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Prohibitins and the Cytoplasmic Domain of CD86 Cooperate To Mediate CD86 Signaling in B Lymphocytes

Christopher R. Lucas,*† Hector M. Cordero-Nieves,*† Robert S. Erbe,* Jaclyn W. McAlees,*† Sumeena Bhatia,‡ Richard J. Hodes,‡ Kerry S. Campbell,§ and Virginia M. Sanders*†

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 Ικβα, which we previously reported leads to NF-κB p50/p65 activation, whereas only Phb1/2 was required for the CD86-induced phosphorylation of phospholipase Cγ and protein kinase Ca/β1, 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.

Also referred to as B7-2, CD86 is a 70-kDa transmembrane glycoprotein expressed primarily on APCs, including macrophages, dendritic cells, and B cells (1, 2). CD86 is a well-known costimulatory molecule that ligates CD28 and CTLA-4 expressed on a CD4+ T cell to increase or decrease, respectively, T cell activation signals (3–6), and it is essential for germinal center formation (7, 8). CD86 expression is low on resting B cells (1), but it increases in response to engagement of the BCR (1), CD40 (9), the IL-4R (10), the LPS receptor (11, 12), or the β2-adrenergic receptor (13, 14). CD86 contains a short cytoplasmic domain that lacks tyrosine phosphorylation sites and was thought not to signal directly. However, the CD86 cytoplasmic domain contains three putative protein kinase C (PKC) serine/threonine phosphorylation sites. Additionally, a proposal by Lenschow et al. (15) reported that the CD86 cytoplasmic domain might become phosphorylated owing to cellular activation stimuli, suggesting that CD86 may signal directly. Studies have reported that CD86 engagement induced a signal directly within the B cell that increased IgG4 production in anti-CD40/IL-4–primed human B cells (16), and the murine IgG4 homolog IgG1 production in CD40L/IL-4– (13, 17–20) or LPS-primed (21) murine B cells in vitro, as well as in B cells from mice immunized with either trinitrophenyl hapten-keyhole limpet hemocyanin (20) or influenza virus (22). It has also been reported that CD86 also signals to regulate other Ig isotopes, including IgE (13, 16), and IgG2a (21), an affect that may be controlled by the priming Ag or stimulus. Collectively, these findings suggested that CD86 on a B cell plays a role in regulating the level of IgG1 produced.

The initial functional results from these studies led to the search for signaling intermediates and transcription factors activated by CD86 engagement to mediate the increase in IgG1 production. CD86 engagement on the surface of a CD40L/IL-4–primed B cell was found to activate two cascades of signaling intermediates that ultimately allowed for NF-κB p50/p65 activation via phosphorylation of Ικβα and p65 phosphorylation, respectively (18). Inhibition or loss of these signaling intermediates in a B cell eliminated the CD86-induced increase in Oct-2 expression (18, 19), Oct-2 binding to the 3′-IgH enhancer (18, 19), the rate of mature IgG1 transcription (17), and the increase in IgG1 protein per cell (13), confirming their roles in mediating CD86 signals to affect the level of IgG1 produced. Importantly, CD86 engagement on primed B cells failed to affect class switch recombination (13, 17–20), indicating that the increase in IgG1 was due to an effect on the amount of IgG1 produced per cell and not the number of cells that switched to IgG1. The increased level of signaling intermediate activation and/or Oct-2 that was induced by CD86 engagement on primed B cells resulted in a 2- to 3-fold increase in

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The online version of this article contains supplemental material.

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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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 (PLCy)2, 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 previously described (27) and was provided by Dr. G. Bishop (University of Iowa, Iowa City, IA). Wild-type (WT), transgenic B cells (line 7) that overexpress CD86 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

Naïve 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 CD40 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 tertbutylamine (Sigma-Aldrich), or 50 µg/ml LMP (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-Toxate (Sigma-Aldrich), a Luminex assay with a level of detection <0.1 U/ml.

#### Immunoprecipitation

Immunoprecipitations were performed using the ProFound mammalian coimmunoprecipitation kit (Pierce). 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), and an intact CD86 cytoplasmic domain are required to mediate CD86 signaling. Such a directly associated protein must exist because one of the most proximal CD86-dependent signaling intermediates, phospholipase C (PLCy)2, 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.

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### Immunoblot densitometric analysis

Specific protein bands detected via immunoblot analysis were quantified via densitometry (Image 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. Biotinylated secondary Abs of each immunoblot 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-KCαββαβ (V636/641), anti-PKCa, anti-phospho-IκBαββ (S32, 14D4), anti-IκBαββ (4D4), anti-phospho-p65 (S536; 9H11), anti-p65 (C22B4), anti-IκBαββ (C161, 14C10), anti–IκB-actin (13E5) (Cell Signaling Technology), and anti–α-tubulin (DM1A1, Sigma-Aldrich).

#### 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 FACSCount II (BD Biosciences). Expression of CD85x-Flag 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

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**Note:** The full manuscript contains additional details and references, including experimental procedures and results pertinent to the CD86 signaling pathway and its role in B cell activation and antibody production. The text provided is a summary of the key findings and methods used in this study.
The Nucleic Acid Shared Resource Facility at The Ohio State University.

To the manufacturer's instructions. The following primers were used to generate a FLAG-CD86 expression construct, murine CD86 cDNA was then subcloned into the pCR2.1 TOPO TA vector. To vector (1 g/106 cells) via nucleofection (Amaxa, program O-003) 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 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 CD40L/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 CD40L/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 locales (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 cytoplasmic CD86 mRNA levels, forward primer, 5′-ATCGTGGCCAAAGCTGTTGTTAGTC-3′, reverse primer, 5′-TACTCGGGGAACTGGGAGAG-3′; Phb2 mRNA levels, forward primer, 5′-GGTACGAAACAGAGGCTGAC-3′, reverse primer, 5′-TTCGACCTCAGACATTGCAGC-3′, cdNA levels, forward primer, 5′-ATCGTGGCCAAAGCTGTTGTTAGTC-3′, reverse primer, 5′-CAGGTTGATGCTCCTCTCAGCT-3′; Oct-2 mRNA levels, forward primer, 5′-ATGCAATGAAAGTTCCTCAGCA-3′, reverse primer, 5′-TGGAGGAGTCTGCTTCTCAGC-3′;igelin1 mRNA levels, forward primer, 5′-CATCCATTGGAGGACATTG-3′, reverse primer, 5′-ATTCGACCTCAGACATTGCAGC-3′. To clone the reverse primer containing an EcoRI site (5′-9CTATCGGGGAACTGGGAGAG-3′; SABiosciences) (1–5 μg/106 cells), or with a FLAG-CD86 expression construct and primary B cells were used (Fig. 1C). To determine whether Phb1 and/or Phb2 could be specifically coimmunoprecipitated from B cells under primed versus resting conditions. Parent CH12.LX cells, CH12.LX cells transfected with FLAG-tagged CD86, or primary B cells were cultured overnight in the presence or absence of CD40L/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 CD40L/IL-4–dependent recruitment event or an increased level of CD86 present in the CD86 immunoprecipitates. Similar results were observed when CH12. LX B 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

**In-cell ELISA**

In-cell ELISAs were performed to detect in-cell levels of phospho-PLCγ2 (Y1217) and total PLCγ2 protein using the 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 CD40L/IL-4 or with a WT or cytoplasmic-deficient (KKPa) FLAG-CD86 expression plasmid followed by priming with CD40L/IL-4 for 4 h. Cells were cultured under serum-free conditions for at least 30 min, followed by ex- generation of either an anti-CD86 Ab with anti-CD86 Abs or CD86 with antibodies to CD86 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.
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 (Fig. 3A). 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.
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 diagramed 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 (KKPD) 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 KKPD 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 KKPD 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 PKCα/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 PKCα/βII (19), we considered PKCα/βII phosphorylation a viable measurement of PLCγ2 activity. We predicted that Phb1/2 alone would be necessary to induce CD86-dependent PKCα/βII phosphorylation independent of the CD86 cytoplasmic domain because Phb1/2 alone were sufficient to allow for CD86-induced PLCγ2 activation. 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 PKCα/βII phosphorylation (pPKCα/βII) relative to total PKCα 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 PKCα/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 KKPD FLAG-CD86 expression plasmids caused an increase in PKCα/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 PKCα/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 PKCα/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...
signaling cascades that converge to activate the p50/p65 subunits of NF-κB (18, 19). Whereas one cascade is essential for CD86-dependent IκBα phosphorylation (pIκBα), 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, IκBα and p65 phosphorylation, CH12.LX B cells were either mock transduced or transfected with scrambled negative control or Phb1/2-specific shRNA 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 IκBα and p65 phosphorylation and the total levels of IκBα protein were determined via immunoblot. We observed

**FIGURE 5.** Phb1 and Phb2 are necessary for the CD86-induced activation of PLCγ2. (A) CH12.LX B cells were either mock transfected with scrambled negative control or Phb1/2-specific shRNA plasmids via nucleofection for 24 h followed by priming with CD40L/IL-4 for 16 h. Phb1/2 protein levels were measured via immunoblot relative to GAPDH. Densitometry was performed and the data are expressed as the mean percentage change ± SEM relative to mock and are representative of three independent experiments. (B) CH12.LX B cells were either mock transfected or transfected with scrambled negative control shRNA (neg ctrl shRNA) via nucleofection for 24 h followed by priming with CD40L/IL-4 for 16 h. A CD86 Ab (anti-CD86) was administered for 5, 15, 30, and 60 min. Levels of phospho-PLCγ2 (pPLCγ2) protein relative to total PLCγ2 were measured via immunoblot. (C and D) Phb1 or Phb2 shRNA plasmids were transfected into CH12.LX B cells followed by CD40L/IL-4 priming, and a CD86 Ab (anti-CD86) was added as described above. Levels of phospho-PLCγ2 protein were measured relative to total PLCγ2 via immunoblot. (E) WT and CD86 cytoplasmic-deficient (KKPΔ) FLAG-CD86 plasmids were transfected into CH12.LX B cells and nuclei were treated with CD40L/IL-4 for 16 h. Either an anti-FLAG Ab or species- and isotype-matched control Ab (iso ctrl Ab) was added for 15 min. Levels of phospho-PLCγ2 protein were measured relative to total PLCγ2 via immunoblot. Representative gels (B–E) are shown from three independent experiments. Densitometry was performed and measured phospho-PLCγ2 relative to PLCγ2 band intensity and the data are presented as the mean fold change in phospho-PLCγ2 from primed B cells where CD86 was engaged relative to priming alone (B–D) or isotype control Ab (E) and expressed as the mean fold change ± SEM from three different experiments. Statistical differences are shown relative to mock (A), priming alone (B–D), or isotype control Ab (E). *p < 0.05. (F) CH12.LX B cells were either mock transfected or transfected with scrambled negative control shRNA (neg ctrl shRNA) via nucleofection for 24 h followed by priming with CD40L/IL-4 for 16 h. A CD86 Ab (anti-CD86) was administered for 30 min. Levels of phospho-PLCγ2 (pPLCγ2) protein relative to total PLCγ2 were measured via in-cell ELISA. (G) WT and 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. Either an anti-FLAG Ab or species- and isotype-matched control Ab (iso ctrl Ab) was added for 15 min. Levels of phospho-PLCγ2 protein were measured relative to total PLCγ2 via in-cell ELISA. (F and G) Phospho-PLCγ2 protein OD values were normalized to total phospho-PLCγ2 protein OD values and the data are represented as a mean fold change in phospho-PLCγ2 protein from CD86-engaged B cells relative to primed alone B cells of each group (F) or isotype control (G) and are expressed as the mean ± SD of quintuplicate samples per condition from at least two independent experiments. Statistical differences are shown relative to priming alone of each group (F) or isotype control (G). *p < 0.05.
FIGURE 6. Phb1 and Phb2 are necessary for the CD86-induced activation of PKCα/βII. (A) CH12.LX B cells were either mock transfected or transfected with a scrambled negative control shRNA (neg ctrl shRNA) or with Phb1 or Phb2 shRNA plasmids into CH12.LX B cells via nucleofection for 24 h followed by priming with CD40L/IL-4 for 16 h. A CD86 Ab (anti-CD86) was administered for 5, 15, 30, and 60 min. Levels of phospho-PKCα/βII (pPKCα/βII) protein relative to total PKCα and PKCβII were measured via immunoblot. (B) WT and CD86 cytoplasmic-deficient (KKPα) FLAG-CD86 plasmids were transfected into CH12.LX B cells via nucleofection and primed with CD40L/IL-4 for 16 h. Either an anti-FLAG Ab or species- and isotype-matched control Ab (iso ctrl Ab) was added for 30 min. Levels of phospho-PKCα/βII protein relative to total PKCα and PKCβII were measured via immunoblot. Representative gels are shown from three independent experiments. Densitometry was performed and measured phospho-PKCα/βII relative to either priming alone (A) or iso ctrl Ab (B) and expressed as the mean fold change ± SEM from three independent experiments. Statistical differences are shown relative to mock priming alone (A) or isotype control Ab (B). *p < 0.05.
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 each required for the CD86-induced phosphorylation of IkBα, which we previously reported leads to NF-kB 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-kB (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.
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 domain was deleted. Alternatively, CD86-induced IκBα 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 IκBα 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 IκBα upon CD86 engagement, suggesting that cross talk may exist between CD86-associated Phb1/2 and the cytoplasmic domain for IκBα 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 IκBα. 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 IκBα 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
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 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 senescence (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 differentiation (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.

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Disclosures

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