Vasoactive Intestinal Peptide Induces Sα/Sμ Switch Circular DNA in Human B Cells

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Abstract

Vasoactive intestinal peptide (VIP), a major neurotransmitter of peripheral nerves, has been suggested to function in host defense by regulating local human immune function. Indirect evidence has been marshaled that VIP can function as a switch factor for IgA in human Ig isotype recombination. In this study we directly tested the ability of VIP to function as a factor driving human B cells into IgA producing cells by assessing its ability to induce switch circular DNA representing direct μ to α switching. In addition we determined the generation of α germ-line transcripts and measured the level of IgA protein produced. Stimulation with VIP and CD40 mAb induced IgA production by human IgD^+ B cells while VIP or CD40 alone failed to do so. Stimulation of purified IgD^+ B cells with VIP plus CD40 mAb induced generation of switch circular DNA representing in vitro driven isotype switching from μ to α. CD40 mAb alone induced α germ-line transcripts but not IgA switch circles. Thus VIP, a neurogenic factor, can induce α-specific switching in CD40-activated human B cells and may thereby play an important role in directing the humoral immune response at mucosal surfaces. (*J. Clin. Invest.* 1996. 98:1527–1532.) Key words: vasoactive intestinal peptide • switch circular DNA • α germ-line transcript • IgA isotype switching • transforming growth factor-β

Introduction

Immunoglobulin (Ig) isotype switching is the process whereby B cells initially expressing IgM and/or IgD on their surface switch to other Ig heavy chain loci and thereby provide antibodies with different effector functions but the same antigen specificity (1, 2). Molecular analyses of Ig isotype switching support a model of alignment of two switch region sequences which are 5′ to each heavy chain region followed by looping out and deletion of the intervening DNA (3). Such excised switch circular DNA has been directly demonstrated in murine (4–8) and human B cells (9, 10). Definitive proof of the ability of a signal to function as an isotype switch factor requires demonstration of it's ability to induce the appropriate deleted switch circular DNA.

TGF-β has been established as a true switch factor for IgA in murine (11) and human systems (12, 13). However, the role of TGF-β in mucosal-associated lymphoid tissue with its high level of IgA production and the large number of IgA-committed B cells is unknown. Low molecular weight B cell growth factor, derived from the supernatant of PHA-activated T cells, also induces IgA production in PWM-stimulated human B cells by a mechanism that apparently does not involve TGF-β, but otherwise this activity is not characterized (14).

Vasoactive intestinal peptide (VIP),[1] a major neuropeptide of the central and peripheral nervous system, has been shown to induce IgA production by human IgA^- B cells (15), fetal B cells, pre-B cells (16), and lamina propria mononuclear cells (17). These finding suggested that VIP might function as a role of switch factor for IgA. To directly test this hypothesis, we tested purified human IgD^+ B cells for their response to VIP. In the presence of CD40 mAb–driven α germ-line transcripts, VIP induced Sα/Sμ switch circular DNA, providing direct evidence of VIP-driven μ to α switching and demonstrating that it functions as an IgA switch factor for human B cells.

Methods

Reagents. CD40 mAb, G28-5, was a gift from Dr. E.A. Clark (University of Washington, Seattle, WA). Mouse (IgG) anti–human IgD mAb was made from the δTA4-1 hybrid cell line obtained from American Type Culture Collection (ATCC HB70, Rockville, MD). VIP and somatostatin (SOM) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and cell cultures. Human B cells were isolated from fresh human tonsil as described previously (9, 10). Purified B cell consisted of > 95% $CD19^+$ cells and < 1% $CD3^+$ cells (10, 18). Resting B cells (IgD^+ B cell) were positively isolated by anti–human IgD mouse mAb and goat anti–mouse IgG coated magnetic beads (Dynal, Great Neck, NY) (10). This enriched population consisted of 95% IgD^+ B cells and 4% IgD^- B cells. IgD^+ B cells (1×10^6 cells/ml) were cultured in RPMI supplemented with 2 mM of glutamine, 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The stimulated cells were harvested for isolation of circular DNA on day 7 and extraction

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1. *Abbreviations used in this paper:* SOM, somatostatin; VIP, vasoactive intestinal peptide.

of RNA on day 5, which should be optimal for generation of switch circular DNA and for expression of germ-line transcripts (9, 10).

Ig measurements. Total immunoglobulin levels were measured by ELISA as described previously in the supernatant of cells cultured for 10 d (18, 19).

Isolation of circular DNA. Circular DNA was prepared from the alkaline lysate of total cultured cells (1×10^7 cells) as described previously (9, 10, 20). The precipitated circular DNA was digested with EcoRI to linearize circular DNA and treated with RNase for 2 h at 37°C to remove RNA. The Sμ, Sα1, and Sα2 regions have no EcoRI sites. The EcoRI-linearized circular DNA provides for far superior amplification compared with undigested supercoiled DNA (9). After linearization, the treated circular DNA was used as PCR templates.

RNA extraction and reverse transcription. Total mRNA was obtained from cultured B cells (1×10^7 cells) using Trizol reagent (Life Technologies, Gaithersburg, MD) (21) and then digested with DNase I (Sigma) to remove contaminating DNA. 2 μg of total RNA was reverse-transcribed to cDNA using oligo $(dT)_{15}$ (Boehringer-Mannheim Co., Indianapolis, IN) as primer and mouse Moloney leukemia virus reverse transcriptase (Life Technologies).

PCR strategy. Nested primer PCR runs for switch fragments were performed on prepared circular DNA as described before (9, 10). Briefly, PCR was run in a 50 μl/reaction with 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 5% DMSO, 50 pmol primer/reaction, and 2.5 U of *Taq* polymerase. The PCR assays were carried out as 1 min denature (94°C), 1 min annealing (65°C on the first PCR, 68°C on the second PCR), and 2 min extension (72°C) for 40 cycles. The primer sequences are as follows: first round PCR: A1, ACAGCAGCCCTCTTGGCAGGCA, and M1, GGTGAGTGTGATGGGGAACGCAGTGTA; second round PCR: A2, CGTGAGGGTGGACCTGCCATGA, and M2, CCATCTATGTCCAACAAGATCATGA.

The μ primers (M1 and M2) are located 3′ from the end of Sμ (22) and the α primers (A1 and A2) are located in the Iα region (23, 24), which is just 5′ to the Sα sequence. The PCR assay for α germ-line transcripts was carried out at 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for 40 cycles. The primers were A1 (above) and Cα1, CGTGAGGTTCGCTTCTGAACCTAAGAG (25, 26).

Cloning and screening. The PCR products of switch circular DNA were cloned by TA cloning (Invitrogen, San Diego, CA). Positive clones were selected by hybridization with an Sμ probe labeled with [^{32}P]αCTP by random priming (10). Hybridization was conducted according to the standard methods with hybridization in 50% formamide performed at 42°C and the washing temperature for blots at 68°C.

DNA sequencing. Nucleotide sequences were determined by the standard dideoxyl chain termination method using a DNA sequencing kit (Sequenase version 2.0; Amersham, Arlington Heights, IL).

Results and Discussion

VIP induced IgA production by IgD$^+$ B cells. Highly purified IgD$^+$ B cells were cultured with various concentrations of VIP in the presence of CD40 mAb (0.1 μg/ml) for 10 d, and the IgA level in the supernatant was measured by ELISA (Fig. 1). Stimulation of IgD$^+$ B cells with VIP ($\geq 10^{-10}$ M) plus CD40 mAb significantly enhanced IgA production in four of four experiments. Stimulation with VIP or CD40 alone had no effect on IgA levels. VIP plus CD40 mAb did not enhance IgG and IgE production from IgD$^+$ B cells (data not shown). Concentrations of SOM ranging from 10^{-12} to 10^{-6} M were used with CD40 mAb but failed to induce IgA production by IgD$^+$ B cells (data not shown).

The combination of TGF-β, IL-10, and CD40 mAb has been shown to induce the greatest IgA production from human IgD$^+$ B cells (12). Indeed, in our experiments, IgA production driven by optimal concentrations of TGF-β1 (1 ng/ml) plus IL-10 (500 U/ml) plus CD40 mAb was about two times higher than IgA production driven by VIP (10^{-10} M) plus IL-10 plus CD40 mAb stimulation (24.7±3.3 ng/ml vs. 12.4±1.8 ng/ml, respectively, $P < 0.01$). However, in the absence of IL-10, VIP (10^{-10} M) plus CD40 mAb stimulation produced 7.4±2.4 ng/ml of IgA while TGF-β1 (1 ng/ml) plus CD40 mAb failed to induce IgA production (0.6±0.4 ng/ml). Anti–TGF-β Ab (10 μg/ml) failed to block IgA production induced by VIP plus CD40 mAb, further showing that TGF-β did not mediate the effects of VIP (data not shown). The addition of VIP antagonist, VIP-[Ac-Tyr1, D-phe]-GRF(1-29) amide (10^{-8} M, Sigma), to the culture medium blocked the IgA induction by VIP plus CD40 mAb (< 0.01 ng/ml), while the SOM antagonist, aminoheptanoyl-phe-D-Trp-Lys-*O*-Benzyl-Thr (10^{-8} M, Sigma), did not (data not shown). The data of neutralization test at the protein level are in agreement with previous findings of IgA production from IgA$^-$ B cells (15).

Induction of α germ-line transcripts in IgD$^+$ B cells. Ig isotype switching is preceded by germ-line transcription from the targeted heavy chain locus (27, 28). The importance of germ-line transcripts in isotype switching has been confirmed recently by gene targeting experiments (29–33). However the exact role of germ-line transcripts/transcription remains controversial as does the requirement for specific I, switch, or constant region sequences (34). The presence of α subclass germ-line transcripts was assessed here by using an RT-PCR strategy.

As shown in Fig. 2 *A*, CD40 mAb stimulation (0.1 μg/ml) alone reproducibly induced α germ-line transcripts in IgD$^+$ B cells in all four experiments with independent tonsil cells. The amplified fragments for α germ-line transcripts were cloned and identified as α germ-line transcripts by DNA sequence analysis (data not shown). On the other hand, stimulation with VIP (10^{-9} M) alone induced α germ-line transcripts in two out of four experiments (50%). Presently, it remains unclear

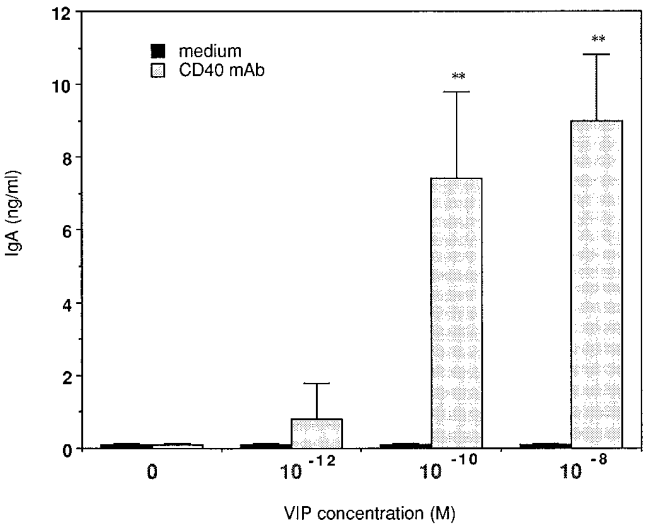

Figure 1. VIP plus CD40 mAb stimulation induces IgA production by IgD$^+$ B cells. Purified IgD$^+$ B cells (1×10^6 cells/ml) were cultured with medium or CD40 mAb (0.1 μg/ml) in the presence of various concentration of VIP as shown. Supernatants were harvested for IgA determination on day 10 of culture. The results were expressed as mean±SD of triplicate cultures (one out of four experiments shown). **Significant difference in the IgA production versus nonstimulated cells ($P < 0.01$).

whether VIP alone can truly and reproducibly induce α germ-line transcript since its ability in this regard appears to depend on the response of the specific cell donor. However, in individual donors, there was definitely no relationship between this VIP-induced expression of α germ-line transcripts and subsequent levels of IgA production driven by VIP plus CD40 mAb. To estimate the sensitivity of our PCR assay for α germ-line transcripts, plasmid DNA containing a Iα-Cα fragment (α germ-line transcripts) were serial diluted and subjected to PCR amplification (Fig. 2 *B*). The sensitivity was determined for 10^5 copies of template per reaction (0.18 pg, cDNA amount per reaction). Control unstimulated IgD^+ B cells showed no expression of α germ-line transcripts as expected. As a positive control for α germ-line transcripts, we used the PCR amplified band from IgD^+ B cells stimulated with TGF-β1 (1 ng/ml) (26). Semiquantitative analysis by the comparison shown in Fig. 2 *B* demonstrated that TGF-β alone induced severalfold more α germ-line transcripts than VIP plus CD40 mAb stimulation.

VIP and CD40 mAb stimulation induced switching from μ to α as determined by switch circle analysis. Gel electrophoresis of nested PCR products from purified IgD^+ B cells stimulated with VIP (10^{-9} M) plus CD40 mAb (0.1 μg/ml) showed amplification of multiple circular DNA fragments (Fig. 3). These multiple bands hybridized on Southern blot with a 3′ Sμ probe, suggesting that amplified fragments represent Sα/Sμ switch circular DNA. Such bands were not detected after B cell stimulation with VIP alone, CD40 mAb alone, or SOM (10^{-9} M) plus CD40 mAb in four independent experiments with four individual sets of tonsil. The sensitivity of this nested PCR assay for detection of switch circular DNA was assessed using a cloned plasmid containing Sα/Sμ fragment. This plasmid was serially diluted and then used as a template for nested PCR assay. As shown in Fig. 3 *C*, our PCR assay could amplify between 1 and 10 copies of template (1.9×10^{-6} pg, DNA amount per reaction).

PCR products from VIP plus CD40 mAb–stimulated B cells were cloned by a TA cloning and six clones were isolated

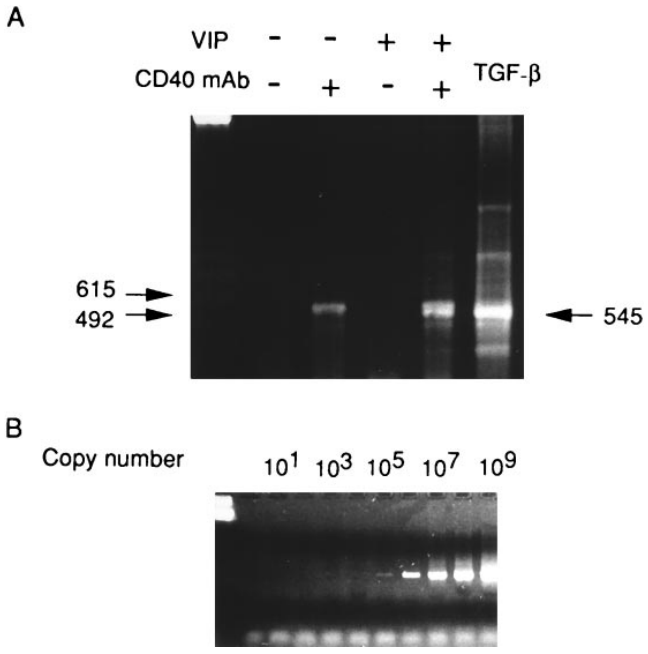

Figure 2. (*A*) Induction of α germ-line transcripts in IgD^+ B cells as assessed by RT-PCR assay. Purified IgD^+ B cells were cultured for 5 d with medium, CD40 mAb (0.1 μg/ml) alone, VIP (10^{-9} M) alone, VIP plus CD40 mAb, or TGF-β (1 ng/ml) alone. Cells stimulated with CD40 mAb, VIP plus CD40 mAb, and TGF-β expressed the predicted 545-bp band for α germ-line transcripts. The lane on the far left is a 123-bp DNA ladder. (*B*) Sensitivity of the PCR assay for α germ-line transcripts. A plasmid containing Iα-Cα fragment was diluted and amplified. 1 gram of this plasmid is equivalent to 5.5×10^{17} copies of template.

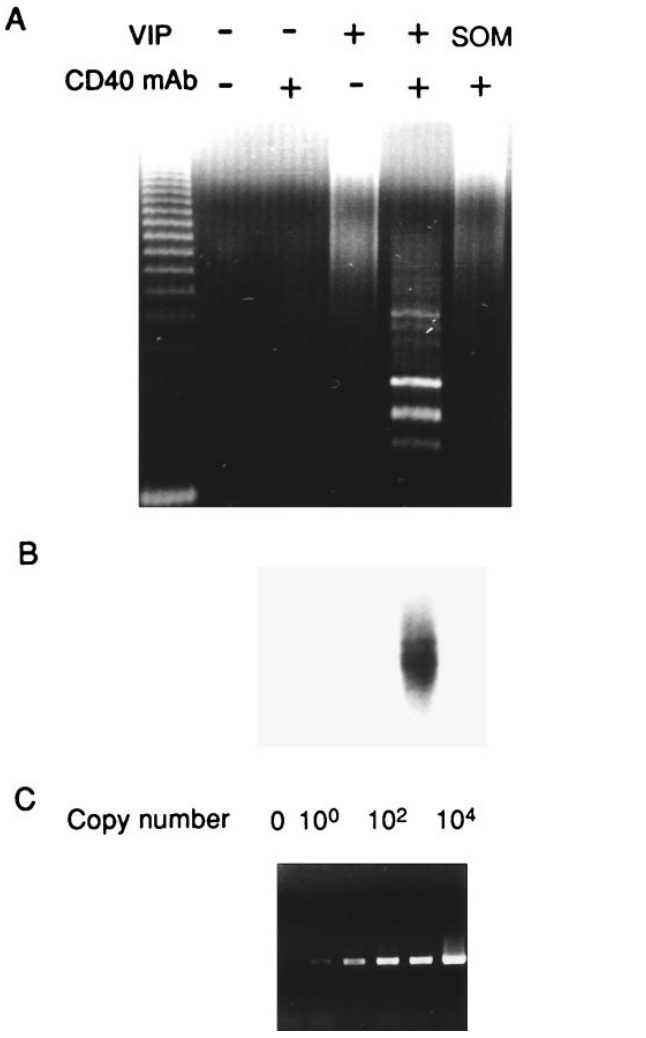

Figure 3. Identification of PCR products representing Sα/Sμ switch circular DNA. (*A*) Agarose gel electrophoresis of PCR-amplified switch circle fragments derived from various cells. Purified IgD^+ B cells were cultured for 7 d with medium alone, CD40 mAb (0.1 μg/ml) alone, VIP (10^{-9} M) alone, VIP plus CD40 mAb, or SOM (10^{-9} M) plus CD40 mAb. Multiple bands were seen in only the fourth lane derived from cells stimulated with VIP plus CD40 mAb. (*B*) Southern blot analysis of PCR products shown in *A*. The sample was transferred to a nylon membrane and hybridized with Sμ probe. Multiple positive bands were seen in the fourth lane. These were shown to be chimeric Sα/Sμ fragments by sequencing as shown in Fig. 4. (*C*) Sensitivity of the nested PCR for the detection of switch circular DNA. A plasmid containing Sα/Sμ switch fragment was serial diluted and amplified. 1 gram of this plasmid is equivalent to 5.2×10^{17} copies of template.

and sequenced. Since the human Sα1 and Sα2 regions are highly homologous (23, 24), we were unable to determine the α-subclass of the Sα fragment in the cloned Sα/Sμ PCR products. The structure of all six clones showed they represented direct class switching from μ to α (Fig. 4). As expected, no consensus sequence was found at the Sα/Sμ breakpoints.

The assay developed in our laboratory for detection of deleted switch circular DNA using the inverted nested PCR technique is not influenced by proliferation or the presence of switched cells. Switch circular DNA does not replicate as episomal DNA (4) and proliferation of cells makes the copy number of circular DNA, in fact, decrease relative to the number of cells. That Sα/Sμ switch circular fragments were not amplified by our PCR method used with unstimulated unfractionated tonsil B cells (data not shown) demonstrates the specificity of this method for active switching (9, 10) as this cell population contains cells having already undergone isotype switch to all isotypes.

Comparison between VIP and TGF-β as α switch factor. Our results demonstrate that VIP induces α switch recombination in the presence of CD40 ligation and there was induction of IgA to readily detectable levels in the cultures. Using our nested PCR method, we also amplified Sα/Sμ switch circular fragments from B cells stimulated with TGF-β plus CD40 mAb in the absence of IL-10 (data not shown), although no significant IgA protein was detected in the supernatant of IgD^+ B cells stimulated with TGF-β and CD40 mAb. Defrance et al. (12) proposed that IgA isotype switching occurs after costimulation of IgD^+ B cells with TGF-β and CD40 mAb, and a subsequent signal, such as IL-10, is necessary for growth and differentiation of the newly IgA-committed B cells. They found that whereas IL-4 and CD40 mAb are sufficient to induce commitment of human B cells to IgE secretion, TGF-β and CD40 mAb consistently failed to elicit IgA synthesis from IgD^+ B cells (12). This is in agreement with our data. Thus, although TGF-β appears to be more effective at driving α germ-line transcriptions than VIP, it is not as effective as a switch and differentiation factor when combined solely with CD40 ligation. A quantitative analysis of α switch recombination comparing VIP and TGF-β theoretically could be determined by the digestion circularization PCR (35); a suitable system has not been established in human fresh cells. Furthermore, IL-10 is very readily found in the airways of humans (36) and mucosal switching to IgA in vivo likely occurs in the presence of a variety of concentrations of this cytokine.

VIP as a regulator of the immune response. Neuropeptides and cytokines are primary mediators of the interaction between cells of the nervous and immune systems (37, 38). VIP has been demonstrated to modulate numerous aspects of the immune response in vitro (39, 40). VIP is detected extensively in the postsynaptic parasympathetic innervation and intrinsic neurons in the lamina propria and submucosa of the upper respiratory system, lung, and gut (40–43). These neurons have been shown to directly innervate mucosal lymphoid tissue (44). VIP can alter the function of the lymphocytes via VIP receptors, the presence of which has been reported on human peripheral B and T cells (45, 46). The concentration (1×10^{-9} M) used by us in the induction of switch circular DNA was fully consistent with the effect being mediated by the high-affinity VIP (VIP1) receptor (38, 39, 45, 47, 48). That the VIP1 receptor could mediate this effect is supported by the recent finding that the VIP1 receptor gene is expressed at readily detectable levels in human peripheral blood lymphocytes (49). The level of VIP in nasal secretions of healthy subjects was reported to be ∼ 40 times higher than that in plasma (50), while another study showed that both allergen and histamine nasal challenge enhanced the release of VIP, substance P, and the calcitonin gene–related peptide, whereas histamine challenge specifically enhanced VIP release in nasal secretions (51). Levels of ∼ 10^{-11} M VIP were measured in very diluted lavage flu-

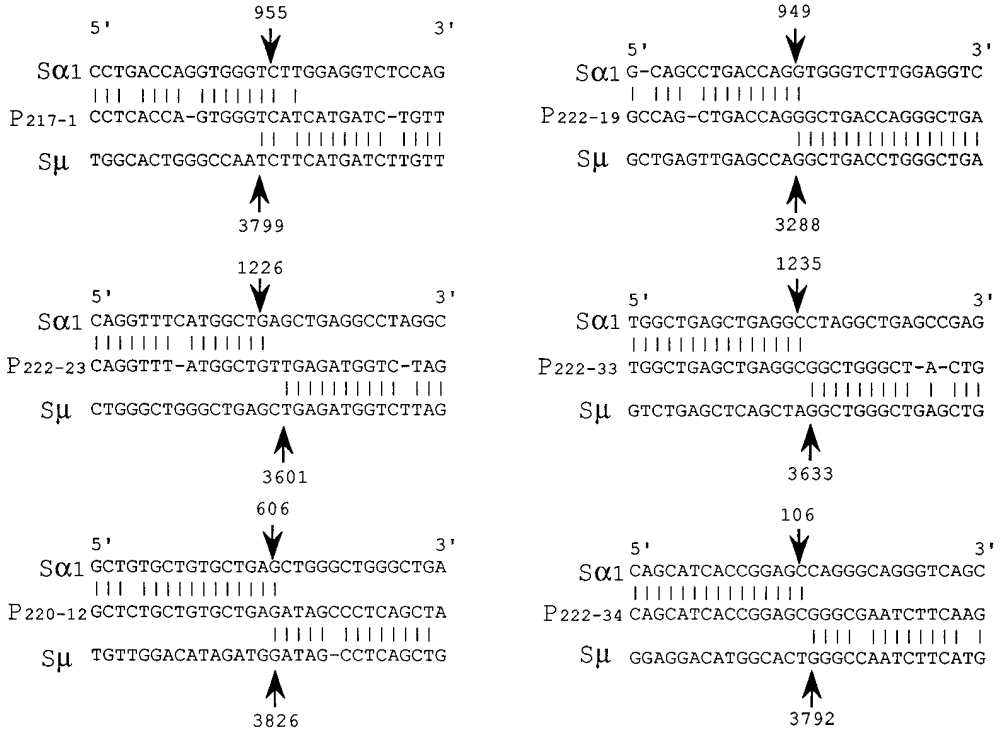

Figure 4. Nucleotide sequences surrounding the breakpoints in six cloned switch circular DNA fragments from cells stimulated with VIP plus CD40 mAb. The arrows indicate the breakpoint site of Sα part (*top*) and Sμ part (*bottom*). The number of breakpoint site indicates corresponding position of Sα1 in reference 23 (clone number: P217-1, P222-19, 23, and 33) or in reference 24 (clone number: P220-12, and P222-34), and of Sμ in reference 22.

ids; the levels at the interface of VIP neurons and lymphoid tissues are likely several logs higher.

VIP release can be brought about by mechanical and chemical stimuli which induce inflammation, e.g., HCl, hypertonic NaCl, and hypertonic glucose, and by antigen–allergen challenge (51–53). Little is known about VIP release in response to early viral or bacterial infection. The ability of the mucosal nervous system to release VIP in a matter of seconds after topical stimulation provides a mechanism by which isotype switching to IgA can be initiated in a far more rapid fashion than possible through the synthesis and release of cytokines. The possible benefit from this is apparent as IgA is the predominant Ig effector molecule of mucosal immunity functioning as the first line of specific immunologic defense against many viral and microbial pathogens (54, 55).

VIP-induced isotype switch is likely to be an important component of a larger immune response orchestrated by VIP, perhaps acting in concert with other neurotransmitters. In this regard, subnanomolar concentrations of VIP have been shown also to alter lymphocyte trafficking in vivo and in vitro and to stimulate the proliferation of cultured lymphocytes (47, 48, 56, 57). VIP also acts upon natural killer cells and macrophages in vitro (58, 59) and protects tissue against a variety of types of inflammatory and other injuries (60, 61). Thus, although it classically acts as a neurotransmitter or neuromodulator, VIP certainly functions as a switch factor for human IgA and likely functions as an important neurogenic mediator of local immune and inflammatory responses.

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