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Inhibition of Interleukin-4-induced Class Switch Recombination by a Human Immunoglobulin Fcγ-Fcε Chimeric Protein*

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Takechiyo Yamada‡§¶‖, Daocheng Zhu‡¶, Ke Zhang‡, and Andrew Saxon‡

From the ‡Hart and Louis Laboratory, Division of Clinical Immunology, Department of Medicine, UCLA School of Medicine, California 90095-1680 and the §Department of Otorhinolaryngology, Fukui Medical University, Fukui, 910-1193, Japan

Immunoglobulin E (IgE) is important in mediating human allergic diseases. We tested the hypothesis that a human Ig Fcγ-Fcε bifunctional chimeric protein, GE2, would inhibit IgE class switch recombination (CSR) by co-aggregating B-cell CD32 and CD23. Indeed, GE2 directly inhibited ε germ-line transcription, subsequent CSR to ε and IgE protein production. This CSR inhibition was dependent on CD23 binding and the phosphorylation of extracellular signal-related kinase (ERK), and it was mediated via suppression of interleukin-4-induced STAT6 phosphorylation. Treatment with PD98059, a specific inhibitor of mitogen-activated protein kinase kinase 1 (MAPKK1 (MEK1)) and MEK2 reversed the ability of GE2 to decrease CSR and STAT6 phosphorylation. GE2 stimulation induced ERK phosphorylation, whereas it did not alter the phosphorylation of c-Jun N-terminal kinase or p38 MAPK. The ability of GE2 to block human isotype switching to ε, in addition to its already demonstrated ability to inhibit mast cell and basophil function, suggests that it will provide an important novel benefit in the treatment of IgE-mediated diseases.

Allergic antibodies (IgE) play an important role in the pathogenesis of allergic asthma, allergic rhinitis, and severe allergic reactions to foods, drugs, and insect stings. These common and important diseases are of increasing concern worldwide. Removal of IgE by anti-IgE therapy has been shown to have clinical utility in the treatment human airway allergic disease (1). However, anti-IgE does not interfere with the underlying issue of ongoing IgE production. Receptors for IgE and IgG are expressed, respectively, on human B cells as the low affinity trimeric IgE receptor (FcεRII[1] (CD23)) and as two isoforms of the Fc gamma receptor type II (FcγRIIA and FcγRIIB (CD32)). CD23 has been proposed to be an important regulator of IgE synthesis (2). Specific IgE antibody titers were increased and sustained in CD23-deficient mice as compared with controls (3). Anti-CD23 antibody (αCD23) treatment of rats inhibited antigen-specific IgE immune response by 90% (4). Human *in vitro* IgE production was also inhibited by monoclonal αCD23, an effect that was shown to require the Fcγ domain, as $F(ab')_2$ αCD23 was ineffective (5). Thus, cross-linking of CD32 with CD23 has been proposed as a therapeutic strategy to down-modulate IgE production. We recently developed a novel human Ig Fcγ-Fcε bifunctional chimeric protein (GE2) (Fig. 1*a*) that inhibits mast cell and basophil FcεRI-mediated function *in vitro* and *in vivo* (6). Because GE2 can cross-link CD32 with CD23, we tested this chimeric protein for its ability to affect IgE class switching and examined the events underlying this effect.

IL-4 is a key participant in human Ig class switching and, in particular, in the production of IgE (7, 8). The α chain of the IL-4 receptor activates the Janus kinase (JAK) family members followed by subsequent phosphorylation and activation of STAT6 (9–12). Phosphorylated STAT6 dimerizes and binds to the STAT6 consensus sequences in the IgH ε germ-line promoter and activates ε germ-line transcription with production of ε germ-line transcripts (εGTs), which themselves are felt to be important for switching to IgE. For optimal ε germ-line transcription and εGT production, synergy between IL-4 stimulation and a second signal through CD40 is required (13). Recently, it has been reported that p38 mitogen-activated protein kinase (p38 MAPK) plays an important role in CD40 induction of εGTs (14) and is related to Ig class switch recombination (CSR) in our system (15).

Here, we have examined whether GE2 could alter induced isotype switching in primary human B cells. Sμ-Sε recombination was measured by digestion-circularization PCR (DC-PCR), and switch circle transcripts (CTs) and εGTs were assessed by RT-PCR. We also examined the effect of GE2 on IL-4 and αCD40-driven phosphorylation and activation of STAT6 as well as on phosphorylation of extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK.

EXPERIMENTAL PROCEDURES

Reagents—Human IL-4 was purchased from R & D Systems. Anti-CD40 mAb G28.5 was purchased from ATCC (Manassas, VA). Anti-human IgE antibody (Mae1) against the Cε1 domain was a kind gift from Dr. Paul Jardieu (Genentech). Anti-ERK antibody (Ab), anti-phosphorylated ERK Ab, anti-JNK Ab, anti-phosphorylated JNK Ab, anti-p38 MAPK Ab, anti-phosphorylated p38 Ab, anti-phosphorylated STAT6 Ab, and U0126 were purchased from Cell Signaling (Beverly, MA). Anti-STAT6 Ab was purchased from Santa Cruz Biotechnology

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¶ These authors contributed equally to this work.
‖ To whom correspondence should be addressed: Dept. of Otorhinolaryngology, Fukui Medical University, Fukui, 910-1193, Japan. Tel.: 81-776-61-8406; Fax: 81-776-61-8118; E-mail: ymdtkcy@fmsrsa.fukui-med.ac.jp.

[1] The abbreviations used are: FcεRII, low affinity trimeric IgE receptor; FcγRII, Fc gamma receptor type II; αCD23, anti-CD23 antibody(s); sCD23, soluble CD23; JAK, Janus kinase; STAT, signal transducer and activator of transcription; GT, germ-line transcript; MAPK, mitogen-activated protein kinase; CSR, class switch recombination; DC-PCR, digestion-circularization PCR; RT-PCR, reverse transcriptase PCR; CTs, switch circle transcripts; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; AID, activation-induced cytidine deaminase; Ab, antibody; ECL, enhanced chemiluminescence; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; Ab, antibody; IL, interleukin; CT, circle transcript; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Santa Cruz, CA). PD98059, SB203580, and SP600125 were obtained from Calbiochem. Restriction endonucleases and ligase were from Promega (Madison, WI).

GE2 Construction and Expression—The construction and expression of the human Fcγ-Fcε Ig chimeric gene has been described in detail previously (6). The expression vector containing the novel Ig Fcγ-Fcε chimeric gene was transfected into SP2/0 cells. GE2 was expressed in cell culture supernatants and purified using an anti-human IgE affinity column. The GE2 protein was reacted with anti-human ε- and γ-chain-specific antibodies.

Cells and Cell Culture—Peripheral blood mononuclear cells were isolated from healthy volunteers by centrifugation on Ficoll-Hypaque. Human B cells were purified from peripheral blood mononuclear cells by T cell depletion after monocytes/macrophages and natural killer cells were removed. Human B cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Omega, Tarzana, CA).

DC-PCR for Human Sμ-Sε Recombination—DC-PCR was performed as described (16) and was used to quantify the levels of Sμ-Sε recombination in stimulated primary human B cells. Genomic DNA was digested with *Bgl*II followed by ligation under conditions favoring self-ligation. The resultant ligated DNA was precipitated, and the appropriate amounts of DNA were used as templates for PCR with 5′ end primer (5′-GATATGCTGTTTGCACAAACTAG-3′) and 3′ end primer (5′-AACAACCCTCATGACCACCAGCT-3′). Amplification for 40 cycles was performed at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The size of the expected PCR product was 222 bp. To verify the amount of ligated DNA between different groups and the efficiency for digestion and ligation of the input DNA sample, the human activation-induced cytidine deaminase (AID) gene was used as an unrelated control gene for the DC-PCR assay. *Bgl*II digestion generated a 4578-bp fragment from the human AID gene (EMBL/GenBank™ accession no. AB040430). 5′ end primer 5′-CCATGGTACAAATCTCAGGACGAATC-3′ and 3′ end primer 5′-AGATGGTGAAACCCCGTCTCTATTAA-3′ were used. This pair of primers amplified a 238-bp product. PCR was conducted with 10 ng of ligated DNA as templates at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min for 40 cycles.

Ig Measurements—Microtiter plates were coated overnight at 4 °C with anti-human IgE Ab against Cε1 and anti-human IgA Ab, respectively. Anti-human IgE Ab against Cε1 was necessary, as standard IgE assays detected the GE2 protein. After the wells were blocked with 0.1% gelatin for at least 1 h, 100 μl of culture supernatants was plated in duplicate wells, and the plates were incubated for 2 h at room temperature. After the plates were washed, alkaline phosphatase-labeled anti-human IgE Ab with anti-human IgA Ab was added for detection. Absorbance was read with a Microplate reader (Molecular Devices, Sunnyvale, CA).

RNA Extraction and RT-PCR—Total mRNA was obtained from stimulated and unstimulated cells using Trizol reagent (Invitrogen). RNA suspended in 0.1% diethylpyrocarbonate-treated water was digested with DNase I (Sigma) to remove possible contaminating DNA and was then extracted with phenol/chloroform followed by precipitation in ethanol. Total RNA (1 μg) was reverse-transcribed to cDNA as described previously (17). All PCR assays were done in 50-μl reaction volumes containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 1 μM of each primer, and 2.5 units of *Taq* polymerase (Promega). For detection of IgH εGTs and GAPDH, PCR was conducted with 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1min. 5′ end primer 5′-AGCTGTCCAGGAACCCGACAGGGAG-3′ and 3′ end primer 5′-GTTGATAGTCCCTGGGGTGTA-3′ were used to amplify εGTs. 5′ end primer 5′-CTGCATCTGACTGTGCTTTCTGAG-3′ and 3′ end primer 5′-TTGTCAGCCTCATCAGGATTAGTG-3′ were used for FcγRIIA. 5′ end primer 5′-ATCACTGTCCAAGTGCCCAGCATG-3′ and 3′ end primer 5′-TTGACATGGTCGTTGGGAGGAAGA-3′ were used for FcγRIIB. The amplification conditions used for FcγRIIA and FcγRIIB PCR were 40 cycles of 94 °C for 1 min, 68 °C for 1 min, and 72 °C for 1 min.

Switch Circle RNA Transcripts—After total mRNA was obtained and reverse-transcribed to cDNA, CTs were amplified with the forward primer Iε 5′-GACGGGCCACACCATCCACAGGCACCAAATGGACGAC-3′ together with the reverse primer Cμ 5′-GTTGCCGTTGGGGTGCTGGAC-3′. Iε-Cμ (408 bp) CTs were amplified for 25 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min (18).

Densitometric Analysis—The images of ethidium-stained gels were recorded using the gel documentation system Speedlight Platinum (Lightools Research, Encinitas, CA). The intensity of each band was measured with the BioImage software Basic Quantifier (Genomic Solutions Inc., Ann Arbor, MI). The samples were diluted to 50, 20, 10, and 5% before PCR amplification. The final quantitative estimates for the densitometric analysis were taken from the linear part of the curve.

Gel Electrophoresis and Western Blot Analysis—Samples containing equal amount of protein were boiled with electrophoresis sample buffer for 3 min and separated using SDS-PAGE. The separated proteins were transferred electrophoretically to the membranes (Millipore, Bedford, MA). The membranes were blocked at room temperature for 1 h in pH 7.4 PBS with 1% bovine serum albumin, incubated with primary Abs for one h at room temperature, and washed and followed by incubation with horseradish peroxidase-labeled secondary Abs for 1 h. The blots were developed using enhanced chemiluminescence reagents (ECL, Amersham Biosciences) and exposed to BioMax film from Eastman Kodak Co.

RESULTS

Inhibition of Sμ-Sε Recombination and IgE Production by Ig Fcγ-Fcε Fusion Protein—We assessed the ability of GE2 to modulate IL-4-induced isotype switching in primary human B cells using DC-PCR. Sμ-Sε recombination was detected in highly purified primary B cells stimulated with IL-4-plus-CD40 (Fig. 1*b*, *2nd lane from left*) but not in unstimulated cells (*1st lane*). IL-4-plus-CD40-induced-Sμ-Sε recombination was inhibited by GE2 in a dose-dependent manner (Fig. 1*b*, *last 4 lanes*). Sμ-Sε recombination was decreased by 85% at 5 μg/ml of GE2 as measured by densitometry of the DC-PCR products. Because cross-linking of CD32 with CD23 has been proposed as a therapeutic strategy to down-modulate IgE production and GE2 suppressed IL-4-induced CSR, we also tested the effects of GE2 on IgE production from human B cells. IL-4-plus-CD40-induced IgE production was decreased by 65 and 75%, respectively, in the presence of 5 or 10 μg/ml GE2, although IgA production was unchanged (Fig. 1*d*).

The Effect of GE2 on CTs—CSR from the *mu* gene generates an extrachromosomal reciprocal switch DNA recombination product, a switch circle with the I_H promoter of the targeted C_H gene, the switched DNA fragment, and Cμ (Fig. 2*a*). The I_H promoter in the switch circle drives transcription of a chimeric I-Cμ product, referred to as CT (19). We also measured the effect of GE2 on IL-4-plus-CD40-induced CSR to ε by measuring the respective switch CTs (Fig. 2*b, Iε-Cμ*). Densitometric analysis provided that the inhibitory effect of GE2 on CSR to ε was 90%.

GE2 Negatively Regulates Production of IgH ε Germ-line Transcripts—Expression of GTs from specific IgH loci is important in Ig CSR preceding the occurrence of isotype switching to those loci (20). Anti-CD23 monoclonal antibody has been reported recently to inhibit ε GT production in B cells (21). Because GE2 binds to CD23, we examined the ability of GE2 to alter IL-4-plus-CD40 induction of εGT transcription. GE2 inhibited IL-4-plus-CD40-induced εGT production in primary human B cells in a dose-dependent manner (Fig. 3*a*). Semi-quantitative analysis comparing these data demonstrated that GE2 at 5 μg/ml reduced IL-4-plus-CD40-driven production of εGTs by 80% (Fig. 3, *a* and *b*). Because CD32 binding is predicted to be involved in the effects of GE2 that we observed, we examined B cell expression of the variant forms of CD32, FcγRIIA and FcγRIIB, using RT-PCR and flow cytometry. This determination is important because FcγRIIA is an "activating" ITAM (immunoreceptor tyrosine-based activation motif)-containing receptor, whereas FcγRIIB is an "inhibitory" ITIM (immunoreceptor tyrosine-based inhibitory motif)-containing receptor. We found that CD32 expressed on resting human B cells is almost exclusively express FcγRIIA. Stimulation with IL-4-plus-CD40 or cross-linking B cell receptors (membrane Ig) using $F(ab')_2$ anti-human Ig for 15 h increased expression of FcγRIIB mRNA more than 6-fold, whereas FcγRIIA expression was unchanged (data not shown).

GE2 Inhibited STAT6 Phosphorylation in Primary Human B Cells—Because activation of STAT6 is essential in mediating the IL-4 response (12) and plays an important role in both

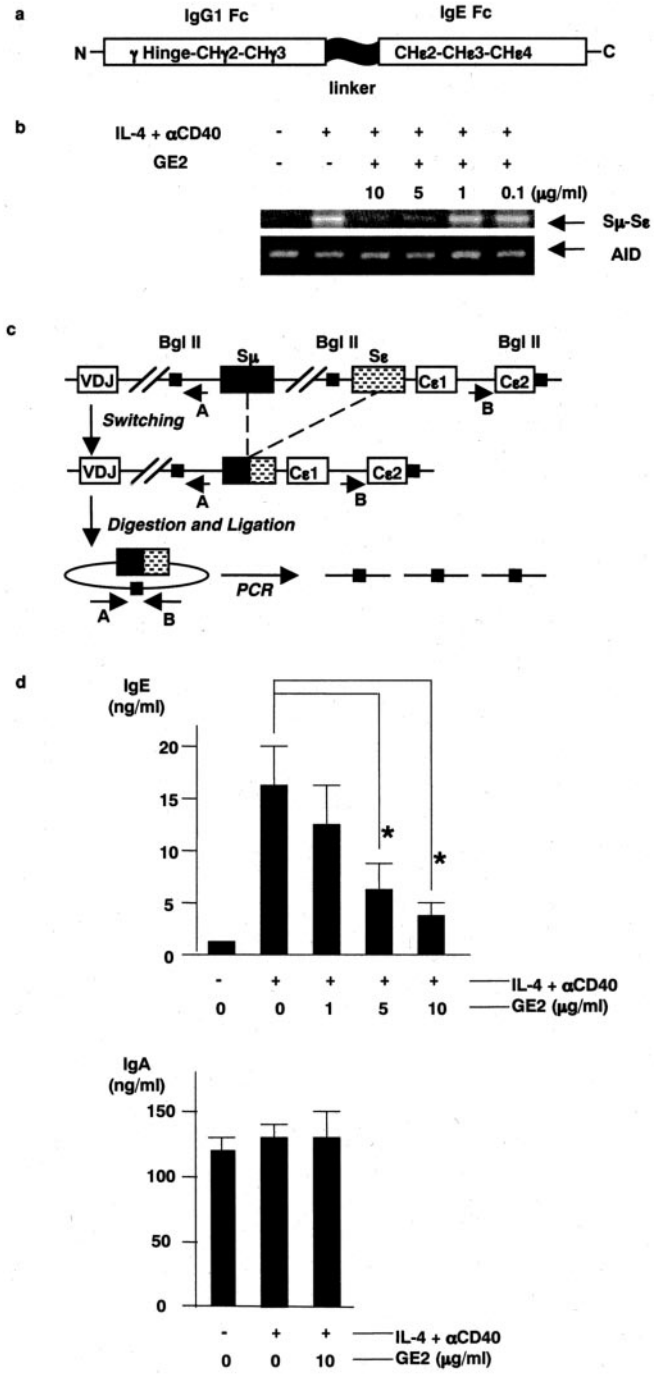

FIG. 1. **GE2 inhibited CSR from Cμ to Cε.** *a*, schematic diagram of a gamma-epsilon fusion protein (*GE2*); the structure is γHinge-CHγ2-CHγ3–15aa linker-CHε2-CHε3-CHε4. *N*, N terminus; *C*, C terminus. *b*, dose response for GE2 inhibition of CSR from Cμ to Cε. DC-PCR was used to quantify the levels of Sμ-Sε recombination. Human B cells (5×10^5 cells/ml) were treated with the noted concentrations of GE2 for 1 h and cultured with or without IL-4 (5 ng/ml) plus αCD40 (0.1 μg/ml) for 5 days. DNA was then prepared, digested with *Bgl*II. and ligated to yield self-ligation products. DC-PCR for Sμ-Sε recombination products was amplified for 40 cycles. DC-PCR for the human AID gene was used as an internal control. *c*, diagram of DC-PCR strategy. The oligonucleotides are marked as 5′μ and 3′ε primer (*arrows A* and *B*). *d*, dose-dependent inhibition by GE2 on IgE production from human B cells. Purified human B cells were treated with the indicated concentrations of GE2 at 37 °C for 1 h and then stimulated with IL-4 (5 ng/ml) plus αCD40 (0.1 μg/ml). Fourteen days later, total IgE and IgA levels in the supernatants of stimulated cells were measured with an enzyme-linked immunosorbent assay. *, $p < 0.05$.

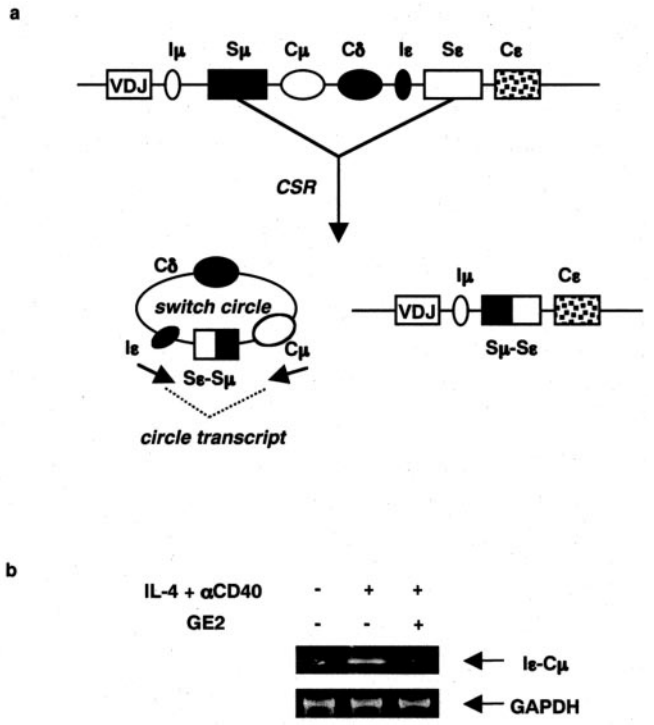

FIG. 2. **GE2 inhibited CTs.** *a*, schematic diagrams of CSR from Cμ to Cε. *Closed* and *open rectangles* are Sμ and Sε, respectively. *V-shaped dotted line* indicates circle transcripts that are amplified using the primers shown by the *arrows*. *b*, effect of GE2 on the expression of CTs. Primary B cells were cultured with or without GE2 (5 μg/ml) for 1 h and then stimulated with IL-4 (5 ng/ml) plus αCD40 (0.1 μg/ml) for 4 days. RNAs were prepared, and CTs (Iε-Cμ) were amplified using synthesized cDNAs by the primers. GAPDH was used as an internal control for RT-PCR.

expression of εGTs and isotype switching to IgE (22), we investigated whether IL-4-induced STAT6 activation was affected by GE2. Samples from appropriately stimulated cells were subjected to Western blot analysis with specific anti-phosphorylated STAT6. Phosphorylated STAT6 was detectable 1 h after IL-4 stimulation and remained elevated throughout 4 h of incubation (Fig. 4). However, IL-4-stimulated B cells treated with GE2 failed to maintain STAT6 phosphorylation beyond 1 h.

PD98059 Reversed GE2-induced Suppression—It has been reported recently that MAPKs inhibit STAT activation and that a MEK inhibitor reversed this effect (23). To examine whether MAPK signaling is involved in the GE2 inhibition of ε CSR and IgE production, we tested the ability of PD98059, a specific inhibitor of MEK1 and MEK2, to reverse the GE2-driven decrease in STAT6 phosphorylation. MEK1 and MEK2 act directly upstream of ERK-1 and ERK-2. Treatment with PD98059 almost completely reversed the ability of GE2 to decrease STAT6 phosphorylation (Fig. 5*a*, *4th lane from left*). This same outcome was also observed using U0126, another specific MEK inhibitor that is structurally unrelated to PD98059, whereas the p38 MAPK inhibitor SB203580 or JNK inhibitor SP600125 did not reverse the GE2 effects (data not shown). To demonstrate the physiological relevance of this MEK inhibition, we examined its effect on GE2-induced suppression of IgE CSR. Pre-incubation of B cells with PD98059 also resulted in a near complete reversal of GE2-mediated inhibition of Sμ-Sε CSR in human primary B cells (Fig. 5*b*, *4th lane*). MEK inhibition also reversed GE2-induced suppression of IL-4-plus-CD40-induced εGTs (Fig. 5*c*, *4th lane*). p38 MAPK inhibition with SB203580 did not alter this suppression of εGT induction (data not shown).

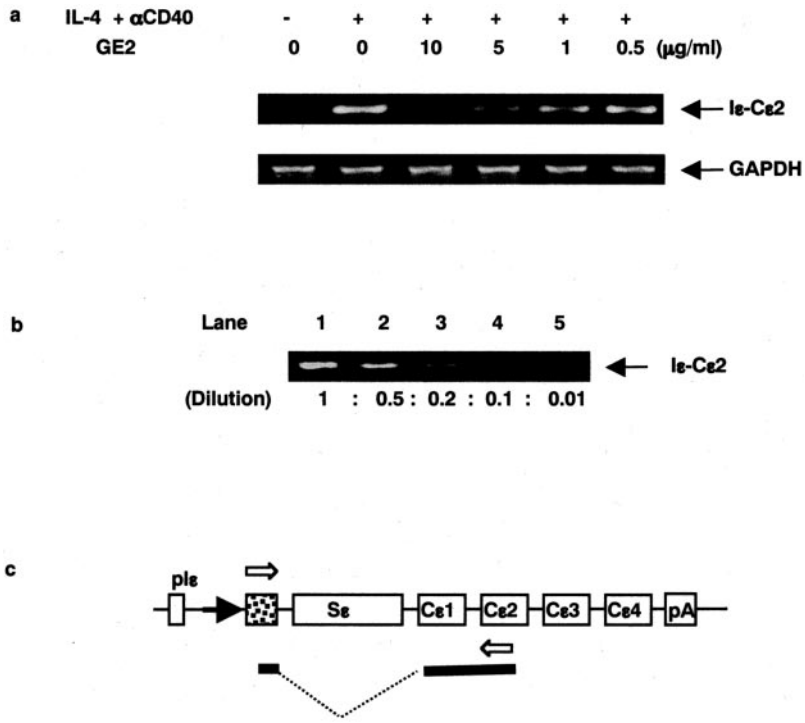

FIG. 3. **GE2 inhibited εGTs in primary human B cells.** *a*, dose dependence of GE2 inhibition of εGTs. GAPDH was used as an internal control for RT-PCR. Primary B cells were cultured with or without GE2 (5 μg/ml) for 1 h and then with stimulated IL-4 (5 ng/ml) plus αCD40 (0.1 μg/ml) for 48 h. RNAs were prepared, and εGTs (Iε-Cε2) were amplified using synthesized cDNAs. *b*, relative quantification for detection of different dilutions of εGTs by RT-PCR assay. The amounts of total diluted RNA containing εGTs used as a template were 5 ng (*lane 1*), 2.5 ng (*lane 2*), 1 ng (*lane 3*), 500 pg (*lane 4*), and 50 pg (*lane 5*). *c*, diagram of the IgH ε locus and the formation of the processed εGTs. The *open arrows* represent the position and orientation of RT-PCR primers (5′μ primer and 3′ε primer). *pIε*, Iε promoter and Iε exon; *pA*, poly(A) site.

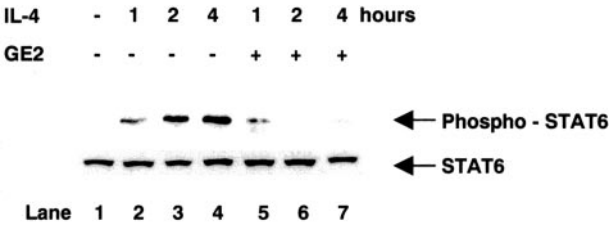

FIG. 4. **The effect of GE2 on STAT6 phosphorylation.** Human B cells were cultured with or without GE2 (5 μg/ml) for 1 h and then stimulated with IL-4 (5 ng/ml) for the indicated times. Equal amounts of lysate from the samples were applied to each lane and blotted with anti-phosphorylated STAT6 Ab (*top*) or with anti-STAT6 Ab (*bottom*).

The Effect of GE2 on MAPK Phosphorylation—As the effects of GE2 on IgE CSR were reversed by specific inhibitors of MEK1 and MEK2, we examined the effect of GE2 on MAPK family signaling, which is critical in a variety of cellular functions. As expected, within 5 min after GE2 stimulation, ERK phosphorylation began to rise, reached a maximum at 15 min, and was maintained for 45 min (Fig. 6*a*). In contrast, GE2 did not alter the phosphorylation of JNK or p38 MAPK, although IL-4-plus-CD40 activated JNK and p38 MAPK (Fig. 6*b*, *lane 3*). We also tested for any effect of GE2 on IL-4-plus-CD40-driven phosphorylation of JNK and p38 MAPK; no effects were detected in our system.

Soluble CD23 Blocked the Effect of GE2—To test whether the GE2-induced ERK phosphorylation and GE2 inhibition of CSR are dependent on CD23 binding, we used a trimeric soluble CD23 (sCD23), which competes with CD23 on B cells for binding to the Fc ε region of GE2. Primary human B cells were cultured with GE2, GE2-plus-sCD23, or the appropriate controls, and the resulting ERK phosphorylation was examined. GE2-induced ERK phosphorylation was blocked by sCD23 in a dose-dependent matter (Fig. 7*a*). Soluble CD23 (10 μg/ml) reversed GE2 (5 μg/ml)-induced phosphorylation by 50%. This treatment also reversed GE2-induced inhibition of εGTs by 40% as determined by densitometry analysis of εGTs (Fig. 7*b*). Treatment with soluble α-chain of the high affinity IgE receptor (FcεRI), which binds with very high affinity to the Fcε region involved in interaction with CD23, also blocked (85%) the ability of GE2 to inhibit εGT production (data not shown). Furthermore, we showed that sCD23 was able to reverse GE2 inhibition of CSR to ε by measuring the production of CTs (Fig. 7*c*, *Iε-Cμ*).

To Optimally Inhibit εGTs and CTs, GE2 Needs To Be Present at the Time of Stimulation—To determine whether the inhibition of εGTs and CSR requires the presence of GE2 at the time of stimulation, cultures were established and GE2 was added at later time points. When GE2 was added 8 and 24 h following IL-4-plus-CD40 stimulation (Fig. 8), there was a marked loss of GE2 inhibitory effects. Densitometric analysis revealed that an 8-h delay led to a 45% decrease in the GE2 inhibitory effect on εGTs and a 20% decrease in the inhibition of CTs. A 24-h delay led to a 50% decrease in the GE2 inhibitory effect on εGTs and a 50% loss in the inhibition of CTs. Conversely, when GE2 was removed after an initial 1-h incubation, its inhibitory effect on the production of εGTs was reduced by 50% (data not shown).

DISCUSSION

In the present study, we demonstrated the ability of GE2 to directly alter B cells isotype switching to ε and examined the mechanisms underlying this effect. IL-4-plus-CD40-induced Sμ-Sε recombination was inhibited by GE2 in a dose-dependent manner. GE2 inhibited in a dose-dependent fashion the induction of IgE production and IL-4-plus-CD40-induced εGT production in primary human B cells. At the same time, GE2 inhibited STAT6 phosphorylation. Treatment with PD98059, a specific inhibitor of MEK1 and MEK2, reversed the ability of GE2 to decrease STAT6 phosphorylation, to inhibit of Sμ-Sε CSR, and to induce εGTs. Furthermore, GE2 induced ERK phosphorylation, although it did not alter the phosphorylation of JNK or p38 MAPK.

IL-4-plus-CD40-induced IgE were decreased by 75% in the presence GE2, whereas IgA production was unchanged. GE2 blocked IL-4-plus-CD40-induced CSR to ε. GE2 also inhibited production of ε GTs. These data are underscored by our results showing that GE2 has an inhibitory effect on IL-4-induced signaling for CSR in B cells and are further supported by the

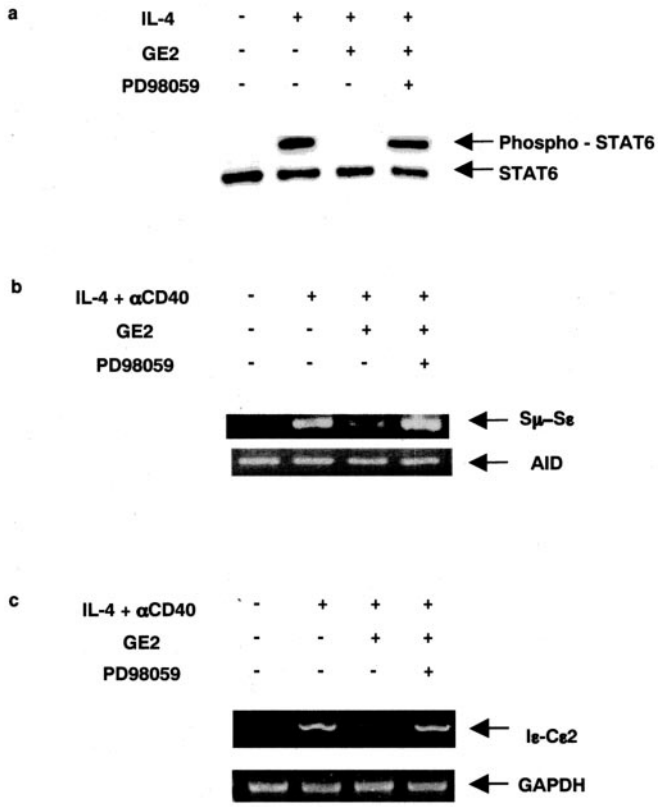

FIG. 5. **PD98059 reversed GE2-induced suppression of IgE CSR.** *a*, GE2 inhibition of STAT6 phosphorylation is reversed by PD98059. After human B cells were pretreated with or without PD58059 (10 μM) for 45 min, the cells were treated with or without GE2 (5 μg/ml) for 1 h and then stimulated with IL-4 (5 ng/ml) plus αCD40 (0.1 μg/ml) for 4 h. The samples were subjected to SDS-PAGE followed by blotting with anti-phosphorylated STAT6 Ab (*top*) or anti-STAT6 Ab (*bottom*). *b*, effect of GE2 and PD98059 on Sμ-Sε recombination. Human B cells were treated under the noted condition and then cultured with or without IL-4 (5 ng/ml) plus αCD40 (0.1 μg/ml) for 5 days. DNA was prepared then and followed by DC-PCR for Sμ-Sε recombination products. *c*, blockage of the GE2 suppression of εGTs by MEK inhibition. RNAs were prepared from the cells harvested 48 h after stimulation with IL-4-plus-αCD40. cDNAs were then synthesized, and εGTs (*Iε-Cε2*) were amplified.

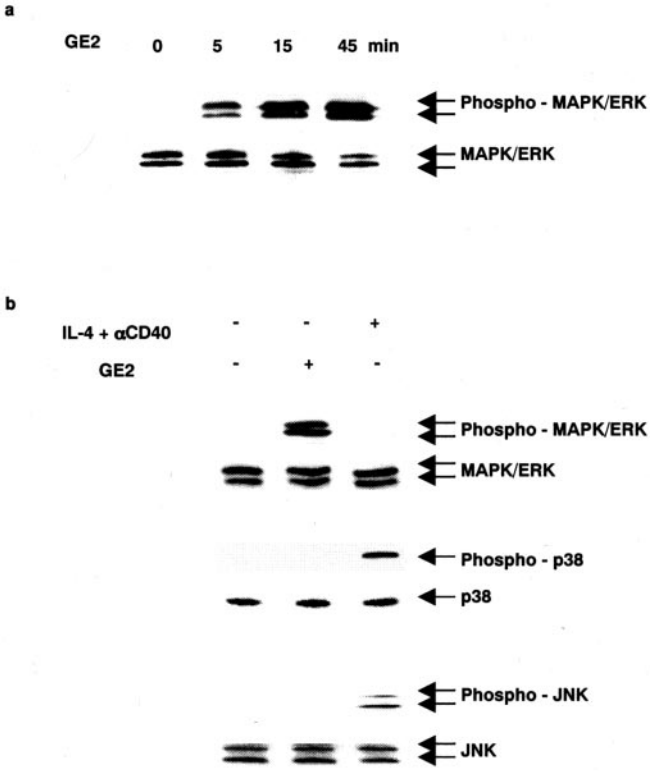

FIG. 6. **The effect of GE2 on ERK phosphorylation.** *a*, primary human B cells were cultured with GE2 (5 μg/ml) for the indicated times. Equal amounts of cell lysate were subjected to SDS-PAGE followed by blotting with anti-phosphorylated ERK Ab. *b*, human B cells were treated with or without GE2 (5 μg/ml) for 30 min. As positive controls for phosphorylated JNK and phosphorylated p38 MAPK, the cells were stimulated with IL-4 (5 ng/ml) plus αCD40 (0.1 μg/ml) for 30 min. Samples were subjected to SDS-PAGE and blotting with anti-phosphorylated ERK Ab, anti-phosphorylated JNK Ab, and anti-phosphorylated p38 MAPK Ab.

ability of GE2 to inhibit STAT6 phosphorylation, features known to play important roles in mediating the IL-4 response.

This report shows that a human Ig Fcγ-Fcε bifunctional chimeric protein inhibits CSR and activates ERK in human B cells, effects not previously reported. At this time, it is difficult to be definitive as to the exact role of CD23 and/or FcγR binding. As will be described below, we feel that the overall data support the hypothesis that binding to both CD23 and FcγR is involved in the inhibitory effect of GE2 on CSR.

CD23 binding appears to play a pivotal role in the inhibitory effect of GE2s, as might be predicted from studies with monoclonal anti-CD23 (21). Although how GE2 induces ERK phosphorylation is unknown, we found that it is also dependent on CD23 binding. Monoclonal anti-CD23 antibody induces intracellular signaling, *e.g.* polyphosphoinositide hydrolysis, and a rise in intracellular Ca^{2+} linked to a GTP-binding protein (24) that controls ERK signaling (25).

Nakamura *et al.* (5) found that the inhibitory effect of αCD23 on human IgE production is dependent on the Fcγ domain and GE2 is expected to function through FcγR binding. However, the role(s) of FcγRIIA *versus* -B in the effects of GE2 will require further dissection. It has been reported recently that the triggering of FcγRIIA activates ERK (26). If GE2 binds to and cross-links CD23 to FcγRIIA on the surface of B cells, this should lead to ERK activation and may be one likely pathway for the effect of GE2. This is further supported by our data in which GE2-induced ERK phosphorylation was observed within 15 min, a time frame wherein detectable FcγRII expression is exclusively of the type A form.

On the other hand, FcγRIIB is highly expressed on activated as opposed to resting B cells. Co-aggregation of FcγRIIB with other receptors (*e.g.* B cell receptor) is well known to have inhibitory effects on B cells by activating an inositol polyphosphate 5′-phosphatase (SHIP) and Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) (27, 28). Thus, a pathway(s) utilizing cross-linking CD23 to FcγRIIB may also be involved in GE2 effects. However, GE2 inhibition of εGT and CSR was markedly but not completely abrogated when the GE2 were added 24 h after IL-4-plus-CD40 stimulation (Fig. 8), a time when FcγRIIB expression was enhanced. Further supporting the interaction of GE2 with FcγRIIA was the loss of 50% of the GE2 effect when it was removed after 1 h. Overall, these data showing that GE2-driven inhibition of CSR mainly occurs early suggests that it is mediated via FcγRIIA, although some lesser effect at later times via other pathway(s) is likely.

An alternative explanation for the effects of GE2 is that it binds to and limits the mobility of FcγRII in the cell membrane. Although possible, we find this explanation less likely, as it has been found from studies with intact monoclonal antibodies to B cell surface molecules other than CD23 (*e.g.* CD19 or CD22), which would be expected to limit the mobility of FcR in the B cell membrane, that they generally fail to inhibit CSR. We have also recently developed a human bifunctional human Ig Fcγ-

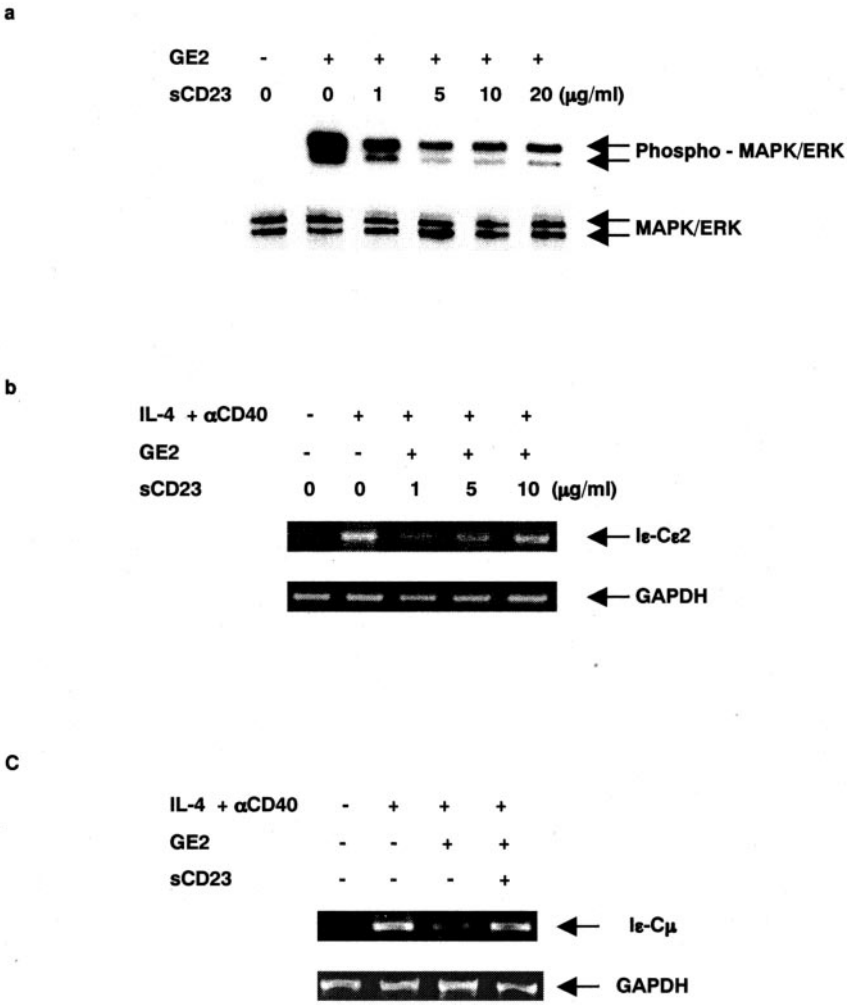

FIG. 7. **Soluble CD23 blocked the effect of GE2.** *a*, GE2 (5 μg/ml) was pretreated with sCD23 indicated, and then primary human B cells were cultured with GE2 and sCD23 under various conditions for 15 min. The cells were harvested, and equal amounts of cell lysate were subjected to SDS-PAGE followed by blotting with anti-phosphorylated ERK Ab and anti-ERK Ab. *b*, after treatment with or without GE2 (5 μg/ml) and sCD23 as indicated, the cells were cultured with IL-4-plus-αCD40 for 48 h. The samples were prepared, and εGTs (*Iε-Cε2*) were amplified. *c*, B cells were treated with or without GE2 (5 μg/ml) and sCD23 (10 μg/ml) and then stimulated for 4 days. The RNAs were prepared, and CTs (*Iε-Cμ*) were amplified. One of three separate experiments that gave similar results is shown.

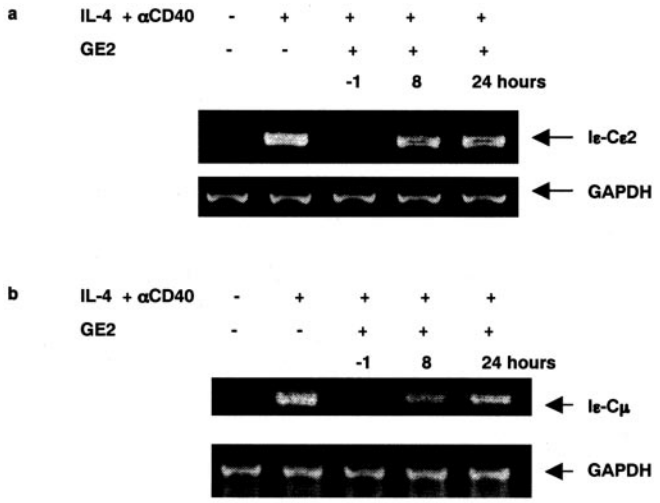

FIG. 8. **The effect of GE2 addition at later time points on εGTs and CTs in primary human B cells.** *a*, GE2 (5 μg/ml) was added 1 h before (−1) or the indicated hours (*8* or *24*) after human B cells were stimulated with IL-4 (5 ng/ml) plus αCD40 (0.1 μg/ml). Two days later after stimulation, RNAs were prepared, and εGTs (*Iε-Cε2*) were amplified. *b*, the cells were cultured for 4 days after the stimulation under various conditions, and then RNAs were prepared. CTs (*Iε-Cμ*) were amplified using synthesized cDNAs. GAPDH was used as an internal control for RT-PCR.

Fcγ protein for other purposes and took the opportunity to test the effect of this protein on CSR. This human Ig Fcγ-Fcγ protein, in doses up to 10 μg/ml, had no effect on primary B cell CSR in our system (data not shown). Although we cannot completely exclude the possibility of GE2 working via altering the mobility of FcγRII, we think it unlikely.

That GE2 appears to work, at least in part, via ERK activation, raises the question as to how ERK activation would effect CSR. Extensive studies on the mechanisms underlying IL-4 signaling have provided insight into how IL-4 regulates immune responses that involve a complex interaction of signaling pathways including the activation of JAK1, JAK3, and STAT6 (29). ERK has been shown to regulate suppressor of cytokine signaling-3 (SOCS-3) (23). Since SOCS-1 and -3 control STAT6 activation (30, 31) via JAK1, this is one plausible pathway as to how GE2, via ERK activation, may participate in CSR.

In this study we have demonstrated that GE2, a novel human Ig Fcγ-Fcε bifunctional chimeric protein that cross-links CD23 and CD32 on the surface of human B cells, is able to directly inhibit CSR to the ε IgH locus. This effect was mediated, at least partially, by an effect on the ERK-STAT pathway. GE2 has recently been shown to inhibit antigen-driven IgE-induced activation of and mediator release in basophils and mast cells by co-aggregating FcεRI with FcγRIIB (6). Thus, our demonstration of the ability of GE2 to inhibit CSR to IgE makes GE2 increasingly interesting as a potential therapy in human allergic diseases.

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