during B cell development (Manz *et al.*, 1988; Ma *et al.*, 1992). The capacity to continue Ig rearrangement is thought to be lost in mature or in plasma stage (Schatz *et al.*, 1992). The process, 'light chain shifting' has been shown in ConA resistant Ig-secreting cells (Tachibana *et al.*, 1996). In mature B cells, sIg can alter their characteristics through various mechanisms that include ongoing rearrangement at the Ig κ locus (Lewis *et al.*, 1982; Feddersen *et al.*, 1990) and somatic hypermutation (Bothwell *et al.*, 1981). The sIg-negative stage may be the interim before a new rearrangement occurs on the λ light chain locus. If the rearrangement is not successful, the sIg-negative condition remains as seen in clone WO. If the rearrangement is successful, a new λ light chain is produced as seen in the other WGA-resistant clones (W26, W29 and W42). This suggests that the new λ light chain is a product of a new VJ rearrangement on a previously excluded allele.

RAG-1 and RAG-2 genes have been found to encode for components of the V(D)J recombinase enzyme (Schatz *et al.*, 1989; Oettinger *et al.*, 1990; Van Gent *et al.*, 1995; Abe *et al.*, 1994). The two genes are expressed during the early stages of B cell differentiation, and have been found in mature cells (Oettinger *et al.*, 1990; Yancopoulos *et al.*, 1990; Mzi *et al.*, 1992), which suggest that control of RAG genes expression is complex. It has been postulated that loss of sIg expression not only interrupts a signal required to terminate RAG genes expression, but also trigger the up-regulation of RAG expression, which in turn stimulates secondary rearrangements thus reconstituting sIg expression (Stiernholm *et al.*, 1993). However, recently RAG genes have been shown to be constitutively expressed in human plasma cells (Tachibana *et al.*, 1996), suggesting that RAG expression is not directly involved in the sIg expression. Interestingly, RAG genes expression is also found in our new λ light chain producing clones-treated with WGA, which are in the plasma stage. Even though the RAG genes are

Figure 4. Analysis of the cell surface. (A) Expression of variant λ light chains were detected by immunoblot analysis with an anti-human λ light chain conjugate to supernatant from WGA of Namalwa and HB4C5 cells continuously stimulated for 0–7 weeks. (B) Flowcytometric analysis of sIg expression on Namalwa and HB4C5 cells using Immunofluorescent staining.

continuously expressed, they alone cannot bring about rearrangement to produce a new λ light chain. This suggests that the expression of a new λ light chain cannot terminate RAG genes expression, and RAG

genes expression is insufficient by itself but is necessary along with other factors for inducing production of the new λ light chain.

Figure 5. Production serine phosphorylated protein in HB4C5. (A) Induction of 70 kDa protein serine phosphorylation in HB4C5 cells stimulated with WGA for the indicated time periods. The lysate from HB4C5 cells was applied to each lane, followed by Western blotting with anti-phosphoserine. Phosphorylated protein bands are indicated by the arrow. (B) Detection of serine phosphorylated protein in Namalwa and HB4C5 before (-) and after (+) incubated with WGA, or with ConA for 40 min. (C) Detection of serine phosphorylated protein in WGA-resistant cells of HB4C5 cells.

There are many kinds of receptors including sIg on the surface of B cells, most of which act as signal transducers. Recently, cross-linking of B cell sIg receptors using an anti-Ig antibody has been shown to induce phosphoinositide hydrolysis of two second messenger molecules (Cambier *et al.*, 1987). One is inositol triphosphate, whose function is to increase intracellular Ca^{2+} . The other one is diacylglycerol, which in conjunction with Ca^{2+} , can activate protein kinase C, thus translocating the enzyme from the cytosol to the plasma membrane (Chen *et al.*, 1986). The sIg cross-linking has been found to result in the phosphorylation of serine/threonine residues which induce nuclear sequence-specific DNA binding proteins acting as third messengers to regulate transcriptional activation of gene expression in mammalian cells (Michell *et al.*, 1989). Many intracellular secondary messenger pathways that activate protein kinase C or increasing the intracellular calcium concentration in mature B cells are affected by ConA (Wamer *et al.*, 1991; Hesketh *et al.*, 1990). ConA is a mannose binding lectin while WGA is a hybrid-type glycoprotein composed of $\text{GlcNAc}\beta 1\text{-4Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc-Asn}$ (Goldstein *et al.*, 1965; Osawa *et al.*, 1987). WGA can induce light chain shifting in HB4C5 cells, but not in Namalwa cells. The new λ light chains variants were detected from the supernatant of HB4C5 cells only during continuous culture in the presence of WGA, but was not detected in Namalwa cells. The induction of lectin-derived light chain shifting suggests that receptor cross-linking on the surface membrane may regulate the reduction of the original light chain transcript. Furthermore, in our present results, a 70 kDa serine phosphorylated protein is observed in HB4C5 cells before an incubation with WGA, but is not observed in WGA-resistant clones or in Namalwa cells both before and after incubation. The same result for both cell lines was seen when ConA replaced WGA. This indicates that both the WGA-resistant clones of HB4C5 cells, which is thought to have completed light chain shifting, and the non-inducible Namalwa cells are unable to activate serine phosphorylation of 70 kDa protein. However, both WGA and ConA can reduce the level of this protein in HB4C5 cells. This indicates that in HB4C5 cells, the phosphorylation of the serine residue on the 70 kDa protein may be one of intracellular factors that work in conjunction with the RAG genes expression to induce light chain shifting upon stimulation with ConA or WGA. However, how the 70 kDa protein is phosphorylated has yet to be fully elucidated. More

studies are required to characterize the structure and function of this molecule.

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Address for correspondence: Sanetaka Shirahata, Graduate school of Genetic Resources Technology, Kyushu University, 6-10-1, Hakoza-ki, Higashi-ku, Fukuoka, 812 Japan.