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Hematopoietic Protein Tyrosine Phosphatase Mediates β_2-Adrenergic Receptor-Induced Regulation of p38 Mitogen-Activated Protein Kinase in B Lymphocytes[▽]

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Stimulation of the β_2-adrenergic receptor (β_2AR) on a CD40L/interleukin-4-activated B lymphocyte increases the level of immunoglobulin E (IgE) in a protein kinase A (PKA)- and p38 mitogen-activated protein kinase (MAPK)-dependent manner. However, the mechanism by which β_2AR stimulation mediates the increase in the level of p38 MAPK activation has remained unclear. Here we show that the β_2AR-induced increase in p38 MAPK activation occurred via a hematopoietic protein tyrosine phosphatase (HePTP)-mediated cross talk between PKA and p38 MAPK. β_2AR agonists, cAMP-elevating agents, and PKA inhibitors were used to show that β_2AR stimulation resulted in a PKA-dependent increase in p38 MAPK phosphorylation. Pharmacological agents and gene-deficient mice revealed that p38 MAPK phosphorylation was regulated by the G-stimulatory (Gs)/cAMP/PKA pathway independently of the G-inhibitory or β-arrestin-2 pathways. Coimmunoprecipitation and Western blot analysis showed that HePTP was phosphorylated in a PKA-dependent manner, which inactivated HePTP and allowed for increased free p38 MAPK to be phosphorylated by the MAPK cascade that was activated by CD40L. HePTP short hairpin RNA confirmed that HePTP played a role in regulating the level of p38 MAPK phosphorylation in a B cell. Thus, β_2AR stimulation on a B cell phosphorylates and inactivates HePTP in a Gs/cAMP/PKA-dependent manner to release bound p38 MAPK, making more available for phosphorylation and subsequent IgE regulation.

Signals received by a cell through surface receptor stimulation or environmental pressures activate a series of upstream dual threonine/tyrosine mitogen-activated protein kinases (MAPKs) that specifically target and activate, through phosphorylation of a Thr-X-Tyr motif, a family of widely expressed MAPKs, namely extracellular signal-regulated kinase (ERK), Jun N-terminal protein kinase, or p38 (reviewed in references 2 and 30). Activation of p38 MAPK is induced by environmental stresses, such as UV light (39) and osmotic shock (12), proinflammatory cytokines (39), growth factors (54), and activation of G-protein-coupled receptors (20–22, 58), all of which lead to changes in survival, proliferation, and/or differentiation of a cell (reviewed in reference 2). In a B cell, p38 MAPK is activated following CD40 (10, 48), B-cell receptor (48, 49), and interleukin 4 (IL-4) receptor stimulation (6), as well as lipopolysaccharide treatment (12), to mediate changes in proliferation and gene expression. Recently data from our laboratory showed that β_2-adrenergic receptor (β_2AR) stimulation on an activated B cell increased the level of p38 MAPK phosphorylation to regulate the level of immunoglobulin E (IgE) produced but not the level of IgG_1 (38).

The β_2AR is a neurotransmitter receptor expressed on the surface of the B cell that binds the neurotransmitter norepinephrine, which is released by sympathetic nerve terminals innervating all lymphoid tissues (reviewed in reference 28). β_2AR stimulation either in vivo following antigen challenge (15) or in vitro at the time of B-cell activation by CD40L and IL-4 results in increased expression of CD86 on the B-cell surface (14), soluble CD23 production (38), and levels of IgE (14, 38), IgG_1 (14, 37), and IgM (16, 40). The molecular mechanism responsible for the increased levels of IgG_1 and IgE produced by CD40L/IL-4-activated B cells exposed to norepinephrine or a β_2AR agonist was due to an increase in the rate of mature mRNA transcription, as determined by nuclear run-on analysis, without an effect on class switch recombination (36, 38). The β_2AR-induced increase in IgG_1 was mediated by protein kinase A (PKA)-dependent phosphorylation of the transcription factor CREB, which translocated to the nucleus to increase the level of the transcriptional coactivator OCA-B, which resulted in increased binding of the OCA-B/Oct-2 complex to the 3′-IgH enhancer (36). In contrast, we recently reported that the β_2AR-induced increase in IgE was mediated by an increase in both PKA and p38 MAPK activity, as well as a p38 MAPK-dependent increase in CD23 mRNA expression and cleavage of CD23 protein from the cell surface (38). These findings were the first to identify the mechanisms by which β_2AR stimulation on a B cell utilizes different signaling intermediates to regulate the level of IgG_1 or IgE, despite the fact that class switch recombination to both isotypes is induced by same signals, i.e., CD40L and IL-4. While both p38 MAPK and PKA were determined to play a pivotal role in the β_2AR-mediated regulation of the level of IgE (38), the mechanism by which β_2AR stimulation regulated the level of p38 MAPK activity, and whether or not there was a link between

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PKA activity and the level of p38 MAPK activity in a B cell, remained unknown.

Studies in vivo and in vitro have shown that β_2AR stimulation regulates the level of p38 MAPK phosphorylation in mouse cardiomyocytes (8, 34) and that this regulation occurs in a PKA-dependent manner (58). However, none of these studies determined the mechanism by which PKA regulated p38 MAPK activity, yet the mechanism may involve a signaling intermediate similar to one described in 1992 as an inducible hematopoiesis-specific protein tyrosine phosphatase in T cells that mediates PKA-dependent regulation of p38 MAPK activity (56), called hematopoietic protein tyrosine phosphatase (HePTP). HePTP is a constitutively active protein tyrosine phosphatase (PTP) in the cytoplasm of lymphocytes that binds to and regulates tyrosine phosphorylation of p38 and ERK1/2 MAPKs through its kinase interaction motif (41), with a preference for p38 MAPK (23). The kinase interaction motif site of HePTP contains a PKA-specific phosphorylation site (Ser^{23}) that when phosphorylated will render HePTP inactive and unable to regulate the level of tyrosine phosphorylation of p38 MAPK (29, 41), which is necessary for p38 MAPK activity. The HePTP-mediated cross talk between PKA and p38 MAPK was shown to occur upon the exposure of T cells to either cAMP analogs or pharmacological agents, such as prostaglandin E2, that elevated endogenous levels of cAMP (29).

We hypothesized that the β_2AR-induced increase in p38 MAPK phosphorylation and activity in B cells also occurs via an HePTP-mediated cross talk between the cAMP/PKA pathway and p38 MAPK. In this study, we show that CD40L/IL-4-activated wild-type (WT) B cells exposed to a β_2AR agonist or a cell-permeating cAMP-elevating agent had increased levels of p38 MAPK phosphorylation, which could be blocked using PKA inhibitors. The use of pharmacological agents revealed that p38 MAPK is a downstream target of the G-stimulatory (Gs)/cAMP/PKA system and not of the PKA-dependent G-inhibitory (Gi) pathway or the G-protein-independent β-arrestin pathway. Western blot analysis showed that activation of PKA by β_2AR stimulation resulted in PKA-specific phosphorylation of HePTP and mutation of this PKA-specific site regulated HePTP binding to p38 MAPK. The free p38 MAPK was then available to be phosphorylated by signals initiated upon activation of the B cell with CD40L and IL-4. Coimmunoprecipitation revealed that HePTP interacted with unphosphorylated but not with phosphorylated p38 MAPK. Using HePTP short hairpin RNA (shRNA), we found that a decrease in HePTP protein levels resulted in an increase in the level of p38 MAPK phosphorylation in unstimulated CH12.LX B cells. Thus, our findings show for the first time that HePTP mediates the β_2AR-induced regulation of the level of p38 MAPK phosphorylation and activity in an activated B cell to play a role in regulating the rate of IgE but not IgG_1 production.

MATERIALS AND METHODS

Animals. Mice were housed under pathogen-free conditions and used at 6 to 12 weeks of age. Female BALB/c mice were purchased from Taconic Farms. Mice were bred and housed within the pathogen-free facility at Taconic Farms until 6 weeks of age and then housed at The Ohio State University (Columbus, OH) in microisolator cages and provided autoclaved food and water ad libitum. CREB-dominant-negative (CREB DN) mice and littermate controls were provided by N. Muthusamy (The Ohio State University). Congenic BALB/c β_2AR-deficient mice were derived from FVB β_2AR-deficient mice kindly provided by B. Kobilka (Stanford University. Stanford, CA). β-Arrestin-2-deficient mice and littermate controls were provided by L. Bohn (The Ohio State University, Columbus, OH). All experiments complied with the Animal Welfare Act and the National Institutes of Health (Bethesda, MD) guidelines for the care and use of animals in biomedical research.

Resting B-cell isolation and cell activation. Resting B-cell isolation and activation were performed as described previously (38). Briefly, naive B cells were isolated from total splenocytes using anti-mouse CD43 magnetic beads and sorted on an AutoMacs machine, following the manufacturer's directions (Miltenyi Biotech). CD43-negative naive B cells were cultured in complete RPMI and activated with CD40L-expressing Sf-9 cells at a B-cell-to-Sf-9-cell ratio of 10:1 and with IL-4 (1 ng/ml) (eBioscience) in the presence or absence of the β_2AR agonist terbutaline (10^{-6} M), forskolin (10^{-5}M), or the β_2AR inverse agonist ICI118551 (10^{-8}M) from Sigma Aldrich. The β_2AR antagonist nadolol (10^{-5}M), PKA inhibitor H-89 (0.2 μM), general protein tyrosine phosphatase inhibitor sodium orthovanadate (1.0 μM), cholera toxin (1 ng/ml), and pertussis toxin (100 ng/ml) are all from Sigma Aldrich. Cells were pretreated for 30 min with nadolol, PKA inhibitors, or sodium orthovanadate or for 3 h with cholera toxin and pertussis toxin prior to activation of the cells with CD40L and IL-4. Experiments were conducted in 1.5-ml tubes in a 37°C water bath, centrifuged at 5,000 × *g* for 30 s in order to accurately obtain the earliest time points. All of the reagents used for B-cell isolation, activation, and pharmacologic treatment tested negative for the presence of endotoxin using E-TOXATE (Sigma Aldrich), a limulus lysates assay with a level of detection of <0.1 U/ml.

Cell lines. CH12.LX is a murine B-cell lymphoma line that has been described previously (13) and was provided by G. Bishop (University of Iowa, Iowa City, IA).

Western blot. Western blot analysis was performed as described previously (38). Briefly, following treatment and activation, B cells were lysed and protein samples (5 to 15 μg) were resolved by electrophoresis on 10% polyacrylamide gels (Bio-Rad), transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore), probed with either anti-p38 MAPK, anti-p38α MAPK, or anti-phospho-(Thr^{180}/Tyr^{182})p38 MAPK (Cell Signaling Technology), anti-HePTP, anti-β-actin (Santa Cruz Biotechnology), or anti-phospho-(Ser^{23})HePTP (21st Century Biotechnology), detected with horseradish peroxidase-labeled secondary antibodies (Santa Cruz), and developed with the LumiGlo detection kit (Cell Signaling Technology). Luminescence was visualized on Kodak Biomax MS film, and densitometry was performed using the NIH Image J 1.61 software program.

Generation of anti-phospho-(Ser^{23})HePTP antibody. Anti-phospho-(Ser^{23})HePTP was manufactured by 21st Century Biochemicals. Briefly, a peptide corresponding to the sequence LQERRG[pS]SVALML was manufactured by Fmoc chemistry and high-performance liquid chromatography purified to >90% and the mass and sequence verified by nanospray mass spectrometry and collision-induced dissociation tandem mass spectrometry, respectively. This peptide was conjugated to a carrier protein and used to immunize two New Zealand rabbits. After multiple boosts and bleeds, the antibody was isolated from serum that detected the target protein via Western blots and was pooled and immunodepleted by passing the serum over cross-linked agarose conjugated with unmodified peptide, followed by affinity purification using phosphopeptide-cross-linked agarose.

Immunoprecipitation. Immunoprecipitations were performed using protein A or protein G agarose beads. B cells were activated as described above and lysed in coimmunoprecipitation lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl with phenylmethylsulfonyl fluoride, protease inhibitor, and phosphatase inhibitor cocktails 1 and 2 [Sigma Aldrich] added fresh) at the indicated time points. Lysates were incubated on ice for 30 min, and then cellular debris was removed by centrifugation at 12,000 × *g* for 10 min. Approximately 80 to 100 μg of total protein was incubated with either anti-total p38α MAPK (Cell Signaling Technology), anti-hemagglutinin (HA) (Abcam), or anti-phospho-(Thr^{180}/Tyr^{182})p38 MAPK (Cell Signaling Technology) overnight at 4°C with gentle rocking. Protein A or Protein G agarose beads were added for 3 h at 4°C with gentle rocking. Beads were washed twice with coimmunoprecipitation lysis buffer and then prepared for Western blot analysis as described previously (38).

p38 MAPK activity assay. p38 MAPK activity was assessed using the p38 MAPK activity assay (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, B cells were activated as described above, and total protein was collected. Phosphorylated (Thr^{180}/Tyr^{182}) p38 MAPK was immunoprecipitated from 80 μg of total protein, followed by an in vitro kinase assay using an ATF-2 fusion protein as a substrate. ATF-2 phosphorylation was detected by Western blot analysis using an anti-phospho-(Thr^{71})ATF-2 antibody. Densitometry was performed using NIH Image J 1.61.

cAMP assay. cAMP accumulation was determined using the Parameter cAMP assay (R&D Systems) according to the manufacturer's directions. Resting B cells

(5×10^6) were pretreated with 1 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Aldrich) for 10 min at 37°C prior to activation in the presence of a β_2AR agonist or forskolin for 1 to 10 min as described previously. Reactions using cholera toxin were pretreated for 3 h with the toxin and then pretreated for an additional 10 min with 1 μM IBMX prior to activation. Following activation, the reactions were terminated by centrifugation at 5,000 × *g*, aspiration of the medium, and then addition of 250 μl of cAMP lysis buffer on ice. Cells were lysed by two freeze/thaw cycles at −20°C, and the total protein content was determined according to the manufacturer's instructions using a bicinchoninic acid protein assay kit (Thermo Scientific). The cAMP assay was performed, optical density was determined using a SpectraMax M2 microplate reader (Molecular Devices), and data were normalized to total protein content and expressed as pmol of cAMP/mg total protein.

Transfections. The HePTP shRNA plasmids and control plasmid were purchased from SuperArray. The HePTP mutant plasmids were provided by T. Mustelin (Burnham Institute for Medical Research, La Jolla, CA) and were described in detail previously (41). Transfections were performed using program K-03 of the Nucleofector device (Amaxa), following the manufacturer's directions. CH12.LX (5×10^6) cells were resuspended in 100 μl of transfection solution (Amaxa) with 10 μg of plasmid DNA. Following transfection, the cells were cultured as described above for 48 h and then collected and resuspended in phosphate-buffered saline for fluorescence-activated cell sorting (BD FACS Aria cell sorter; The Ohio State University) or prepared for coimmunoprecipitation as described above.

Quantitative real-time PCR. Quantitative real-time PCR was performed as described previously (38). The following primers were used: β-actin (5′-TACA GCTTCACCACCACAGC-3′ and 5′-AAGGAAGGCTGGAAAAGAGC-3′) (annealing temperature, 60°C); mature IgE (5′-TGGGCATGAATTAATGGT TACTAGAG-3′ and 5′-TTACAGGGCTTCAAGGGGTAGAGC-3′) (annealing temperature, 60°C); mature IgG_1 (5′-TATGGACTACTGGGGTCAAG-3′ and 5′-CCTGGGCACAATTTTCTTGT-3′) (annealing temperature, 63°C).

Statistics. Data were analyzed by analysis of variance to determine whether an overall statistically significant change existed. Certain *P* values were calculated using either a Bonferroni post hoc test for comparison of more than two treatment groups or a Student *t* test for comparison between two treatment groups. A *P* value of ≤0.05 indicated statistically significant results.

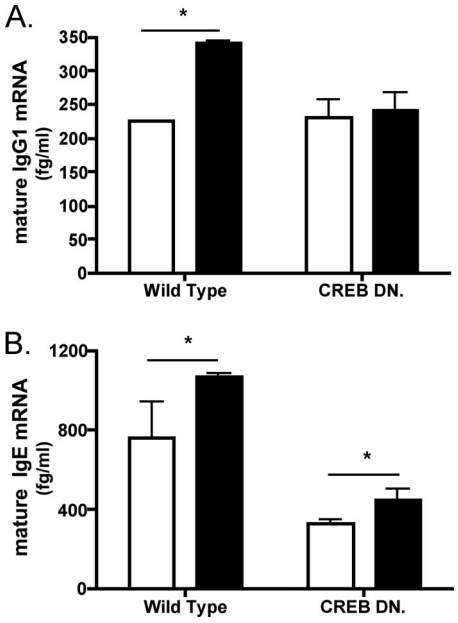

FIG. 1. CREB is required for the β_2AR-induced increase in IgG_1 but not for the increase in IgE. Naive B cells were isolated from the spleens of WT and CREB DN mice and then activated by CD40L/IL-4 in the absence (open bars) or presence (filled bars) of terbutaline. After 5 days of culture, cells were collected, total mRNA was isolated, and the level of mature IgG_1 (A) or mature IgE (B) mRNA was determined using real-time PCR analysis. The data were normalized to β-actin, and each bar represents the mean fg/ml ± standard error of the mean from three independent experiments, (*, $P \leq 0.05$).

RESULTS

CREB is required for the β_2AR-induced increase in IgG_1 but not for the increase in IgE. Activation of PKA following β_2AR stimulation is required for the increase in both IgG_1 and IgE (14, 38). A known downstream target for PKA is the transcription factor CREB, which was shown to be required for the β_2AR-induced increase in IgG_1 (36). Therefore, to determine if CREB activation was also required for the β_2AR-induced increase in IgE and to confirm its requirement for the increase in IgG_1, WT B cells and CREB DN B cells, which contain a serine-to-alanine substitution at position 133 in the PKA-specific phosphorylation site, were activated with CD40L and IL-4 in the absence or presence of the β_2AR agonist, terbutaline. The B cells were collected after 5 days in culture, and total mRNA was isolated and analyzed by quantitative real-time PCR for mature IgG_1 and IgE mRNA. As shown in Fig. 1A, the WT B cells activated and exposed to terbutaline were able to significantly increase the level of mature IgG_1 mRNA above that induced by CD40L/IL-4 activation alone, while the CREB DN B cells were unable to regulate the level of mature IgG1 mRNA in response to terbutaline. In contrast, both the WT and CREB DN B cells exposed to terbutaline were able to significantly increase the level of mature IgE mRNA above that induced by CD40L/IL-4 activation alone (Fig. 1B), even with the expected decrease in the baseline level of IgE measured in the CREB DN B cells (44, 57). Thus, these data show that β_2AR stimulation on an activated B cell regulates IgG_1 via a CREB-dependent mechanism while IgE is regulated independently of CREB activation.

PKA mediates the β_2AR-induced increase in p38 MAPK phosphorylation. β_2AR regulation of the level of IgE appears to be mediated by the activation of a different signaling intermediate than that involved in regulating the level of IgG_1, despite the fact that the same B-cell activation stimuli, i.e., CD40L and IL-4, induce class switch recombination to either IgG_1 or IgE in a naive B cell. Previously published data using PKA and p38 MAPK inhibitors showed that both kinases were activated following β_2AR stimulation and both were required for the β_2AR-induced increase in the level of IgE, while only PKA was required for the increase in the level of IgG_1 (38). However, the mechanism responsible for differential regulation of the isotypes was unknown. Because there is a report showing that p38 MAPK phosphorylation by β_2AR stimulation on mouse cardiac myocytes is dependent on PKA activation (58) and because the classic β_2AR signaling pathway, cAMP/PKA, was shown to increase p38 MAPK phosphorylation in a T cell (41), we sought to determine if PKA mediated the β_2AR-induced increase in p38 MAPK phosphorylation in a B cell, as well. B cells from WT and β_2AR-deficient mice were activated and exposed to terbutaline or the cell-permeating cAMP-elevating agent forskolin to activate the cAMP/PKA pathway independently of β_2AR stimulation. Total protein was collected over a 15-min time course and analyzed by Western blotting for the level of Thr^{180}/Tyr^{182} phosphorylation of p38 MAPK, since the phosphorylation of both sites is required for activation of p38 MAPK (discussed in reference 2). As shown

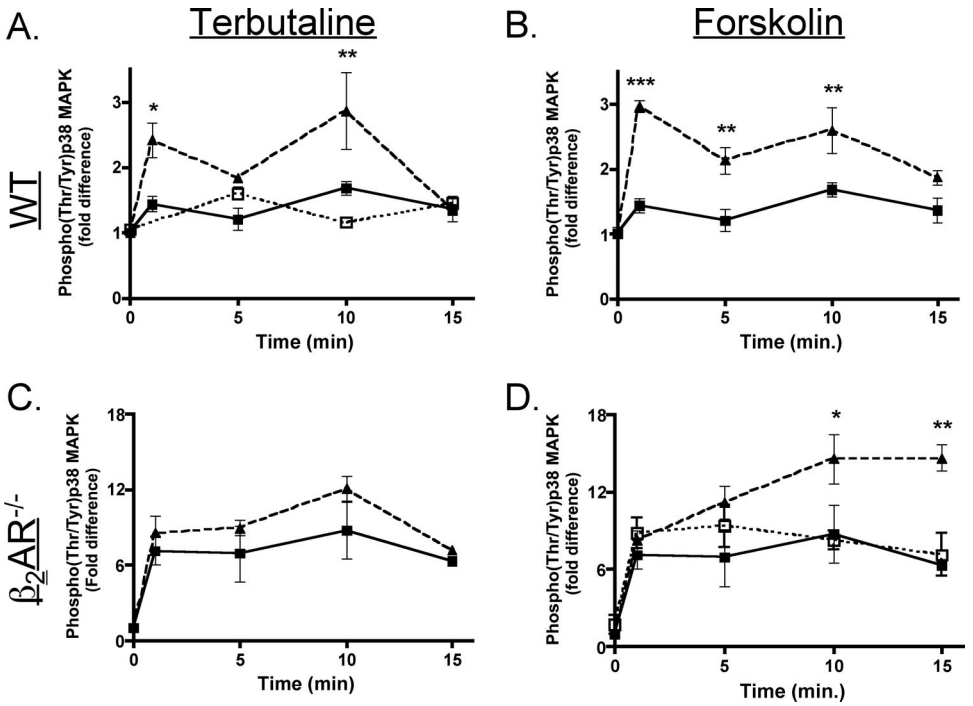

FIG. 2. β_2AR stimulation and PKA activation result in an increase in p38 MAPK phosphorylation in activated B cells. Naive B cells were isolated from the spleens of WT (A and B) and β_2AR-deficient mice (C and D) and activated by CD40L/IL-4 in the absence or presence of terbutaline (A and C) or forskolin (B and D). CD40L/IL-4-activated cells (solid squares), terbutaline- or forskolin-exposed cells (triangles), and cells pretreated with H-89 prior to exposure to terbutaline or forskolin (open squares) are analyzed. Total protein was isolated at the indicated time points, and the level of total and phospho-(Thr^{180}/Tyr^{182})p38 MAPK was analyzed by Western blotting. Band density was determined using densitometry, and each data point represents the mean difference of phospho-(Thr/Tyr)p38 MAPK normalized to total p38 MAPK and compared to resting levels ± standard error of the mean from three independent experiments. Exposure groups were compared to CD40L/IL-4-activated cells for statistical significance, (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

in the upper panels of Fig. 2, the activated WT B cells exposed to either terbutaline or forskolin showed a similar two- to threefold increase in p38 MAPK phosphorylation 1 to 10 min following activation, returning to baseline levels by 15 min, similar to the time course previously shown for MAPK phosphorylation (45). In contrast, as shown in the lower panels of Fig. 2, B cells lacking expression of a functional β_2AR showed a two- to threefold increase in p38 MAPK phosphorylation only after activation in the presence of forskolin, indicating that a cAMP-dependent mechanism was necessary to regulate the level of p38 MAPK phosphorylation following β_2AR stimulation on a B cell and that terbutaline was specifically stimulating the β_2AR to mediate this affect. Pretreatment with a selective PKA inhibitor blocked the terbutaline- and forskolin-induced increase in p38 MAPK phosphorylation in WT and β_2AR-deficient B cells, respectively, and a β_2AR antagonist, nadolol, blocked the terbutaline-induced increase in p38 MAPK phosphorylation in WT B cells (data not shown), indicating that terbutaline stimulates the β_2AR to activate the cAMP/PKA system to induce an increase in the level of p38 MAPK phosphorylation. Collectively, these data support a mechanism by which β_2AR stimulation on an activated B cell results in a PKA-dependent phosphorylation of CREB to regulate the level of IgG_1 (36) or a PKA-dependent increase in the level of p38 MAPK phosphorylation to regulate the level of IgE (38).

Gs/cAMP/PKA pathway mediates β_2AR-induced increase in p38 MAPK phosphorylation. Agonist stimulation of the β_2AR results in a high-affinity interaction between the receptor and the stimulatory G-protein (Gs), which leads to an increase in cAMP and activation of PKA. Concurrently, active PKA phosphorylates multiple sites on the intracellular loops of the β_2AR itself, which switches receptor binding from the Gs protein to the inhibitory G protein (Gi) to activate a negative feedback loop. This inhibitory pathway reduces signaling through the Gs/cAMP/PKA pathway and subsequently allows for MAPK activation (55). To determine if PKA-dependent phosphorylation of p38 MAPK is mediated by the Gs protein, cAMP, and PKA activation, we bypassed the β_2AR and used cholera toxin to induce activation of Gs independently of β_2AR stimulation, which is necessary for the Gs-to-Gi switch. After 10 min of exposure, cells were collected and analyzed for the level of cAMP accumulation, PKA activation, and/or p38 MAPK phosphorylation and activity. Figure 3A shows that in comparison to the level of cAMP accumulation in CD40L/IL-4-activated B cells, cells exposed to terbutaline, cholera toxin, and forskolin showed a three- to fivefold increase in the level of cAMP accumulation. Likewise, the same exposure groups showed elevated levels of PKA activity, which positively correlated with the level of cAMP accumulation (Fig. 3B). Lastly, the level of Thr^{180}/Tyr^{182} phosphorylation of p38 MAPK (Fig. 3C), the sites essential for p38 MAPK activation, and p38 MAPK ac-

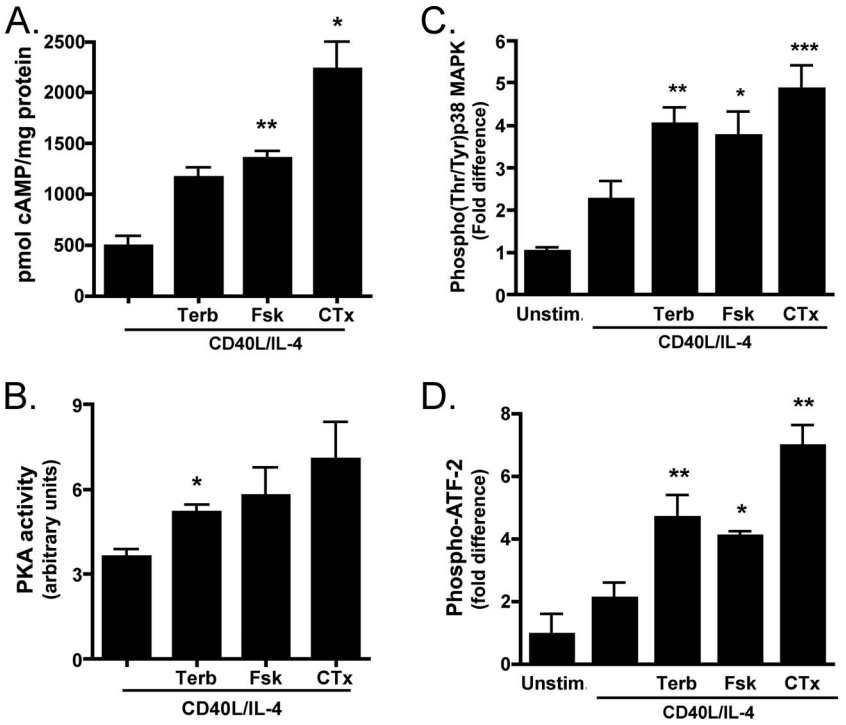

FIG. 3. β_2AR stimulation activates the Gs/cAMP/PKA pathway to increase the level of p38 MAPK phosphorylation and activity in an activated B cell. Naive B cells from the spleens of WT mice were activated with CD40L/IL-4 in the absence or presence of terbutaline (Terb), forskolin (Fsk), or cholera toxin (CTx). All data were collected after 10 min of activation, and exposure groups were compared to CD40L/IL-4-activated cells for statistical significance (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). (A) B cells were preincubated with IBMX for 10 min and then activated as described above. Cells were lysed, and cAMP accumulation was measured and normalized to total protein isolated. Each bar represents the mean pmol cAMP/mg of protein ± standard error of the mean from three independent experiments. (B) B cells were activated as described and then lysed and assayed for PKA activity. Each bar represents the mean PKA activity in arbitrary units ± standard error of the mean from three independent experiments. (C) Total protein was collected from B cells activated as described and analyzed by Western blotting for total and phospho-(Thr/Tyr)p38 MAPK. Band density was determined using densitometry, and each bar represents the mean difference in phospho-p38 MAPK normalized to total p38 MAPK ± standard error of the mean from three independent experiments. (D) B cells were activated as described above, total protein was collected, and phospho-(Thr/Tyr)p38 MAPK was immunoprecipitated from 80 μg of protein and then assayed for p38 MAPK activity using an in vitro kinase assay and an ATF-2 fusion protein as a target. The level of ATF-2 phosphorylation was determined by Western blot analysis, and the band density was determined using densitometry. Each bar represents the mean difference in phospho-ATF-2 normalized to total p38 MAPK ± standard error of the mean from three independent experiments.

tivity (Fig. 3D) were increased compared to the basal level measured in unstimulated B cells. Activation with CD40L/IL-4 alone increased the levels of p38 MAPK phosphorylation and activity two- to threefold, while cholera toxin, terbutaline, or forskolin resulted in a four- to sixfold increase. Thus, these data indicate that the Gs/cAMP/PKA pathway mediates the β_2AR-induced increase in p38 MAPK phosphorylation and activity.

The Gs-to-Gi switch is not necessary for increased p38 MAPK phosphorylation. Our findings indicate that activation of the Gs/cAMP/PKA pathway is important for the β_2AR-induced increase in p38 MAPK phosphorylation. Active PKA phosphorylates the β_2AR to inhibit receptor binding to the Gs protein and induce a high-affinity interaction with the Gi protein and activate a negative feedback loop. Because this pathway is PKA dependent and can lead to MAPK activation (55), it is possible that the Gi protein may be mediating the PKA-dependent activation of p38 MAPK. To determine if Gi activation is necessary for the β_2AR-induced increase in p38 MAPK phosphorylation, we pretreated cells with pertussis toxin to inhibit Gi activation and then activated the cells in the presence of terbutaline. Following 10 min of activation, total protein was isolated and the levels of p38 MAPK phosphorylation (Fig. 4A) and activation (Fig. 4B) were determined. Cells that were pretreated with pertussis toxin were still able to increase p38 MAPK phosphorylation and activation to levels comparable to those of cells activated and exposed to terbutaline. Thus, these data indicate that p38 MAPK activation is directly downstream of the Gs/cAMP/PKA pathway and not the PKA-dependent Gi pathway.

β-Arrestin activation is not necessary for β_2AR-induced increase in p38 MAPK phosphorylation. While our findings indicate that p38 MAPK phosphorylation induced by β_2AR stimulation on an activated B cell is regulated primarily by Gs/cAMP/PKA and not the Gi pathway, another G-protein-independent β_2AR-activated pathway might also contribute. Upon binding of ligand to the β_2AR, G-protein-coupled receptor kinases (GRKs) are recruited to the receptor to phosphorylate specific sequences on the cytoplasmic loops that are revealed only when ligand is bound to the receptor (18, 51). The phosphorylation allows for the recruitment of β-arrestins to the β_2AR, which not only mediate internalization of the

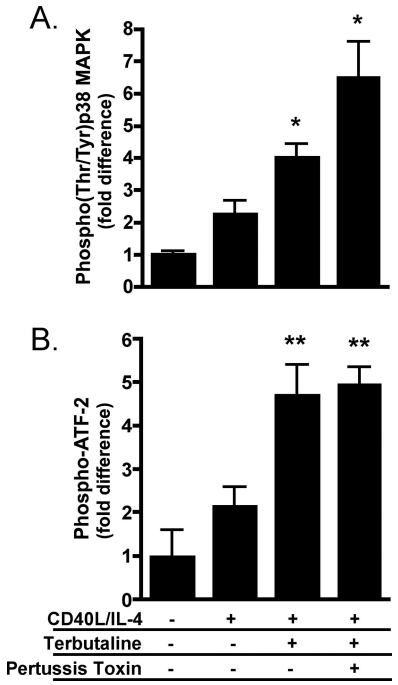

FIG. 4. Gi activation is not necessary for the β_2AR-induced increase in p38 MAPK phosphorylation in an activated B cell. Naive B cells from the spleens of WT mice were activated with CD40L/IL-4 in the absence or presence of terbutaline or pertussis toxin. All exposure groups were compared to CD40L/IL-4-activated cells for statistical significance (*, $P \leq 0.05$; **, $P \leq 0.01$). (A) Total protein was collected 10 min after activation and analyzed by Western blotting for total and phospho-(Thr/Tyr)p38 MAPK. Band density was determined using densitometry, and each bar represents the mean difference in phospho-p38 MAPK normalized to total p38 MAPK ± standard error of the mean from three independent experiments. (B) B cells were activated as described, total protein was collected after 10 min of activation, and phospho-(Thr/Tyr)p38 MAPK was immunoprecipitated from 80 μg of protein and assayed for p38 MAPK activity as described for Fig. 3. The level of ATF-2 phosphorylation was determined by Western blot analysis, and band density was determined using densitometry. Each bar represents the mean difference in phospho-ATF-2 normalized to total p38 MAPK ± standard error of the mean from three independent experiments.

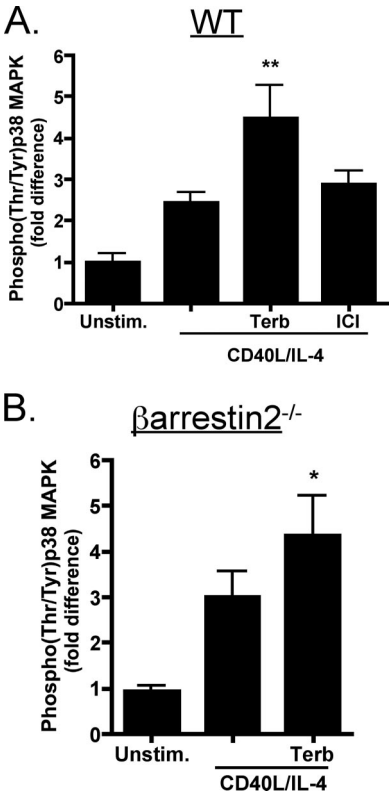

FIG. 5. β-Arrestin activation is not necessary for a β_2AR-induced increase in p38 MAPK phosphorylation in an activated B cell. Naive B cells from the spleens of WT (top panel) or β-arrestin-2-deficient (bottom panel) mice were activated by CD40L/IL-4 in the absence or presence of either terbutaline or ICI118551. Total protein was collected, and total and phospho-(Thr/Tyr)p38 MAPK levels were determined by Western blot analysis. Band density was determined using densitometry, and each bar represents the mean difference in phospho-p38 MAPK normalized to total p38 MAPK ± standard error of the mean from four independent experiments. Exposure groups were compared to CD40L/IL-4-activated cells for statistical significance (*, $P \leq 0.05$; **, $P \leq 0.01$).

receptor but also act as scaffolding proteins for signaling intermediates, such as molecules in the MAPK cascade (45). Recruitment of, and receptor internalization by, GRK/β-arrestins is dependent upon ligand binding to the receptor but independent of any G-protein activation (discussed in reference 46) and could play a role in mediating the β_2AR-induced regulation of the level of p38 MAPK phosphorylation. To determine if GRK/β-arrestin-mediated activation of MAPKs contributed to the β_2AR-induced increase in p38 MAPK phosphorylation, we used a β_2AR inverse agonist that has been reported to stabilize the receptor in a conformation that allows for activation of the GRK/β-arrestin pathway independently of G-protein pathways (3) while preventing activation of the G proteins. In addition to WT B cells, cells from β-arrestin-2-deficient mice were also used, because β-arrestin-2 has been shown to bind the β_2AR in several cell types and to mediate activation of MAPKs (reviewed in reference 19). WT B cells were activated and exposed to either terbutaline or the inverse agonist, ICI118551, and then analyzed for levels of total and Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK by Western blotting. In contrast to the increased levels of phosphorylated p38 MAPK measured when terbutaline was added to activated B cells, treatment with ICI118551 did not increase the level of p38 MAPK phosphorylation above that induced by CD40L/IL-4 activation alone (Fig. 5A), suggesting that β-arrestins do not play a role in mediating the β_2AR-induced increase in the level of p38 MAPK phosphorylation. To confirm the findings with ICI118551, β-arrestin-2-deficient B cells that were activated and exposed to terbutaline were able to increase the level of p38 MAPK phosphorylation above the levels measured in unstimulated and activated cells to levels comparable to the terbutaline-induced response measured in WT B cells (Fig. 5B), suggesting that β-arrestin-2 recruitment is not necessary for the β_2AR-induced increase in the level of p38 MAPK phosphorylation. Thus, these data indicate that β_2AR stimulation on a CD40L/IL-4-activated B cell increases the level of p38 MAPK phosphorylation and activation in a Gs/cAMP/PKA-

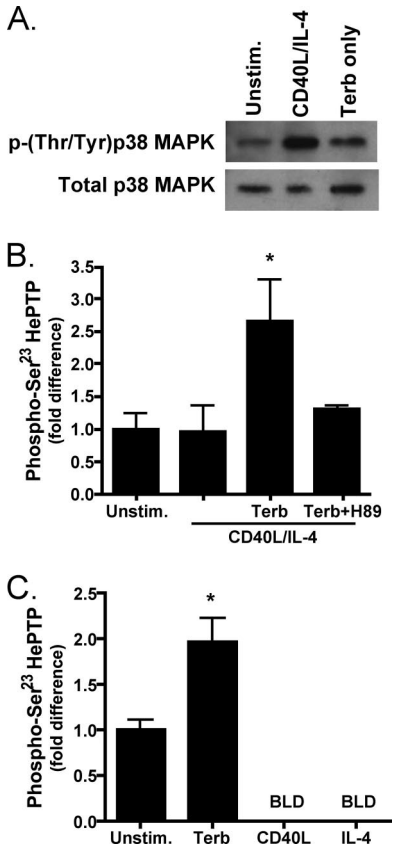

FIG. 6. β_2AR stimulation alone and in the presence of CD40L/IL-4 activation induces PKA-specific phosphorylation of HePTP in a B cell. (A) Naive B cells were either not activated (Unstim.) or activated with CD40L/IL-4 or terbutaline (Terb) only. Total protein was collected after 10 min of culture and analyzed for total and phospho(Thr/Tyr)-p38 MAPK by Western blotting. One representative blot from three independent experiments is shown. Naive B cells from the spleens of WT mice either were not activated or were activated with CD40L/IL-4 in the presence of terbutaline with or without H-89 pretreatment (B) or CD40L, IL-4, or terbutaline alone (C). Total protein was collected after 10 min of culture and analyzed for total and PKA-specific phospho-(Ser^{23})HePTP by Western blotting. Band density was determined using densitometry, and each bar represents the mean difference in PKA-specific phospho-(Ser^{23})HePTP normalized to total HePTP ± standard error of the mean from three independent experiments. Exposure groups were compared to CD40L/IL-4-activated cells (B) or resting cells (C) for statistical significance (*, $P \leq 0.05$).

dependent manner, independently of Gi activation or β-arrestin recruitment.

HePTP is phosphorylated by PKA following β_2AR stimulation. Our data indicate that the Gs/cAMP/PKA pathway regulates the level of p38 MAPK phosphorylated at Thr^{180}/Tyr^{182}, both of which are required for activation of p38 MAPK. However, PKA is a serine/threonine kinase and therefore is unable to directly phosphorylate the Tyr^{182} residue to fully activate p38 MAPK. We hypothesized that another kinase or phosphatase was responsible for mediating the cross talk between the cAMP/PKA system and p38 MAPK. While PKA has been reported to regulate small GTPases that may result in changes in p38 MAPK phosphorylation (7, 53), these molecules are capable of activating the MAPK signaling cascade to phosphorylate p38 MAPK independently of any other activating signals. Our data showed that CD40L/IL-4 activation of B cells induced a baseline level of p38 MAPK phosphorylation and that β_2AR stimulation alone was unable to increase the level of p38 MAPK phosphorylation above the level measured in resting cells (Fig. 6A), suggesting that PKA activation of the upstream MAPK cascade via small GTPase activation is not involved in the β_2AR-induced increase in the level of p38 MAPK phosphorylation. However, PKA has been reported to regulate a PTP, called HePTP, that binds to and regulates tyrosine phosphorylation of p38 MAPK in T cells (41). To test the role of PTPs in regulating p38 MAPK phosphorylation after β_2AR stimulation on an activated B cell, we pretreated the cells with a general PTP inhibitor, sodium orthovanadate, prior to activation and terbutaline exposure. Treatment with sodium orthovanadate resulted in an increase in the level of p38 MAPK phosphorylation in B cells activated with CD40L/IL-4 in the absence or presence of terbutaline (data not shown), suggesting that regulation of a PTP may play a role in regulating the level of p38 MAPK phosphorylation.

HePTP is a constitutively active protein tyrosine phosphatase that regulates of the level of p38 or ERK MAPK phosphorylation in total splenocytes (56) with a preference for p38 MAPK (23). In the T cell, HePTP can be phosphorylated by PKA, which inactivates the phosphatase and causes it to release p38 MAPK into the cytoplasm, where it is phosphorylated by other signaling cascades in the cell (41). Taken together with our findings that a PTP played a role in regulating the level of p38 MAPK phosphorylation in an activated and β_2AR-stimulated B cell, we hypothesized that HePTP may mediate cross talk between the Gs/cAMP/PKA system and p38 MAPK activation following β_2AR stimulation. In order for HePTP to mediate this cross talk, it would need to be a downstream target for PKA-mediated phosphorylation and it would need to bind to p38 MAPK. To determine if HePTP is phosphorylated by PKA following β_2AR stimulation on an activated B cell, WT B cells were activated and exposed to terbutaline or H-89 and then analyzed by Western blotting for PKA-specific Ser^{23} phosphorylation of HePTP, which has been shown to inactivate HePTP (29, 41). Unstimulated and CD40L/IL-4-activated cells had low levels of PKA-specific phosphorylation of HePTP at Ser^{23}, while cells activated in the presence of terbutaline had significantly increased levels of Ser^{23}-phosphorylated HePTP, which could be inhibited with H-89 pretreatment (Fig. 6B). These data show that HePTP is phosphorylated by PKA following β_2AR stimulation on an activated B cell. To determine if CD40L/IL-4 activation of the B cell was necessary for PKA-specific phosphorylation of HePTP to occur, WT cells were exposed to CD40L, IL-4, or terbutaline alone. Terbutaline-only-exposed B cells had significantly increased levels of Ser^{23}-phosphorylated HePTP, while the level in CD40L- and IL-4-only-exposed cells was below the level of detection, suggesting that CD40L/IL-4 activation was not necessary for β_2AR-mediated phosphorylation of HePTP (Fig. 6C). Nonetheless, CD40L/IL-4 stimulation was necessary for p38 MAPK phosphorylation to occur (Fig. 6A), suggesting that Gs/cAMP/PKA activation and HePTP phosphorylation did not mediate phosphorylation of p38 MAPK but merely regulated the level of p38 MAPK phosphorylation that occurs through B-cell activation signals. Thus, these data show that

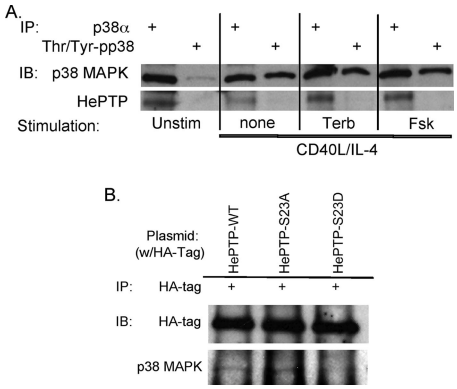

FIG. 7. Unphosphorylated forms of HePTP and p38 MAPK interact constitutively, while phosphorylated forms do not interact in a resting or activated B cell. (A) Naive B cells from WT mice were activated with CD40L/IL-4 in the absence or presence of terbutaline or forskolin. Total protein was isolated after 10 min of culture, and coimmunoprecipitation was performed with antibodies to either total p38 MAPKα or phospho-(Thr/Tyr)p38 MAPK. Immunoprecipitates were analyzed by Western blotting for HePTP or total p38 MAPK. One representative blot from three independent experiments is shown. (B) Unactivated CH12.LX B cells were transfected with plasmids containing HA-tagged HePTP-WT, HePTP-S23A, or HePTP-S23D for 48 h, and then the cells were lysed and total protein was collected. Coimmunoprecipitation was performed using an anti-HA antibody, and immunoprecipitates were analyzed by Western blotting for HA-tagged HePTP and total p38 MAPK. One representative blot from three independent experiments is shown.

β_2AR stimulation activates the Gs/cAMP/PKA pathway to phosphorylate the PKA-specific Ser^{23} residue of HePTP and that this phosphorylation is independent of CD40L/IL-4 activation of the B cell.

Regulation of the HePTP-p38 MAPK interaction. HePTP has a kinase interaction motif that allows it to bind to and dephosphorylate the Tyr^{182} residue in p38 MAPK (26, 41). PKA-dependent Ser^{23} phosphorylation of HePTP, which is located in the kinase interaction motif, inactivates HePTP and causes it to dissociate from p38 MAPK (41), allowing phosphorylation by the MAPK signaling cascade activated upon CD40L/IL-4 exposure. Therefore, we hypothesized that HePTP would be bound to unphosphorylated p38 MAPK and not to Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK in an activated and terbutaline-exposed B cell. To determine if HePTP interacts with unphosphorylated and/or phosphorylated p38 MAPK in a B cell, we used antibodies to total p38α MAPK and Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK to coimmunoprecipitate the HePTP-p38 MAPK complex from resting, activated, and terbutaline- or forskolin-treated B cells. Western blot analysis was used to determine if HePTP was bound to the immunoprecipitated p38 MAPK (Fig. 7A). Under all conditions, HePTP immunoprecipitated with total p38α MAPK but not with Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK, suggesting that HePTP is bound to unphosphorylated p38 MAPK and not to Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK in a B cell.

To determine if the PKA-dependent phosphorylation of Ser^{23} in the HePTP kinase interaction motif regulated HePTP binding to p38 MAPK, mutant HA-tagged HePTP molecules mimicking WT (HePTP-WT), unphosphorylated (serine-to-alanine mutation at position 23 [HePTP-S23A]), and PKA-phosphorylated HePTP (serine-to-aspartic acid mutation at position 23 [HePTP-S23D]) were transiently transfected into the murine B-cell line, CH12.LX. The cells were lysed, total protein was collected 48 h after transfection, and the mutant HePTP molecules were immunoprecipitated. The immunoprecipitates were analyzed by Western blotting for the amount of p38 MAPK bound to the mutant HePTP molecules, which showed that p38 MAPK coimmunoprecipitated with HePTP-WT and HePTP-S23A and not with HePTP-S23D (Fig. 7B), suggesting that PKA-dependent Ser^{23} phosphorylation of HePTP regulates its ability to bind to p38 MAPK in a B cell. Taken together, these results suggest that HePTP and p38 MAPK interact in a B cell and that PKA-dependent Ser^{23} phosphorylation of HePTP and/or Thr^{180}/Tyr^{182} phosphorylation of p38 MAPK results in dissociation of the complex.

HePTP regulates the level of p38 MAPK phosphorylation in a B cell. The data thus far show that PKA regulates the level of HePTP and p38 MAPK phosphorylation and that HePTP binds to unphosphorylated p38 MAPK, but Ser^{23}-phosphorylated HePTP and Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK are unable to interact. However, these data fail to indicate whether or not HePTP regulated the level of p38 MAPK phosphorylation. To test the role of HePTP in regulating the level of p38 MAPK phosphorylation in a B cell, we transfected the CH12.LX B-cell line with an shRNA plasmid containing HePTP small interfering RNA (siRNA) and a green fluorescent protein (GFP) marker so that the cells expressing HePTP siRNA could be sorted from the cells not expressing HePTP siRNA. CH12.LX cells were transfected with the shRNA and sorted for GFP-positive and GFP-negative cells (Fig. 8A). Compared to primary B cells, the CH12.LX B cell line has a relatively high basal level of p38 MAPK phosphorylation (data not shown); therefore, the cells do not need to be activated to induced p38 MAPK phosphorylation. Unstimulated GFP-positive and GFP-negative cells were lysed and assayed for total levels of HePTP protein and Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK. The GFP-positive cells, which expressed HePTP siRNA, showed a 25% reduction in total HePTP protein levels compared to GFP-negative cells not expressing HePTP siRNA (Fig. 8B), which correlated to an average fivefold increase in the level of Thr^{180}/Tyr^{182} phosphorylation of p38 MAPK (Fig. 8C). Thus, these findings indicate that HePTP directly regulates the level of p38 MAPK phosphorylation in the CH12.LX B cell line.

DISCUSSION

The goal of the present study was to identify the mechanism by which β_2AR stimulation on a CD40L/IL-4-activated B cell increased the level of p38 MAPK activation. We report that unphosphorylated p38 MAPK is bound to, and regulated by, HePTP in a B cell. Upon β_2AR stimulation, HePTP is phosphorylated in a PKA-dependent manner at a PKA-specific phosphorylation site, causing HePTP to become inactive and allowing p38 MAPK to be phosphorylated by the CD40L-activated MAPK cascade (Fig. 9). These data are the first to indicate that HePTP functions in a B cell. The relevance of this finding with regard to the IgE response will be discussed.

β_2AR stimulation has been shown to activate many signaling pathways, some of which lead to MAPK regulation (reviewed

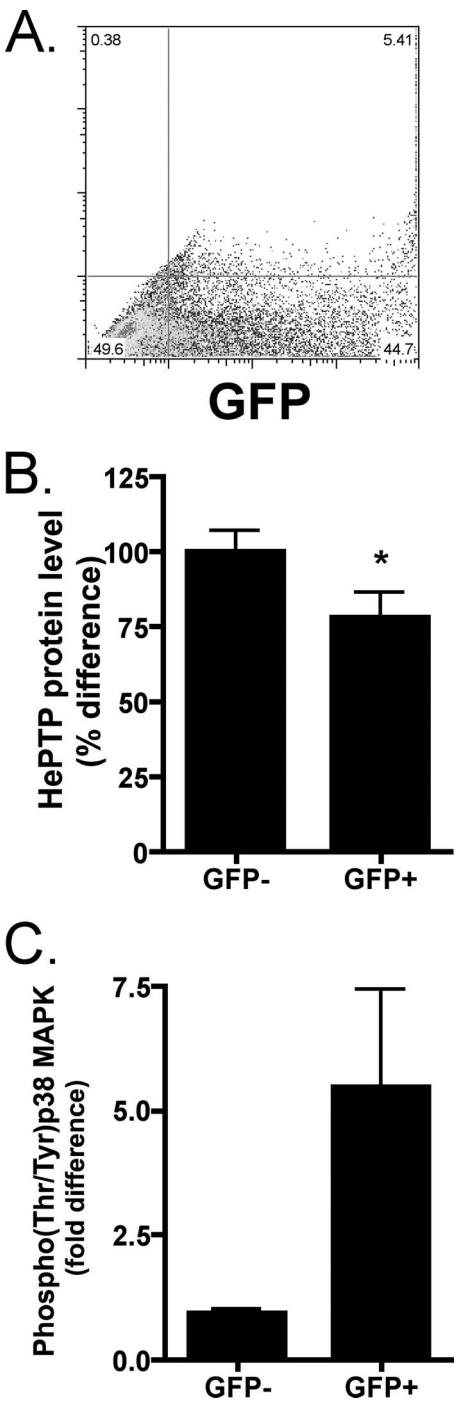

FIG. 8. HePTP regulates the level of p38 MAPK phosphorylation in a B cell. Unactivated CH12.LX B cells were transfected with HePTP shRNA plasmids and cultured for 48 h. (A) The cells were collected and sorted on a BD FACS Aria cell sorter for GFP-positive (HePTP siRNA positive) and GFP negative (HePTP siRNA negative) cells. Total protein was isolated and analyzed for actin and total HePTP (B) or total and phospho-(Thr/Tyr)p38 MAPK (C) levels by Western blotting. Band density was determined using densitometry, and the data represent the percent difference in total HePTP normalized to actin or the mean difference in phospho-p38 MAPK normalized to total p38 MAPK ± standard error of the mean from three independent experiments. (*, $P \leq 0.05$).

in reference 35). Therefore, it was important to consider each pathway as a possible mechanism by which β_2AR stimulation increased the level of p38 MAPK phosphorylation. The classic β_2AR-signaling pathway involves activation of PKA, which our data showed was necessary for the increase in the level of p38 MAPK phosphorylation. Other than HePTP phosphorylation, PKA has been shown to regulate a small GTPase called B-Raf, which is part of a signaling cascade capable of directly mediating p38 MAPK phosphorylation in neurons and lung endothelial cells (7, 53). It was possible that PKA activation mediated the β_2AR-induced increase in p38 MAPK through phosphorylation of a small GTPase. However, if this were the case, then β_2AR stimulation should be able to induce p38 MAPK phosphorylation in the absence of other activation signals, which was not the case in the present study. Our data showed that β_2AR stimulation alone failed to induce p38 MAPK phosphorylation in WT B cells in the absence of CD40/IL-4 activation, suggesting that β_2AR stimulation and PKA activation alone did not induce p38 MAPK phosphorylation but regulated the level of p38 MAPK available for phosphorylation by the CD40L-activated MAPK cascade. Therefore, we think it is unlikely that PKA activation of small GTPases is involved in the β_2AR-induced increase in p38 MAPK phosphorylation. Alternatively, PKA has been shown to induce a switch from Gs to Gi binding of the β_2AR to mediate activation of the MAPK cascade and direct phosphorylation of downstream MAPKs. However, the Gi inhibitor, pertussis toxin, which inhibits Gi activation after β_2AR stimulation, was unable to prevent the β_2AR-induced increase in p38 MAPK phosphorylation, suggesting that Gi activation was not involved.

Other possible β_2AR-activated pathways have been described, and each could potentially induce and/or regulate the level of p38 MAPK phosphorylation, but in a PKA-independent manner. Exchange protein activated by cAMP (Epac) is a signaling molecule directly activated by cAMP (reviewed in references 4 and 43) and has been reported to regulate the levels of p38 MAPK phosphorylation in neurons as quickly as 10 min after activation independently of PKA (47), similar to the time course shown in Fig. 2. However, our data showed that the increase in the level of p38 MAPK was PKA dependent. Also, recruitment and activation of β-arrestin-2 has been shown to regulate β_2AR-dependent ERK phosphorylation (45) and explain our findings. However, our data using an inverse agonist and β-arrestin-2-deficient mice showed that the β_2AR-induced increase in the level of p38 MAPK was independent of β-arrestin-2 activity. Thus, our findings suggest that EPAC and β-arrestins do not play a role in the β_2AR-induced increase in the level of p38 MAPK phosphorylation because they function in a PKA-independent manner.

A previous report showed in T lymphocytes that cross talk between the cAMP/PKA pathway and p38 MAPK was mediated by HePTP (41), and another study showed that HePTP was expressed in lymphocytes (56). Although our data showed that a change in the level of HePTP protein expression in a B cell was associated with changes in the level of p38 MAPK activation, we were unable to show that the level of HePTP-p38 MAPK physical interaction changed following either agonist stimulation of the β_2AR or activation of cAMP/PKA with cell-permeating agents that act independently of the receptor.

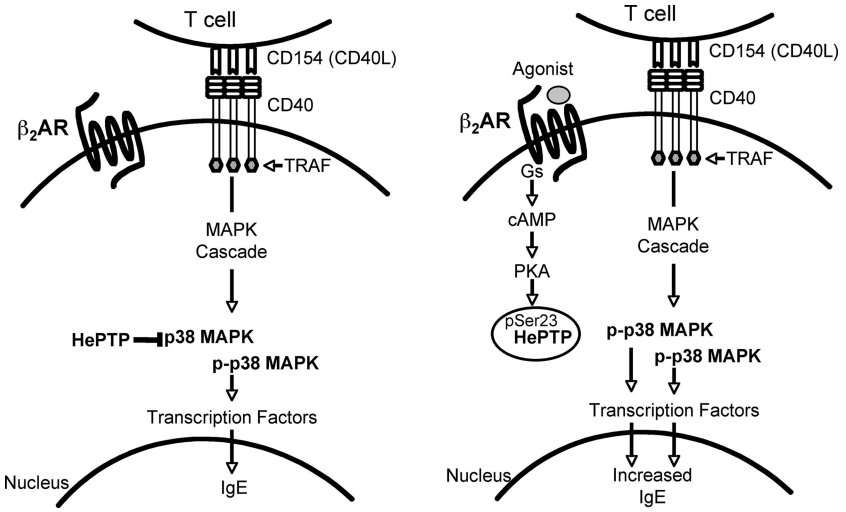

FIG. 9. Model of the mechanism by which β_2AR mediates an increase in p38 MAPK phosphorylation to increase the level of IgE. B-cell CD40 engagement by CD40L (CD154) on a T cell induces activation of the MAPK cascade and results in phosphorylation and activation of p38 MAPK. The active p38 MAPK translocates to the nucleus and, along with molecules activated by IL-4 receptor stimulation by IL-4 (not shown), induces class switch recombination and the production of IgE. In the absence of β_2AR stimulation (left model), the level of p38 MAPK phosphorylation is regulated by the protein tyrosine phosphatase HePTP. In the presence of β_2AR stimulation (right model), HePTP is phosphorylated in a PKA-specific manner, which inactivates the phosphatase and results in the release of p38 MAPK. The free p38 MAPK is phosphorylated by molecules of the MAPK cascade to result in increased levels of phosphorylated p38 MAPK and downstream effects, such as increased production of IgE.

The inability to detect a change in the level of physical interaction between HePTP and p38 MAPK is likely due to a combination of factors, including the limited sensitivity of the assay, the low two- to threefold change induced by β_2AR agonists and pharmacological agents in the level of p38 MAPK and HePTP phosphorylation, the rate at which these changes occurred, and the dynamics of the physical interaction that occurs between HePTP and p38 MAPK. However, we know that when Ser^{23} on HePTP is phosphorylated by PKA, it can no longer bind p38 MAPK (41). We took advantage of this finding to circumvent the above-mentioned limitations and measured the level of physical interaction directly by using a Ser^{23} HePTP mutant that mimicked the constitutively phosphorylated form transfected into a B-cell line to show that the PKA-specific phosphorylation site in the HePTP-p38 MAPK interaction motif of HePTP inhibited its ability to bind to p38 MAPK, thus supporting indirectly that a physical interaction occurs between HePTP and p38 MAPK in B cells. In addition, we used HePTP shRNA to show that a 25% decrease in the total HePTP protein level was sufficient to induce a fivefold change in the level of p38 MAPK phosphorylation, supporting the hypothesis that HePTP in a B cell regulates the level of p38 MAPK phosphorylation. However, the latter data are limited by the use of a B-cell line, since primary B cells cannot be transfected efficiently. Definitive proof of our findings will require the use of primary B cells from HePTP-deficient mice (11), which unfortunately are no longer available (B. Zanke, personal communication). Taken together, findings derived from several different experimental approaches suggest that HePTP physically interacts with p38 MAPK and plays a role in regulating the level of p38 MAPK phosphorylation in an activated B cell.

Protein tyrosine phosphatases have been difficult to study due to their variety, multiple cell type and pathway specificities, and the lack of selective inhibitors (reviewed in reference 52). For example, approximately 60 to 70 different PTPs are expressed in T cells, and it is hypothesized that B cells express a similar number (reviewed in references 27 and 52). Also, selective PTP inhibitors that could be used to distinguish among the PTPs are not commercially available (50). However, several general PTP inhibitors exist, such as pervanadate (33, 42), phenylarsine oxide (31), and sodium orthovanadate (25), the last of which suggested to us initially that a PTP was playing a role in regulating the β_2AR-induced increase in the level of p38 MAPK phosphorylation in a B cell (data not shown). However, as many as 20 PTPs are thought to positively and negatively regulate the signaling pathway that is activated by T-cell receptor stimulation in a T cell (24, 27). Here we have demonstrated that HePTP physically interacts with p38 MAPK in primary B cells and that β_2AR stimulation leads to a PKA-dependent phosphorylation of HePTP to regulate the level of p38 MAPK phosphorylation in a B-cell line. Taken together, our data suggest that β_2AR stimulation and PKA activation regulate HePTP activity to increase the amount of free p38 MAPK available for phosphorylation in an activated B cell.

The relevance of the present findings becomes evident when we consider a previous finding in our laboratory that the β_2AR-induced increase in the level of p38 MAPK phosphorylation played a role in mediating an increase in the level of IgE but not IgG_1, even though both isotypes are produced in response to the same stimuli, i.e., CD40L and IL-4 (38). IgG_1 is important for protection from bacterial pathogens, while IgE plays a critical role in mediating protection from parasites and in the clearance of allergens. During an immune challenge by a bacterial pathogen, parasite, or allergen, nerve terminals that reside within the parenchyma of lymphoid tissues release nor-

epinephrine (17) to stimulate the β_2AR on a B cell to increase the level of IgG_1 or IgE (14) to ensure that an appropriate level of antibody is maintained for the benefit of protection. However, physiological conditions and therapeutic drugs exist that cause the β_2AR to be overstimulated, potentially inducing an excess of antibody to be produced and increasing the risk of disease development. For example, data from humans and mice show that a strong correlation exists between the level of IgE measured in both serum and bronchoalveolar lavage fluid and the severity of allergic asthma symptoms, which includes difficulty breathing due to bronchoconstriction (5, 9, 32). Individuals with asthma relieve bronchoconstriction with β_2AR agonists that stimulate the β_2AR on the smooth muscle cells of the lung. However, the β_2AR agonists lose efficacy over time and increase the risk of succumbing to an asthma-related death (1). As yet, it remains unknown why these β_2AR agonists lose their ability to relieve bronchoconstriction after prolonged use, but the findings from our laboratory would suggest that β_2AR stimulation on B cells in the lung increases the level of IgE over time to exacerbate the allergic asthma symptoms of the patient and intensify bronchoconstriction to a point that β_2AR agonist drugs are no longer effective. It is interesting that corticosteroids are introduced at this point in the asthma profile to suppress immune cell activity and lessen the level of inflammation in the lung. We would suggest that the corticosteroids are also suppressing the ability of B cells to make excess IgE, which is induced by β_2AR overstimulation. Also, allergic asthma patients under high levels of stress, a condition that increases the release of norepinephrine to stimulate the β_2AR, experience exacerbated asthma symptoms, which we predict occur via a similar mechanism to that described above.

Thus, if the β_2AR agonist effect on IgE could be dissociated from the effect on bronchodialation, therapies that inhibit the increase in IgE but still allow for the induction of bronchodialation and the production of IgG_1 could be developed. The present data show that the mechanism by which β_2AR stimulation on a B cell increases the level of IgE involves HePTP and is unique to the IgE response, suggesting that HePTP in a B cell may be a possible therapeutic target for allergic asthma therapy. This type of targeted therapy for HePTP would block the increased production of IgE, which we predict would occur with long-term β_2AR agonist therapy or stress, without interfering with the action of β_2AR agonists to relieve bronchoconstriction or the level of IgE and IgG_1 that is necessary for the clearance of allergens or bacterial pathogens. Targeting HePTP with selective inhibitors would also lend tissue specificity to allergic asthma therapy, because HePTP is expressed mainly in lymphocytes (56), in contrast to the use of a β_2AR antagonist that would bind to the β_2AR expressed on cells residing in almost every organ system. Likewise, because our data indicate that the 25% change in the level of HePTP that was induced by shRNA was sufficient to cause a fivefold change in the level of p38 MAPK phosphorylation, it is possible that genetic differences or disease states that might cause small changes in either the HePTP level or function could play a role in the development of allergic asthma.

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