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The role of protein phosphorylation in the regulation of class switch recombination

KANO TANABE[1]; RYUTARO KAJIHARA[2,3,*]

[1] Department of Medical Technology, Faculty of Health Science, Kumamoto Health Science University, Kumamoto, 861-5598, Japan
[2] Department of Biomedical Laboratory Sciences, Faculty of Life Sciences, Kumamoto University, Kumamoto, 862-0976, Japan
[3] Center for Immunotherapy, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263, USA

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Abstract: Antibody is an important part of adaptive immune system and is produced only by B cells. There are five main classes (IgM, IgD, IgG, IgA, IgE) and some subclasses in antibodies. IgM and IgD are produced by mature naïve B cells. On the other hand, IgG, IgA and IgE are produced by activated antigen-specific B cells via class switch recombination (CSR). CSR is the irreversible DNA rearrangement from upstream to downstream classes in immunoglobulin heavy chain genes. Co-stimulations of CD40 ligand (CD40L) and cytokines are required for induction of CSR by activating several transcription factors. These signal transduction pathways involve many protein phosphorylation. Phosphorylation or dephosphorylation of cellular protein is an important kind of post-translational protein modification in intracellular signal transduction. In the fact, more than one third of the intracellular proteins are said to be transiently phosphorylated in human. A protein kinase is an enzyme that catalyzes the addition of phosphate to substrate protein. Whereas, a protein phosphatase catalyzes the removal of phosphate from the substrate. This review focuses on the mechanism of CSR controlled by protein phosphorylation and dephosphorylation. We provide the role of protein kinase and phosphatase in the regulation of class switch recombination.

Introduction

Antibodies, which are a type of glycoprotein produced by B lymphocytes, play a critical role in the biophylactic mechanism. When B lymphocytes recognize specific antigens, they become activated, leading to the production and release of secretory immunoglobulins. Antibodies are classified into five isotypes, and some isotypes can be further divided into subclasses (Ballieux *et al.*, 1964; Ishizaka and Ishizaka, 1967; Ishizaka *et al.*, 1964; Terry and Fahey, 1964). Despite the varying functions and characteristics of each antibody class, all antibodies can be produced from the same B cells without changing their antigen specificities. Antibodies are divided into two parts, known as the variable region and the constant region, based on their structure and function (Hozumi and Tonegawa, 1976). The former is important for antigen recognition, while the latter defines the class of antibody. Irreversible gene rearrangement enables a change in the constant region, which is known as class switch recombination (CSR) (Sakano *et al.*, 1980). During class switching, there are many changes in intracellular molecules, and intracellular signal transduction occurs in various cascade formats, leading to final changes. Various post-translational protein modifications play a role in cellular modulation. Phosphorylation, in particular, is a reversible reaction involving numerous proteins. Enzymes directly involved in phosphorylation occupy approximately 2% of genomic DNA (Cohen, 1985; Krebs and Fischer, 1955).

This review will explain the fundamental mechanism of class switching, as well as discuss the important changes in controlling signal transduction during class switching, with a focus on protein phosphorylation. In recent years, kinase inhibitors have been used as molecularly targeted drugs for cancer treatment, and the control of phosphorylation has become increasingly important (Fabian *et al.*, 2005). Class switching is essential in a wide range of immune responses involving antibodies, including infections, autoimmune diseases, and allergies, and deficiencies of class switching can cause diseases such as hyper-IgM syndrome (Allen *et al.*, 1993; Aruffo *et al.*, 1993). Thus, understanding phosphorylation or dephosphorylation in class switching is fundamental in gaining new insights into disease regulation.

*Address correspondence to: Ryutaro Kajihara, kajihara@kumamoto-u.ac.jp
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Class Switch Recombination

Antibody structure and types

Antibodies play a key role in humoral immunity and have a Y-shaped structure where 2 H-chains and 2 L-chains, totaling 4 glycoproteins, are coupled by SS-bonding (Marquart *et al.*, 1980; Watt and Voss, 1979). Individual H-chains and L-chains can be divided into the variable region and the constant region, with the variable region being important for antigen recognition. Meanwhile, the constant region in H-chains defines the antibody class. Antibody functions vary depending on their class. Antibodies are classified into five isotypes, IgM, IgD, IgG, IgA, and IgE. IgG has four subclasses in both humans and mice, but the subclasses in humans are IgG1, IgG2, IgG3, and IgG4, whereas mice have IgG1, IgG2a (BALB/c) or IgG2c (C57BL/6), IgG2b, and IgG3 (Ballieux *et al.*, 1964; Fahey *et al.*, 1964; Grey *et al.*, 1971). IgA differs between humans and mice in terms of whether or not there are subclasses. In humans, subclasses IgA1 and IgA2 exist, but no subclasses exist for IgA in mice (Tab. 1).

The H-chain in antibodies is encoded by the long arm of chromosome 14 (14q32) in human, and the downstream variable region is composed of variable segments (V), diversity segments (D), and joining segments (J), known as the VDJ region (Hozumi and Tonegawa, 1976). The constant domain (CH) encodes the constant region. All sequences encoding each class exist in the constant region, and in each class, the layout of the I region, the switch (S) region, and the constant (C) region are arranged starting upstream. However, only Cδ that encodes IgD does not have the specific I and S regions (Lennon and Perry, 1998). In the C region of the other immunoglobulins, Cμ, Cδ, Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε, and Cα2 are sequentially encoded, and each of them has specific I and S regions upstream nearby.

Antibody production in naïve B cells

When producing antibodies, B cells do not randomly select the constant region of the H-chain, but they first transcribe and translate the nearest constant region downstream of the VDJ region. Therefore, in the case of naïve B cells, since Cμ exists in the immediate downstream of the VDJ region, the membrane form of IgM is expressed on the cell membrane as a B cell receptor (BCR). Unlike other classes, IgD does not have specific I or S regions, can only be translated when Cμ and Cδ are transcribed, and is regulated by alternative splicing. As a result, IgM and IgD are expressed on the membrane surface of mature B cells. This means that B cells can only produce IgM and IgD unless there are specific changes (Kluin *et al.*, 1995; Li *et al.*, 1994).

Molecular mechanism and control of class switching

When B cells recognize a specific antigen through a B cell receptor, they become activated and receive several stimuli from $CD4^+$ T cells, which recognize the antigen. These stimuli induce B cells to perform an irreversible gene rearrangement called class switching. This reaction removes certain genomic DNA to enable any class of antibodies to be produced. Class switching is referred to as a reaction that removes DNA between Sμ and the downstream S region (Hozumi and Tonegawa, 1976; Sakano *et al.*, 1980). This makes the C region, apart from Cμ and Cδ, the most adjacent to the VDJ region. For example, in the case where DNA between Sμ-Sε is removed, Cε becomes adjacent, and B cells start producing IgE (Fig. 1).

Once the coding DNA between the S regions has been cleaved off, B cells completely lose their ability to produce the corresponding class of antibodies existing in the removed DNA. In general, the binding of CD40L (CD145) expressed mainly on the surface of $CD4^+$ T cells to CD40 expressed on the cell membrane of B cells is important for B

TABLE 1

Class switching in human and mice

Human									
Isotypes	IgM	IgD	IgG				IgA		IgE
Subclasses	None	None	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	None
Heavy chains	μ	δ	γ1	γ2	γ3	γ4	α1	α2	ε
Responsible cytokines			IL-10		IL-10	IL-4, IL-13	IL-10+TGF-β		IL-4, IL-13
Transcription factors									STAT6, NF-κB, PAX5, PU.1

Mouse								
Isotypes	IgM	IgD	IgG				IgA	IgE
Subclasses	None	None	IgG1	IgG2a (BALB/c) IgG2c (C57BL/6)	IgG2b	IgG3	None	None
Heavy chains	μ	δ	γ1	γ2a (BALB/c) γ2c (C57B/6)	γ2b	γ3	α	ε
Responsible cytokines			IL-4, IL-13	IFN-γ (IgG2a)	TGF-β		TGF-β	IL-4, IL-13
Transcription factors			STAT6, NF-κB	T-bet (IgG2a)			RUNX3, R-SMAD, Co-SMAD	STAT6, NF-κB, PAX5, PU.1, AP1

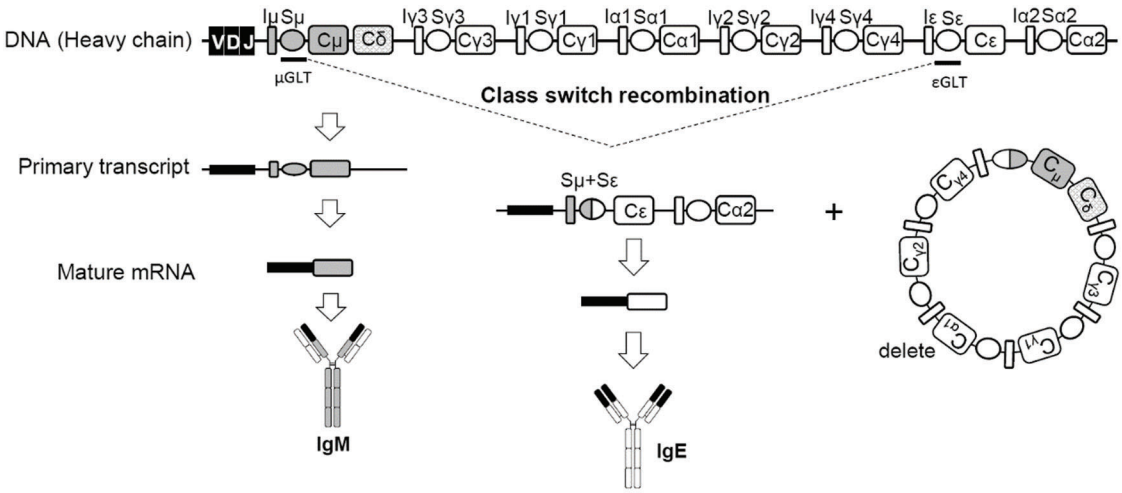

FIGURE 1. Immunoglobulin gene diversification and class switch recombination.
The heavy chain gene regions of human antibodies are shown. Downstream of the VDJ region, which encodes the variable region of the antibody, there are C regions that determine the class of each antibody. Except for Cδ, there are specific I and S regions in each upstream of the C region. Therefore, in naïve B-cell DNA, downstream of the VDJ is Iμ-Sμ-Cμ-Cδ, Iγ3-Sγ3-Cγ3, Iγ1-Sγ1-Cγ1, Iα1-Sα1-Cα1, Iγ2-Sγ2-Cγ2, Iγ4-Sγ4-Cγ4, Iε-Sε-Cε, and Iα2-Sα2-Cα2 in that order. These specific C regions encode IgM or IgD, IgG3, IgG1, IgA1, IgG2, IgG4, IgE, and IgA2, respectively. In naïve B cells, IgG, IgA, and IgE-encoding C regions are located downstream of the VDJ region that determines antigen specificity. Naïve B cells translate Cμ or Cδ, which are located next to the VDJ, and synthesize IgM and IgD. The I and S regions are located upstream of all the C regions except Cδ, and in the event of a class switching, a single-stranded RNA called germline transcript (GLT) is synthesized in each S region. The GLT triggers the class switching to proceed. When IgE class switching occurs, μGLT and εGLT are synthesized in the Sμ and Sε regions of the B cell, respectively, and the DNA region between the two S regions is completely removed. This results in the presence of Cε in the immediate downstream of the VDJ region, allowing the B cell to produce IgE. The removal of this DNA sequence is an irreversible reaction, making it impossible for the B cell to make antibodies of the class on the removed sequence.

cell activation (Gordon *et al.*, 1989; Gordon *et al.*, 1988; Hollenbaugh *et al.*, 1992; Noelle *et al.*, 1992; Paulie *et al.*, 1989). CD40-CD40L binding is a necessary stimulus for B cell survival as well as induction of class switching (Kawabe *et al.*, 1994; Marriott *et al.*, 1999). If there is a mutation in CD40 or CD40L, class switching is inhibited, presenting as a hyper-IgM syndrome (Allen *et al.*, 1993; Aruffo *et al.*, 1993; DiSanto *et al.*, 1993). In humans, most mutations are found in CD40L and rarely found in CD40 (Jhamnani *et al.*, 2018; Murguia-Favelaa *et al.*, 2017).

It is known that the direction of class switching is determined by cytokines. For example, in humans, interleukin (IL) -4 and IL-13 induce class switching to IgG4 and IgE, and IL-10 induces class switching to IgG1 and IgG3 (Fujieda *et al.*, 1996; Gascan *et al.*, 1991; Malisan *et al.*, 1996; Punnonen *et al.*, 1993). Furthermore, the application of IL-10 at the same time as transforming growth factor-β (TGF-β) induces class switching to IgA1 and IgA2 (Defrance *et al.*, 1992; Kitani and Strober, 1994; Tangye *et al.*, 2002; Zan *et al.*, 1998). It has been suggested that the transcription factor activated by the cytokine plays a significant role. However, how this interaction contributes to class switching remains unclear. As one of the reasons, some cytokines affect the regulation of different class switches and their actions overlap. This relates to transcription factor binding sites in the I region upstream of the S region that is specific to each CH domain. There are several transcription factor-binding sites in individual specifical I regions. The Iε region has many transcription factor binding sites such as signal transducer and the activator of transcription 6 (STAT6), nuclear factor-κB (NF-κB), PU.1, B-cell-specific activator protein (BSAP, also called as PAX5), CCAAT/enhancer-binding protein (C/EBP) 10, and activator protein 1 (AP1) (Delphin and Stavnezer, 1995; Dryer and Covey, 2005; Linehan *et al.*, 1998; Messner *et al.*, 1997; Mao and Stavnezer, 2001; Shen and Stavnezer, 2001; Stütz and Woisetschläger, 1999; Thienes *et al.*, 1997). Furthermore, binding sites for NF-κB are present at least in Iε, Iα, and Iγ1 domains. Also, it is not completely clear how NF-κB is involved in the specific regulation of class switches, as it has been reported that NF-κB deficiency affects not only IgE and IgA class switching but also class switching of the IgG subclass (Bhattacharya *et al.*, 2002). NF-κB is composed of p50, p52, p65 (RelA), c-Rel, and RelB, and has been variously reported, as the pathways it activates and the combinations it functions as a transcription factor vary depending on the cells and the stimuli. For example, overexpression of RelB suppresses IgG1 CSRs but not IgE CSRs under IL-4 stimulation (Bhattacharya *et al.*, 2002). In contrast, it has been reported that both STAT6 and NF-κB are crucial for IL-4-induced IgE CSR in humans (Messner *et al.*, 1997). It is possible that they are more complexly regulated by multiple transcription factors rather than being regulated by a single factor. This suggests that the transcription factor is important for class switching, but it is difficult to conclude that transcription factor regulates only one CSR in a specific way.

As a fact already known, B cells stimulated by CD40L and cytokines activate transcription factors bound to specific I regions through various signal transduction pathways. Upon transcription factor binding, a complementary single-stranded RNA called germline transcript (GLT) is

synthesized in the downstream area of the S region, which triggers class switching (Flanagan and Rabbitts, 1982; Islam *et al.*, 1994; Sakano *et al.*, 1980; Stavnezer-Nordgren and Sirlin, 1986; Wang *et al.*, 2009). The synthesized GLT forms a DNA-RNA hybrid with a complementary strand. Thus, a single-stranded DNA (ssDNA) is formed in the S region. An enzyme called activation-induced cytidine deaminase (AID) acts on the ssDNA, and cytosine in the ssDNA is replaced with uracil (Muramatsu *et al.*, 2000; Muramatsu *et al.*, 1999). Since this then results in the appearance of U-contained DNA strands, a base excision repair enzyme called uracil-N-glycosylase (UNG) recognizes U and eliminates it. Furthermore, apurinic/apyrimidinic endonuclease 1 (APE1) makes a cut in the position where U was and creates a nick (Guikema *et al.*, 2007; Masani *et al.*, 2013). Although the details of this reaction are still unknown, it also occurs where cytosines are: A DNA-RNA hybrid is formed, and ultimately a nick is created with both DNA strands in the S region, resulting in a double-strand break (DSB). The same reaction simultaneously happens with Sμ, and when a DSB occurs in two places of the S region, the arrangement in between is removed as a circular DNA. However, both cut sections in the S region are reconnected by the non-homologous end-joining pathway (NHEJ) in order for the targeted CH domain to be consequently positioned proximate to the VDJ region. The S region is very important in class switching. In mice, CD40 and IL-4 induce class switching to IgG1 or IgE. Sγ1 deficiency completely inhibits class switching to IgG1. In addition, IL-4 induces class switching to IgG1 or IgE as explained above, while Sγ1 deficiency increases class switching to IgE from approximately 3% to more than 40% (Matthews *et al.*, 2014; Misaghi *et al.*, 2010).

Furthermore, class switching occurs several times. One study using mice reported that class switching in which there was direct switching from IgM to IgE, as well as switching once to IgG1 and then to IgE in stages (Yoshida *et al.*, 1990). However, class switching to IgG1 never happens through IgE. The opportunity for class switching to IgG1 is lost since prior class switching to IgE creates a situation whereby Cγ1 has already been eliminated, due to the Cε coded IgE position in the downstream side of Cγ1 coded IgG1.

IgA class switch is triggered by stimulation with TGF-β in both humans and mice (Coffman *et al.*, 1989; Defrance *et al.*, 1992; Harriman *et al.*, 1996; Islam *et al.*, 1991; Nilsson *et al.*, 1991). TGF-β-deficient or TGF-β receptor (TGFβR) II-deficient mice have lower IgA levels, indicating that stimulation from TGF-β is important for IgA class switching (Cazac and Roes, 2000; van Ginkel *et al.*, 1999).

Class switching to IgG depends on specific cytokines and transcription factors; however, the exact mechanism of class switching is still not clear. In mice, cytokines that induce class switching to IgG1, IgG2a, and IgG2b have been identified. For example, class switching to IgG1 is induced by the stimulation of IL-4/IL-13 (however, the regulatory mechanism of class switching to IgE or IgG1 induced by the same stimulus is not well understood). IgG2a and IgG2b are reported to be induced by interferon-γ (IFN-γ) and TGF-β, respectively (Snapper *et al.*, 1988; Deenick *et al.*, 1999). Also, class switching of human IgG4 is induced by IL-4/IL-13 stimulation as in mice IgG1 (Cocks *et al.*, 1993; Gascan *et al.*, 1991). This stimulus also induces a class switching to IgE, which is the same as in mice. Furthermore, human IgG1 and IgG3 are induced by IL-10 (Briere *et al.*, 1994; Malisan *et al.*, 1996). The transcription factors involved in these processes are poorly understood, and it is thought that T-bet is required for IgG2a induction, as the deletion of T-bet represses IFN-γ-induced IgG2a (Peng *et al.*, 2002).

Although the full picture of regulation by transcription factors is not yet clear, the fact that serum IgG1 and IgE are severely impaired in B cells of STAT6 knockout (KO) mice and that IFN-γ-induced IgG2a class switch is inhibited in T-bet-deficient B lymphocytes suggests that transcription factors activated by each cytokine contribute to the specificity of class switching.

Phosphorylation and Dephosphorylation

Phosphorylation or dephosphorylation of cellular protein is an important kind of post-translational protein modification in intracellular signal transduction. The existence of phosphorylation changes protein behavior (Ardito *et al.*, 2017). Protein phosphorylation/dephosphorylation is a reversible reaction. Protein kinases catalyze phosphorylation while protein phosphatases catalyze dephosphorylation. Phosphorylation in eukaryotes occurs when a phosphate group in ATP is transferred and added to the hydroxyl group of serine, threonine, and/or tyrosine residues. Protein phosphorylation/dephosphorylation impacts a wide variety of actions such as protein synthesis and regulation, protein-protein interactions, cell division, cellular differentiation, and apoptosis (Ardito *et al.*, 2017; Hubbard and Cohen, 1993). In addition, phosphorylation triggers ubiquitination. More than 1/3 of intracellular proteins are phosphorylated, in which serines, threonines, and tyrosines are respectively phosphorylated at 86.4%, 11.8%, and 1.8%, indicating that the majority of phosphorylation occurs at serine/threonines (Olsen *et al.*, 2006). The human genome contains more than 500 kinases, approximately 2% of the human genome.

The majority of kinases can be classified into serine-threonine kinases and tyrosine kinases, and they are distributed and function in the cytoplasm and the nucleus. In addition, receptor tyrosine kinases are expressed on the cell membrane. Tyrosine kinases can be divided into receptor tyrosine kinases (RTKs), which reside at the cell membrane, and non-RTKs (NRTKs), which exist in the cytoplasm. RTK plays an important role in various biological activities, including cell proliferation, differentiation, and survival. Therefore, gain-of-function mutations in RTK are associated with diseases, such as cancer and leukemia (Greenman *et al.*, 2007; Khan *et al.*, 2017; Stephens *et al.*, 2005; Zhou *et al.*, 2017). In contrast, there are four Janus kinase (JAK) isoforms: JAK1, JAK2, JAK3, and TYK2, which are typical NRTKs, and different JAKs are specifically bound to various cytokine receptors. Activated by cytokine stimulation, a JAK first phosphorylates tyrosine residues of the receptor. Then, STAT, a transcription factor with an SH2 domain, is recruited and binds to the phosphorylated tyrosine residue

of the receptor via SH2. JAK then phosphorylates the tyrosine residue of STAT bound to the receptor. As a result, activated STATs form a dimer, which leaves the receptor and translocates to the nucleus, where it functions as a transcription factor. This is called the JAK-STAT signaling pathway, which is important for the immune response. For example, stimulation with IFN-γ activates STAT1 via JAK1/JAK2 and exhibits an antiviral effect. Various other combinations exist: IL-4 stimulation activating JAK1/JAK3-STAT6 and IL-6 stimulation activating JAK1/JAK2/TYK2-STAT1/STAT3. Similarly, Abl, another NRTK, localizes and binds to actin filaments in the cytoplasm and chromatin in the nucleus. Breakpoint cluster region-Abelson 1 (BCR-ABL1) is a well-known genetic mutation in chronic myelogenous leukemia (CML), and unlike the original ABL, its tyrosine kinase activity is constitutively active. This activates various intracellular signaling pathways involved in cell proliferation, transformation, and inhibition of apoptosis, leading to the development of CML (McWhirter *et al.*, 1993). Furthermore, an insulin receptor is a tyrosine kinase; many tyrosine kinases play important roles in cell division, migration, and survival (Hunter and Sefton, 1980; Kasuga *et al.*, 1982; Tonks *et al.*, 2002; Ushiro and Cohen, 1980; Wilks *et al.*, 1991). There are many kinds of serine-threonine kinases, such as mitogen-activated protein kinase (MAPK), protein kinase A (PKA), protein kinase C (PKC), and Ca^{2+}/calmodulin-dependent protein kinases (CaMKII), the substrates of which are composed of transcription factors and cell cycle regulators. Abnormalities in these kinases are related to diseases (Aronowski and Grotta, 1996; Aronowski *et al.*, 2000; Chen *et al.*, 2003). For example, X-linked agammaglobulinemia is caused by a mutation in the Bruton tyrosine kinase (BTK) gene that inhibits the maturation of B cells (Ponader and Burger, 2014). BTK is a cytoplasmic NRTK and belongs to the Tec kinase family. BTK is widely expressed on B cells (except plasmatic cells), monocytes, granulocytes, platelets, etc., but it is particularly important for the differentiation of pre-B cells to immature B cells (Tsukada *et al.*, 1993). Downstream of the pre-B cell receptor, Lyn, Syk, SLP65, BTK, and PLCγ2 are activated to induce B cell differentiation. Therefore, in X-linked agammaglobulinemia, which results from a genetic mutation in the BTK gene, B cells cannot mature from pre-B cells, and eventually, antibodies cannot be produced as the number of mature B cells decreases (Conley, 1985). In mature B cells, BTK also acts downstream of the B cell receptor and induces the activation of the transcription factor NF-κB, which is important for cell survival and proliferation. An association with MAPK and phosphoinositide 3-kinases (PI3K) has been reported in asthma, and MAPK is thought to play an important role in the pathogenesis of the disease, as its inhibition has been reported to suppress allergic airway inflammation (Liu *et al.*, 2008; Sousa *et al.*, 1999). Indeed, p38 MAPK is strongly activated on alveolar macrophages in some asthmatics (Bhavsar *et al.*, 2010; Wuyts *et al.*, 2003). PI3K (the details of the molecular mechanism will be described later) causes bronchodilation, and PI3K inhibitor suppresses eosinophil accumulation in asthmatic mice, suggesting that PI3K is important in the pathogenesis of asthma (Duan *et al.*, 2005; Koziol-White *et al.*, 2016). Also, kinase has been reported to be associated with many cancers, and epidermal growth factor receptor (EGFR), an RTK, has been linked to various cancers such as non-small cell, colon, and pancreatic cancers, and abnormal activation by EGFR mutation causes cancer cell growth (Greenman *et al.*, 2007; Khan *et al.*, 2017; Stephens *et al.*, 2005; Zhou *et al.*, 2017). Furthermore, serine/threonine kinase AKT2, which is important for cell proliferation and survival, is known to be overexpressed in pancreatic and ovarian cancers (Cheng *et al.*, 1992; Miwa *et al.*, 1996). Thus, kinase inhibitors, such as EGFR and Bcr-Abl inhibitors, are used as molecularly targeted drugs for cancer treatment (Gambacorti-Passerini *et al.*, 1997; Shepherd *et al.*, 2005). Inhibitors of these kinases are now being used as anticancer drugs. Compared to kinases, there are fewer phosphatases; the human genome contains approximately 200 phosphatases (Sacco *et al.*, 2012). Like kinases, phosphatases are divided into serine-threonine phosphatases and tyrosine phosphatases.

Serine-threonine phosphatases are further classified into the phosphoprotein phosphatase (PPP) family and the metallo-dependent protein phosphatase (PPM) family; the former contains protein phosphatase (PP) 1, PP2A, PP2B, PP4, PP5, PP6, and PP7, and the latter contains PP2C (Johnson, 2009; Shi, 2009). The PPP family members possess a catalytic subunit and regulatory subunits. PPM family members are composed of a monomer without a regulatory subunit and contain a catalytic domain and a domain regulating substrate specificity. PPM family members also have Mn^{2+} or Mg^{2+} -dependent functions. PPP family members are divided into various groups based on chemical properties, and among them, PP2A, PP4, and PP6 are highly homologous and known as the PP2A family. The PP2A family has subunit A as a foothold and core enzymes consisting of subunit C, which changes the substrate and localization depending on which regulatory subunit B is bound to (holoenzyme). This makeup of phosphatases enables specific regulation patterns to dephosphorylation (Brautigan, 2013; Lillo *et al.*, 2014). Studies of the PP2A family highlight the involvement of PP2A in autoimmune diseases. For example, in systemic lupus erythematosus (SLE), there is an abnormal response to the T-cell receptor (TCR)-mediated stimulation of T cells and a loss of the CD3ζ chain, which induces the expression of the Fc receptor γ (FcRγ) chain, ultimately leading to abnormal T cell activation (Liossis *et al.*, 1998; Nambiar *et al.*, 2001). It has been reported that PP2A expression and activity are increased in T cells of SLE patients, and CD3ζ expression, as well as IL-2 production, are suppressed (Katsiari *et al.*, 2005; Sunahori *et al.*, 2011). Moreover, mice lacking PP2A in peripheral blood T cells showed a decrease in Th17 cells, indicating that PP2A is important for Th17 cell differentiation (Xu *et al.*, 2019). Th17 cells have been reported to be associated with autoimmune diseases, such as Crohn's disease and rheumatoid arthritis, suggesting that PP2A is associated with various autoimmune diseases as well as SLE.

Cyclin-dependent kinases (CDK) were originally discovered as kinases that regulate the cell cycle, but they are now known to be involved in the regulation of transcription factors and metabolism as well as the cell

cycle. CDK1, CDK2, CDK4, and CDK6 are involved in the regulation of the cell cycle, and CDK1 is important in the transition from the G2 to the M phase of the cell cycle. CDK1 inactivation has been shown to lead to the induction of apoptosis. CDK4/CDK6, cyclin D complex, CDK2, and cyclin E complex are involved in the transition from G1 to S phase, called the R-point, which is important for cell proliferation, and CDK4/6 inhibitors are used as anticancer drugs (Mukhopadhyay *et al.*, 2002; Wolter *et al.*, 2001).

Kinase and Class Switching

Class switching establishment and regulation is via various, intricate signal transduction pathways. Kinases play roles in class switching modulation/regulation. PI3K has four subclasses known as IA, IB, II, and III and phosphorylate the third position of the inositol ring of PtdIns (4, 5,) P2 (PIP2) to produce PtdIns (3, 4, 5,) P3 (PIP3), which acts as a second messenger within cells. In particular, Class IA PI3K plays an important role in signal transduction. PI3Kα, PI3Kβ, and PI3Kδ belong to Class IA PI3K and consist of each catalytic subunit (p110α, p110β, and p110δ) in combination with regulatory subunits (p85α, p55α, p50α, p85β, and p55γ). PI3Kδ consists of the catalytic subunit p110δ and a regulatory subunit, distributed in the blood and the immune system, and is important for the activation of T and B cells (Okkenhaug *et al.*, 2014).

The autosomal dominant gain-of-function mutation in p110δ coding PIK3CD induces hyperactivation on PI3Kδ, which causes immunodeficiency accompanied by hyper-IgM syndrome due to a failure of class switching (Lucas *et al.*, 2014). This disease is called activated PI3Kδ syndrome (APDS) and is related to aging T cells. Since class switching in mice splenic B cells is enhanced when treated with a PI3K inhibitor, LY294002, or a PI3Kδ inhibitor, IC87114, it is suggested that activated PI3K suppressively controls class switching (Omori *et al.*, 2006). The activation of PI3K in B cells is known to be important for B-cell differentiation and survival and to be involved in the transcription of different molecules depending on the stages of differentiation in B cells (Omori and Rickert, 2007). In peripheral blood B cells, PI3K/Akt (the serine-threonine kinase; also known as protein kinase B) activity, such as that induced by CD19 stimulation, is important for cell survival, but the transcription factor forkhead box protein O1 (FOXO1), it is transcriptional regulation downstream of PI(3)K, is not thought to be involved in the mechanism of B-cell survival (Dengler *et al.*, 2009). However, FOXO1 regulated by PI3K/Akt signaling has been reported to repress L-selectin expression and class switching in response to FOXO1 reduction (Dengler *et al.*, 2009). This means that FOXO1 may contribute to L-selectin and AID expression in peripheral blood B cells. This is supported by the fact that the generation of a FOXO1 T24A mutant whose activity is not suppressed by Akt1/2 increases AID expression and class switching. As a result of PI3K activation, PIP3 produced by the phosphorylation of PIP2 induces subsequent phosphorylation of PDK1, which leads to the activation of Akt that inhibits the transcription factor FOXO1. Since FOXO1 has been reported to exacerbate the expression of AID genes, activated PI3K may ultimately inhibit AID (Dengler *et al.*, 2008; Omori and Rickert, 2007) (Fig. 2). However, in addition, to the FOXO1-mediated pathway, several other transcription factors, such as NF-κB, which is a downstream molecule of CD40L stimulation, are involved in the activation of AIDs. Thus, although we have introduced PI3K/Akt-mediated production of AIDs via FOXO1 regulation, it is difficult to explain the specificity using this activation pathway alone.

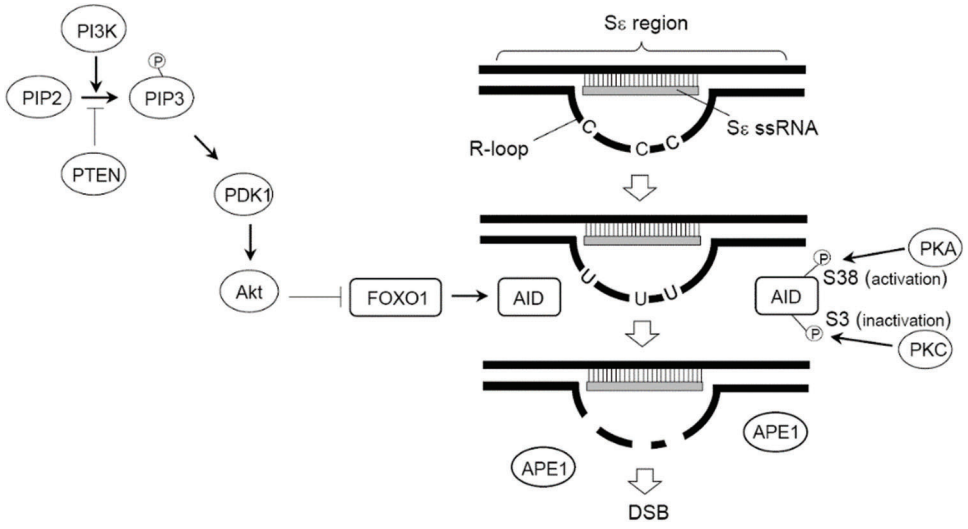

FIGURE 2. CSR regulated by AID.
In class switching, a complementary ssRNA, GLT, is synthesized in one strand of the target S regions. That results in the formation of DNA-RNA hybrids in the S region. Thereafter, AID targets the other strand of DNA that does not form DNA-RNA hybrids and converts its cytosines to uracils. The converted uracil of ssDNA is removed by APE1, resulting in a double-strand break (DSB) and a class switching. The regulation of AID involves PI3K as an indirect control of kinase. PI3K represses the transcription factor FOXO1 via the PI3K/Akt pathway. FOXO1 upregulates AID production and, therefore, PI3K activation has an inhibitory effect on CSR. On the other hand, PKA and PKC directly regulate AID by phosphorylating S38 and S3 in AID, respectively. Phosphorylation of S38 activates AID, and phosphorylation of S3 inhibits its activation.

AID is an essential enzyme in class switching and is also important for somatic hypermutation (SHM) during class switching. It has been reported that AID deficiency causes hyper-IgM syndrome (Revy *et al.*, 2000). Phosphorylation on serine 38 (S38) in AID is critical for class switching, and in mice, class switching was inhibited when a mutant in which S38 in AID was substituted with alanine (S38A) was prepared (Cheng *et al.*, 2009). A serine-threonine kinase, protein kinase A (PKA), is responsible for this phosphorylation (Basu *et al.*, 2005; Chen *et al.*, 2015; McBride *et al.*, 2006). Furthermore, phosphorylation by protein kinase C (PKC) on serine 3 (S3) in AID is also considered significant for class switching (Gazumyan *et al.*, 2011). Since class switching increases when an excessive quantity of mutants, where S3 in AID is substituted with alanine (S3A), are expressed in AID-deficient B cells, phosphorylation on S3 suppressively controls class switching (Gazumyan *et al.*, 2011) (Fig. 2).

When TGF-β binds to TGFβRII, it associates with TGFβRI to form a heterotetramer in IgA class switching. Both TGFβRI and TGFβRII have serine/threonine kinase activation sites, and TGFβRII phosphorylates and activates TGFβRI. Activated TGFβRI is activated by the phosphorylation of TGFβRI-bound receptor-activated Smad (R-SMAD) to form a multimer with common mediator Smad (Co-SMAD). This multimer is transferred to the nucleus, where it cooperates with runt-related transcription factor 3 (RUNX3) to induce a class switch to IgA (Hanai *et al.*, 1999; Lin and Stavnezer, 1992; Shi and Stavnezer, 1998; Zhang and Derynck, 2000). In particular, RUNX3 is considered to be an important transcription factor in the IgA class switch because TGF-β and retinoic acid (RA)-stimulated production of αGLT is completely inhibited in RUNX2/3 KO mice (Watanabe *et al.*, 2010) (Fig. 3A).

With regard to switching to a specific antibody class, it has been reported that TANK Binding Kinase 1 (TBK1) suppressively controls IgA class switching (Jin *et al.*, 2012). TBK1 is known as a kinase that induces the production of type 1 IFN by phosphorylating transcription factor IRF-3. TBK1-deficient mice, specifically deficient in B cells, present increased IgA production and pathological symptoms similar to nephropathy. TBK1 controls IgA class switching byinhibiting activation on the NF-κB alternative pathway (Fig. 3A). Specifically, TBK1 phosphorylates S862 in NIK that is important for the NF-κB alternative pathway, which facilitates the decomposition of NF-κB-inducing kinase (NIK) and inhibits the activating pathway.

JAK is involved in IgE class switching. In particular, JAK3 is highly expressed in lymphocytes and plays an important role in the signal transduction of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors using a common γ-chain (Johnston *et al.*, 1994). IL-4 plays a significant role in IgE class switching, and the IL-4 receptor forming a heterodimer upon activation further activates JAK1/3. The activated JAK1/3 phosphorylates transcription factor STAT6 and forms a dimer. The activated STAT6 is then transferred to the nucleus and induces IgE class switching by binding with the Iε promotor (Jiang *et al.*, 2000) (Fig. 3B). STAT6 is known as an important transcription factor in IgE class switching since its deficiency impairs IgE production (Goenka and Kaplan, 2011). However, STAT6 is not atranscription factor that acts only on the I domain of IgE, as it also induces a class switch to IgG1 in mice. However, STAT6 is known to be activated in B cells by stimulation by

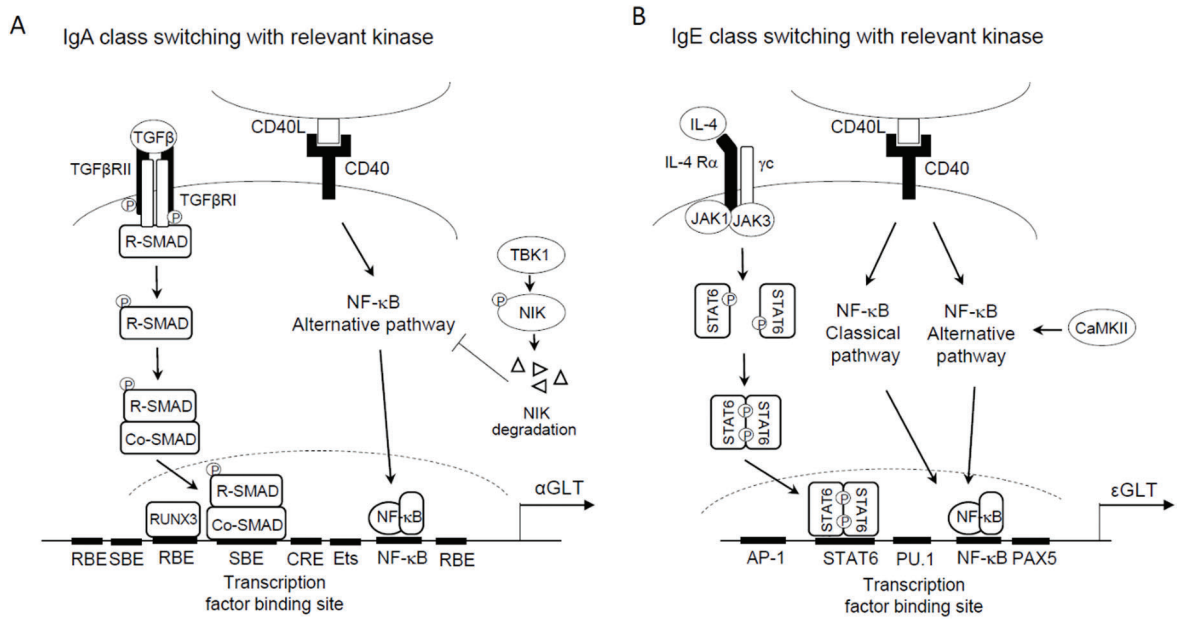

FIGURE 3. Class switching with relevant kinase.
(A) In IgA class switching, the tyrosine kinase activity of TGFβRI/II causes R-SMAD to be phosphorylated and bind to Co-SMAD, which functions as a transcription factor. The NF-κB alternative pathway downstream of CD40 is also involved in IgA CSR. TBK1 inhibits IgA CSRs by phosphorylating NIK, which is important for the NF-κB alterative pathway, leading to its degradation. (B) Stimulation of IL-4 activates JAK1/JAK3 and phosphorylates STAT6. The complex of STAT functions as a transcription factor. Also, downstream of CD40, NF-κB classical and alternative pathways are activated and contribute to IgE CSR. It is possible that CaMKII promotes NF-κB alternative pathway by CD40L stimulation.

IL-4 and IL-13 and is thought to be a highly specific transcription factor for the class switching induced by these stimuli. The importance of STAT6 for IgE and IgG1 CSRs is also demonstrated by the fact that B cells from STAT6-deficient mice showed the lack of the production of εGLT and γ1GLT by IL-4 stimulation (Shimoda *et al.*, 1996; Linehan *et al.*, 1998). It is also known that JAK1/TYK2-STAT1, STAT3, and STAT5 are activated downstream of IL-10, and these JAK-STAT pathways may also play an important role in the production of IgG1 and IgG3, as IL-10 is known to induce human IgG1 and IgG3 class switching (Briere *et al.*, 1994).

We have reported that serine-threonine kinase CaMKII is important for IgE class switching (Tanabe *et al.*, 2016). CaMKII has four subtypes known as CaMKIIα, CaMKIIβ, CaMKIIγ, and CaMKIIδ, and they are all activated in a complex bound with calmodulin (CaM) in accordance with an increase in the intracellular Ca^{2+} concentration level. CaMKII characteristically maintains activity through autophosphorylation, even when Ca^{2+}/CaM is isolated. CaMKII is widely known to play a vital function in memory related to the central nervous system, and learning disorders occur in CaMKII knockout mice (Silva *et al.*, 1992). Furthermore, CaMKII is linked to arrhythmia and cardiac insufficiency (Rokita and Anderson, 2012; Swaminathan *et al.*, 2012). Although CaMKII is expressed in lymphocytes, its function has not yet been clarified. We found that upon treatment with CaMKII inhibitor KN-93, while IgE class switching is induced by stimulating mouse B cell strain M12 and mouse splenic B cells, with IL-4 and an anti-CD40 antibody, the index for IgE class switching, εGLT, is suppressed. Furthermore, it was suggested that suppression by CaMKII enhances IgE class switching while the NF-κB alternative pathway is activated by CaMKII, facilitating the ubiquitination of tumor necrosis factor receptor-associated factor 3 (TRAF3) molecules that are inhibitors in the NF-κB alternative pathway (Tanabe *et al.*, 2016) (Fig. 3B).

Phosphatase and Class Switching

There are fewer studies on phosphatases and class switching than studies on kinases and class switching; however, some key phosphatases have been identified. PP4 of the PP2A family is a serine-threonine phosphatase known to be involved in microtubule growth, DNA repair, apoptosis, and tumor necrosis factor-α (TNF-α) signaling (Shui *et al.*, 2007). In a study using B cell-specific PP4 deficient mice, PP4 was demonstrated to be important for B-cell differentiation, the formation of germinal centers, and class switching (Chen *et al.*, 2014; Su *et al.*, 2013). Class switching is inhibited by inducing DNA replication stress under PP4 deficiency (Chen *et al.*, 2019). PP6 is also a serine-threonine phosphatase belonging to the PP2A family. PP6c, PPP6R1 (SAPS1), PPP6R2 (SAPS2), and PPP6R3 (SAPS3), consisting of PP6, are known for the large number of mRNA expressed in immune cells and tissues (Ziembik *et al.*, 2017). In particular, there is a very abundant expression of PP6c mRNA in B cells, natural killer (NK) cells, and dendritic cells. PP6c protein expression in lymphocytes is also abundant. Regarding the relationship between PP6 and class switching, it has been reported that while there is no direct control over B cells, PP6 does affect T cells, which control class switching (Ziembik *et al.*, 2017). This was discovered when serum IgE concentration increased by 100 to 1000 times when mice deficient in SAPS1, which is a PP6 control subunit, were compared with *Ppp6r1* f/f mice and C57BL/6 mice. Thus, IL-4 producing $CD4^+$ T cells are significantly increased in SAPS1-deficient mice. PP6c protein expression in lymphocytes is also abundant. Regarding the relationship between PP6 and class switching, it has been reported that, although there is no direct control on B cells, PP6 does affect T cells, which control class switching. Furthermore, an excessive expression of phosphatase and tensin homolog (PTEN), known as a tumor suppressor gene, enhances class switching (Chen *et al.*, 2015). It has also been reported that class switching is suppressed in PTEN- deficient mice, leading to hyper-IgM syndrome (Omori *et al.*, 2006). PTEN is considered to retain a normal balance in class switching through inhibition of Akt signal transduction pathways, while dephosphorylating PIP3 produced by activated PI3Kδ, resulting in PIP2 (Fig. 2).

Conclusion

In this review, we have presented the regulation of antibody class switching, which plays a vital role in biophylaxis, via phosphorylation. Although there have been quite a few findings that revealed how class switching is controlled, many points remain to be clarified, including the relationship between cytokines and transcription factors. Although not outlined in this article, the class switching control mechanism by infectious diseases cannot be underestimated. For example, it is well known that the bacterial component lipopolysaccharide (LPS) induces a class switch in a T-cell-independent manner (Deenick *et al.*, 1999; Stavnezer *et al.*, 1988). There have also been reports of class-switching control mechanisms by specific bacterial, viral, and parasitic infections. A holistic interpretation that includes these factors is inherently important for the elucidation of mechanisms of class switching. In this review, there have been several reports on the control by kinases, but only a few reports on the control by phosphatases. However, as phosphorylation is reversible, phosphatases may likely be more involved in the control of class switching than previously thought. Phosphorylation and dephosphorylation of proteins are reversible post-translational modifications, and their status is changing every second. This fact makes it difficult to elucidate the mechanism involving protein phosphorylation and dephosphorylation, as they are easily influenced by stimulation time, sample collection method, and detection system. Also, many phosphate-specific antibodies have low sensitivity, which also makes detection difficult. Furthermore, low concentrations of phosphorylated proteins also create the need for enrichment. Therefore, there are still many unclear points about the detailed mechanism. However, research in protein phosphorylation is one step ahead in the field of oncology, and the use of kinase inhibitors as molecular targets is becoming more widespread. Although there are still many unknowns in the

regulation of class switching, this field has great potential for the development of new allergy drugs and efficient methods of inducing the production of antibodies using vaccines if research is carried out from the perspective of the control of protein phosphorylation.

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