The PHB1/2 Phosphocomplex Is Required for Mitochondrial Homeostasis and Survival of Human T Cells*

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Many immune pathologies are the result of aberrant regulation of T lymphocytes. A functional proteomics approach utilizing two-dimensional gel electrophoresis coupled with mass spectrometry was employed to identify differentially expressed proteins in response to T cell activation. Two members of the prohibitin family of proteins, Phb1 and Phb2, were determined to be up-regulated 4–5-fold upon activation of primary human T cells. Furthermore, their expression was dependent upon CD3 and CD28 signaling pathways that synergistically led to the up-regulation (13–15-fold) of Phb1 and Phb2 mRNA levels as early as 48 h after activation. Additionally, orthophosphate labeling coupled with phosphoamino acid analysis identified Phb1 to be serine and Phb2 serine and tyrosine phosphorylated. Tyrosine phosphorylation of Phb2 was mapped to Tyr248 using mass spectrometry and confirmed by mutagenesis and phosphospecific antibodies. In contrast to previous reports of Phb1 and Phb2 being nuclear localized, subcellular fractionation, immunofluorescent, and electron microscopy revealed both proteins to localize to the mitochondrial inner membrane of human T cells. Accordingly, small interfering RNA-mediated knockdown of Phbs in Kit225 cells resulted in disruption of mitochondrial membrane potential. Additionally, Phb1 and Phb2 protein levels were up-regulated 2.5-fold during cytokine deprivation-mediated apoptosis of Kit225 cells, suggesting this complex plays a protective role in human T cells. Taken together, Phb1 and Phb2 are novel phosphoproteins up-regulated during T cell activation that function to maintain mitochondrial integrity and thus represent previously unrecognized therapeutic targets for regulating T cell activation, differentiation, viability, and function.

Complete activation of T cells requires three threshold-limited sequential signals. Naive T cells receive induction signals through engagement of the T cell receptor complex (TCR/CD3) via specific antigens (signal 1). This signal is amplified by co-stimulatory molecules such as B7-1/CD28 (signal 2), which promotes the synthesis and secretion of cytokines, which activate cell surface receptors (signal 3) to drive clonal expansion and functional differentiation. Each extracellular signal induces an intracellular cascade of tyrosine, serine, threonine, and lipid kinases. Early TCR signaling is mediated by the tyrosine kinase p56lck, which phosphorylates immunoreceptor tyrosine-based activation motifs within the cytoplasmic domains of the TCR subunits (1–3). These phosphorylated immunoreceptor tyrosine-based activation motifs recruit Zap70, which in turn propagates the signal by phosphorylating multiple proteins including linker of activated T cells (4). This protein acts as a scaffold to recruit a number of downstream signaling molecules, including Grb2 to drive Ras signaling (5), and phospholipase C-γ1 to produce inositol 1,4,5-triphosphate and diacylglycerol from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (6, 7). These effector pathways ultimately promote activation of key T cell transcription factors (NFAT via inositol 1,4,5-triphosphate, NF-κB via diacylglycerol, and AP-1 via Ras) to regulate the expression of genes required for proliferation and differentiation including interleukin 2 (IL-2), which serves as an autocrine growth factor (8, 9).

Signaling from IL-2 through its receptor is primarily delivered by two molecular families, namely Janus tyrosine kinases (Jaks) and signal transducers and activators of transcription (STATS) (10). Jak3, which is required for T cell proliferation in response to IL-2, is differentially expressed upon activation (11–13). Additionally, the IL-2 receptor α chain (CD25), which is required for a high affinity IL2R complex, is also up-regulated upon T cell activation (14). These findings have provided a molecular rationale for therapeutic strategies targeting the IL-2 signaling pathway to treat lymphoid-derived diseases (15). Additional insight into other proteins up-regulated or phosphorylated during T cell activation will likely harbor yet to be realized therapeutic strategies.

To identify these potential regulatory proteins, two-dimensional gel electrophoresis coupled with mass spectrometry have been critical. Using these technologies, we have identified the highly conserved prohibitin (Phb) family of proteins, Phb1 and Phb2, to be differentially expressed upon T cell activation. The Phbs have been found in multiple cellular compartments and possess diverse functions ranging from acting as scaffolding proteins at the plasma membrane to transcriptional regulators in the nucleus.

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2 The abbreviations used are: IL-2, interleukin 2; ConA, concanavalin A; Jak, Janus tyrosine kinase; LAT, linker of activated T cells; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; Phb, prohibitin; PMA, phorbol 12-myristate 13-acetate; STAT, signal transducers and activators of transcription; TCR, T cell receptor; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; RT, reverse transcriptase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; siRNA, small interfering RNA; PI, phosphatidylinositol; PARP, poly(ADP-ribose) polymerase; WT, wild type.
nucleus. Phb2 was originally identified as a B cell receptor-associated protein termed Bap37 (16). More recently, Phb1 was shown to be required for activation of c-Raf by Ras in modulating epithelial cell adhesion and migration, thus providing evidence for a signaling component to the Phb mechanism of action (17). Conversely, Phb nuclear localization has been described in a variety of cell lines predominantly from breast and prostate cancers (18, 19). Prohibitions were shown to modulate the transcriptional activity of various transcription factors, including steroid hormone receptors, either directly or through interactions with chromatin remodeling proteins (20–22). Interestingly, numerous studies in Saccharomyces cerevisiae and Caenorhabditis elegans suggest the evolutionarily conserved Phb mechanism of action is as chaperone proteins in mitochondria, which has been extensively reviewed (2, 23–25). It is worth noting that Phb1 and Phb2 null yeast strains have a reduced lifespan (25), however, higher eukaryotes are more dependent upon their presence, suggesting enhanced biological function(s). For example, the Phb homologue in Drosophila is reported to be in a lethal complementation group (26) and genetic deletion of Phb1 or Phb2 in mice is lethal before embryonic day 9.0, implying these proteins play a critical role in the early stages of development (27, 28).

Little is known about the Phb family in lymphocytes. Phb1 has been proposed to inhibit cell proliferation and to promote differentiation of lymphocytes. Indeed, its expression is increased during pregnancy-associated thymic involution with a pattern of limited tissue distribution localized to the medulla of the thymus that primarily contains only mature, non-proliferating thymocytes (29). The present study sought to identify proteins differentially expressed upon T cell activation. Evidence is provided that Phb1 and Phb2 form a phosphocomplex in the mitochondrial inner membrane of primary human T cells. Furthermore, functional analysis reveals that this complex is required for mitochondrial homeostasis that may be critical for differentiated T cell survival.

EXPERIMENTAL PROCEDURES

T Cell Purification, Activation, and Cell Culture

Peripheral blood mononuclear cells (PBMCs) were collected from buffy coats obtained from the Gulf Coast Regional Blood Bank or WBF2 filters (Pall Corporation) obtained from El Paso United Blood Services and purified by isocentrifugation (Ficoll-Hypaque). PBMCs were grown in RPMI 1640 supplemented with 10% fetal calf serum (Atlanta Biologicals), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin (complete RPMI). Explanted normal human T lymphocytes (3 × 10^6/ml) were activated for 72 h using one of the following agents: anti-CD3-coated 96-well plates (BD Biosciences) in the presence or absence of soluble anti-CD28 (5 µg/ml), phytohemagglutinin (PHA) (10 µg/ml), concanavalin A (ConA) (10 µg/ml), or phorbol 12-myristate 13-acetate (PMA) (100 nM) and ionomycin (500 ng/ml). CD3+ T cells were purified by negative selection (R&D Systems) and their purity and activation status determined by flow cytometry (Cytofics FC500, Beckman Coulter) using directly conjugated anti-CD3-PE and anti-CD25-FITC, respectively. Jurkat (30), MT-2 (31), and T47D (32) cells were maintained in complete RPMI. The IL-2-dependent human T cell line Kit225 (33) (kindly provided by Dr. J. Johnston, Queens University, UK) was maintained in complete RPMI plus 100 IU/ml recombinant IL-2.

Cell Extracts, Immunoprecipitation, and Western Blot Analysis

Nuclear and cytoplasmic fractions were obtained by hypotonic lysis as previously described (34). For subcellular fractionation, activated primary human T cells (2 × 10^6) were suspended in isotonic buffer (10 mM HEPES, pH 8.0, 10 mM potassium chloride, 2 mM magnesium chloride, 250 mM sucrose, 1.0 mM EDTA, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, 5 µg/ml aprotinin, and 1 mM phenylmethylsulfonfyl fluoride) and mechanically disrupted with passage through a 30-gauge needle until >90% were trypan blue positive. Nuclei and broken cells were pelleted by centrifugation at 1500 × g for 10 min. The resulting supernatant was collected and centrifuged at 10,000 × g for 15 min to obtain the soluble cytoplasmic (supernatant) and mitochondrial (pellet) fractions.

For whole cell lysis, frozen cell pellets were thawed on ice and solubilized in 1% Triton X-100 containing lysis buffer as previously described (11). Immunoprecipitation and Western blot analysis was performed as described previously (35). A novel and specific affinity purified rabbit polyclonal antibody was generated against the extreme C-terminal 15 amino acids of human Phb2. Monoclonal anti-Phb1 (Molecular Probes), anti-actin (Sigma), anti-GAPDH (Research Diagnostics), and the affinity purified anti-Phb2 were used at a dilution of 1/1000 for Western blot. For all samples, total protein was determined by the bicinchoninic acid method (Pierce).

Two-dimensional Gel Electrophoresis and Protein Identification by Mass Spectrometry

First Dimension Separation—CD3+ naïve or PHA-activated whole cell lysates (500 µg) were precipitated using trichloroacetic acid and washed twice with cold acetone before dissolving in sample rehydration buffer (7 M urea, 2 M thiourea, 50 mM dithiothreitol, 2% Triton X-100, 0.2% carrier ampholytes (pH 3–10), and 0.001% bromphenol blue). Lysates were loaded onto 7-cm IPG ReadyStrips (Bio-Rad) through passive rehydration overnight at room temperature. Following rehydration, these proteins were isoelectrically focused on the Bio-Rad Protein IEF Cell with the following step protocol: 250 V for 15 min, 4000 V for 150 min, and maintained at 4000 V for a total of 10,000 V-h. ReadyStrip IPG strips were stored at −80 °C until second dimension processing.

Second Dimension Separation—IPG ReadyStrips from the first dimension separation were equilibrated in succession with buffer I (375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 130 mM dithiothreitol, 20% glycerol) and buffer II (375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 135 mM iodoacetamide, 20% glycerol) for 10 min each. The IPG strips were then washed in Tris-glycine SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3), secured on top of 12% SDS-PAGE gels using low melting point agarose, and electrophoresed at 200 V for 1 h. The resulting gels were stained using the mass spectrometry compatible Silver Stain Plus kit from Bio-Rad according to the manufacturer’s protocol and gel images collected in a 16-bit
grayscale format using an Epson 1680 Professional dual bed scanner at 600 dpi resolution. Protein spots of interest were excised and submitted to the Center for Functional Genomics (University at Albany) or the Border Biomedical Research Center Biomolecule Analysis Core Facility (University of Texas, El Paso) for identification following their standard procedure.

Quantitative RT-PCR

PBMCs were plated at a concentration of $3 \times 10^5$ cell per well in either uncoated or anti-CD3-coated 96-well plates (BD Biosciences) in the presence or absence of anti-CD28 (5 µg/ml). Total RNA was isolated from ~5 $\times 10^6$ cells using the RNeasy kit (Qiagen), DNase treated, and reverse transcribed (100 ng, 50 °C for 30 min, Superscript II; Invitrogen) with specific reverse primers. Quantification based on real-time monitoring of amplification was determined using a Bio-Rad IQ5 with SYBR Green dye. Absolute numbers of mRNA molecules were normalized to 18 S rRNA to correct for RNA concentration differences. Samples were run in triplicates with one control reaction containing no reverse transcriptase enzyme to test for potential DNA contamination. Values of transcripts in unknown samples were obtained by interpolating threshold cycle (PCR cycle number at threshold) values on a standard curve. Standard curves were prepared from known amounts of purified, PCR-amplified amplicon. Primer sets for human Phb1 and Phb2 were as follows: Phb1 forward, GGAGGCGTGGT-GAACCTCCTG; Phb1 reverse, CTGGCACCATTACGTGTC-GAG; Phb2 forward, CTTGGTCCAGTACCCCATTATC; Phb2 reverse, CGAGACAACACTCGCAGGG. Primer sets for human CD25 (ID number 4557667a1) were obtained from PrimerBank.

Immunofluorescent Confocal Microscopy and Transmission Electron Microscopy

For confocal microscopy, primary human T cells were cyto-centrifuged and fixed on glass slides with cold methanol and permeabilized with 0.2% Triton X-100 for 5 min. Immunofluorescent staining was performed at room temperature. Slides were blocked with 5% normal donkey serum and incubated with either mouse monoclonal anti-Phb1 (Neomarkers), and affinity purified rabbit polyclonal anti-Phb2 for 1 h. Cells were washed three times with PBS-T (0.05% Tween 20 in PBS) and incubated with secondary Cy2-conjugated donkey anti-mouse antibody or Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) for 1 h at a 1:50 dilution. After three washes with PBS, sections were blocked with 5% normal donkey serum for 1 h and incubated with 1:50 dilution of mouse monoclonal anti-Phb1 (Neomarkers) and 1:50 dilution of affinity purified rabbit polyclonal anti-Phb2 for 1 h. The sections were washed five times with 1% bovine serum albumin and 0.05% Tween 20 in PBS and incubated with secondary 6-nm gold particle-conjugated donkey anti-mouse antibody or 12-nm gold particle-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) for 1 h at a 1:50 dilution. Post-fixation was performed with 2.5% glutaraldehyde for 5 min. The sections were post-stained using the Kai Chien procedure with 2% uranyl acetate and Reynold’s (36) lead citrate for 6 min each. The sections were observed using a Zeiss EM-10 transmission electron microscope operating at 60 – 80 kV in the Border Biomedical Research Center Analytical Cytology Core Facility (University of Texas, El Paso). Photographs were obtained with Kodak SO-163 film, and negatives were scanned with a Nikon Super Coolscan 5000 ED scanner to obtain digital images.

Phosphoamino Acid Analysis and Phosphopeptide Mapping

Naïve and PHA-activated primary human T cells and Kit225 cells were metabolically labeled with 1 mCi/ml $[^{32}]$Porthophosphate (PerkinElmer Life Sciences) overnight at 37 °C. The cells were lysed and Phb2 was immunoprecipitated as described above. The corresponding proteins were visualized by Coomassie Blue R-250 stain (Bio-Rad) and autoradiography, excised, and subjected to limited hydrolysis in 6 N HCl at 100 °C for 30 min. The samples were then dried and resuspended in pH 1.9 buffer (formic acid:acetic acid:water at 50:156:1794 ratio) containing 1 µg of phosphoamino acid standards. The samples were spotted on a thin layer cellulose-acetate gel and electrophoresis was performed in the first dimension at 1500 V for 30 min in pH 1.9 buffer and in the second dimension at 1300 V for 15 min in pH 3.9 buffer (pyridine:acetic acid:water at 10:100:1890 ratio) using the Hunter Thin Layer Electrophoresis apparatus. Standards were visualized with ninhydrin and radiolabeled samples detected by autoradiography. Phosphopeptide mapping of immunoaffinity purified Phb2 by MALDI-TOF mass spectrometry was performed by the proteomics core facility at the Center for Functional Genomics (University at Albany).

Phosphospecific Phb2 Tyr(P)248 Antibody and Peptide Competition Assay

A rabbit polyclonal antibody was generated (Sigma Genosys) against the Phb2 phosphopeptide, KCKNGpYIKLR. Anti-Phb2
**Prohibitin Cloning, Site-directed Mutagenesis, and Transfection**

Phb2 was PCR amplified from mRNA purified from the tumor T cell line, Kit225. The pLenti6/V5-D-TOPO-Phb2-WT was constructed by subcloning Phb2 cDNA from pcDNA3.1-Phb2 donor plasmid using TOPO technology. The Phb2 Y248F mutant was prepared using the QuikChange (Stratagene) site-directed mutagenesis kit according to the manufacturer’s instructions. The following primer (sense strand) was used for Phb2 Y248F mutation: 5’-AGCAAGAACCTGCTCATCAAACCTGCGAAC-3’. Before use, subclones and mutations were verified by DNA sequencing. The pLenti6-Phb2WT-V5 (2.5 μg) or pLenti6-Phb2Y248F-V5 (2.5 μg) plasmid transfections were performed in Kit225 (5 × 10⁶) cells using Amazix nucleofection technology using protocol X-001 according to the manufacturer’s instructions. Cells were harvested 36 h post-nucleofection and total cell lysates or anti-V5 (Invitrogen) immunoprecipitated proteins were analyzed by SDS-PAGE and Western blot as described above.

**siRNA-mediated Silencing of Phb1 and Phb2**

Phb1 (SMARTpool catalog number M-010530-00) and Phb2 (SMARTpool catalog number M-018703-00) specific siRNA as well as control non-targeting (siControl pool catalog number D-001206-13) siRNA were purchased from Dharmacon. Transfection of Kit225 cells was carried out by electroporation using the Nucleofection system by Amazix. Briefly, Kit225 cells (5 × 10⁶) were suspended in 100 μl of transfection solution V and transfected with 1.5 μg of Phb1, Phb2, or control siRNA using the X-001 program. Transfected cells were immediately diluted with pre-warmed complete RPMI containing IL-2 (100 IU/ml) and cultured for the time indicated.

**Mitochondrial Transmembrane Potential, ΔΨm Analysis**

Mitochondrial membrane potential was analyzed using the lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (DiPsipher, R&D Systems). Kit225 cells (1 × 10⁶) were electroporated alone or with control non-targeting siRNA (500 nM) or Phb1/Phb2 siRNA (250 nM each) and cultured for 36 h. The potassium ionophore valinomycin was used as a positive control for disruption of ΔΨm. Kit225 cells (1 × 10⁶) were treated with valinomycin (100 nM) for 6 h at 37 °C and 5% CO₂. The cells were harvested by centrifugation at 500 × g for 5 min, resuspended in DiPsipher reagent (5 μg/ml), and incubated for 15 min at 37 °C and 5% CO₂. The cells were then washed twice with PBS, and fluorescence observed by flow cytometry (Cytomics FC500, Beckman Coulter) and quantitated with CXP analysis software version 2.2 (Beckman Coulter).

**Assessment of Apoptotic Cell Death**

Kit225 cells (1 × 10⁶) were washed with PBS and centrifuged at 200 × g for 5 min. Cell pellets were resuspended in 100 μl of Annexin-V-FLUOS staining solution (for 10 assays, 20 μl of Annexin V-Fluorescein, and 20 μl of PI in 1000 μl of HEPES buffer) (Roche) and incubated for 15 min at room temperature. Stained cells were then analyzed by flow cytometry (Cytomics FC500, Beckman Coulter) and quantitated with CXP analysis software version 2.2 (Beckman Coulter) using 488-nm excitation and 515-nm band pass filter for fluorescein detection and a filter >600 nm for PI detection. Additionally, caspase activation was determined by detection of PARP degradation by Western blot analysis with rabbit polyclonal anti-PARP (Cell Signaling).

**RESULTS**

Proteomic Identification of Phb2 as a Differentially Expressed Protein during T Cell Activation—In an effort to identify differentially expressed proteins during T cell activation, two-dimensional gel electrophoresis coupled with mass spectrometry was employed. Lysates from primary human naïve or PHA activated CD3⁺ T cells were separated by two-dimensional gel electrophoresis and visualized with silver stain. Landmark spots were selected to confirm equal loading and proper gel alignment. Several proteins were differentially expressed upon T cell activation including a 37-kDa protein, with a pi of 9.0, was greater in PHA activated compared with naïve T cells (Fig. 1, A and B). Two distinct spots were evident for p37, due to variance in isoelectric points. The identification of p37 was achieved by searching the National Center for Biotechnology Information (NCBI) data base with spectra obtained from MALDI-TOF mass spectrometry on the excised spots using the Mascot algorithm (Matrix Science) (Fig. 1C). The data base search suggested three putative proteins, however, only Phb2 had a significant E-value and matched the theoretical molecular weight and isoelectric point (37). The remaining differentially expressed proteins remain unknown, but are being investigated for their identity.

Phb1 and Phb2 Protein Levels Are Up-regulated during T Cell Activation—Phb1 and Phb2 are thought to be important for the regulation of cell proliferation and differentiation in various distinct cell types, however, no function has been ascribed to T cells. To characterize these proteins within a T cell system, specific polyclonal antibodies were generated to the extreme C-terminal 15 amino acids of Phb2 and subsequently purified by peptide affinity chromatography. Western blot analysis of PBMC extracts during a PHA activation time course spanning 96 h revealed that both Phb1 and Phb2 protein levels were up-regulated during T cell activation that was readily detected between 48 and 96 h (Fig. 2A). This membrane was stripped and rebotted for actin to ensure equal loading, whereas Jak3 expression confirmed efficient T cell activation. Densitometric analysis indicated Phb1 and Phb2 were up-regulated 4–5-fold.
after 72 h and 6–8-fold after 96 h of PHA activation when normalized to actin levels (Fig. 2B).

The process of PHA-mediated activation of primary human T cells occurs through nonspecific cross-linking of cell surface receptors, primarily the TCR and costimulatory molecules. To determine whether other methods would generate the up-regulation of Phb1 and Phb2 protein levels, several approaches were performed including anti-CD3 to specifically drive TCR signaling pathways, lectin ConA to agglutinate cells, or PMA and ionomycin. After 72 h of treatment with the various activating agents, Western blot analysis was performed to detect Phb1, Phb2, and GAPDH protein levels (Fig. 3A). Phb1 and Phb2 protein levels were again up-regulated 2–2.5-fold with all activation agents, however, PMA and ionomycin resulted in the greatest increase (3–4-fold) relative to the GAPDH levels (Fig. 3B). Purity and activation of PBMCs before and after treatment was determined by flow cytometry utilizing anti-CD3-FITC or anti-CD25-FITC-conjugated antibodies, respectively. PBMCs were typically 70% CD3+ T cells, whereas PHA, anti-CD3, or PMA/ionomycin treatment resulted in a homogenous population of CD25 expressing (70%) activated human T cells. Conversely, ConA treatment yielded a mixed population of CD25 expressing cells (62%) (Fig. 3C). The different mitogenic properties of PHA and ConA have been previously described in detail, which may explain the observed variation in these activation profiles (38).

TCR and Costimulatory Signaling Pathways Result in the Up-regulation of Phb1 and Phb2 mRNA Levels during T Cell Activation—To determine Phb1 and Phb2 mRNA levels during T cell activation, Q-RT-PCR analysis of RNA extracted from untreated or anti-CD3- and/or anti-CD28-stimulated primary human T cells was performed. Phb1 mRNA levels were

FIGURE 1. Proteomic identification of Phb2 as a differentially expressed protein during T cell activation. Two-dimensional gel electrophoresis silver-stained images of naive (A) or PHA activated (B) primary human CD3+ T cell extracts separated over pI range 3–10 and then 12% SDS-PAGE. Landmark protein spots (circles) and protein spot of interest (square) are indicated. C, MALDI-TOF mass spectrum obtained from p37 analysis and resulting scored protein hits from the NCBI data base search. See “Experimental Procedures” for details.

FIGURE 2. Up-regulation of Phb1 and Phb2 protein levels during T cell activation. A, PBMCs were activated with PHA (10 μg/ml) and harvested at the time points indicated. Phb1, Phb2, Actin, and Jak3 protein levels were determined by Western blot (WB) analysis. Phb1 and Phb2 band intensities were normalized to Actin using densitometric analysis and -fold induction plotted for each time point. Representative data from three independent experiments are shown.
detected at 48 h, and declined at 96 h of activation with anti-CD3 and anti-CD3/anti-CD28 (Fig. 4A). There was no significant increase in Phb1 mRNA levels with anti-CD28 treatment alone, however, anti-CD3 and anti-CD28 synergistically led to up-regulated Phb1 mRNA. Similarly, Phb2 mRNA levels increased after 48 h and declined after 96 h of activation with anti-CD3 and anti-CD3/anti-CD28 (Fig. 4B). Only after 96 h of anti-CD28 treatment did Phb2 mRNA levels show an increase. Similar to Phb1, anti-CD3 and anti-CD28 synergistically up-regulated Phb2 mRNA levels after 48 h. CD25 (IL2Rα) up-regulation was used as a positive control for anti-CD3 and anti-CD28 signaling. CD25 mRNA levels increased as early as 12 h, and declined at 96 h post-activation with anti-CD3 or anti-CD3/anti-CD28 (Fig. 4C). As a positive control, CD25 was found to be up-regulated following CD3 and CD28 activation after 24 h of each treatment.

**FIGURE 3.** Phb1 and Phb2 protein levels are induced by different T cell activating protocols. A, PBMCs were left untreated (lane a) or treated with anti-CD3 (lane b), PHA (lane c), ConA (lane d), or PMA/ionomycin (lane e) for 72 h and protein levels detected by Western blot (WB) using the antibodies indicated. B, Phb1 and Phb2 band intensities were normalized to GAPDH using densitometric analysis and the fold induction plotted for each activation agent. C, PBMCs were analyzed by flow cytometry for the T cell marker CD3 (left panel) and activation marker CD25 (right panel). Percentages of positive cells are shown in the appropriate quadrants. Representative data from two independent experiments are shown.
primary human T cells, lysates were immunoprecipitated with either Phb1 or Phb2 antibodies. Phb1 or Phb2 immunoprecipitated complexes from PHA-activated primary human T cells were subjected to Western blot analysis for their association. Indeed, the opposing Phb co-precipitated with either Phb antibody indicating that Phb1 and Phb2 form a complex in activated human T cells (Fig. 5A).

Our initial two-dimensional gel electrophoresis experiments (Fig. 1) suggested Phb2 was post-translationally modified in primary human T cells as evident by an acidic and basic form during isoelectric focusing. To determine the global phosphorylation state of Phb proteins, phosphoamino acid analysis of $^{32}$P-labeled immunopurified Phb1 and Phb2 from naïve and PHA-activated primary human T cells and the T cell leukemia cell line, Kit225, was performed. Phb2 was immunoprecipitated from cells radiolabeled overnight with 1 mCi of $[^{32}P]$orthophosphate and subjected to separation by 10% SDS-PAGE and transfer to polyvinylidene difluoride membrane. Phb1 and Phb2 were visualized by Coomassie Blue stain (Fig. 5B) before the membrane was subjected to autoradiography. To obtain a sufficient amount of Phb proteins from naïve human T cells, a 2-fold greater number of cells were assayed compared with Kit225 and PHA-activated T cells. Autoradiography showed both Phb1 and Phb2 were phosphorylated in Kit225 cells and PHA-activated primary human T cells (Fig. 5C, lanes a and c), however, radiolabeled protein was not present in naïve human T cells (Fig. 5C, lane b), which could be due to the quiescent nature of these cells. Phb1 and Phb2 bands were excised and subjected to phosphoamino acid analysis. Under these conditions, Phb1 was determined to be phosphorylated on serine residue(s), whereas Phb2 was phosphorylated on serine and tyrosine residues in primary human T cells (Fig. 5D). Similar results were obtained in the Kit225 cells (data not shown). This finding provides the first evidence that Phb function can be regulated by tyrosine kinase signaling pathways.

Mass Spectrometry Analysis and Phosphospecific Antibodies Identify Tyr248 as a Novel Phosphosite in Phb2—To identify the specific phosphorylation sites in Phb2, phosphopeptide mapping with MALDI-TOF mass spectrometry was performed. Briefly, Phb2 was immunoprecipitated from activated human T cells, separated by SDS-PAGE, and subjected to Coomassie Blue stain (Fig. 6A, lane a). A duplicate sample was transferred to polyvinylidene difluoride membrane and Western blotted with anti-Tyr(P) to confirm tyrosine phosphorylation (Fig. 6A, lane b). The Phb2 corresponding band was excised, trypsin digested, and subjected to analysis by MALDI-TOF mass spectrometry. Two novel Phb2 phosphosites were identified from five phosphopeptides (Fig. 6B): 1) MLGEALSK, containing Ser243 (underlined); and 2) NPGYIKLR, containing Tyr 248 (underlined). Additionally, six other phosphorylated residues were identified, however, the specific phosphoacceptor site could not be confirmed by mass spectroscopy/mass spectrometry (Fig. 6E). A high level of protein coverage (81%) was achieved during the mapping by MALDI-TOF mass spectrometry (Fig. 6E). Interestingly, although Phb1 and Phb2 share 48% identity and 67% similarity at the amino acid level, Tyr248 and Ser243 are not conserved in Phb1 (Fig. 6E).

To confirm Tyr248 as a novel Phb2 phosphorylation site, phosphospecific antibodies against the Phb2 phosphopeptide CKNPgpYIKLR were generated. The resulting antiserum was purified by negative selection using the non-phosphopeptide, and peptide competition experiments were performed to determine the specificity of this antiserum. Phb2 was immunopre-
Phb1 and Phb2 form a phosphocomplex in primary human T cells and the T cell leukemia cell line, Kit225. A, lysates from PHA (10 μg/ml) activated human T cells were immunoprecipitated for either Phb1 or Phb2, separated by 10% SDS-PAGE and subsequently analyzed by Western blot (WB) by the antibodies indicated. B, Kit225 cells (lane a), naïve (lane b) or PHA (10 μg/ml) activated primary human T cells (lane c) were $^{32}\text{P}$-radiolabeled overnight under normal culturing conditions. Phb2 was immunoprecipitated, separated by SDS-PAGE, and subjected to Coomassie Blue staining. C, autoradiography of the membrane after 8 days exposure is presented. D, phosphoamino acid analysis was performed on both Phb1 and Phb2 from PHA-activated human T cells (lanes c in panels A and B). Phospho standards were detected by ninhydrin (left panel), and migration of Phb phosphoamino acids by autoradiography (right panel) is shown. Arrows denote locations of Phb1 and Phb2. Brackets denote the locations of the immunoglobulin G heavy chains (IG HC) and light chains (IG LC). IP denotes immunoprecipitation.

cipitated from PHA-activated human T cell lysates, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with the affinity purified anti-Phb2 Tyr(P)248 (1:5000) in the presence of either 500 μM phosphopeptide or non-phosphopeptide, followed by re-probing the membrane with anti-Phb2. The anti-Phb2 Tyr(P)248 phosphoantibody was specifically blocked by the phosphopeptide and not the non-phosphopeptide, thus confirming antibody specificity (Fig. 6C). Additionally, phosphopeptide inhibition of anti-Phb2 Tyr(P)248 was dose dependent relative to the non-phosphopeptide (Fig. 6D).

Phb2 Tyr(P)248 Phosphorylation Is Not Required for Phb Complex Formation But Is Present in Several Human Tumor Cell Lines—Tyrosine phosphorylation can affect protein activity, localization, and protein-protein interactions. To determine whether phosphorylation of Phb2 Tyr(P)248 is required for complex formation with Phb1, plasmids were constructed encoding V5 tagged WT Phb2 or Y248F Phb2. The plasmids were transfected into Kit225 cells and recombinant proteins immunoprecipitated using a monoclonal anti-V5 antibody followed by SDS-PAGE separation and Western blot analysis. Phenylala-

T Cell Mitochondrial Integrity and Function Requires Phb1/2

cnine substitution of Phb2 Tyr248 did not affect its ability to form a complex with endogenous Phb1, as indicated by the presence of Phb1 in both Y248F (lane b) and WT V5 (lane c) immunocapture assays (Fig. 7A). Total cell lysate Western blotted with anti-V5 and anti-Phb1 confirmed equal protein input amounts. Western blot analysis with anti-Tyr(P) indicated Tyr248 is the major tyrosine phosphorylation site of Phb2 in Kit225 cells. The phosphospecific Phb2 Tyr(P)248 Western blot shows the antiserum primarily recognizes the WT Phb2, however, it does cross-react with the Y248F mutant Phb2. To determine the extent of cross-reaction, we performed densitometry analysis on the Phb2 Tyr(P)248 band intensities from the Y248F and WT recombinant Phb2 protein Western blots normalized to the V5 band intensities (Fig. 7B). The Phb2 Tyr(P)248 antiserum has a 2.67-fold increase in affinity for the WT Phb2 relative to the Y248F Phb2.

To determine whether Phb2 is tyrosine phosphorylated in other human tumor cell lines, Phb2 was assessed in an acute lymphoblastic leukemia cell line (Jurkat), human T cell leukemia virus 1 transformed cell line (MT2), NK-like acute lymphoblastic lymphoma cell line (YT), and a breast cancer cell line derived from a ductal carcinoma (T47D). Western blot analysis (Fig. 7C) with anti-Tyr(P) and anti-Phb2 Tyr(P)248 revealed that Phb2 is indeed tyrosine phosphorylated in these tumor cell lines, specifically at residue 248. Additionally, Phb1 was present in each of the Phb2 immunoprecipitation reactions, also indicating a heterocomplex formation in these tumor cell lines.

In Primary Human T Cells, Phb1 and Phb2 Co-localize to the Mitochondrial Inner Membrane—Prohibitins have been found to localize to many regions of the cell, including the plasma membrane, mitochondria, and nucleus (17, 20, 40). Identification of the cellular localization of the Phb complex is a critical step in understanding its function in human T cells. To assess their subcellular localization, immunofluorescence confocal microscopy, subcellular fractionation, and immunoelectron microscopy was performed. Phb1 and Phb2 were determined to primarily co-localize to polarized perinuclear regions in PHA-activated human T cells (Fig. 8A). There were no detectable levels of Phb1 or Phb2 at the plasma membrane and only limited amounts nuclear localized.

To confirm and further define Phb localization, subcellular fractionation of PHA-activated primary human T cells was per-
formed using differential centrifugation. Western blot analysis of nuclear and cytoplasmic fractions detected Phb1 (lane b) and Phb2 (lane d) to be present only in the cytoplasmic fraction (Fig. 8B). The cytoplasmic tyrosine kinase Jak3 and nuclear DNA repair protein PARP were used for fractionation controls. The mitochondrial fraction was separated from the cytoplasmic fraction using high speed centrifugation. Western blot analysis of these fractions detected Phb1 (lane b) and Phb2 (lane d) only in the mitochondria (Fig. 8C). The mitochondrial localized OxPhos CII protein and cytoplasmic and mitochondrial localized GAPDH were used as fractionation controls for these studies.

Transmission electron microscopy was utilized to provide the ultrastructural resolution required to determine the location of Phb1 and Phb2 within the mitochondria. Immunogold labeling of Phb1 and Phb2 was performed using monoclonal anti-Phb1 and affinity purified polyclonal anti-Phb2 in combination with gold particle-conjugated secondary antibodies. CD3+ human T cells were either left untreated or PHA activated for 72 h. Representative whole cell electron micrographs were taken at ×5,000 magnification (Fig. 9, upper panel). To resolve the gold particles, ×3,150 magnification electron micrograph images where taken of cell sections enriched in mitochondria. Phb1 (6-nm gold) and Phb2 (12-nm gold) localize to the inner mitochondrial membrane in PHA-activated primary human T cells (Fig. 9, lower panel). Furthermore, the gold particles are in groups of two or three supporting the concept of a multimeric Phb ring complex (42, 43). Interestingly, although Phb1 and Phb2 have been shown to form a complex in a number of cell types, including this work, immunogold labeling did not show Phb1 to be in complex with Phb2. The reason for this in not clear, however, it may be due to steric hindrance between the Phb1 and Phb2 antibodies, which is exacerbated by the proposed Phb ring structure.

siRNA-mediated Knockdown of Individual Phbs Results in Degradation of the Homologous Phb Protein in Kit225 Cells—To gain insight into the Phb mechanism of action in human T cells, siRNA mediated knockdown of Phb1 and Phb2 in Kit225 cells was performed. Phb1, Phb2, or non-targeting control siRNA were delivered into Kit225 cells via electroporation and protein knockdown was determined by Western blot analysis of total cell lysates after 48 h (Fig. 10A). Interestingly, when Kit225 cells were electroporated with Phb1 (lane b) or Phb2 (lane c) siRNA, a decrease in both protein levels compared with the non-targeting control siRNA (lane a) was detected. To determine specificity of the siRNA, Q-RT-PCR analysis was performed on RNA isolated from Kit225 cells treated with control, Phb1, or Phb2 siRNA (Fig. 10B). Phb1- and Phb2-specific siRNA and control siRNA were delivered into Kit225 cells via electroporation and RNA was isolated after 24 h incubation. Phb1 siRNA significantly (p < 0.05) reduced Phb1 mRNA levels 52%, whereas Phb2 mRNA levels remained unchanged. Additionally, Phb2 siRNA significantly (p < 0.01) reduced Phb2 mRNA levels 81%, whereas Phb1 mRNA levels slightly increased, indicating the Phb siRNA are specific. These findings demonstrate the independent relationship between Phb1 and Phb2 in T cells.

Loss of the Prohibitin Complex in Kit225 Cells Results in Disruption of Mitochondrial Membrane Potential—Subcellular fractionation in combination with immunofluorescent and electron microscopy have established the localization of Phb1 and Phb2 to the mitochondria in human T cells (Figs. 8 and 9). siRNA-mediated knockdown of Phb1 and Phb2 in Kit225 cells results in cell death. Additionally, previous reports indicated that the mitochondrial Phb complex functions as a molecular chaperone to stabilize newly imported proteins, including subunits of mitochondrial respiratory enzymes (23, 25, 44). To determine the effect of Phb1 and Phb2 knockdown on mitochondrial membrane potential in human T cells, Kit225 cells were treated with Phb1 and Phb2 siRNA or non-targeting control siRNA for 36 h and the fluorescence of the mitochondrial potential detector dye DePsipher (R&D Systems) was detected. The potassium selective ionophore valinomycin, which uncouples oxidative phosphorylation, was used as a positive control for depolarization. Knockdown of Phb1 and Phb2 resulted in an ~50% decrease in DePsipher aggregation as detected by flow cytometry (Fig. 10C, panel D). Treatment of Kit225 cells with valinomycin for 6 h resulted in complete mitochondrial depolarization (Fig. 10C, panel E), whereas electroporation alone or with non-targeting siRNA did not affect the mitochondrial membrane potential (Fig. 10C, panels B and C). Non-DePsipher treated Kit225 cells were used as a negative control for fluorescence detection (Fig. 10C, panel A).

Phb1 and Phb2 Are Up-regulated during IL-2 Deprivation-mediated Apoptosis in Kit225 Cells—Cells respond to a variety of insults, including growth factor withdrawal, by up-regulating stress response proteins that provide protection based primarily upon their chaperoning ability (45, 46). Kit225 cells are dependent on the T cell growth factor IL-2. To determine whether Phb1 and Phb2 expression is induced upon growth factor deprivation-mediated apoptosis, Western blot analysis of lysates from IL-2-deprived Kit225 cells was assessed over 5 days with collection time points every 24 h (Fig. 11A). Reprobing the membrane for GAPDH levels confirmed equal loading, whereas caspase activation was detected by Western blot via detection of PARP degradation. Densitometric analysis indicated Phb1 protein levels increased 2.0-fold after 72 and 96 h post-IL-2 withdrawal. Similarly, Phb2 protein levels increased 2.0-fold at 48 h and 2.5-fold at 96 h after IL-2 withdrawal (Fig. 11B). Apoptosis of Kit225 cells was monitored by Annexin V/PI staining at 24, 48, 72, 96, and 120 h (Fig. 11C). Kit225 cells showed minimal Annexin V staining (12.2%) after 24 h IL-2 withdrawal, however, significant staining was observed after 48 (33.9%), 72 (37.4%), 96 (34.5%), and 120 h (42.9%).

DISCUSSION

In an effort to gain insight into the complex molecular mechanisms of T cell activation, a functional proteomics approach was used that identified the Phb family of proteins to be differentially expressed. Further characterization revealed that engagement specifically through the TCR complex and CD28 costimulatory molecule led to an increase of Phb1 and Phb2

3 J. A. Ross, Z. S. Nagy, and R. A. Kirken, unpublished observations.
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**A**

![Image A](image1)

**B**

| Start–End | Peak Observed | Peak Expected | # of Phosphates | Sequence  |
|-----------|---------------|---------------|-----------------|-----------|
| 38–48     | 1232.76       | 1231.76       | 1               | ESYTVEHGHR |
| 90–97     | 1016.52       | 1015.51       | 3               | ISSPTGSK   |
| 237–244   | 927.64        | 926.63        | 1               | MLGEALSK   |
| 245–252   | 1040.55       | 1039.54       | 1               | NPGYKLVR   |
| 256–270   | 1761.95       | 1760.96       | 2               | AAQNISKTTSONR |

**C**

![Image C](image2)

**D**

![Graph D](image3)

**E**

Phb1

MAAKVFE5IGK--GLAVAVGVVNS-ALYNA Diploma GAV FeRFGVR-DIVVGE8TFHFLIPWSQKII 66

Phb2

MAQHLDLAGE1LPAGPR363STALKLLGAGAVAYGVK585SETVE8GSHRAFFNIRGIVQ3TIBEA1B9HPFQPIII 80

Phb1

EDCRSFRNPVIVGSDC1LQMVNTLRLFIPVAGSQLPFRITSIGSDEYERLPSITTELKSVVARFDGELTQRELV 146

Phb2

YDIREPARK1SSPTGSDLQMV1ISLRVLRSPQAEAMSITMYLGLDYEERLPSITNEVLSVVA 160

Phb1

SRQWSDD|TERRAATFGLIDDVS|THLTFKEFTEAVAEK|VAQ|EAEARF|VEK|E|QQ|KAAAI|SUB|| 226

Phb2

SLLIR|REL|TP|EKDSL|IL|L|DAV|TELSFSREYTAHAVEK|VAAQEEAQAQF|F|VE|KE|QQ|Q|TVQ|A|EOE|E|A|AA|AM 240

Phb1

SL|ATAGDL|L|E|LKLEA|E|D|AYQLS|S|R|NY|LPAGQ|SV|LL|Q|F| 272

Phb2

ALSKN--PGYIKIKTKRAOQ|ISKTIATSONR|Y|LT|ADDNLVNLQ|D|F|RG|DSL|IK 299

488 Identical

678 Similar
Phb1 and Phb2 sequence alignment showing conserved (*) and similar (: or .) residues, Phb2 peptide coverage during mass spectrometry analysis (B).

**FIGURE 6.** Phb2 is phosphorylated on tyrosine 248. A, Phb2 was immunoaffinity purified from PHA (10 μg/ml) activated primary human T cells, analyzed by Coomassie Blue staining (lane a) and anti-Tyr(P) Western blot (lane b), and subjected to phosphopeptide mapping. Arrows denote location of IgG heavy chain (HC), Phb1, and Phb2. B, MALDI-TOF mass spectrometry identified five putative Phb2 phosphorylation sites. The primary amino acid sequences returned are underlined, and the antibody confirmed phosphosites (gray). Phosphopeptides identified (underlined), and the antibody confirmed phosphotyrosine 248 (+). WB, Western blot.

**FIGURE 7.** Tyr(P)\textsuperscript{248} is not required for Phb complex formation, but is constitutively phosphorylated in several human tumor cell lines. A, Kit225 cells were transfected alone (lane a), with Phb2 Y248F-V5 (lane b), or Phb2 WT-V5 (lane c) plasmids and the resulting proteins immunoprecipitated using the anti-V5 antibody and separated by 10% SDS-PAGE. Western blot (WB) analysis was performed using the indicated antibodies. For input protein detection, total cell lysate (10 μg) was separated by 10% SDS-PAGE and Western blotted for the indicated proteins. B, densitometric analysis of the Phb2 Tyr(P)\textsuperscript{248} band intensity normalized to the V5 band from both the Phb2 Y248F mutant and Phb2 WT proteins. C, equal amounts of protein from Kit225 (lane a), Jurkat (lane b), MT2 (lane c), YT (lane d), and T47D (lane e) cells were immunoprecipitated for Phb2 and separated by 10% SDS-PAGE. Western blot (WB) analysis was performed with the antibodies indicated. Arrows denote location of IgG heavy chain (HC), IgG LC, Phb1, and Phb2. IP denotes immunoprecipitation.

T cell activation is the product of a highly coordinated network of signal transduction pathways induced by the TCR complex and costimulatory molecules, which result in the regulation of proteins required for costimulation, migration, differentiation, proliferation, and apoptosis. To date, the majority of proteins found differentially expressed upon T cell activation are cell surface molecules. The effector molecules involved in this response are not well established, therefore it is critical to expand studies to identify these proteins. Indeed, we detected the differential expression of Phb2 after 72 h PHA activation of CD3\textsuperscript{+} primary human T cells using two-dimensional gel electrophoresis (Fig. 1). Phb2, together with Phb1, belong to a superfamily of proteins that share a structurally related domain referred to as the SPFH (stomatin, prohibitin, flotillin, hflK) domain, also known as the Phb domain (23). The up-regulation of both Phb1 and Phb2 protein levels during T cell activation was confirmed by Western blot analysis of PBMCs activated with immobilized anti-CD3, PHA, ConA, or PMA and ionomycin for 72 h (Fig. 3). The increase in Phb1 and Phb2 protein levels was detected as early as 48 h and continued through 96 h (Fig. 2), which closely paralleled the induction of Jak3 (48 to 60 h). The differential expression of Jak3 during T cell activation is primarily due to the TCR-mediated activation of the transcription factors ETS-1 and AP1 (47). Indeed, activation through TCR and CD28 signal transduction pathways results in an increase of Phb1 and Phb2 mRNA levels after 48 h, indicating that the control of Phb protein levels in this cell is at least partially at the level of transcription (Fig. 4). Transcriptional control of Phb expression by ETS-1 and AP-1 remains to be investigated, however, the regulation of Phb levels in response to various stimuli has been reported in a number of cell types. For example, IL-6 signaling through STAT3 was shown to modulate Phb1 expression in intestinal epithelial cells where it was shown to protect against oxidative stress (48, 49). Additionally, Phb1 expression was induced upon phorbol ester treat...
ment of chronic lymphocytic leukemia-derived B lymphocytes, suggesting Phb1 may facilitate proliferation or maturation of B cells (50). In support of this notion, an increase in the oncoprotein Myc, which is commonly activated in proliferating cells, induced the expression of Phb1 and Phb2 (40). However, the androgen, dihydrotestosterone, was shown to down-regulate Phb1 expression in the prostate cancer cell line LNCaP suggesting an anti-proliferative function in this cell type (19). Phb1 has also been shown to be preferentially expressed in non-proliferating thymocytes and is induced in thymi during pregnancy (29). The different expression patterns of Phb1 and Phb2 may be due to the apparent pleiotropic functions of this family of proteins.

Phb1 was originally cloned from cDNAs derived from transcripts that were more abundantly expressed in non-dividing rat liver cells, thus suggesting a negative regulatory function on cell cycle progression (51). When microinjected into normal human fibroblasts, Phb1 mRNA attenuated DNA synthesis, however, this effect was subsequently shown to be mediated by the 3'-untranslated region rather than the coding region of the cDNA (52, 53). The mechanism of cell cycle regulation was determined by Wang et al. (54, 55) who showed Phb1 could bind Rb as well as E2F1 to repress their transcriptional activity (56). Phb2 was originally identified as a 37-kDa protein associated with the IgM receptor in B cells, and therefore initially named B cell receptor-associated protein 37 (Bap37) (16). Phb2 was found to repress the transcriptional activity of the estrogen receptor in breast cancer cell lines by competing for coactivator binding sites on estrogen receptor in the nucleus (21) (57). Due to this inhibitory action, the protein was named repressor of estrogen receptor activity, however, it has recently been shown to interact with histone deacetylases HDAC1 and HDAC5 to mediate repression of COUP-TFs, suggesting a more general nuclear receptor corepressor function (58). Interestingly, nuclear Phb2 was recently shown to protect sister chromatid cohesion during mitosis in the cervical carcinoma cell line, HeLa (59).

Phosphorylation is a primary protein regulatory mechanism for controlling activity, stability, localization, and cofactor interactions. It is estimated that 30% of all cellular proteins contain covalently bound phosphate at a ratio of 1800:200:1 for...
Ser(P), Thr(P), and Tyr(P), respectively (60). Evidence has emerged that suggests prohibitins can be regulated by phosphorylation. Indeed, several studies have noted the presence of multiple isoforms for Phb1, which was proposed to be phosphorylated derivatives (61). Recently, a global phosphoproteomic mass spectrometry study on epidermal growth factor stimulation of HeLa cells identified Ser252 and Ser254 as acceptor sites in Phb1, however, phosphorylation of these sites in vitro or in vivo has not been validated (62). We provide direct evidence of Phb phosphorylation using the incorporation of \[^{32}P\]orthophosphate and subsequent phosphoamino acid analysis to determine Phb1 serine and Phb2 serine and tyrosine residues are phosphorylated (Fig. 5). Interestingly, multiple labeling attempts (4 h) did not result in significant incorporation, however, prolonged incubation (18 h) with radiolabel was successful, suggesting slow phosphorylation kinetics. Indeed, overnight labeling of naïve human T cells did not result in phosphate incorporation into either Phb1 or Phb2. Tyrosine phosphorylation of Phb2 was confirmed by Western blot using an anti-phosphotyrosine antibody. Furthermore, phosphopeptide mapping by mass spectrometry suggested Tyr248 was an acceptor site in Phb2. Phosphoproteins antibodies made to this region, and site-directed mutagenesis confirmed this notion (Fig. 6). Interestingly, Tyr248 resides within a known phosphotyrosine binding domain called a NPXYY motif and is presumably the reason this residue is not required for complex formation with Phb1, which does not contain a known phosphotyrosine binding domain. Phb2 Tyr248 is evolutionarily conserved in human, mouse, rat, Xenopus, zebrafish, and Drosophila, however, C. elegans and Schizosaccharomyces pombe contain a phenylalanine at this position, suggesting a gain of function at this point of divergence. Sequence comparison of Phb1 and Phb2 reveal that although there is 45% identity and 74% similarity at the amino acid level, the NPXYY motif is not conserved in Phb1. Tyr248 is present in a putative coiled coil domain (amino acids 190–264), which is C-terminal to the conserved Phb domain (amino acids 39–201) (42). Collectively, these findings support the hypothesis that phosphorylation can regulate Phb function possibly through the binding of novel cofactors, however, its exact role in T cell function remains to be determined.

Prohibitin subcellular localization appears to be cell type dependent. Phb1 and Phb2 have been reported at the plasma membrane in human B cells (16), intestinal epithelial cells (16, 63), and vascular endothelial cells (64). Nuclear Phb1 and Phb2 have been described in human breast cancer (18) and prostate cancer (19) cell lines. Mitochondrial prohibitins have been described in detail in yeast (43, 44, 65), C. elegans (66), and human fibroblasts (40). Utilizing immunofluorescent confocal microscopy, Phb1 and Phb2 were shown to primarily co-localize to perinuclear regions in PHA-activated primary human T
Phb in Kit225 cells resulted in disruption of mitochondrial membrane potential (Fig. 10). This is in accordance with recent findings of Kasashima et al. (68) who reported loss of mitochondrial integrity upon knockdown of Phb2 in HeLa cells. Furthermore, Phb1 and Phb2 expression was induced upon IL-2 deprivation-mediated cell death indicating these proteins play an anti-apoptotic or survival function in Kit225 cells (Figs. 11). Our findings support the hypothesis that Phb1 and Phb2 function as molecular chaperones in human T cells to protect mitochondrial integrity during cellular stress that may occur during events of T cell activation or cell death.

In conclusion, using a proteomics based approach, we have identified the Phb family of proteins, Phb1 and Phb2, to be up-regulated during T cell activation. Evidence is provided that phosphorylation is a potential regulator of the Phb mechanism of action. Specifically, tyrosine phosphorylation of Phb2 Tyr248, which lies within a conserved NPXY motif, occurs in primary and tumor cell lines where it may be important in protein-protein interactions, however, is not required for Phb1/Phb2 association. Mitochondria play a critical role in providing ATP derived from the electron transport chain and oxidative phosphorylation. Phb1 and Phb2 were determined to localize to the mitochondrial inner membrane of human T cells and function to maintain mitochondrial integrity, indicating this complex facilitates T cell survival through stabilization of mitochondrial electron transport enzymes during the increased metabolic demand required for T cell proliferation. Their additional roles for cell signaling events also cannot be ruled out. In addition to serving as intracellular biomarkers for T cell activation, Phb1 and Phb2 may hold therapeutic value to regulate T cell-mediated diseases by manipulating mitochondrial integrity and function.

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