Hck Enhances the Adherence of Lipopolysaccharide-stimulated Macrophages via Cbl and Phosphatidylinositol 3-Kinase*

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Src family tyrosine kinases have previously been proposed to mediate some of the biological effects of lipopolysaccharide on macrophages. Accordingly, we have sought to identify substrates of Src family kinases in lipopolysaccharide-stimulated macrophages. Stimulation of Bac1.2F5 macrophage cells with lipopolysaccharide was found to induce gradual and persistent tyrosine phosphorylation of Cbl in an Src family kinase-dependent manner. Immunoprecipitation experiments revealed that Cbl associates with Hck in Bac1.2F5 cells, while expression of an activated form of Hck in Bac1.2F5 cells induces tyrosine phosphorylation of Cbl in the absence of lipopolysaccharide stimulation. The Src homolog 3 domain of Hck can directly bind Cbl, and this interaction is important for phosphorylation of Cbl. Association of the p85 subunit of phosphatidylinositol (PI) 3-kinase with Cbl is enhanced following lipopolysaccharide stimulation of Bac1.2F5 cells, and transient expression experiments indicate that phosphorylation of Cbl by Hck can facilitate the association of p85 with Cbl. Lipopolysaccharide treatment also stimulates the partial translocation of Hck to the cytoskeleton of Bac1.2F5 cells. Notably, lipopolysaccharide enhances the adherence of Bac1.2F5 cells, an effect that is dependent on the activity of Src family kinases and PI 3-kinase. Thus, we postulate that Hck enhances the adherence of lipopolysaccharide-stimulated macrophages, at least in part, via Cbl and PI 3-kinase.

Macrophages play a critical role in the host response to inflammation and bacterial infection (1). During these processes, macrophages are primarily involved in the phagocytosis of bacteria and host cell debris, antigen processing and presentation, and the secretion of reactive nitrogen intermediates and inflammatory cytokines (e.g. tumor necrosis factor α, interleukin-1, and interleukin-6) (1). In vitro exposure of macrophages to lipopolysaccharide (LPS),1 a major component of the outer wall of Gram-negative bacteria, mimics many of the effects bacteria have on macrophages in vivo, namely inducing the secretion of reactive nitrites and inflammatory cytokines and enhancing their tumoricidal activity (1).

The activation of macrophages by LPS is mediated by the binding of LPS to CD14, a glycosylphosphatidylinositol-anchored protein found on the surface of monocytes/macrophages and neutrophils (2). Binding of LPS to CD14 is enhanced by LBP, an LPS-binding protein found in serum (3). Since CD14 lacks an intracellular domain, it has previously been unclear how CD14 transduces signals across the plasma membrane in response to the binding of LPS. Recent studies suggest that members of the Toll-like receptor family, and in particular Toll-like receptor 4, may serve as cell surface co-receptors with CD14 to mediate transmembrane signal transduction (4–11). Members of the Toll-like receptor family are structurally characterized by an extracellular domain containing leucine-rich repeats, a transmembrane domain, and an intracellular domain with sequence homology to the intracelluar domain of the interleukin-1 receptor (12).

LPS stimulation of macrophages leads to the activation of a variety of proteins involved in signal transduction, including protein kinase C (13, 14), Raf (15), mitogen-activated protein kinase (15, 16), p38 stress-activated protein kinase (17), Jun kinase (18), and ceramide-activated protein kinase (19). However, it is still unclear how the activation of these various signal-transducing proteins mediates the variety of biological responses of macrophages to LPS. Significantly, both in vitro and in vivo studies with tyrosine kinase inhibitors have revealed that the activation of tyrosine kinases is necessary for a number of the biological responses of macrophages to LPS (e.g. tumoricidal activation) (20–22).

The Src family tyrosine kinases Hck, Lyn, and Fgr have all been implicated in playing a role in the biological response of macrophages to LPS. Stimulation of monocytes/macrophages with LPS induces a rapid increase in the specific kinase activity of Hck, Lyn, and Fgr and physical association of Lyn with CD14 (23, 24). Moreover, enforced expression of an activated form of Hck in Bac1.2F5 macrophage cells augments tumor necrosis factor α production in response to LPS stimulation (24). Chronic exposure of bone marrow-derived macrophages to LPS induces an increase in the expression of both Hck and Lyn (25). Analysis of the promoter region of the hck gene has facilitated the identification of an element that confers LPS responsiveness (26). Although these observations suggest that Src family kinases play a role in the response of macrophages to LPS, and hence in the response of macrophages to bacterial infection, the critical substrates of Src family kinases that mediate the various biological responses of macrophage to LPS have yet to be identified.

In the present study we have sought to identify proteins that are phosphorylated by Src family kinases following LPS stimulation of the Bac1.2F5 macrophage cell line. We show that Cbl is a substrate of Hck in LPS-stimulated Bac1.2F5 cells. Further, we show that the phosphorylation of Cbl facilitates the physical association of the p85 subunit of PI 3-kinase with Cbl. Notably, LPS stimulation enhances the adherence of Bac1.2F5...
cells, an effect that is dependent on the activity of Src family kinases and PI 3-kinase. On the basis of these findings, we postulate that Hck, at least in part, enhances the adherence of LPS-stimulated macrophages via Cbl and PI 3-kinase.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cell culture medium and G418 were from Trace Biosciences Ltd. (Melbourne, Australia) and Life Technologies, Inc., respectively. Fetal calf serum (low endotoxin) was from P. A. Biologicals (Hawera, New Zealand). A rabbit anti-Hck polyclonal antibody was a generous gift from Dr. Clifford Lowell, while a rat anti-murine Hck monoclonal antibody was developed in this laboratory. The anti-phosphothreonine monoclonal antibody (4G10) and anti-phosphorylcalton antibody were from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal rabbit anti-Cbl antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Dr. Wallace Langdon. Protein A-Sepharose and glutathione-Sepharose were from Amersham Pharmacia Biotech. [32P]ATP (300 Ci/mmol) was obtained from Brescattc, Ltd. (Adelaide, Australia). Lipopolysaccharide (Escherichia coli 0111:B4) and mitomycin C were purchased from Sigma. ECL reagents were from Amersham Pharmacia Biotech. Pfu DNA polymerase was obtained from Stratagene. EZ-Link sulfo-N-hydroxysuccinimide-biotin was from Pierce. P22, P33, and Wortmannin were obtained from Calbiochem-Novabiochem. All other reagents were of the highest grade available.

**Plasmid Construction**—A cDNA encoding activated murine Hck (i.e., Hck499F) was excised from the plasmid pCDMS Hck499F (a gift from Dr. Margaret Hibbs) with XbaI and subcloned into the XbaI sites of pEF-BOS (27) to create pEF-Hck499F or “blunted” and subcloned into the PvuI site of the retroviral vector pRFu Neo (a generous gift from Dr. Tom Gonda (28)) to create pRFu Hck499F. The mammalian expression vectors pEF-Hck267M (in which lysine 267 is replaced with methionine) and pEF-Hck91A499F (in which tryptophan 91 and tyrosine 499 are replaced with alanine and phenylalanine, respectively) were constructed by polymerase chain reaction. The mammalian expression vectors pEF-Cbl (encoding wild type human Cbl) or pEF-Cbl731F (a generous gift from Dr. Wallace Langdon), respectively, and subcloning the fragment into the XhoI sites of pEF-BOS.

**Cell Culture, Retroviral Infection, and Transient Transfection—** Bac1.2F5 cells were grown in Hepes-buffered Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum and 25% L-cell conditioned medium (as a source of colony-stimulating factor) for 3 days. Cytoskeleton stabilization experiments were performed with cell lines grown in the above medium, but lacking L-cell conditioned medium, for 16 h prior to stimulation. pEF-Cbl cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The retroviral constructs pRFu Neo and pRFu Hck499F were introduced into pEF-Cbl cells by calcium phosphate-mediated transfection and subjected to selection with 500 μg/ml G418 for 2 weeks. For infection of Bac1.2F5 cells, 2 × 10⁶ retrovirus-producing cells were seeded in 10-cm tissue culture dishes. The following day, the cells were treated with 10 μg/ml mitomycin C for 4 h and then washed extensively with Hepes-buffered Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Bac1.2F5 cells (2.5 × 10⁶) were co-cultivated with the mitotically inactivated pEF-Cbl cells in the presence of 4 μg/ml polybrene for 2 days and then subjected to selection with 500 μg/ml G418 for 3 weeks. Individual clones were isolated by single cell cloning. Human 293T cells were grown in RPMI medium supplemented with 10% fetal calf serum and transfected with plasmid DNA using polyethylenimine (29).

**Cell Lysis, Western Blotting, and Immunoprecipitation—** Bac1.2F5 cells were seeded in 10-cm tissue culture dishes. They were then treated with either 20 μg/ml LPS for 30 min or 4 °C for 4 h with mixing. Beads were then washed four times with 1% Nonidet P-40 buffer and subjected to Western blotting by standard techniques. For Western blotting was performed by probing filters with biotinylated GST or GST-HckU32 (100 ng/ml) for 4 h at 4 °C. Filters were washed with TBST (Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and then stored at -70 °C in the presence of 15% glycerol. Binding assays were conducted by incubating 20 μl of glutathione-Sepharose beads containing 2 μg of recombinant Hck with 100 μg of cell lysate containing 1 μg of protein for 2 h at 4 °C with mixing. Beads were then washed four times with 1% Nonidet P-40 buffer and subjected to Western blotting by standard techniques. For Western blotting was performed by probing filters with biotinylated GST or GST-HckU32 (100 ng/ml) for 4 h at 4 °C. Filters were washed with TBST (Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and then incubated for 1 h at 4 °C with streptavidin- horseradish peroxidase in TBST. Following extensive washing, filters were developed with ECL reagents (Amersham Pharmacia Biotech).

**Adhesion Assays—** Bac1.2F5 cells were seeded in six-well tissue culture plates and grown until approximately 90% confluent. The cells were then treated with either 20 μg/ml PP3 (an inactive Src family kinase inhibitor), 20 μg/ml PP2 (a specific Src family kinase inhibitor), or 100 nM wortmannin (a PI 3-kinase inhibitor) for 60 min, followed by stimulation with 1 μg/ml LPS for 30 min. Differences in the adherence of the cells were then assessed by incubating the cells in 2 ml of PBS containing 10 mM EDTA (PBS/EDTA) for 30 min on a rotating platform. Following this treatment, the nonadherent cells (i.e. the “nonadherent” fraction) were collected and counted with the aid of a hemocytometer. Cells that remained attached to the dishes (i.e. the “adherent” fraction) were collected by vigorous pipetting in PBS/EDTA and counted.

**RESULTS**

**LPS Induces the Src Family Kinase-dependent Phosphorylation of Cbl in Bac1.2F5 Cells—** Bac1.2F5 cells, which were derived from SV40-transformed mature splenic macrophages (30), exhibit many of the immunological properties of mature primary macrophages, including the ability to secrete reactive nitrogen intermediates and tumor necrosis factor α when stimulated with LPS as well as the ability to kill bacterial and tumor cells (32). Thus, Bac1.2F5 cells represent a good model to investigate the role of Src family kinases in the regulation of inflammatory responses.
system to study LPS-induced signal transduction. Stimulation of Bac1.2F5 cells with LPS induced a time-dependent increase in tyrosine phosphorylation of a number of cellular proteins (p190, p150, p120, p110, and p66) (see Fig. 1A). Since tyrosine phosphorylation of Cbl has previously been reported to occur in response to a variety of stimuli (33–45), we were interested in determining if the p120 protein that became tyrosine-phosphorylated in response to LPS was Cbl. Accordingly, Cbl was immunoprecipitated from lysates of Bac1.2F5 cells that had been stimulated with LPS for various periods of time, and the immunoprecipitates were Western blotted with anti-phosphotyrosine antibody and anti-Cbl antibodies. Western blotting of the immunoprecipitates with an anti-phosphotyrosine antibody revealed that Cbl is indeed highly tyrosine-phosphorylated in Bac1.2F5 cells expressing an activated form of Hck (Fig. 2D). Although this finding indicates that Hck can induce tyrosine phosphorylation of Cbl in Bac1.2F5 cells, it does not allow us to conclude that Hck directly mediates its phosphorylation. Thus, an in vitro phosphorylation assay was performed to determine if Hck can directly phosphorylate Cbl. Specifically, Cbl was immunoprecipitated from transiently transfected 293T cells and then incubated in the absence or presence of ATP and purified recombinant Hck. The data shown in Fig. 2E clearly indicate that Hck can phosphorylate Cbl. In an attempt to demonstrate that Hck is directly involved in the LPS-induced tyrosine phosphorylation of Cbl in Bac1.2F5 cells, we generated clonal cell lines expressing a dominant-negative form of Hck (i.e., Hck499F) following infection of the parental Bac1.2F5 cells with the retroviral vector pRuf-Hck499F. However, LPS-induced tyrosine phosphorylation of Cbl was not significantly perturbed in the Bac-Hck499F cell lines tested.2 Notably though, Western blotting of lysates derived from various Bac-Hck499F cell lines with an anti-Hck antibody revealed that the kinase-inactive mutant of Hck was only expressed at levels that were approximately 2–3-fold above that of endogenous Hck.2 It seems unlikely that this level of overexpression of kinase-inactive Hck would be sufficient to exert a dominant-negative effect over endogenous Hck.

**The SH3 Domain of Hck Directly Mediates the Physical Association of Hck with Cbl**—We next sought to identify which domain of Hck was responsible for mediating the interaction of Hck with Cbl in Bac1.2F5 cells and, further, whether this is a direct physical interaction between the two proteins. To address the first of these two questions, the ability of Cbl in
lysates of unstimulated or LPS-stimulated Bac1.2F5 cells to associate with GST fusion proteins of Hck domains was examined. The results shown in Fig. 3A indicate that the isolated SH3 domain of Hck can mediate the association of Cbl with Hck. It was estimated that at least 20% of the total Cbl present in the cell lysates was capable of associating with GST fusion proteins containing the SH3 domain of Hck under the conditions employed.2 In agreement with our co-immunoprecipitation experiments, Cbl from both unstimulated and LPS-stimulated Bac1.2F5 cells associated equally well with GST fusion proteins containing the SH3 domain of Hck. No association of Cbl with the GST-Hck SH2 domain fusion protein was observed, although Cbl had become tyrosine-phosphorylated in response to LPS stimulation (Fig. 3A). Since this apparent lack of binding of tyrosine-phosphorylated Cbl to the SH2 domain of Hck may have simply been due to limited sensitivity of our assay, Cbl and Hck499F were transiently co-expressed in 293T cells. Cell lysates derived from 293T cells transfected with a plasmid encoding Cbl alone or co-transfected with plasmids encoding Cbl and Hck499F were then incubated with GST fusion proteins of Hck domains. As shown in Fig. 3B (upper panel), even under these conditions only a very small fraction of the total Cbl present in the cell lysates associated with the GST-Hck SH2 domain fusion protein. It was estimated that greater than 50% of the total Cbl present in the lysates associated with the GST-Hck SH3 domain fusion protein.2 The filter was reprobed with anti-phototyrosine antibodies in order to determine if tyrosine-phosphorylated Cbl binds the SH3 domain of Hck in preference to the SH2. The results shown in the lower panel of Fig. 3B revealed that approximately 2–3-fold more tyrosine-phosphorylated Cbl associated with the GST-Hck SH3 domain fusion protein when compared with that which associated with the GST-Hck SH2 domain fusion protein. To determine if the association of Cbl with the SH3 domain of Hck is direct, Cbl was immunoprecipitated from lysates of unstimulated Bac1.2F5 cells. The immunoprecipitates were then subjected to far-Western blotting in which a biotinylated GST fusion protein containing the unique, SH3, and SH2 domains of Hck (referred to as GST-HckU32) was used as a probe. This experiment revealed that Cbl could directly bind to Hck (Fig. 3C).

The SH3 Domain of Hck Is Important for Phosphorylation of Cbl—Given the above findings, we wanted to establish if Hck requires a functional SH3 domain to phosphorylate Cbl in vivo. Accordingly, an SH3 domain mutant of Hck499F was created by mutating tryptophan 91 to alanine. GST binding experiments confirmed that the mutation abolished the ability of the Hck SH3 domain to bind Cbl (Fig. 4A). The relative ability of Hck499F and the SH3 domain mutant (i.e. Hck91A/499F) to phosphorylate Cbl was tested by transiently co-expressing the proteins in 293T cells. Significantly, Hck91A/499F was found to be approximately 4–5-fold less efficient than Hck499F in phosphorylating Cbl under these conditions (Fig. 4B). Western blotting of the cell lysates with an anti-Hck antibody revealed that Hck499F and Hck91A/499F were expressed to similar levels (Fig. 4B). Intriguingly, Hck91A/499F exhibited a slightly reduced electrophoretic mobility in comparison with Hck499F (Fig. 4B). To exclude the possibility that the mutation may detrimentally affect its specific activity, the ability of Hck91A/499F to phosphorylate other proteins was investigated. Expression of Hck91A/499F in 293T cells induced equivalent levels of tyrosine phosphorylation of endogenous cellular proteins (Fig. 4C) or co-transfected paxillin (Fig. 4D) as did Hck499F.

LPS Induces the Association of Cbl with p85—It has previously been reported that the p85 regulatory subunit of PI 3-kinase can physically associate with tyrosine-phosphorylated Cbl (35, 37, 39, 40, 42, 46). Moreover, it has been reported that phosphorylated tyrosine 731 serves as a binding site on Cbl for p85 (37, 46). We were therefore interested in determining if Cbl bound p85 following LPS stimulation of Bac1.2F5 cells. It can be seen from Fig. 5A that p85 is physically associated with Cbl prior to LPS stimulation of Bac1.2F5 cells but that association of p85 with Cbl is quantitatively enhanced following LPS stimulation of the cells. Notably, physical association of p85 with Cbl (both basal and LPS-stimulated) was completely abolished by pretreatment of the cells with the Src family kinase inhibitor PP2 (Fig. 5A).

To ascertain if Hck can create a binding site on Cbl for p85, wild type Cbl or Cbl731F (in which tyrosine 731 is replaced with phenylalanine) was expressed either alone, or together...
The SH3 domain of Hck directly mediates the physical association of Hck with Cbl. A, Bac1.2F5 cells were either untreated (−) or stimulated (+) with 1 μg/ml LPS for 60 min and then lysed. Aliquots of cell lysate were incubated with glutathione-Sepharose beads containing immobilized GST (GST); GST-Hck unique domain (GST-unique); GST-Hck SH3 domain (GST-SH3); GST-Hck SH2 domain (GST-SH2); or GST-Hck unique, SH3, and SH2 domains (GST-U32). The ability of Cbl to bind to the various fusion proteins was then determined by Western blotting with anti-Cbl antibodies. B, Cbl was expressed in the absence (−) or presence (+) of Hck499F in 293T cells. Lysates of the transfected cells were then incubated with glutathione-Sepharose beads containing immobilized GST, GST-Hck SH3 domain, or GST-Hck SH2 domain. The ability of Cbl to bind to the fusion proteins was determined by Western blotting with anti-Cbl antibodies. C, lysates of unstimulated Bac1.2F5 cells were subjected to immunoprecipitation reactions in the absence (−) or presence (+) of anti-Cbl antibodies, and the immunoprecipitates were subjected to Far-Western blotting (FWB) with a biotinylated GST-HckU32 fusion protein. The filter was then stripped and reprobed with anti-Cbl antibodies.

with a kinase-active (i.e. Hck499F) or kinase-inactive (i.e. Hck267M) form of Hck in 293T cells. The Cbl proteins were then immunoprecipitated from lysates of the transiently transfected cells using anti-Cbl-specific antibodies. Both wild type Cbl and Cbl731F became tyrosine-phosphorylated when co-expressed with kinase-active Hck, although wild type Cbl exhibited a somewhat higher degree of tyrosine phosphorylation when compared with Cbl731F (Fig. 5B). This finding suggests that in addition to being capable of phosphorylating Cbl on tyrosine 731, Hck is also capable of phosphorylating Cbl at another site(s). The ability of endogenous p85 to physically associate with transfected wild type Cbl or Cbl701F was tested by determining if p85 was present within the anti-Cbl immunoprecipitates. As shown in Fig. 5B, co-expression of wild type Cbl with an active, but not with an inactive, form of Hck resulted in co-immunoprecipitation of p85 with Cbl. In contrast, no co-immunoprecipitation of p85 with the Cbl731F mutant was detected, although the mutant form of Cbl had become tyrosine-phosphorylated when co-expressed with an active form of Hck (Fig. 5B). We have found that co-expression of wild type Cbl with an activated form of Lyn in 293T cells also facilitates the co-immunoprecipitation of endogenous p85 with Cbl.2

LPS Induces Partial Translocation of Hck to the Cytoskeleton of Bac1.2F5 Cells—LPS stimulation of Bac1.2F5 cells induces significant cell spreading, which presumably involves reorganization of the cell’s cytoskeleton. We were therefore curious to determine if Hck, Cbl, or p85 became associated with the cytoskeleton of Bac1.2F5 cells in response to LPS stimulation. Partial translocation of both the p59 and p56 isoforms of Hck from the soluble fraction to the cytoskeletal fraction of Bac1.2F5 cells was observed within 30 min of LPS stimulation and increased further by 60 min (see Fig. 6A). Although it is not yet clear why the p56 isoform of Hck found in the cytoskeletal fraction exhibits a reduced electrophoretic mobility on SDS-polyacrylamide gel electrophoresis gels in comparison with the p56 isoform in the soluble fraction, it is most likely to be due to differences in their phosphorylation status. Although there is a suggestion from Fig. 6A that Cbl and p85 may also partially translocate to the cytoskeleton, we have been unable to conclusively demonstrate translocation of these two proteins in response to LPS stimulation of Bac1.2F5 cells.

It is possible that the partial translocation of Hck may be due, at least in part, to the formation of Hck-binding sites in the cytoskeleton of LPS-stimulated Bac1.2F5 cells. The presence of Hck-binding proteins in the cytoskeleton of Bac1.2F5 cells was investigated by subjecting the soluble and cytoskeletal fractions of unstimulated and LPS-stimulated cells to Far-Western blotting with a GST-Hck fusion protein. Whereas GST only bound two major proteins (p125 and p75), the GST-Hck

**Fig. 4.** The SH3 domain of Hck is important for the in vivo phosphorylation of Cbl. A, lysates of 293T cells transiently expressing Cbl were incubated with glutathione-Sepharose beads containing immobilized GST, GST-Hck SH3 domain (GST-SH3), or GST-Hck SH3 domain mutant (GST-SH3 mutant). The ability of Cbl to bind to the fusion proteins was determined by Western blotting with anti-Cbl antibodies. B, Cbl was immunoprecipitated from lysates of 293T cells transiently expressing Cbl alone or together with Hck499F or Hck91A/499F. The immunoprecipitates were then sequentially Western blotted with anti-phosphotyrosine (α-pY) and anti-Cbl antibodies. Whole cell lysates (WCL) of the transfected cells were then subjected to Western blotting with an anti-Hck antibody. C, whole cell lysates of 293T cells transiently expressing Hck499F or Hck91A/499F were subjected to Western blotting with anti-phosphotyrosine and anti-Hck antibodies, respectively. D, paxillin alone or together with Hck499F or Hck91A/499F was transiently expressed in 293T cells. Paxillin was immunoprecipitated from lysates of the 293T cells and sequentially Western blotted with anti-phosphotyrosine and anti-paxillin antibodies. Whole cell lysates of the transfected cells were then subjected to Western blotting with an anti-phosphotyrosine antibody.
fusion protein (i.e. GST-HckU32) bound a number of additional proteins (p120, p68, p62, p60, and p44) (Fig. 6B). Binding of GST-HckU32 to p68 was more pronounced in the cytoskeletal fraction, and notably, binding of the fusion protein to p68 increased in response to LPS stimulation of the cells. Likewise, binding of GST-HckU32 to p44 also increased in response to LPS stimulation; however, p44 binding occurred exclusively in the cytoskeletal fraction (see Fig. 6B). The identity of p68 and p44 remains to be determined.

**LPS Enhances the Adherence of Bac1.2F5 Cells in a Src Family Kinase and PI 3-Kinase-dependent Manner—Phospho-rylation of Cbl by Src family kinases and the binding of PI 3-kinase to tyrosine-phosphorylated Cbl have previously been shown to be important for β1-integrin-mediated macrophage adhesion (47). To determine if LPS alters the adherence of Bac1.2F5 cells, an adhesion assay that relied upon the ability of EDTA coupled with mechanical shaking to promote detachment of cells from tissue culture dishes was used to assess the relative adherence of unstimulated and LPS-stimulated Bac1.2F5 cells. Using this assay, LPS stimulation induces an approximately 2.5-fold increase in the adherence of Bac1.2F5 cells (Fig. 7A). Significantly, the ability of LPS to enhance the adherence of Bac1.2F5 cells could be completely abolished by pretreating the cells with the Src family kinase inhibitor PP2 prior to LPS stimulation (Fig. 7A). Pretreating the cells with PP2 was also found to perturb the basal adherence of unstimulated Bac1.2F5 cells, suggesting that both basal and LPS-enhanced Bac1.2F5 cell adhesion are dependent on Src family kinase activity (Fig. 7A). To determine if PI 3-kinase activity is required for the observed enhancement in the adherence of LPS-stimulated Bac1.2F5 cells, adhesion assays were performed on cells that had been treated with the PI 3-kinase inhibitor wortmannin prior to stimulation with LPS. As was the case for PP2, pretreatment with wortmannin suppressed...
both the basal and LPS-enhanced adherence of Bac1.2F5 cells (Fig. 7B).

**DISCUSSION**

In the present study, we sought to identify proteins that are phosphorylated by Src family kinases following LPS stimulation of Bac1.2F5 cells, since these proteins may play a critical role in the biological response of macrophages to LPS. We have shown that one of the proteins that became tyrosine-phosphorylated in an Src family kinase-dependent manner following LPS stimulation of Bac1.2F5 cells was Cbl. Tyrosine phosphorylation of Cbl was found to be both gradual and persistent. Maximal tyrosine phosphorylation of Cbl did not occur until 15–30 min after LPS stimulation and remained elevated for at least 2 h. Such kinetics of tyrosine phosphorylation of Cbl contrast with the rapid and transient tyrosine phosphorylation of Cbl following colony-stimulating factor-1 stimulation of Bac1.2F5 cells (34) but are somewhat similar to those observed upon plating macrophages onto fibrobrin-coated tissue culture dishes (43). Significantly, we found that Cbl is physically associated with Hck in Bac1.2F5 cells and that enforced expression of a constitutively activated form of Hck in Bac1.2F5 cells induces tyrosine phosphorylation of Cbl in the absence of LPS stimulation. Additionally, Hck was shown to be capable of directly phosphorylating Cbl in vitro. Taken together, these findings are consistent with the notion that Hck directly mediates, at least in part, the phosphorylation of Cbl in LPS-stimulated Bac1.2F5 cells.

By employing GST fusion proteins of Hck, we have been able to demonstrate that the association of Cbl with Hck is mediated by a direct interaction of Cbl with the SH3 domain of Hck. The interaction of Cbl with the SH3 domain of Hck is likely to be important for its subsequent phosphorylation, since an SH3 domain mutant of Hck499F was found to be 4–5-fold less efficient than Hck499F in phosphorylating Cbl in transiently transfected 293T cells. The reduced ability of the SH3 domain mutant to phosphorylate Cbl does not appear to be a consequence of the mutation negatively impacting on its specific activity, since its ability to phosphorylate other proteins (e.g. endogenous cellular proteins or co-transfected paxillin) was comparable with that of Hck499F. Since these experiments were performed in an overexpression system (i.e. 293T cells), the 4–5-fold lower phosphorylation of Cbl by the Hck499F SH3 domain mutant may actually underestimate the contribution of the SH3 domain of Hck to the phosphorylation of Cbl when the proteins are expressed at physiologically relevant levels (e.g. in Bac1.2F5 cells).

Our finding that the SH3 domain of Hck is sufficient to bind Cbl contrasts with two previous reports describing interactions between Hek and Cbl (35, 48). The GST-SH3 domain fusion protein utilized in this study encompassed amino acids 72–140 of murine Hck, whereas the GST-SH3 domain fusion proteins employed in the previous studies encompassed amino acids 87–137 (35, 48). X-ray crystallographic and NMR studies, however, have revealed that the SH3 domain of Hck is formed by amino acids 80–135 (49, 50). Thus, the fact that the GST fusion proteins employed in the prior studies lacked amino acids 80–86 may potentially explain the inability of those fusion proteins to bind Cbl. Our observation that the SH3 domain of Hck is capable of binding Cbl is consistent with previous reports that GST fusion proteins encompassing just the SH3 domain of other Src family kinases (e.g. Fyn, Lck, and Lyn) are capable of binding Cbl (38, 51).

Even when tyrosine-phosphorylated, 2–3-fold more Cbl bound the SH3 domain of Hck when compared with that which bound the SH2 domain of Hck. Preferential binding of tyrosine-phosphorylated Cbl to the SH3 domain of Hck would have at least two important consequences. First, it may allow Hck to simultaneously bind another protein via its SH2 domain; second, the tyrosine residue(s) on Cbl that is phosphorylated by Hck may remain accessible to bind SH2 domain-containing proteins, thus potentially mediating the formation of a multi-protein-signaling complex. Tyrosine phosphorylation of Cbl has previously been reported to occur in response to a variety of stimuli, including cytokine stimulation (33–37), activation of the T-cell and B-cell receptors (38–42), cell adhesion (43), and oncogenic transformation (44, 45). Cbl is able to bind a number of SH3 domain-containing proteins involved in signal transduction (e.g. Grb2), and when tyrosine-phosphorylated bind SH2 domain-containing proteins (e.g. p85 subunit of PI 3-kinase) (33, 35–37, 39–43, 45). These observations have led to the suggestion that Cbl may serve as a docking protein to facilitate assembly of multiprotein signaling complexes. Our finding that Hck phosphorylates Cbl in response to LPS stimulation of Bac1.2F5 cells suggests that Hck may mediate the formation of such a multiprotein-signaling complex in LPS-stimulated macrophages. Indeed, we have found that association of the p85 subunit of PI 3-kinase with Cbl is enhanced following LPS stimulation of Bac1.2F5 cells and that this association of p85 with Cbl is dependent on Src family kinase activity. Transient expression experiments in 293T cells have allowed us to demonstrate that phosphorylation of Cbl by Hck can facilitate the physical association of Cbl with p85. Moreover, we have been able to demonstrate that phosphorylation of Cbl on tyrosine 731 is necessary for its physical association with p85. Tyrosine 731 is found within the sequence CTYEA, which conforms to the minimal consensus binding sequence of YXXM for the SH2 domains of p85 (52).
cells via a Hck-Cbl-PI 3-kinase signal transduction pathway. Specifically, we propose that Hck directly phosphorylates Cbl on tyrosine 731 in response to LPS stimulation, thus facilitating the physical association of Cbl with p85 and leading to the coordinated activation of PI 3-kinase. However, since Lyn is also capable of phosphorylating Cbl in 293T cells and facilitating the physical association of p85 with Cbl, we cannot exclude the possibility that Lyn (and possibly Fgr) might also contribute to the tyrosine phosphorylation of Cbl and its association with p85 in LPS-stimulated Bac1.2F5 cells. The fact that a similar mechanism has been proposed for β1-integrin-mediated macrophage cell adhesion (47) suggests that Src family kinases, Cbl, and PI 3-kinase may regulate the adherence of macrophages in response to various stimuli.

Further investigation will be required to elucidate how activation of this Hck-Cbl-PI 3-kinase signal transduction pathway enhances the adherence of LPS-stimulated Bac1.2F5 cells. However, since the phosphorylation products of PI 3-kinase (e.g., PI 3,4,5-trisphosphate) can interact with a subset of pleckstrin homology domains (55), it seems likely that a pleckstrin homology domain-containing protein might be involved in regulating the adherence of macrophages in response to LPS. Notably, Hmama et al. (56) have recently proposed a model in which LPS-induced monocyte adherence is mediated by the pleckstrin homology domain-containing protein cytohesin-1 (57, 58). Specifically, Hmama et al. have postulated that the activation of PI 3-kinase following LPS stimulation leads to the generation of PI 3,4,5-P3, which binds to, and modifies the properties of, cytohesin-1 (56). Engagement of the cytoplasmic tail of CD18 by cytohesin-1 would then lead to the conversion of low avidity LFA-1 (CD11a/CD18) molecules into high avidity LFA-1 (CD11a/CD18) molecules capable of increased binding to intercellular adhesion molecule 1 (56). This model for LPS-induced monocyte/macrophage cell adhesion can possibly now be extended further to incorporate the signal transduction events we have described in this report, namely that the activation of PI 3-kinase in response to LPS stimulation might be mediated by the phosphorylation of Cbl by Hck.

We have been unable to maintain the phenotype of cloned derivate Bac1-Hck499F cells. Upon extended passages, the cells (i) became morphology heterogeneous and (ii) exhibited levels of morphology (56), this model for LPS-induced monocyte/macrophage cell adhesion can possibly now be extended further to incorporate the signal transduction events we have described in this report, namely that the activation of PI 3-kinase in response to LPS stimulation might be mediated by the phosphorylation of Cbl by Hck.

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Cbl Is a Target of Hck in LPS-stimulated Macrophages

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