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*J Immunol* 2013; 190:723-736; Prepublished online 12 December 2012; doi: 10.4049/jimmunol.1201646

http://www.jimmunol.org/content/190/2/723
Prohibitins and the Cytoplasmic Domain of CD86 Cooperate To Mediate CD86 Signaling in B Lymphocytes

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CD86 engagement on a CD40L/IL-4–primed murine B cell activates signaling intermediates that promote NF-κB activation to increase Oct-2 and mature IgG1 mRNA and protein expression, as well as the rate of IgG1 transcription, without affecting class switch recombination. One of the most proximal signaling intermediates identified is phospholipase Cγ2, a protein reported to bind tyrosine residues, which are absent in the cytoplasmic domain of CD86. Using a proteomics-based identification approach, we show that the tyrosine-containing transmembrane adaptor proteins prohibitin (Phb)1 and Phb2 bind to CD86. The basal expression of Phb1/2 and association with CD86 was low in resting B cells, whereas the level of expression and association increased primarily after priming with CD40. The CD86-induced increase in Oct-2 and IgG1 was less when either Phb1/2 expression was reduced by short hairpin RNA or the cytoplasmic domain of CD86 was truncated or mutated at serine/threonine protein kinase C phosphorylation sites, which did not affect Phb1/2 binding to CD86. Using this approach, we also show that Phb1/2 and the CD86 cytoplasmic domain are required for the CD86-induced phosphorylation of IκBα, which we previously reported leads to NF-κB p50/p65 activation, whereas only Phb1/2 was required for the CD86-induced phosphorylation of phospholipase Cγ2 and protein kinase Ca/β1a, which we have previously reported leads to NF-κB (p65) phosphorylation and subsequent nuclear translocation. Taken together, these findings suggest that Phb1/2 and the CD86 cytoplasmic domain cooperate to mediate CD86 signaling in a B cell through differential phosphorylation of distal signaling intermediates required to increase IgG1. The Journal of Immunology, 2013, 190: 723–736.

Abbreviations used in this article: Phb, prohibitin; PKC, protein kinase C; PLC, phospholipase C; qRT-PCR, quantitative real-time PCR; shRNA, short hairpin RNA; Syk, spleen tyrosine kinase; WT, wild-type.

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Received for publication June 14, 2012. Accepted for publication November 11, 2012.

This work was supported by National Institutes of Health Grants R01-AI037326 and R56-AI073663.

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The online version of this article contains supplemental material.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201646

The Journal of Immunology
IgG1 as compared with primed B cells in the absence of CD86 engagement. Notably, clinical findings have shown that a 2- to 3-fold increase in serum IgG correlates to a 3- to 9-fold increase in protection against *Streptococcus pneumoniae* and pertussis (23, 24), suggesting that increases of this magnitude in the level of Ab produced are potentially relevant clinically.

In this study we sought to identify potential signaling intermediates associated directly with CD86 and to verify its role in mediating CD86 signaling. Such a directly associated protein must exist because one of the most proximal CD86-dependent signaling intermediates, phospholipase C (PLC)γ2, functions classically by recruitment to proteins containing tyrosine residues (25, 26), which are lacking in the CD86 cytoplasmic domain. Using a proteomics approach, we demonstrate a novel protein/protein interaction that occurred in a CD40L/IL-4–primed B cell between CD86 and prohibitin (Phb) 1 and Phb2, which are transmembrane adaptor proteins that contain tyrosine residues. Furthermore, we report that both Phb1/2 and an intact CD86 cytoplasmic domain are required to mediate CD86 signaling that regulates the level of IgG1 produced by a B cell via differential activation of distal signaling intermediates.

**Materials and Methods**

**Animals**

Female pathogen-free BALB/c mice were obtained from Taconic (Germantown, NY) and were housed in the American Association Accreditation of Laboratory Animal Care–accredited Animal Research Facility at The Ohio State University (Columbus, OH). All mice were provided autoclaved food and deionized water ad libitum and used at 8–10 wk age.

**Cell lines/transgenic B cells**

CH12.LX is a murine B cell lymphoma line previously described (27) and an intact CD86 cytoplasmic truncation (line I2) within a CD80/CD86 double-deficient mouse on a C57BL/6 background were previously described (28). Transgenic B cells that express a CD86 cytoplasmic truncation (line I2) within a CD80/CD86 double-deficient mouse on a C57BL/6 background were previously described (29).

**B cell isolation/priming**

Naive B cells were isolated using MACS anti-mouse CD43 beads (Miltenyi Biotec, Auburn, CA) following the manufacturer’s directions. Cells were primed with CD40L-expressing Sf9 cells or control Sf9 cells lacking CD40L expression at a B cell/Sf9 cell ratio of 10:1, 1 ng/ml IL-4 (eBioscience), 0.1 μg/ml anti-IgM, 10−6 M terbutaline (Sigma-Aldrich), or 0.5 μg/ml LPS (Sigma-Aldrich) for 16 h. Total mRNA and total protein were collected and analyzed via quantitative real-time PCR (qRT-PCR) and immunoblot, respectively. All reagents were used for the presence of endotoxin, as determined by E-Toxase (Sigma-Aldrich), a Limulus lysate assay with a level of detection <0.1 U/ml.

**Immunoprecipitation**

Immunoprecipitations were performed using the ProFound mammalian coimmunoprecipitation kit (Fierce). Briefly, 25 × 10^6 CH12.LX B cells were cultured and primed as described above, lysed with 0.5% Triton X-100 buffer, immunoprecipitated with an anti-CD86 GL1 (eBioscience) or species- and isotype-matched control Ab (rat IgG2a; eBioscience), 0.1 μg/ml anti-IgM, 10−6 M terbutaline (Sigma-Aldrich), or 0.5 μg/ml LPS (Sigma-Aldrich) for 16 h. Total mRNA and total protein were collected and analyzed via quantitative real-time PCR (qRT-PCR) and immunoblot, respectively. All reagents were used for the presence of endotoxin, as determined by E-Toxase (Sigma-Aldrich), a Limulus lysate assay with a level of detection <0.1 U/ml.

**Mass spectrometry**

Mass spectrometry and protein identification were performed by the Mass Spectrometry and Proteomics Core Facility at The Ohio State University. The unique band present at ∼35 kDa in an anti-CD86 immunoprecipitate of CH12.LX cells from a gel stained with SYPRO Ruby (Invitrogen) was excised and digested with sequencing-grade trypsin from Promega (Madison, WI) using the MultiScreen Solvinent filter plates from Millipore (Bedford, MA). Capillary/liquid chromatography/nanospray tandem mass spectrometry was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The liquid chromatography system was an EliteMate 3000 system from Dionex (Sunnyvale, CA). Sequence information from the tandem mass spectrometry data were processed by converting the .raw files into a merged file (.mgf) using an in-house program, RAW2MZXML_n_MGF_batch (merge.pl, a Perl script). The resulting .mgf files were searched using Mascot Daemon by Matrix Science (Boston, MA) version 2.3.2.

**Immunoblot analysis**

Resident B cells (5–7 × 10^6) were primed as described above. CH12.LX B cells were transfected with scrambled negative control short hairpin RNA (shRNA) or Phb1/2-specific shRNA plasmids (1–5 μg/10^6 cells) Phb1 (clones 1, 3, and 4) and Phb2 (clones 1 and 3) (SABiosciences) for 24 h following priming with CD40L/IL-4 for 16 h or with a FLAG-CD86 expression plasmid (1 μg/10^6 cells) following priming with CD40L/IL-4 for 16 h. In some experiments, cells were cultured under serum-free conditions for at least 30 min and an intact CD86 cytoplasmic truncation (line I2) within a CD80/CD86 double-deficient mouse on a C57BL/6 background were previously described (28). Transgenic B cells that express a CD86 cytoplasmic truncation (line I2) within a CD80/CD86 double-deficient mouse on a C57BL/6 background were previously described (29).

**Immunoblot densitometric analysis**

Specific protein bands detected via immunoblot analysis were quantitated via densitometry (ImageJ software, National Institutes of Health). The scanned image was inverted to measure the OD of a specific protein band. A measurement box was created around the broadest band on a given gel and used to measure each band on the gel. The numerical value was recorded as an OD. ODs of each band were recorded from the corresponding gel lanes and subtracted from the original ODs. The ODs of Phb1/2 total protein bands were normalized to GAPDH protein loading control band ODs. The ODs of phospho-PLCγ2 (Y1217) protein bands were normalized to ODs of corresponding total PLCγ2 protein bands. ODs from phospho-PKCαβ2 (T638/641), anti–PKCα, anti–phospho-IκB (S32), and total IκB protein bands were normalized to GAPDH total protein band ODs. The ODs recorded from phospho-p65 (S536) protein bands were normalized to both total p65 protein bands and ODs obtained from GAPDH protein bands. The ODs measured from nuclear phospho-p65 (S536) and nuclear p65 were normalized to both total Lamin A and Lamin C protein band ODs. The data are expressed as mean fold change ± SD/SEM relative to resting, CD40L/IL-4–priming alone, or species- and isotype-matched control Ab conditions.

**Flow cytometry**

The level of CD86 on GFP^+ Phb1/2-shRNA^+ CH12.LX B cells was determined as previously described (13) using allophycocyanin-conjugated rat anti-mouse CD86 (GL1). Samples were analyzed using a FACSCanto II (BD Biosciences). Expression of CD86-shRNA was measured via FITC-conjugated anti-mouse FLAG (M2) (Sigma-Aldrich). The data were analyzed using FlowJo software (Tree Star).

**RNA-interfering Phb1 and Phb2 knockdown and qRT-PCR analysis**

CH12.LX B cells were transfected with scrambled negative control shRNA, Phb1 (clones 1, 3, and 4) or Phb2 (clones 1 and 3)–specific shRNA
CD86 engagement on a CD40/IL-4–primed B cell is known to activate PLCγ2 (19), a signaling intermediate that requires binding to proteins containing phospho-tyrosine residues for recruitment/activation and its ability to implement receptor function. We reasoned that CD86, which does not contain tyrosine residues in the cytoplasmic domain, must associate with a tyrosine-containing adaptor/scaffolding protein to mediate the activation of PLCγ2. Therefore, a proteomics-based approach was used to identify any protein that associated with CD86 in primed B cells. The murine B cell line CH12.LX was cultured overnight in the presence of CD40/IL-4 and then exposed to either an anti-CD86 or species- and isotype-matched control Ab. CD86 was immunoprecipitated from cell lysates, and proteins were separated via SDS-PAGE and stained with SYPRO Ruby (Fig. 1A). Three unique bands were present at molecular masses of ∼50, 35, and 28 kDa in the anti-CD86 Ab immunoprecipitate, but they were absent when the control Ab was used. The unknown ∼35-kDa protein was excised because it was clearly isolated from other bands and identified by mass spectrometry as the adaptor protein Phb2 (Fig. 1B). In all, 14 non-redundant tryptic peptide fragments of murine Phb2 were identified.

Although sequence analysis was not performed on the other two bands, we predict that the CD86-associated ∼28 kDa protein band is Phb1 because the literature suggests that Phb2 associates closely with the highly related Phb1 in a variety of intracellular loci (30). The identity of the CD86-associated protein band at ∼50 kDa remains unclear, but it may be another adaptor/scaffolding protein similar to that reported for other B cell surface receptors, including CD40 (31–34) and the BCR (35–39) that are also devoid of tyrosine residues within the cytoplasmic domain, but that use adaptor/scaffolding complexes to function.

We tested whether Phb1 and/or Phb2 could specifically coimmunoprecipitate from B cells under primed versus resting conditions. Parent CH12.LX cells, CH12.LX cells transfected with CD86–flagged CD86, or primary B cells were cultured overnight in the presence or absence of CD40/IL-4 priming. Lysates were prepared and immunoprecipitated with control and anti-CD86 Abs and then analyzed for level of Phb1/2 and CD86 by immunoblot. As shown in Fig. 1C, levels of Phb1 and Phb2 were low or undetectable in CD86 immunoprecipitates from resting CH12.LX B cells, whereas coimmunoprecipitation of both proteins increased substantially in primed B cells. This increase in Phb1/2 binding to CD86 may be due to a specific CD40/IL-4–dependent recruitment event or an increased level of CD86 present in the CD86 immunoprecipitates. Similar results were observed when CH12. LXB cells were transfected with a FLAG epitope–tagged CD86 construct and primary B cells were used (Fig. 1C). To determine whether Phb1/2 associated with the cytoplasmic domain of CD86, we tested CH12.LX B cells transfected with a cytoplasmic truncated form of FLAG-CD86 (KKPA), as well as primary B cells from transgenic mice that express a truncated form of the CD86 cytoplasmic domain. Phb1 and Phb2 were found to associate with the

In-cell ELISA

In-cell ELISAs were performed to detect in-cell levels of phospho-PLCγ2 (Y1217) and total PLCγ2 protein using an in-cell colorimetric ELISA kit (Pierce). Briefly, 1 × 10^6 CH12.LX B cells were transfected under mock conditions or with either scrambled negative control shRNA or Phb1/2–specific shRNA plasmids for 24 h followed by priming with CD40/IL-4 or with a WT or cytoplasmic-deficient (KKPA) FLAG-CD86 expression plasmid followed by priming with CD40/IL-4 for 4 h. Cells were cultured under serum-free conditions for at least 30 min, followed by exogenerate of either CD86 or with anti-CD86 (clone 2D8) with anti-TCRα/β Abs and appropriate controls (as described above) for 15–30 min. The cells were then washed in 1 × PBS. Cells (2 × 10^6) were plated on a 96-well culture plate and fixed with 8% formaldehyde. The remainder of the assay was performed according to the manufacturer’s instructions. Abs used to detect phospho-PLCγ2 (Y1217) and total PLCγ2 in-cell protein levels were anti-phospho-PLCγ2 (Y1217) and anti-PLCγ2 (Cell Signaling Technology). The HRP-conjugate Ab was used at a 1:2000 dilution.

Statistical analysis

Data from multiple treatment groups were analyzed using a one-way ANOVA to determine whether an overall statistical change existed. Certain p values were calculated using a Bonferroni post hoc analysis or a two-sided Student t test for comparison of two treatment groups. A p value ≤0.05 indicated statistically significant results.

Results

CD86 associates with Phb1 and Phb2 in B lymphocytes

CD86 engagement on a CD40/IL-4–primed B cell is known to activate PLCγ2 (19), a signaling intermediate that requires binding to proteins containing phospho-tyrosine residues for recruitment/activation and its ability to implement receptor function. We reasoned that CD86, which does not contain tyrosine residues in the cytoplasmic domain, must associate with a tyrosine-containing adaptor/scaffolding protein to mediate the activation of PLCγ2. Therefore, a proteomics-based approach was used to identify any protein that associated with CD86 in primed B cells. The murine B cell line CH12.LX was cultured overnight in the presence of CD40/IL-4 and then exposed to either an anti-CD86 or species- and isotype-matched control Ab. CD86 was immunoprecipitated from cell lysates, and proteins were separated via SDS-PAGE and stained with SYPRO Ruby (Fig. 1A). Three unique bands were present at molecular masses of ∼50, 35, and 28 kDa in the anti-CD86 Ab immunoprecipitate, but they were absent when the control Ab was used. The unknown ∼35-kDa protein was excised because it was clearly isolated from other bands and identified by mass spectrometry as the adaptor protein Phb2 (Fig. 1B). In all, 14 non-redundant tryptic peptide fragments of murine Phb2 were identified.

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CD86 in the absence or presence of the cytoplasmic domain (Fig. 1D), suggesting that a transmembrane-specific association occurred between CD86 and Phb1/2. Taken together, these findings indicated that low levels of Phb1 and Phb2 were associated with CD86 in resting B cells, but that the levels increased after CD40L/IL-4 priming and were independent of most of the CD86 cytoplasmic domain.

The expression of Phb1 and Phb2 is regulated primarily by CD40 engagement

A number of studies have reported that Phb1 and Phb2 associate with each other to form a large complex within the inner mitochondrial membrane and that they function by stabilizing proteins of the electron transport chain (40). If a similar stabilizer/molecular chaperone function existed for Phb1/2 to facilitate CD86 stability, transport, and insertion into the plasma membrane, then Phb1/2 protein expression would be expected to increase concomitantly with CD86 protein expression when the B cell was primed with CD40L/IL-4, as well as with other B cell stimuli that are known to increase CD86 protein expression. Primary resting B cells were cultured in the absence or presence of IL-4 overnight with either CD40L/anti-IgM to engage the BCR, the β2-adrenergic receptor agonist terbutaline, or LPS, all of which have been reported to increase CD86, that is, IgM and β2-adrenergic receptor engagement on a B cell, were unable to upregulate Phb1/2 expression to any significant level in comparison with resting levels. Importantly, control SF9 cells lacking CD40L expression failed to induce Phb1/2 mRNA or protein levels (Fig. 2C). Taken together, these findings suggested that Phb1/2 protein expression may be regulated primarily by a CD40-dependent mechanism that is independent from the mechanism involved in upregulating expression of CD86.

Phb1 and Phb2 play a role in mediating the effect of CD86 engagement on B cell function

Although the data thus far showed that Phb1/2 associated with CD86, the functional significance of this association was unclear. Recent studies have reported that Phb1 interacts with various signaling intermediates to modulate cellular processes, and thus the possibility was supported that by associating with CD86 in primed B cells, Phb1 and Phb2 could be providing CD86 with a cytoplasmic signaling platform to stimulate B cell responses. To test this possibility directly, CH12.LX B cells were transfected with GFP
containing shRNA expression vectors designed to suppress Phb1/2 expression. The effect of transfection on B cell CD86 expression was measured before functional studies were performed. Flow cytometric analysis showed that CD86 surface expression following CD40L/IL-4 priming was comparable in the absence or presence of Phb1/2 knockdown (Supplemental Fig. 1B). qRT-PCR analysis of FACS-sorted GFP+ and GFP− cells showed that both Phb1 and Phb2 expression was reduced by at least 50% in shRNA-expressing GFP+ cells (Fig. 3A), where reduction of Phb1 resulted in the loss of Phb2 and vice versa (Supplemental Fig. 2).

We next determined whether both Phb1 and Phb2 play roles in mediating the CD86-induced increases in Oct-2 and IgG1 without a change in class switch recombination, as reported previously (13, 17–20). CD86 engagement on primed GFP+ B cells resulted in comparable levels of germline IgG1, Oct-2, and mature IgG1 mRNA relative to isotype control–treated B cells, whereas significant increases in Oct-2 and mature IgG1 mRNA were observed owing to CD86 engagement in GFP− B cells (Fig. 3B). To ensure that the comparable levels of Oct-2 and mature IgG1 mRNA were not due to an off target effect of the Phb1/2 shRNA plasmids, scrambled negative control shRNA plasmids were transfected into CH12.LX B cells as described. CD86 engagement promoted significant increases in the level of Oct-2 and mature IgG1 mRNA produced (Fig. 3B), whereas germline IgG1 mRNA remained unchanged. Taken together, these findings indicated that Phb1 and Phb2 are both necessary for CD86 engagement on a primed B cell to enhance the level of B cell function.
FIGURE 3. Phb1 and Phb2 play a role in mediating the effect of CD86 engagement on B cell function. (A and B) CH12.LX B cells were transfected with either a GFP-containing Phb1, Phb2, or scrambled, negative control shRNA (neg ctrl shRNA) vector for 36 h to silence Phb1 and Phb2 gene expression. At 24 h after transfection, cells were primed with CD40L/IL-4 for 16 h. GFP cells containing the Phb1 or Phb2 shRNA vector were separated by FACS then recultured with either an anti-CD86 Ab or a species- and isotype-matched control Ab (iso ctrl). On day 2 or day 6 after cell priming, Phb1/Phb2 mRNA (A) or Oct-2/germline IgG1 and mature IgG1 mRNA (B), respectively, were measured via qRT-PCR analysis and normalized to actin. GFP* cells and scrambled negative control shRNA are depicted with gray bars and GFP+ cells containing Phb1/2 shRNA are depicted with black bars. Data are expressed as mean fold change ± SEM relative to either no GFP (A) or isotype control of each group (B) and represent at least quadruplicate samples per condition from three independent experiments. Statistical differences are shown relative to no GFP (A) or isotype control of each group (B). *p < 0.05.

The cytoplasmic domain of CD86 also plays a role in mediating the effect of CD86 engagement on B cell function

The evidence thus far suggested that CD86 associated with Phb1/2 through transmembrane-specific interactions and that Phb1 and Phb2 were essential to couple CD86 engagement to a downstream functional increase in IgG1. However, it remained unclear whether the cytoplasmic domain of CD86 played any role in mediating the CD86-dependent increase in the B cell functional response. It was proposed previously that the cytoplasmic domain of CD86 had the potential to be phosphorylated at one or more of several putative PKC phosphorylation sites (15), which might provide a mechanism for CD86 to recruit and/or activate downstream signaling intermediates that are known to participate in the CD86-induced increases in Oct-2 and IgG1.

To test this possibility, a series of mutations/truncations were introduced into the FLAG-CD86 cytoplasmic domain that included single alanine point mutations of the three putative PKC serine/threonine phosphorylation sites, as well as either partial or full truncation of the cytoplasmic domain as diagrammed in Fig. 4A. CH12.LX B cells were transfected with the FLAG-CD86 plasmids, and cells were primed with CD40L/IL-4 in culture for 16 h, at which time either a species- and isotype-matched control Ab or anti-FLAG Ab (M2) was added. Cells were assessed, via qRT-PCR analysis, for levels of Oct-2/germline IgG1 mRNA on day 2 or mature IgG1 mRNA on day 5 (Fig. 4B). The introduction of alanine point mutations at all PKC phosphorylation sites had no effect on germline IgG1, but significantly reduced Oct-2 and mature IgG1 mRNA levels, except for Oct-2 induction by the T291A and S285A mutants, as compared with WT controls. Partial and full truncation of the CD86 cytoplasmic domain resulted in significant reductions of Oct-2 and mature IgG1 mRNA levels when compared with WT controls. Importantly, levels of either WT or mutant FLAG-CD86 expressed on the B cell surface remained comparable (data not shown). These results emphasize that PKC phosphorylation of the cytoplasmic domain of CD86 is important for signaling function.

In addition to the use of CD86-transfected B cells, B cells were also used that had been isolated from mice transgenic for either WT CD86 that overexpressed CD86 or a truncated form of CD86 that lacked the cytoplasmic domain. In B cells from the truncated form of CD86 mice that were primed, Phb1/2 proteins remained associated with CD86 (Fig. 1D), but the level of mRNA expressed for the cytoplasmic domain of CD86, Oct-2, and mature IgG1 was decreased in comparison with WT-expressing B cells, whereas germline IgG1 was comparable (Supplemental Fig. 3). Thus, to our knowledge, these data collectively provide evidence that, in addition to Phb1/2, the cytoplasmic domain of CD86 is also required to mediate CD86 signaling to regulate B cell function, likely via phosphorylation of the cytoplasmic domain at serine and/or threonine residues, as the present site-directed mutagenesis data show.

Phb1 and Phb2 are necessary for the CD86-induced activation of PLCγ2

Although findings thus far suggested that both Phb1/2 and the CD86 cytoplasmic domain are required to mediate CD86 signaling to regulate a B cell functional response, the proximal molecular mechanism was unclear. One of the most proximal CD86 signaling intermediates to date, PLCγ2 (19), is classically recruited to phosphorylated tyrosine-containing protein/protein complexes (25, 26). Additionally, it was reported that Phb1 binds the protein tyrosine kinase spleen tyrosine kinase (Syk) upon BCR engagement in murine B cells (41), indicating that Phb1 may be involved with the activation of protein tyrosine kinases specifically in B cells, and perhaps other membrane-associated signaling intermediates after receptor engagement. To address this, CH12.LX B cells were transfected with scrambled negative control or Phb1/2-specific shRNA plasmids and either a WT or
cytoplasmic-deficient FLAG-CD86 expression plasmid (KKPΔ) followed by priming with CD40L/IL-4 and CD86 or FLAG-CD86 engagement. Phb1/2 protein knockdown efficiency and the level of PLCγ2 phosphorylation were measured by immunoblot. The presence of Phb1/2-specific shRNA resulted in modest reductions of Phb1/2 protein where reduction of Phb1 resulted in the loss of Phb2 (Fig. 5A) and vice versa. Importantly, levels of Phb1/2 among mock-transfected cells versus cells transfected with scrambled negative control shRNA were comparable (Fig. 5A). Whereas mock- and scrambled negative control shRNA–transfected CH12.LX B cells promoted a time-dependent CD86-induced phosphorylation of PLCγ2 relative to primed cells alone (Fig. 5B), PLCγ2 phosphorylation upon CD86 engagement in the presence of Phb1/2 shRNA remained comparable to priming alone (Figs. 5C, 5D). Engagement of FLAG-CD86 on B cells transfected with both WT and KKPΔ FLAG-CD86 expression plasmids caused an increase in PLCγ2 phosphorylation relative to a species- and isotype-matched control Ab (Fig. 5E). To extend our immunoblot findings, the level of PLCγ2 phosphorylation was measured via in-cell ELISA. Whereas mock- and scrambled negative control shRNA–transfected CH12.LX B cells allowed for a CD86-induced increase in PLCγ2 phosphorylation relative to priming alone, PLCγ2 phosphorylation in the presence of Phb1/2 shRNA remained comparable to priming alone (Fig. 5F). Similar to our immunoblot findings, engagement of FLAG-CD86 on B cells transfected with both WT and KKPΔ FLAG-CD86 expression plasmids caused an increase in PLCγ2 phosphorylation relative to a species- and isotype-matched control Abs (Fig. 5G). Taken together, these findings suggested that whereas Phb1 and Phb2 are critical for the CD86-induced activation of PLCγ2, the CD86 cytoplasmic domain is not involved, indicating that Phb1/2 and the CD86 cytoplasmic domain must differentially activate signaling intermediates upon CD86 engagement.

Phb1 and Phb2 are necessary for the CD86-induced activation of PKCa/βII

Although our evidence suggested that Phb1 and Phb2 are necessary for the CD86-induced activation of PLCγ2 independent of the CD86 cytoplasmic domain, whether Phb1 and Phb2 were essential for the CD86-dependent downstream activity of PLCγ2 remained unknown. Because PLC classically allows for the activation of PKC via cleavage of phosphoinositides into inositol-3-phosphate and diacylglycerol, leading to an increase in Ca2+ (42), and because our previous findings showed that CD86 engagement on a CD40L/IL-4–primed B cell promoted the activation of PLCγ2 and the downstream activation of PKCa/βII (19), we considered PKCa/βII phosphorylation a viable measurement of PLCγ2 activity. We predicted that Phb1/2 alone would be necessary to induce CD86-dependent PKCa/βII phosphorylation independent of the CD86 cytoplasmic domain because Phb1/2 alone were sufficient to allow for CD86-induced PLCγ2 phosphorylation. To address this issue, CH12.LX B cells were either mock transfected or transfected with scrambled negative control shRNA or with Phb1/2-specific shRNA plasmids followed by priming with CD40L/IL-4 and the addition of a CD86 Ab during a 60-min time course. The levels of PKCa/βII phosphorylation (pPKCa/βII) relative to total PKCa and total PKCβII protein were measured via immunoblot. Whereas CH12.LX B cells that were mock transfected and scrambled negative control shRNA produced a time-dependent CD86-induced PKCa/βII phosphorylation relative to priming alone, the effect was prevented in the presence of Phb1/2-specific shRNA relative to priming alone (Fig. 6A). Engagement of FLAG-CD86 on B cells transfected with both WT and KKPΔ FLAG-CD86 expression plasmids caused an increase in PKCa/βII phosphorylation relative to a species- and isotype-matched control Ab (Fig. 6B). Collectively, these findings indicate that whereas Phb1 and Phb2 are necessary for CD86-dependent activation of PKCa/βII, a downstream signaling intermediate promoted through PLCγ2 activity, the CD86 cytoplasmic domain is not involved, providing further support that CD86 engagement allows for differential activation of distal signaling intermediates.

Phb1/2 and the CD86 cytoplasmic domain are each required for the CD86-dependent activation of NF-κB

Although our findings suggested that Phb1/2 alone is sufficient to promote CD86-dependent PLCγ2 and PKCa/βII activation, the molecular role of the CD86 cytoplasmic domain remained unknown. Previous reports demonstrated that engagement of CD86 on the surface of a CD40L/IL-4–primed B cell initiates two

FIGURE 4. The cytoplasmic domain of CD86 also plays a role in mediating the effect of CD86 engagement on B cell function. (A) A series of single-alanine point mutations (S285A, T291A, S303A) or cytoplasmic truncations (KKPΔ and 282Δ) were introduced into a FLAG-CD86 expression plasmid. (B) Either WT FLAG-CD86 or mutant/truncated plasmids were transfected into CH12.LX B cells before the cells were primed with CD40L/IL-4. Cells were cultured for 16 h before an anti-FLAG Ab (M2) or species- and isotype-matched control Ab (mouse IgG1 iso ctrl) was added to the culture. Twenty-four hours and 4 d later, respectively, the levels of germline IgG1/Oct-2 mRNA and mature IgG1 mRNA were determined by qRT-PCR. Values were normalized to actin and data are expressed as mean fold change ± SEM relative to WT mouse IgG1 isotype control and represent data of quadruplicate replicates per condition from three independent experiments. Statistical analysis was used to determine statistical significance relative to WT anti-FLAG. *p < 0.05.
signaling cascades that converge to activate the p50/p65 subunits of NF-κB (18, 19). Whereas one cascade is essential for CD86-dependent IκB phosphorylation (pIκB), the other promotes the phosphorylation of p65 (p-p65) in a PKCα/βII- and PLCγ2-dependent manner (18, 19). To determine which CD86-induced signaling intermediate controlled NF-κB activation, that is, IκB and p65 phosphorylation, CH12.LX B cells were either mock transfected or transfected with scrambled negative control or Phb1/2-specific shRNA plasmids and either WT-FLAG CD86 or CD86 cytoplasmic domain-deficient (KKPΔ) expression plasmids followed by CD40L/IL-4 priming and engagement of either CD86 or FLAG-CD86 during a 60- to 90-min time course. The level of IκB and p65 phosphorylation and the total levels of IκBα protein were determined via immunoblot. We observed
a time-dependent increase in the level of phosphorylated IkBα and a time-dependent decrease in the level of total IkBα protein upon engagement of CD86 when both Phb1/2 and the CD86 cytoplasmic domain were intact, whereas Phb1/2 depletion and the lack of the CD86 cytoplasmic domain prevented a CD86-dependent increase in the level of IkBα phosphorylation and decrease in the level of total IkBα protein (Fig. 7). Furthermore, the CD86-induced increase in p65 phosphorylation was prevented when Phb1/2 was depleted (Fig. 7A) and when CD86 lacked the cytoplasmic domain (Fig. 7B) relative to either priming alone or isotype control Ab, respectively. However, we speculated that the signaling intermediates Phb1 and Phb2, which were essential for PLCγ2 and PKCo/βII activation, may also prevent CD86-induced p65 phosphorylation because previous findings showed that PKCo/βII-dependent p65 phosphorylation occurred distal to IkBα phosphorylation (18). Taken together, these findings indicated that both Phb1/2 and the CD86 cytoplasmic domain are necessary for the CD86-dependent activation of NF-κB via IkBα phosphorylation/degradation, followed by p65 phosphorylation.

Phb1/2 and the CD86 cytoplasmic domain are each required for the CD86-dependent nuclear localization of NF-κB (p65)

Although our findings suggested that both Phb1/2 and the CD86 cytoplasmic domain were required for the CD86-induced activation of NF-κB, what remained unclear was whether the intermediates were necessary for NF-κB (p65) nuclear entry promoted via CD86 engagement. Because previous findings from our laboratory showed that CD86 engagement promoted an increase in the level of p65 phosphorylation present in the nucleus (18), and findings in the present study indicated that both Phb1/2 and the CD86 cytoplasmic domain were required to promote CD86-dependent NF-κB activation, it was likely that both Phb1/2 and the CD86 cytoplasmic domain were required to promote CD86-dependent p65 nuclear localization. To address this issue, CH12.LX B cells were either mock transfected or transfected with scrambled negative control shRNA and Phb1/2-specific shRNA plasmids to deplete Phb1/2, and either WT-FLAG CD86 or CD86 cytoplasmic domain-deficient (KKPΔ) FLAG-CD86 plasmids followed by CD40L/IL-4-priming and either CD86 or FLAG-CD86 engagement for 90 min. Nuclear enriched protein lysates were prepared and levels of p65 phosphorylation and total p65 protein levels relative to nuclear membrane proteins Lamin A/C were measured via immunoblot. Whereas phosphorylated and total p65 nuclear localization increased upon CD86 engagement when both Phb1/2 and the CD86 cytoplasmic were intact, the effect failed to occur upon CD86 engagement in the absence of the intermediates (Fig. 8). Furthermore, successful cytoplasmic- and nuclear-enriched protein lysate fractionation was demonstrated via the presence of α-tubulin, β-actin, and GAPDH in cytoplasmic-enriched protein lysates and the presence of Lamin A/C in nuclear-enriched protein lysates (Supplemental Fig. 4). Thus, our findings indicated that Phb1/2 and the CD86 cytoplasmic domain were necessary to promote the CD86-dependent nuclear localization of p65 and, therefore, extended the finding that both Phb1/2 and the CD86 cytoplasmic domain are each required for the CD86-dependent NF-κB activation.

Based on the findings of the present study and our previous reports, we propose the following CD86 signaling model (Fig. 9): Upon CD86 engagement, Phb1/2 and the CD86 cytoplasmic domain promote the phosphorylation of IkBα, allowing for the activation of NF-κB p50/p65. Concurrently, Phb1/2 alone promote CD86-induced PLCγ2 phosphorylation, allowing for subsequent PKCa/βII activation and phosphorylation of p65. NF-κB p50/p65
then initiates Oct-2 transcription, which ultimately regulates 3′-IgH enhancer activity to elevate the rate of IgG1 transcription and the level of IgG1 produced by a B cell.

Discussion
In this study, we show for the first time, to our knowledge, that expression of the tyrosine-containing adaptor/scaffolding proteins Phb1 and Phb2 increases in a CD40L/IL-4–primed B cell to associate with CD86, and that depletion of these proteins renders CD86 unable to mediate an enhancement in the level of Oct-2 and IgG1 produced. To our knowledge, we also show for the first time that Phb1/2 and the CD86 cytoplasmic domain are required for the CD86-induced phosphorylation of IκBa, which we previously reported leads to NF-κB p50/p65 activation (18), whereas only Phb1 and Phb2 were required for the CD86-induced phosphorylation of PLCγ2, which we previously reported leads to PKCα/βII activation and NF-κB (p65) phosphorylation (19). Thus, Phb1/2 association with CD86 and expression of an intact CD86 cytoplasmic domain are each necessary to mediate CD86 signaling function to enhance the level of the Oct-2/IgG1 response in a primed B cell through the differential activation of distal signaling intermediates.

Phb2 and its closely related homolog Phb1 are evolutionarily conserved, multifunctional proteins that are expressed in most cell types and typically reside in a variety of cellular compartments, including the inner mitochondrial and plasma membranes (30). Both Phbs have previously been found to be associated with the BCR (43) and they were recently found to colocalize with CD3 of the TCR complex (44). Phb2 is also known as either Bap37 (BCR-associated protein of 37 kDa) or REA (nuclear repressor of estrogen receptor activity), whereas Phb1 is also referred to as Bap32 (BCR-associated protein of 32 kDa). Phbs are a family of proteins that possess various cellular and molecular functions (30, 45, 46) that have been found to function independently of each other (30, 45), except within the context of the mitochondrial membrane, where they seem to function together (40, 47–49), and when they associate with the BCR and CD3 of the TCR complex, where their function remains less well understood. Taken together with findings that Phb1/2 residues can be phosphorylated, including tyrosines (50–53), we considered Phb1 and Phb2 as viable candidates to mediate CD86 signaling function.

Previous reports demonstrated that NF-κB (p65) phosphorylation is regulated via PKCα/βII, and that PKCα/βII activation is
PLCγ2-dependent on CD86 engagement on a B cell (18, 19). Data in the present study showed that the CD86-induced PLCγ2 phosphorylation failed to occur when Phb1/2 was reduced, but not when the CD86 cytoplasmic tail was deleted. Alternatively, CD86-induced IkBα phosphorylation failed to occur when either Phb1/2 was reduced or the CD86 cytoplasmic domain was deleted, indicating that both were required for NF-κB activation. The present evidence indicated that both Phb1/2 and the cytoplasmic domain of CD86 on a B cell, although not directly associated, appear to each be necessary to elicit CD86-induced back signaling to enhance the level of Oct-2 and IgG1 responses. Previous reports showed in B cells that engagement of cell surface receptors that lack cytoplasmic tyrosine residues, including the BCR (54, 55) and CD40 (56, 57), used adaptor protein complexes to signal intracellularly to activate NF-κB. Because the CD86 cytoplasmic domain is devoid of tyrosine residues, and because our evidence suggested that a novel association exists between CD86 and Phb1/2, which does express tyrosine residues, we initially thought it was possible that Phb1/2 alone might mediate CD86-dependent IkBα phosphorylation, independently of the CD86 cytoplasmic domain. However, data in this study showed that both an intact CD86 cytoplasmic domain and Phb1/2 are required to activate IkBα upon CD86 engagement, suggesting that cross talk may exist between CD86-associated Phb1/2 and the cytoplasmic domain for IkBα activation. Data also showed that all alanine substitutions of the PKC phosphorylation sites within the CD86 cytoplasmic domain impaired CD86 function, with S303A causing the most significant reduction of CD86-induced Oct-2 and mature IgG1 mRNA, suggesting that phosphorylation of the CD86 cytoplasmic domain may serve as a potential docking site for an unknown common signaling intermediate that may also interact with Phb1/2 to regulate phosphorylation of IkBα. Although the mechanistic link between Phb1/2 and the CD86 cytoplasmic domain is unclear at present, each appears to be required to promote CD86-dependent phosphorylation of IkBα and the activation of NF-κB p50/p65.

Previous reports showed that both Phb1 and Phb2 become tyrosine phosphorylated within their C-terminal domains upon

FIGURE 8. Phb1/2 and the CD86 cytoplasmic domain are each required for the CD86-dependent nuclear localization of NF-κB (p65). (A) CH12LX B cells were transfected either under mock conditions or with scrambled negative control shRNA or Phb1/2-specific shRNA plasmids via nucleofection for 24 h followed by CD40L/IL-4 priming for an additional 16 h. The cells were then resuspended in serum-free conditions for at least 30 min. A CD86 Ab (anti-CD86) was added to cell cultures for 90 min. Nuclear-enriched protein lysates were prepared and the level of p65 phosphorylation (p-p65) and total levels of p65 protein present in the nucleus were measured via immunoblot relative to Lamin A/C. (B) WT or CD86-cytoplasmic deficient (KKPΔ) FLAG-CD86 plasmids were transfected into CH12.LX B cells via nucleofection and primed with CD40L/IL-4 for 16 h. An anti-FLAG Ab was added for 90 min relative to a species- and isotype-matched control Ab (iso ctrl Ab). The level of p65 phosphorylation and total levels of p65 present in the nucleus were determined via immunoblot. Densitometry was performed and measured p-p65 and total p65 band intensity present in the nucleus relative to Lamin A/C and the data are presented as the mean fold change in p-p65 or total p65 from primed B cells where CD86 was engaged relative to priming alone (A) or isotype control Ab (B) and expressed as the mean fold change ± SEM from three independent experiments. Statistical differences are shown relative to priming alone (A) or isotype control Ab (B). *p < 0.05.
engagement of cell surface receptors such as the TCR and CD28 (50), whereas Phb1 alone was shown to undergo tyrosine phosphorylation upon insulin receptor engagement (51–53, 58). Recently, it was found that Phb1 and Phb2 are linked to the MAPK phosphorylation upon insulin receptor engagement (51–53, 58). Re- cently, it was found that Phb1 and Phb2 are linked to the MAPK signaling cascade upon CD3 engagement in T cells (44). Therefore, it was possible that Phb1/2 may serve as critical intermediates for the CD86-induced activation of PLCγ2. We also proposed that the CD86 cytoplasmic domain may not be involved with the CD86 induction of PLCγ2 owing to the lack of tyrosine residues within this domain that would be necessary for PLCγ2 binding. Because prior reports revealed that PLCγ2 becomes recruited to tyrosine-containing proteins upon cell surface receptor engagement (25, 26), our findings indicate a potential role for Phb1/2 alone in mediating CD86-induced PLCγ2 phosphorylation, independent of the CD86 cytoplasmic domain, because Phb1 and Phb2 contain tyrosine residues, whereas the cytoplasmic domain does not. Also, it was reported that Phb1 associates with Syk upon BCR engagement (41). Because Syk has been reported to regulate the activation of PLCγ2 via the adaptor protein B cell linker protein upon BCR engagement (59), it is possible that Phb1 and Phb2 serve as a scaffolding complex to link CD86 engagement to PLCγ2 activation. Nonetheless, the modest interdependent reduction in Phb1/2 protein levels in the present study was sufficient to prevent CD86 induction of IgG1 and proximal signaling intermediates, suggesting that Phb1 and Phb2 are each required to mediate CD86-induced signaling in B cells.

In this study, we used RNA interference to silence Phb1/2 because both Phb1- and Phb2-deficient mice are embryonic lethal. Previous reports revealed that Phb1 and Phb2 protein expression levels were interdependent, with depletion of one causing the loss of the other (60, 61), which is consistent with findings in the present study. Phb1/2 mRNA levels, however, were reported to be comparable in the presence of non-gene–specific Phb1/2 siRNA (61), which is in contrast with findings of the present study. The discrepancy could be due to a mixed population of Phb1/2 shRNA plasmids, as were used in the present study that targeted multiple regions of Phb1/2 transcripts, because Phb1 and Phb2 are highly homologous (40), thus promoting a higher likelihood of suppressing mRNA translation.

To our knowledge, the present findings using RNA interference are the first to identify a role for Phb1 and Phb2 in mediating the CD86-induced enhancement of the levels of Oct-2 and IgG1 produced by a CD40L/IL-4–primed B cell. Phb1/2 shRNA-mediated depletion in the present study led to modest reductions in Phb1/2 protein levels, suggesting that only a modest amount of Phb1/2 protein in the cell is required to mediate signaling events induced by CD86, whereas a greater proportion is necessary to regulate mitochondrial function. This is supported by previous reports that showed Phb1/2 depletion resulted in the loss of mitochondrial membrane potential (50, 62), even though other reports have shown Phb1/2 depletion did not affect mitochondrial membrane potential (61, 63). Also, by using gene silencing, Phb1 was shown to have functional roles in cell migration (64), regulation of angiogenic activity and cellular se- nescence (62), and cellular metabolic activity (65) upon engagement of various cell surface receptors. Likewise, gene silencing of Phb2 indicated a functional role for Phb2 in muscle cell differ- entiation (66). Also, Phb1 and Phb2 have been reported to associate with the BCR (43) and, recently, CD3 of the TCR complex (44), although the exact functional roles for the associations remain unclear. Furthermore, a direct protein/protein interaction has been shown to occur between Phb1 and Syk upon BCR engagement (41), whereas another report showed a decrease in MAPK signaling upon CD3 engagement when Phb1/2 function was blocked, indicating that Phb1/2 may serve functional roles in BCR/TCR signaling. Thus, the present findings using RNA interference provide further support that Phb1 and Phb2 participate in mediating cell surface receptor–induced regulation of cellular functions.

We originally hypothesized that the cytoplasmic domain of CD86 associated directly with Phb1/2 to mediate CD86 signaling function. However, our data showed that Phb1/2 associated with CD86 even in the absence of the CD86 cytoplasmic domain, suggesting a transmembrane-specific interaction. The ability of CD86 to bind to transmembrane proteins is supported by a recent study in dendritic cells where it was shown that CD86 associated with an E3 ubiquitin ligase in the presence or absence of the CD86 cytoplasmic domain (67). It is also supported by previous reports showing that Phb1 and Phb2 exist as transmembrane proteins that function to stabilize proteins in the electron transport chain and to mediate functional changes induced by cell surface receptors (40, 47–49, 62, 64–66). Taken together, these findings support the proposal that CD86 associates with Phb1/2 via transmembrane-specific interactions in B cells.

Findings from the present study may help us to understand the mechanism by which CD86 signals directly to a B cell to increase not only IgG1, but also IgE production, as shown previously (13, 16). Additionally, understanding this novel signaling mechanism may help direct the rational design of CD86-targeted therapeutic strategies to increase the level of protective Ab in immunocompromised patients. Conversely, CD86-targeted therapeutic strategies might also be developed to decrease the level of harmful Abs produced in certain cases of autoimmunity or allergic asthma.

Acknowledgments
We thank Dr. Alan Smith and Matthew Gormley for critical experimental support and Todd Shawler for assistance with flow cytometry. We also thank Caroline Padro and Sara McMaken for critical evaluation of the manuscript.
Disclosures

The authors have no financial conflicts of interest.

References


SUPPLEMENTAL MATERIAL

Fig. S1. CD86 expression in B cells exposed to different stimuli. (A) Naïve splenic B cells were treated as described previously in Fig. 2. Total mRNA was collected at 16 hours and analyzed via qRT-PCR analysis for CD86 and GAPDH mRNA levels. CD86 mRNA values were normalized to GAPDH and the data are presented as a mean Fold Change in CD86 from primed B cells relative to resting B cells and are expressed as the mean Fold Change ± SEM from quadruplicate samples/condition of either representative (Resting vs. Terb) or from two independent experiments. Statistical analysis was used to determine significant differences between groups. *, $p < 0.05$. (B) Phb1 or Phb2 GFP-containing shRNA plasmids were transfected into CH12.LX B cells and primed as described in Fig. 3. Surface expression of CD86 on GFP+ and GFP− cell populations were determined by FACS analysis. Isotype ctrl histograms are depicted as (gray lines), and CD86 histograms are depicted as (black lines). Representative histograms are shown from three independent experiments.

Fig. S2. Phb1 and Phb2 expression in B cells treated with Phb1 and Phb2 shRNA. CH12.LX B cells were transfected with either a GFP-containing Phb1 or Phb2 shRNA plasmids for 36 hours to silence Phb1 and Phb2 gene expression. At 24 hours post transfection, cells were primed with CD40L/IL-4 for 16 hours. GFP cells containing the Phb1 or Phb2 shRNA vector were separated by FACS. Phb1/Phb2 mRNA were measured via qRT-PCR analysis and normalized to actin. Data are expressed as mean ± SEM Fold Change relative to No GFP of quadruplicate samples/condition from 1-3 independent experiments. No GFP cells are depicted as (gray bars) and GFP cells are shown as (black bars). Statistical differences are shown between groups. *, $p < 0.05$. 
Fig. S3. Oct-2 and mature IgG1 expression is regulated via the cytoplasmic domain of CD86. (A) WT-transgenic B cells (WT Tg) or Truncated CD86 (Trunc CD86) B cells were primed with CD40L/IL-4 for 16 hours followed by the addition of CD28/Ig to engage CD86. At 16 hours, levels of cytoplasmic CD86 mRNA were measured by qRT-PCR analysis between WT Tg CD86 and Trunc CD86 groups. Data are expressed as mean Fold Change ± SEM relative to WT Tg resting and are pooled from two independent experiments. (B, C) On day 2 post cell priming, levels of Oct-2 and germline IgG1 mRNA were quantified by qRT-PCR between WT Tg CD86 and Trunc CD86. Data are expressed as mean Fold Change ± SEM relative to WT Tg CD40L/IL-4 and are pooled from two independent experiments. (D) On day 5 post cell priming, levels of mature IgG1 mRNA were measured by qRT-PCR analysis between WT Tg CD86 and Trunc CD86. Data are expressed as mean Fold Change ± SEM relative to WT Tg CD40L/IL-4 and represent 1-2 independent experiments. Statistical analysis was performed to determine significant differences among groups (A), or compared to WT Tg CD40L/IL-4 (B to D) *, p < 0.05.

Fig. S4. Cytoplasmic- and Nuclear-enriched protein lysate fractionation in CH12.LX B cells. In order to demonstrate sufficient Cytoplasmic- and Nuclear-enriched protein fractionation, CH12.LX B cells were treated with 0.5% NP-40-based lysis buffer, followed by treatment with 1% Triton-X100-based lysis buffer as described in Materials and Methods. Either Cytoplasmic (C)- or Nuclear (N)-enriched protein lysates were analyzed for the presence of Cytoplasmic-specific proteins including α-tubulin, β-Actin, and GAPDH, or Nuclear-specific proteins Lamin A and Lamin C. Gels are representative of three independent experiments.