CD45 plays a critical regulatory role in receptor signaling through its protein tyrosine phosphatase and Janus kinase (JAK) phosphatase activities. To investigate whether CD45 also plays a regulatory role in Ig class switching in human B cells, we examined the effects of CD45 triggering on Ig class switching to IgE and its relationship with CD45 JAK phosphatase activity. Anti-CD45 triggering of CD45 significantly inhibited interleukin-4 (+anti-CD40-induced switch recombination in a switch recombination vector assay in stably transfected Ramos 2G6 human B cells, as well as IgF germ-line transcription and Sµ-Sε switch recombination in primary human B cells. These negative regulatory effects on Ig class switching were concomitant with the ability of CD45 to dephosphorylate the induced phosphorylation of JAK1, JAK3, and signal transducer and activator of transcription 6, but not on stress-activated/mitogen-activated protein kinases. We also showed that phosphorylated JAK1 and JAK3 were directly dephosphorylated by recombinant CD45 in vitro. These results indicate that CD45 is able to function as JAK phosphatase in human B cells and that this activity is directly associated with the negative regulation of the class switch recombination to IgE. CD45 may be an appropriate target drug for modulating IgE in allergic diseases.

CD45 is a type I transmembrane molecule and a prototypic transmembrane protein tyrosine phosphatase that has been shown to play a critical role in controlling the activation and development of lymphocytes. Anti-CD45 antibody (aCD45) has been reported to alter signaling in B cells (1–3), T cells (4, 5), basophils (6), and microglial cells (7). Anti-CD45RB is a potent immunosuppressive agent that can prevent transplant rejection in animals (8). CD45 is well known to couple to and directly regulate the activity of Src family tyrosine kinases (9).

It has been suspected that CD45 might have broader biological effects by acting on additional substrates (10). Indeed, it has been reported recently that (a) CD45 negatively regulates cytokine receptor signaling as a Janus kinase (JAK) phosphatase in hematopoietic cells (11) and (b) aCD45 can control cytokine-mediated signal transducers and activators of transcription (STAT3 and -5) to inhibit cytokine-driven lymphocyte proliferation at an early activation stage (12). These discoveries suggest that CD45 plays a wider role in cytokine- and JAK kinase-mediated B cell function, prompting us to investigate the effect of aCD45 on IL-4 signaling and Ig class switch recombination (CSR) in human B cells.

IL-4 is among the most important factors in determining Ig class switching in humans and, in particular, in the production of IgE (13, 14). The α chain of the IL-4 receptor activates the JAK family members JAK1 and JAK3 via induction of tyrosine phosphorylation, a process that is required for the subsequent tyrosine phosphorylation and activation of STAT6 (15–18). Phosphorylated STAT6 demerits and is translocated to the nucleus, where it binds to the STAT6 consensus sequences in the IgF germ-line promoter and activates germ-line transcription with production of germ-line transcripts (εGTs).

Both the process of germ-line transcription and εGTs themselves are felt to be important for switching, although the exact roles of each in Ig CSR remain to be determined. For optimal germ-line transcription and εGTs production, synergy between IL-4 stimulation and a second signal through CD40 is required (19).

In this study, we examined whether activation of CD45 could alter isotype switching in human B cells. Switch recombination was quantified employing a recently established switch vector assay (20), Sµ-Sε recombination in primary B cells was measured by digestion-circularization-PCR (DC-PCR), and εGTs were assessed by RT-PCR. We also examined the effect of aCD45 on IL-4- and aCD40-driven phosphorylation and activation of JAK1, JAK3, and STAT6, as well as phosphorylation of ε-jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), and extracellular-signal-related kinase (ERK).

EXPERIMENTAL PROCEDURES

Reagents—Human IL-4 was purchased from R&D System. Anti-CD40 mAb G28.5 were purchased from ATCC (Manassas, VA). Anti-CD45 mAb HI30, anti-CD45RA mAb HI100, anti-CD45RB mAb MT4, and anti-CD45RO mAb UCHL1 were purchased from Pharmingen (San Diego, CA). Anti-JAK1 Ab, anti-JAK3 Ab, anti-STAT6 Ab, anti- phospho-tyrosine Ab, and anti-phosphorylated STAT6 Ab were purchased from Cell Signaling (Beverly, MA). Anti-JNK Ab, anti-phosphorylated JNK Ab, anti-p38 MAPK Ab, anti-phosphorylated p38 Ab, anti-ERK Ab, and anti-phosphorylated ERK Ab were purchased from Santa Cruz.
Biotechnology (Santa Cruz, CA). Recombinant CD45 was obtained from Calbiochem (San Diego, CA). Restriction endonucleases, ligase, and mung bean nuclease used for construction of switch vector came from Promega (Madison, WI) and New England Biolabs (Beverly, MA).

**Cells, Cell Lines, and Cell Culture**—The human B lymphoma cell line Ramos 2G6 (ATCC) was maintained as a frozen cell line cultured in complete RPMI 1640. For transfection, 10 μg of plasmid DNA that had been predigested with Asel was mixed with 1 million cells in 0.2 ml and then subjected to electroporation (200 V, 0.975 millifarads). Selection of stable transfected cell lines was achieved by Geneticin (Invitrogen) selection beginning 2 days later with concentration being increased over a period of 4 weeks to 1.5 mg/ml. The switch construct XF-5a has been described previously (20).

Peripheral blood mononuclear cells were isolated from healthy volunteers by centrifugation on Ficol-Hypaque. Human B cells were purified from peripheral blood mononuclear cells by T cell depletion after monocytes/macrophages and NK 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).

**RT-PCR and RNA Extraction**—Total RNA was obtained from stimulated and unstimulated cells using Trizol reagent (Invitrogen). RNA suspended in 0.1% diethyl pyrocarbonate-treated water was digested with RNase-free RNase-free DNase (Promega (Madison, WI)) and New England Biolabs (Beverly, MA). RNA was used as templates for PCR with primer a (5'-AGCTGGTGTGAGAGG-3') and b (5'-ACACCCCTCATGACCAGAC-3'). The resultant ligated DNA was precipitated and the appropriate amounts of DNA were used for PCR with primers a (5'-GATGTGCG- TTTGGCACAATACTG-3') and b (5'-AACACCCCTCATGACCAGAC- T-3'). Amplification for 40 cycles was performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 40 cycles.

**FACS Analysis**—The expression of green fluorescence protein (GFP) in cell lines stably transfected with the switch vector after the stimulated or unstimulated culture conditions was measured by either single-color or dual-color flow cytometry (FACS Core laboratory, UCLA) as described (20). FACS data were analyzed with FCS expression software (Deno Nova software Inc., Thornhill, Ontario, Canada).

**Statistical Analyses**—Statistical analysis was performed using Mann-Whitney's U test in levels of Ig class switch recombination. Macintosh computers (StatView software, Abacus Concepts, Berkeley, CA) were used for all statistical analyses.

**RESULTS**

**Triggering of CD45 Inhibits Switch Recombination Activity in Switching Constructs**—We tested the ability of different αCD45 reagents, αCD45 (H130), αCD45RA (H1100), and αCD45RB (MT4), to affect IL-4 + αCD40-driven human Ig isotype switching. We have previously established a switch recombination assay by using GFP as an indicator for SSR in the Ramos 2G6 human B cell line (20). More than 95% of these Ramos cells express readily detectable levels of CD45, CD45RA, and CD45RB as determined by flow cytometry (data not shown). All three αCD45 attenuated IL-4 + αCD40-induced SSR (as indicated by GFP expression) from the switch construct (XF-5a) with αCD45 H130 showing the greatest inhibition (Fig. 1A). The optimal concentration of all three mAbs was 5–10 μg/ml (data not shown). Thus, αCD45 H130 was used in most of the following experiments.

As shown in Fig. 1B, IL-4 + αCD40-induced SSR was inhibited by αCD45 in a dose-dependent fashion with the maximal inhibition generally being reached at 5 μg/ml or higher. At these concentrations, αCD45 alone did not significantly alter cell viability nor did it alter viability in IL-4 + αCD40-stimulated cultures (data not shown).

It should be noted that GFP expression measures both deletional and inversional recombination events in the constructs (Fig. 1C) (20). Thus, αCD45-mediated inhibition of the GFP expression in the SSR assay reflects the suppression of recombination activity including both deletional and inversional recombination.

**CD45 Suppresses Sμ-Sε Switch Recombination in Primary B Cells**—As αCD45 was able to inhibit SSR in the human B cell line, we determined the effects of CD45 stimulation on Sμ-Sε switch recombination in human primary B cells as assessed through a quantitative DC-PCR assay. As shown in Fig. 2, Sμ-Sε recombination was readily detectable in IL4 + αCD40-stimulated primary B cells (lane 2) but was not detectable in unstimulated cells (lane 1). The induction of Sμ-Sε recombination was inhibited by αCD45 in a dose-dependent manner, with significant inhibition being achieved at 5 μg/ml (lanes 3–6). It was decreased 85% as measured by semi-quantitative analysis using densitometry.

**CD45 Negatively Regulates e Germ-line Transcripts**—To investigate the mechanism by which CD45 triggering interferes with Ig CSR, we examined the effects of CD45 (H130) on IL4 + αCD40 induction of eGTS in primary B cells and human B cell lines. Expression of IgH germ-line transcripts from Ig heavy-chain loci precedes the occurrence of isotype switching and has been shown to be important in Ig CSR (23). αCD45 inhibited IL4 + αCD40-induced eGTS in a dose-dependent manner in both primary human B cells and two human B cell lines, Ramos 2G6 and 2C4/F3 (Fig. 3, A and B). The maximum human activation-induced cytidine deaminase (AID) gene was used as an unrelated control gene for the DC-PCR assay. By12 digestion would generate a 4578-bp fragment from the human AID gene (EMBL/GenBank databases, accession No. AA704030). Primer pairs AID5 (5'-CCATGGTC- CACATCCTGACAGCAATG-3') and AID6 (5'-AGATGGTGAAACCC- GTCTTCTATTAA-3') were used. This pair of primer would amplify a 238-bp product. PCR was conducted using 20 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.
CD45 Controls IgE Class Switch Recombination

anti-CD45 leads to a decrease in IL-4-induced STAT6 phosphorylation—Because activation of STAT6 by JAK kinase is essential in mediating the IL-4 response (18) and plays an important role in both expression of eGTs and isotype switching to IgE (24), we investigated whether IL-4-induced STAT6 activation was also regulated by αCD45. Samples from appropriately stimulated cells were subjected to Western blot analysis with specific anti-phosphorylated STAT6. As shown in Fig. 5, phosphorylated STAT6 was readily detected after the stimulation. Within 5 min of IL-4 + αCD40 stimulation, STAT6 phosphorylation increased and remained elevated throughout the 20-min incubation. However, after αCD45 treatment, IL-4 + αCD40 failed to induce STAT6 phosphorylation, indicating that triggering of αCD45 strongly inhibited IL-4 + αCD40-induced STAT6 phosphorylation and activation.

Recombinant CD45 Dephosphorylates JAK1 and JAK3—The inhibition of STAT6 phosphorylation by αCD45 was likely mediated via its effect on JAK kinases. To confirm that CD45...
functions as a JAK phosphatase to negatively regulate cytokine receptor signaling in our system, we tested the phosphatase activity of recombinant CD45 in a phosphatase assay for the immunoprecipitated JAK kinases from stimulated Ramos 2G6 cells. Recombinant CD45, which contains the intracellular phosphatase domain but lacked the extracellular portion of CD45, directly dephosphorylated JAK1 and JAK3 in a dose-dependent manner (Fig. 6). The addition of vanadate, a potent inhibitor of protein tyrosine phosphatases, blocked the ability of recombinant CD45 to dephosphorylate both JAK1 and JAK3 in a dose- dependent manner (Fig. 6). The addition of vanadate, a potent inhibitor of protein tyrosine phosphatases, blocked the ability of recombinant CD45 to dephosphorylate both JAK1 and JAK3 in a dose-dependent manner (Fig. 6).

The effect of aCD45 on Phosphorylation of JNK, p38 MAPK, and ERK—We used IL-4 + aCD40 to stimulate human B cells in order to test the effects of CD45 on eGTs induction, Sp5sehen switch recombination, and the relationship of these outcomes to JAK phosphatase activity. CD40 cross-linking in B cells also increased JNK, p38 MAPK, and ERK (25–28); p38 MAPK has been reported as potentially playing an important role in eGTs (29). Therefore we also examined whether aCD45 plays a role in regulating the induced activation of these kinases. Thus we measured the effects of aCD45 on IL-4 + aCD40-induced phosphorylation of JNK, p38 MAPK, and ERK using anti-phosphorylated JNK Ab, anti-phosphorylated p38 MAPK Ab, and anti-phosphorylated ERK Ab.

JNK was activated by IL-4 + aCD40, resulting in increased JNK phosphorylation on Thr-183 and Tyr-185 (Fig. 7A). Induced JNK phosphorylation was not inhibited by the same concentration of aCD45 that strongly inhibited JAK phosphorylation (Figs. 4 and 7A). Similarly, the activation of p38 MAPK phosphorylated on Tyr-182 was not significantly altered by aCD45 treatment (Fig. 7B). ERK was only modestly phosphorylated on Tyr-204 in response to IL-4 + aCD40 and was unaffected by aCD45 treatment (Fig. 7C). aCD45 itself had no effect on activating these three kinases.
ANTI-ERK, as shown in the
The same samples were probed with anti-JNK, anti-p38 MAPK, and
switching to
/H9280
CD40 plays an important role in B cell proliferation, sur-
the induced IgE class switching, they did not prove that
CD45-dependent class
that CD45 may alter other CD40-activated or -dependent signaling pathways, e.g. CD40-
involved JAK3 and Lyn (35, 36), that are not known to be involved in class switching processes.

Isotype switching is a key event in generation of humoral immunity. IL-4 and CD40 play critical roles in this process. The
CD45 might function as a JAK phosphatase, as
reported recently (11), was further supported by our results
that recombinant CD45 directly dephosphorylated the phospho-
-STAT6 phosphorylation, provide strong evidence that
can activate multiple kinases and signal pathways (26

though we showed that the intracellular portion of CD45 pos-
phosphorylation or dephosphorylation of proteins organized in
numerous signaling intermediates and negative modulation of dif-
different parts of the IL-4 cascade remain to be elucidated. Irie-
Sasaki et al. (11) recently reported in murine system that CD45
CD45 has been suggested to be an important gatekeeper in
dephosphorylation of JAK1, JAK3, and
STAT6, molecules that play crucial roles in IL-4-induced iso-
type switching (24, 30). We also demonstrated that recombi-
CD45 directly dephosphorylated JAK1 and JAK3. At the
the same time, we did not detect any effect of CD45 on CD40-
driven activation of JNK, p38 MAPK, and ERK. These findings
suggest that CD45 controls isotype switching in human B cells
primarily through its function as a JAK phosphatase.

The biologic responses induced by IL-4 involve a complex
interaction of signaling pathways including the activation of
JAK1 and JAK3 and
STAT6. Although phosphatases, including
SHIP and SHP-1, may regulate IL-4 signaling (31, 32), the
molecular mechanisms responsible for the dephosphorylation
of key signaling intermediates and negative modulation of dif-
by CD45 engagement, suggesting that CD45-mediated JAK
phosphatase activity is responsible for the suppression, be-
cause STAT6 activation by phospho-JAK1 and -JAK3 is re-
quired for IL-4-dependent eGTs production and subsequent
SROWSER recombination. However, inhibition of SROWSER recombina-
tion might not be the sole consequence of inhibition of the
IL-4-dependent e germ-line transcription, although the latter
may well participate in the inhibition of SROWSER recombina-
For example, CD45 might inhibit SROWSER recombination through
other steps in CSR, e.g. by altering the activation and/or induc-
ment of the putative switch recombinase activity, which is also
dependent on cytokine activation (37).

CD45 has been suggested to be an important gatekeeper in
determining early intracellular signaling events by influencing
phosphorylation or dephosphorylation of proteins organized in
lipid microdomains (rafts) (38). It is expressed throughout B
these pathways are the exception of ter-
arginated plasma cells. CD45 has been shown to
play an important role in modulating the signal that is trans-
duced via the B cell antigen receptor by regulating Lyn (39, 40).
However, the role of CD45, specifically in regulating B cell class
switching, which we have studied, has not been investigated in
detail previously. By showing that CD45 negatively regulates
the phosphorylation of JAK1 and
STAT6, signal transduction
molecules known to be critical for IL-4-induced isotype switching,
we concluded that CD45 suppressed IgE isotype switching
through its JAK phosphatase activity. At the same time, we
cannot exclude the possible effects of CD45 on unknown or
other molecules including Lyn, for which involvement in the
class switching process has not been elucidated. Although re-
combinant CD45 strongly dephosphorylated JAK1 and JAK3,
it is still not clear how oCD45 modulates the protein tyrosine
phosphatase activity of CD45 so as to decrease IL-4-induced
phosphorylation of JAK1 and JAK3. Whether this is a confor-
mational effect and/or requires cross-linking is unknown. Even
though we showed that the intracellular portion of CD45 pos-
sessed JAK phosphatase activity in vitro, how CD45 acts as a
JAK phosphatase in vivo is also unknown. Finally, development-
ally regulated alternative splicing of the single CD45 gene
results in multiple isoforms with differences in their extracel-

\footnote{K. Zhang, T. Yamada, D. Zhu, and A. Saxon, unpublished data.}
lular portions and these distinct isoforms are likely associated with differential functions.

It has become increasing evident that CD45 can have both positive and negative effects in regulating receptor thresholds and in the resulting biologic outcomes. Thus it is not surprising that the role of CD45 may vary according to cell lineage and developmental stage. Indeed, CD45 activation has been implicated in such disparate events as transplant rejection (8), cell adhesion-specific signaling events (41), the pathogenesis of systemic lupus erythematosus (42–44), and Alzheimer’s disease (45, 46). Only by careful analysis in different cell types and at different developmental stages will the central role of CD45 as a regulator in specific diseases be determined. Our data shows that CD45 signaling via its JAK phosphatase activity can regulate IL-4 + αCD40-induced Ig class switching in human B cells, an event that plays a central role in the humoral immune reactivity associated with the TH2 responses seen in allergic disease and raises the possibility of CD45 as a drug target.

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REFERENCES

1. Morikawa, K., Oseko, F., and Morikawa, M. (1991) Scand. J. Immunol. 34, 273–283
2. Hasegawa, K., Nishimura, H., Ogawa, S., Hirose, S., Sato, H., and Shirai, T. (1990) Int. Immunol. 2, 367–375
3. Faries, M., Gaskin, F., Parsons, J. T., and Fu, S. M. (1994) J. Exp. Med. 179, 1923–1931
4. Mittler, R. S., Rankin, B. M., and Kiener, P. A. (1991) J. Immunol. 147, 3434–3440
5. Mittler, R. S., Schiene, G. L., Dubois, P. M., Klussman, K., O’Connell, M. P., Kiener, P. A., and Herndon, V. (1994) J. Immunol. 153, 84–96
6. Hock, W. A., Berenstein, E. H., Zanisser, F. U., Fischer, C., and Siragian, E. P. (1991) J. Immunol. 147, 2670–2676
7. Tan, J., Town, T., Mori, T., Wu, Y., Saxe, M., Crawford, F., and Mullan, M. (1995) J. Clin. Invest. 96, 907–914
8. Takekawa, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashihara, M., Nakanishi, K., Yoshiha, N., Kishimoto, T., and Akira, S. (1996) Nature 380, 627–630
9. Shapira, S. K., Verrelli, D., Jabara, H. H., Fu, S. M., and Ghea, R. S. (1992) J. Exp. Med. 175, 289–292
10. Suzuki, M., Tsuru, K., and Kishi, M. (1998) Science 282, 273–276
11. Takeuchi, T., Pang, M., Amano, K., Koide, J., and Abe, T. (1997) Clin. Exp. Immunol. 109, 44–50
12. Blank, N., Kriegel, M., Hieronymus, T., Geller, T., Winkler, P., Kalden, J. R., and Lorenz, H. M. (2001) J. Immunol. 167, 2921–2929
13. Lebman, D. A., and Coffman, R. L. (1988) J. Exp. Med. 167, 269–284
14. Berton, M. T., Uhr, J. W., and Vitetta, E. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2829–2833
15. Lehman, D. A., and Coffman, R. L. (1988) J. Exp. Med. 168, 853–862
16. Berton, M. T., Uhr, J. W., and Vitetta, E. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2829–2833
17. Fenghao, X., Saxon, A., Nguyen, A., Zhang, K., Diaz-Sanchez, D., and Nei, A. (1995) J. Clin. Immunol. 15, 249–256
18. Takekawa, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashihara, M., Nakanishi, K., Yoshiha, N., Kishimoto, T., and Akira, S. (1996) Nature 380, 627–630
19. Shapira, S. K., Verrelli, D., Jabara, H. H., Fu, S. M., and Ghea, R. S. (1992) J. Exp. Med. 175, 289–292
20. Suzuki, M., Tsuru, K., and Kishi, M. (1998) Science 282, 273–276
21. Takeuchi, T., Pang, M., Amano, K., Koide, J., and Abe, T. (1997) Clin. Exp. Immunol. 109, 44–50
22. Blank, N., Kriegel, M., Hieronymus, T., Geiler, T., Winkler, S., Kalden, J. R., and Lorenz, H. M. (2001) J. Immunol. 167, 2921–2929
23. Lebman, D. A., and Coffman, R. L. (1988) J. Exp. Med. 167, 269–284
24. Berton, M. T., Uhr, J. W., and Vitetta, E. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2829–2833
25. Lehman, D. A., and Coffman, R. L. (1988) J. Exp. Med. 168, 853–862
26. Berton, M. T., Uhr, J. W., and Vitetta, E. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2829–2833