p59Hck Isoform Induces F-actin Reorganization to Form Protrusions of the Plasma Membrane in a Cdc42- and Rac-dependent Manner*

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Sébastien Carréno, Emmanuelle Caron§§, Céline Cougoule, Laurent J. Emarone®,
and Isabelle Maridonneau-Parini∥

From the Institut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique UMR 5089, 205 route de Narbonne, Toulouse cedex 31077, France and Medical Research Council Laboratory for Molecular Cell Biology, CRC Oncogene and Signal Transduction Group, University College London, Gower Street, London WC1E 6BT, United Kingdom

Hck is a protein kinase of the Src family specifically expressed in phagocytes as two isoforms, p59Hck and p61Hck, localized at the plasma membrane and lysosomes, respectively. Their individual involvement in functions ascribed to Hck, phagocytosis, cell migration, and lysosome mobilization, is still unclarified. To investigate the specific role of p59Hck, a constitutively active variant in fusion with green fluorescent protein (p59HckGFp) was expressed in HeLa cells. p59Hck was found at focal adhesion sites and triggered reorganization of the actin cytoskeleton, leading to plasma membrane protrusions where it co-localized with F-actin. Similarly, microinjection of p59Hck cDNA in J774.A1 macrophages induced membrane protrusions. Whereas kinase activity and membrane association of p59Hck were dispensable for location at focal adhesions, p59Hck-induced membrane protrusions were dependent on kinase activity, plasma membrane association, and Src homology 2 but not Src homology 3 domain and were inhibited by dominant-negative forms of Cdc42 or Rac but not by blocking Rho activity. A dominant negative form of p59Hck inhibited the Cdc42- and Rac-dependent FcγRIIa-mediated phagocytosis. Expression of the Cdc42/Rac-interacting domain of p21-activated kinase in macrophages abolished the p59Hck-induced morphological changes. Therefore, p59Hck-triggered remodeling of the actin cytoskeleton depends upon the activity of Cdc42 and Rac to promote formation of membrane protrusions necessary for phagocytosis and cell migration.

Hck is a protein-tyrosine kinase (PTK) of the Src family mainly expressed in phagocytes (1). Src PTKs are key elements of diverse signaling cascades, which act via at least two different ways: a tyrosine kinase activity and/or an adaptor function (for a review, see Ref. 2). These two roles are achieved by three highly conserved domains shared by all members of the Src family. The Src homology domain 1 (SH1) lying at the C terminus of the protein supports the catalytic activity of the enzyme, and the SH2 and SH3 domains allow interactions with cellular proteins through recognition of phosphotyrosine and proline-rich motif, respectively (3, 4). Src PTKs are regulated by the phosphorylation state of a conserved carboxyl-terminal tyrosine. When phosphorylated, this tyrosine is recognized by the SH2 domain, leading to intramolecular interactions that stabilize the kinase under its inactive form. Its dephosphorylation disrupts these intramolecular interactions and subsequently unmask the SH3, SH2, and catalytic domains. Therefore, this protein “opening” activates both the adaptor and the kinase functions of Src PTKs (5).

Hck is the unique example among the Src PTKs to be expressed as two isoforms generated in equal amounts by alternative translation. p61Hck translation is initiated at a CTG codon 21 codons upstream from the p59Hck ATG codon (6). Like other members of the Src family, both Hck isoforms have a unique amino-terminal domain comprising about 80 amino acids with acylation motifs (7). p61Hck has 21 additional N-terminal amino acids containing the Met-X-Gly-X-X-Ser/Thr-N-terminal sequence that supports covalent myristoylation of glycine 2, whereas the Met-X-Gly-X-X-Ser/Thr-N terminus of p59Hck guides permanent myristoylation of glycine 2 and reversible palmitoylation of cysteine 3 (8). These different acylations govern association of both isoforms with distinct cellular membranes; while the double acylated form of p59Hck is anchored at the plasma membrane, the monoacylated forms (p61Hck and the nonpalmitoylated form of p59Hck) are associated with lysosomal membranes, both isoforms being present at the Golgi apparatus (9).

These distinct subcellular localizations are probably a key element of the differential functions of Hck isoforms by offering them access to different substrates. Since Hck has been involved in the lysosome mobilization process (10, 11) and in the signaling of membrane receptors such as phagocytic ones (12–14), we have proposed that the monoacylated lysosomal p61Hck and p59Hck could control lysosome exocytosis, whereas the plasma membrane-associated isoform p59Hck could transduce signals from membrane receptors (9).

However, no attempt has been made to identify the respective functions of each isoform. In this work, we focused on p59Hck and investigated the function devoted to this plasma membrane-associated isoform. Since we have previously shown...
that ectopic expression of Hck isoforms in HeLa cells leads to the same subcellular distribution as the endogenous kinase in human neutrophils and monocytes-macrophages (9), we first decided to study p59Hck function in these human epithelial cells. We took advantage of the nonexpression of Hck in these cells to examine whether expression of this phagocyte-specific kinase would trigger a phagocyte-specific phenotype. Ectopic expression of a constitutively active form of p59Hck in fusion with GFP in these cells led to reorganization of the cell cytoskeleton, which triggered the formation of plasma membrane protrusions. When the Hck-GFP construct was microinjected into J774A.1 macrophages, a similar phenotype was observed. Using targeted mutagenesis and deletion constructs, we showed that these cytoskeletal changes were strictly dependent on the association of p59Hck with the plasma membrane and involved both its tyrosine kinase activity and its SH2 adaptor domain. Furthermore, we showed that a dominant negative form of p59Hck co-transfected in HeLa cells with the FcyRIIa was able to inhibit phagocytosis mediated by this receptor. Since Cdc42 and Rac, two small GTP-binding proteins of the Rho subfamily that control the actin cytoskeleton, have been involved in FcyR-mediated phagocytosis (15, 16), we investigated their role in the p59Hck-mediated membrane protrusion. Using dominant negative Cdc42 or Rac or the Cdc42/Rac-interacting domain of p21 (Cdc42/Rac-activated kinase) (PAK), we showed that p59Hck acted upstream of the Cdc42/Rac pathway to promote these cytoskeletal changes both in HeLa cells and in macrophages. We thus propose that p59Hck is part of a signaling pathway between plasma membrane receptors and Cdc42/Rac that promotes actin cytoskeleton rearrangements necessary for phagocytosis or cell migration.

MATERIALS AND METHODS

Plasmid Construction and Mutagenesis—The wild-type Hck cDNA was a gift from N. Quintrell (1). Construction of p59Hck in fusion with GFP has been described (9). p59Hck* was obtained by point mutation of the carboxyl-terminal tyrosine 505 (TAC) into phenylalanine (TTC) and of the stop codon TGA into CGA by PCR using primers that generate XbaI and NotI sites at the 5′- and 3′-ends, respectively. The PCR products were ligated into the XbaI and NotI sites of pEGFP-N3 (CLONTECH, Palo Alto, CA), preserving the 28-amino acid polylinker of the vector between p59Hck and GFP. The C3S point mutation was introduced into the p59Hck*-GFP vector by inverse PCR mutating the cysteine (TGC) into serine (AGC) and introducing a Ncol site. The p59Hck*GFP was obtained by inverse PCR on the p59Hck*-GFP vector mutating the lysine 381 (AAG) responsible of ATP binding into glutamic acid (GAG) and introducing an XcmI site. p61Hck and p61Hck* fused with GFP were obtained by the same strategy using the p61Hck-GFP vector as template (9).

p59(K9SH2-SH3)Hck*, p59(K9SH2-SH3)Hck, and p59(3SH2-SH3)Hck* were obtained by inverse PCR on the p59Hck*-GFP vector using primers allowing the deletion of amino acids 57–220, 123–220, and 57–117, respectively. Conformation of these mutations was verified by sequencing (Genome Express, Grenoble, France). In addition, all constructs were tested for expression by Western blotting (see below).

For phagocytic assay we used the plasmid pKc3 encoding the human FcyRIIa provided by C. Sautes-Fridman (17).

cDNAs coding for Myc-tagged Cdc42N17 and RacN17, subcloned into pRK5 vector, were provided by A. Hall (15).

The Cdc42/Rac binding domain of Pak PKACRIB (18) was subcloned into the eukaryotic expression vector pRK5myc (19).

Cell Culture and Transfection—HeLa cells were cultured at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. HeLa cells were seeded in 24-well plates (2 × 105 cells/well on glass coverslips) for immunofluorescence experiments or in 9-cm dishes for immunoblotting experiments. The following day, phosphate precipitate was added to the cells (1 µg of DNA per 40 µl of DNA/calcium per well containing 360 µl of fresh complete medium). For double transfection experiments, DNA/calcium phosphate precipitates were made with 500 ng of each cDNA. Cells were washed free of DNA/calcium phosphate precipitates after 16–18 h and incubated in fresh medium for an additional period of 60 h before analyses.

The murine macrophage cell line J774.A1 was maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat inactivated fetal calf serum and penicillin/streptomycin (100 units/ml and 100 µg/ml).

Indirect Immunofluorescence—Transfected cells grown on glass coverslips were washed twice with PBS and fixed in 3.7% paraformaldehyde for 30 min at room temperature, and unreacted aldehyde groups were neutralized in 50 mM NH4Cl for 1 min. After washing and permeabilization (0.3% Triton X-100 in PBS, 5 min), cells were blocked for 10 min in PBS containing 1% bovine serum albumin (PBS-BSA). Coverslips were then overlaid as described with 20 µl of one of the following antibodies diluted in PBS-BSA: monoclonal anti-vinculin antibody (1:50; Sigma), monoclonal anti-FcγRIIa IV.3 antibody (300 ng/ml; kindly provided by C. Sautes-Fridman (20)), monoclonal anti-Golgi CTTR33 (kindly provided by M. Bornens (9)), and monoclonal anti-Myc 9E10 (1:100; Sigma). After a 30-min incubation period, the coverslips were washed three times in PBS and incubated for 30 min at room temperature with a 1:100 dilution of affinity-purified TRITC-conjugated secondary antibodies (Sigma) directed against mouse IgG. The coverslips were washed three times in PBS.

Labeling of F-actin was performed on cells fixed as described above, after permeabilization (0.3% Triton X-100, 5 min), and coverslips were overlaid with 20 µl of permeabilization buffer supplemented with 0.3 units of rhodamine-phallolidin (Molecular Probes, Leiden, The Netherlands).

In some experiments, cells were incubated for 24 h with 20 µg/ml recombinant C3 exoenzyme from Clostridium botulinum kindly provided by P. Boquet and prepared as previously described (21).

All coverslips were mounted in Mowiol and viewed using a Leica DM-RB fluorescence microscope or a Leica TCS-SP2 confocal scanning microscope. Epifluorescence images were captured, and negatives were digitalized with a Nikon LS2000. All images were prepared for publication using Adobe Photoshop software.

Microinjection and Immunofluorescence—J774 macrophages were used for microinjection as previously described (15). Briefly, macrophages were seeded on glass coverslips at a density of 1 × 106 cells/ml. Immediately prior to injection, cells were transferred to 10 mM Hepes-buffered, serum-free Dulbecco’s modified Eagle’s medium. cDNA constructs prepared for microinjection by standard CsCl gradient methods were injected (0.1 µg/ml) into the nucleus of 50–100 cells in a temperature of 37 °C and CO2 (10%-controlled chamber using phase-contrast microscopy. Cells were returned to the incubator for 2–2.5 h for optimal expression. Cells were fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature prior to permeabilization with 0.1% Triton X-100/ phosphate-buffered saline (PBS). Cells were permeabilized (0.3% Triton X-100/PBS for 20 min). For immunostaining, cells were blocked with 0.5% BSA for 30 min and then incubated with antibodies diluted in PBS for 30 min. Where appropriate, all antibody mixes contained excess human IgG (Sigma) to prevent nonspecific binding to the Fc receptors. Myc-tagged constructs were visualized using mouse monoclonal anti-Myc (9E10) followed by Cy5-conjugated anti-mouse IgG. F-actin was stained using rhodamine-conjugated phalloidin. Coverslips were mounted in Mowiol (Calbiochem) containing p-phenylenediamine as an antibleaching agent. Cells were examined with a Zeiss Axioskop microscope using a Zeiss 63 × 1.4 oil immersion objective. Fluorescence images were captured using a Hamamatsu C5985–10 video camera and Openlab software and processed using Adobe Photoshop.

Cell Lysis and Immunoblotting—After transfection, HeLa cells (3 × 105 cells) were washed in PBS, lysed in 1 ml of Laemmli buffer, and boiled for 5 min. Neutrophils from healthy donors were isolated (22) by Dextran T500 sedimentation (Amershams Biosciences) and Ficoll centrifugation (Eurobio) and were resuspended in boiling Laemmli buffer. Proteins were electrophoresed through 8% SDS-PAGE, transferred to nitrocellulose membrane, which was blocked in Tris-buffered saline buffer containing 5% nonfat milk, and then incubated with anti-Hck antibodies (1:2000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The primary antibody was revealed with horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:10,000; Bio-Rad, Hercules, CA) followed by ECL (Amershams Biosciences).

Labeling and Oposinization of Zymosan Particles—Zymosan particles (Sigma) were swollen in PBS for 30 min. They were then washed, dried, and resuspended in 0.2 M Na2CO3/NaHCO3, pH 9.2, with 250 µg/ml rhodamine B isothiocyanate (Sigma) for 1 h at room temperature, and the reaction was stopped by 50 mM NH4Cl. Labeled zymosan was washed several times in PBS, resuspended, and opsonized in pooled human sera for 30 min at 37 °C. Opsonized and rhodamine-labeled zymosan was washed twice in PBS, and opsonized in pooled human sera for 30 min at 37 °C. Opsonized and rhodamine-labeled zymosan was washed twice and resuspended in PBS.

Phagocytosis Assay—HeLa cells were used 60 h after transfection.
Fig. 1. p59Hck triggers the formation of membrane protrusions. p59Hck (A and B) and p59Hckca (C and D) in fusion with GFP were transiently expressed in HeLa cells. Cells were fixed and observed using direct fluorescence microscopy (A and C) or by interferential Nomarsky phase optics (B and D). The arrows show association of both p59Hck constructs with the plasma membrane. Only p59Hckca triggers membrane protrusions (arrowheads in C and D).

Cells were starved in serum for 3 h, and then opsonized zymosan was added at a concentration of 100 particles/cell and incubated at 37 °C for 3 h. Cells were then extensively washed to remove adherent zymosan, fixed in paraformaldehyde. External particles were labeled using fluorescein-conjugated anti-human IgG (1:100; Diagnostics Pasteur, Paris, France) and then appeared as doubly green and red fluorescence. FcRIIa was detected using a monoclonal anti-FcRIIa antibody (Becton Dickinson, San José, CA) and then appeared as red fluorescence. Cells positive for both GFP fluorescence and FcRIIa labeling were then counted by fluorescence microscopy for the presence of at least one particle inside the cell.

RESULTS
Expression of a Constitutively Active Form of p59Hck Leads to Formation of Plasma Membrane Protrusions—To study the cellular effects triggered by p59Hck, we made use of a constitutively active form of p59Hck (p59Hckca), obtained by point-mutating p59Hck cDNA to replace the C-terminal regulatory tyrosine (responsible for the intramolecular regulation) by a phenylalanine (23). Subcellular localization and estimation of the expression level of p59Hckca were allowed by a C-terminal fusion with GFP, which has been previously shown to not interfere with the kinase activity of Hck or with its localization (9). Direct confocal fluorescence analysis of HeLa cells transiently expressing p59Hck or p59Hckca showed the association of both proteins with the plasma membrane (Fig. 1) (9).

The shape of cells expressing p59Hckca was dramatically modified by the formation of plasma membrane protrusions (Fig. 1). This phenotype was observed in 57.2 ± 5.3% of the cells expressing p59Hckca (488 total cells counted out of three representative experiments). It is notable that this phenotype was obtained even at low levels of p59Hckca expression as assessed by the heterogeneous intensity of fluorescence in cells forming protrusions (see the cell shown by an arrow in Fig. 1, C and D). Because membrane extensions are associated with reorganization of the actin cytoskeleton, the effects of activated p59Hck on F-actin organization were examined by rhodamine-phalloidin labeling. Cells expressing p59Hck showed a similar pattern of F-actin as nontransfected cells, whereas cells expressing p59Hckca displayed a clear reorganization of their actin filaments with disappearance of stress fibers and actin polymerization at the periphery in GFP-enriched membrane protrusions corresponding to sites of p59Hckca localization (Fig. 2, D–I).

It can also be noted that, as previously described, the Golgi apparatus was stained by p59Hck (Figs. 1A and 2A) (9). p59Hckca was also associated with the Golgi, which was dispersed as vesicles into the cytoplasm (Fig. 2, J–L). A similar Golgi phenotype is observed in cells treated with microtubule depolymerizing agents (24). However, the microtubule network was not affected in cells expressing the constitutively active p59Hckca (data not shown). The mechanisms involved in disruption of the Golgi apparatus by the action of p59Hckca are presently under study in the laboratory.

Because Hck is a phagocyte-specific Src-like kinase, we also studied the effect of its overexpression in the murine macrophage cell line, J774.A1. When J774 cells are grown on glass coverslips, their morphology is generally reminiscent of that of motile cells (i.e., they adopt an elongated, polarized shape that shows an F-actin-rich leading edge). cDNA constructs were microinjected in the nucleus of cells. As shown in Fig. 3, microinjection per se did not affect cell morphology. In some cases, GFP-expressing cells displayed numerous, long filopodia, contrasting with the few short filopodia associated with control cells (12.8 ± 1.8% of 226 cells counted of three experiments). By contrast, overexpression of GFP-tagged p59Hck (data not shown) or p59Hckca induced the formation of membrane protrusions and localized actin-rich ruffles in which Hck-GFP accumulated (Fig. 3) in 53.1 ± 1.9% (252 cells counted of three experiments) or 57.9 ± 3.7% (90 cells counted of three experiments) of the cells, respectively.

Kinase Activity and Plasma Membrane Association of p59Hck Are Both Necessary to Promote Formation of Membrane Protrusions but Dispensable for Association with Focal Adhesions—Since the association of p59Hck with the plasma membrane is dependent on palmitoylation (9), we constructed a palmitoylation mutant of p59Hckca to determine whether formation of membrane protrusions is due to its presence at the plasma membrane. The palmitoylated cysteine residue at position 3 of p59Hckca was substituted by a serine (p59C38Hckca). This mutant was not associated with the plasma membrane but was redistributed into cytoplasmic vesicles previously characterized as lysosomes (9). Whereas about 60% of the cells expressing p59Hckca had membrane protrusions, none of the cells expressing p59C38Hckca presented these structures (Fig. 4, A–C).

Src kinases comprise a tyrosine kinase activity and SH2 and SH3 adaptor functions. To distinguish which of these two properties was involved in the formation of plasma membrane protrusions, the lysine residue responsible for ATP binding was substituted by a glutamic acid in p59Hckca. This mutation has previously been shown to lead to a kinase-less, adaptor-plus form of Src kinases with dominant negative properties for kinase activity (p59Hckdn) (25, 26). HeLa cells expressing p59Hckdn never showed formation of membrane protrusions or disorganization of stress fibers (Fig. 4, D–F). We noticed that p59C38Hckca or p59Hckdn mutants were redistributed to focal adhesion structures, where they colocalized with vinculin, a usual marker of these sites (27) (Fig. 4, G–L). Similarly, p59Hckca co-localized with vinculin, mostly at the edge of membrane extensions (Fig. 4, M–O). Therefore, neither kinase activity nor plasma membrane attachment is required for association to focal adhesions. The presence of p59Hckca at focal adhesion sites was not isoform-specific, since we observed that...
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The Golgi apparatus was stained by CTR33 antibodies revealed by TRITC-conjugated secondary antibodies (E) and H9253, and p59(Hckα) was found in focal adhesion plaques, indicating that the adaptor function of the protein is sufficient for this targeting.

The SH2 and Not the SH3 Domain of p59Hck Is Necessary to Trigger Plasma Membrane Protrusions—The SH2 domain is responsible for phosphotyrosine recognition and the SH3 domain for binding to proline-rich domains. To determine whether adaptor domains were implicated in formation of membrane protrusions, several deletions were made in p59Hckα cDNA (Fig. 6A). The deletion mutants expressed in HeLa cells migrated in SDS gels at the expected molecular weights (p59ΔSH2-SH3Hckα, p59ΔSH2Hckα, and p59ΔSH3Hckα) (Fig. 6B). Each of these mutants remained localized at the plasma membrane, but those deleted for both SH2 and SH3 domains failed to promote membrane protrusions (Fig. 6C) and modifications of the actin cytoskeleton (data not shown). Similarly, the p59ΔSH2Hckα was unable to promote membrane protrusions, whereas cells expressing p59ΔSH3Hckα showed these cytoskeletal modifications at a similar rate as cells transfected by p59Hckα (Fig. 6C), indicating that interaction of the kinase with its partners involved in formation of membrane protrusions requires the SH2 but not the SH3 domain.

FcγRIIa-dependent Phagocytosis Is Regulated by p59Hck—Pseudopodia are membrane structures involved in phagocytosis of IgG-coated particles by FcγRs (28). Src PTKs are activated upon FcγR aggregation (29), and Hck has been described to be physically associated with the FcγRIIa receptor (13). Since we have shown above that constitutive activation of p59Hck is sufficient to trigger actin rearrangements leading to formation of membrane protrusions, this led us to hypothesize that p59Hck might link the Fcγ receptors to the actin cytoskeleton.

Expression of the FcγRIIa receptor confers phagocytic capacities to non-phagocytic cells in contact with IgG-coated particles (30). Because of the well-known functional redundancy of Src PTKs, it is likely that ubiquitous members of the Src PTK family replace the phagocyte-specific kinases that are lacking in these cells. Indeed, in macrophages from Hck, Lyn, and Fgr triple knockout mice, phagocytosis of IgG-coated particles is still driven by PTKs of the Src family (31). Expression of a plasmid encoding the human FcγRIIa in HeLa cells that are normally devoid of phagocytic receptors allowed 30% of these cells to ingest serum-opsonized zymosan, whereas mock-transfected HeLa cells were not able to engulf particles. Co-expression of the receptor with the wild type p59Hck did not increase the rate of phagocytosis (Fig. 7), suggesting that Src PTKs are not rate-limiting in HeLa cells. By contrast, co-expression of the receptor with p59Hck<sup>dn</sup> strongly inhibited particle internalization. No inhibition of the phagocytic process was obtained with p61Hck<sup>dn</sup>, indicating that the two isoforms are involved in distinct biological functions (Fig. 7).

To verify that the effect of p59Hck<sup>dn</sup> was not due to mislocalization of FcγRIIa, cells co-expressing the receptor with the dominant negative form or with p59Hck as a control were examined by confocal microscopy. In both cases, FcγRIIa was present at the plasma membrane (data not shown).

When opsonized zymosan was added to cells expressing p59Hckα but not FcγRIIa, the membrane protrusions triggered by the kinase were unable to internalize particles in the absence of the phagocytic receptor (data not shown).

p59Hck Directs Formation of Membrane Protrusions through Cdc42 and Rac Activation—Cdc42 and Rac are two small GTP-binding proteins of the Rho subfamily that control actin polymerization. They are known as key regulators of filopodia and lamellipodia formation, respectively (32), and both proteins have been implicated in FcγR-mediated phagocytosis (15, 16). Therefore, we tested the implication of Cdc42 and Rac in the formation of membrane protrusions initiated by p59Hck activation. To this aim, a dominant negative form of Cdc42 (Cdc42<sub>N17</sub>) or Rac (Rac<sub>N17</sub>) was co-expressed with p59Hckα in HeLa cells. Under either of these conditions, the formation of membrane protrusions was significantly decreased (Fig. 8A). Thus, we propose that the cytoskeletal changes induced by p59Hckα require the Cdc42 and Rac GTPases. Rho was not involved in this process, since co-incubation of p59Hckα-expressing cells with the purified C3 exoenzyme did not change their phenotype (Fig. 8B).

Since in HeLa cells and macrophages, overexpression of p59Hck induced a similar phenotype (32, 34), we examined whether membrane protrusions induced by p59Hck in J774 cells are Cdc42- and Rac-dependent.

For this purpose, we made use of the Cdc42/Rac-interacting binding (CRIB) domain of PAK, known to bind specifically to the effector region of active, GTP-bound Rac and Cdc42. The PAK-CRIB glutathione S-transferase fusion protein is widely used in pull-down assays for monitoring Cdc42 and Rac activation in cell lysates (18, 35). The PAK-CRIB fragment, expected to prevent newly activated Rac and Cdc42 from interacting with their downstream targets and elicit their cellular effects, was transferred into a eukaryotic expression vector and used in microinjection studies (35). When expressed alone, the PAK-CRIB fragment did not exert any noticeable effect on macrophage morphology (data not shown). However, coexpre-
FIG. 4. Both plasma membrane localization and kinase activity of p59Hck are necessary to induce protrusions. Palmitoylation p59_{c_{3S}}Hck^{ca} (A–C and G–I) and kinase-defective p59Hck^{dn} (D–F and J–L) mutants were transiently expressed as GFP fusion proteins. HeLa cells were fixed, permeabilized, and labeled for F-actin by rhodamine-phalloidin (B and E) or for focal adhesion with a monoclonal anti-vinculin antibody.
sion of PAK-CRIB abolished p59Hckca-induced morphological changes (Fig. 8C), suggesting that in macrophages, p59Hck-induced morphological changes and remodeling of the actin cytoskeleton are dependent upon the activity of Rac and/or Cdc42.

DISCUSSION

Hck has been implicated in several phagocyte-specific functions such as mediation of signals from phagocytic (12, 13, 36) and chemotactic receptors (37–39), cellular adhesion and migration (23, 40–42), and control of lysosome mobilization (10, 11, 43, 44). In a recent work, we have proposed that each Hck isoform may exert a specific function related to the differential subcellular localization of p59Hck in the plasma membrane and p61Hck in the lysosomal compartment (9).

Investigation of the function of a given Src PTK is problematic. Pharmacological inhibitors available for Src tyrosine kinases are not specific for any member of the family, and gene knockout approaches revealed a high degree of redundancy among these proteins, as illustrated by a recent work showing that in Hck−/−Fgr−/− macrophages other Src kinases could replace the phagocyte-specific ones in IgG-dependent phagocytosis (31). Another difficulty has to be faced in determining the function of Hck, since it is expressed under two isoforms (6). Therefore, we decided to investigate the function of p59Hck by expressing a constitutively active form in the human epithelial HeLa cell line. During this work, we found that p59Hckca induced the formation of plasma membrane protrusions. This is certainly an intrinsic feature of activated p59Hck, since it was observed not only in HeLa cells but also in NIH3T3 fibroblasts, Chinese hamster ovary epithelial cells (data not shown), and macrophages, cells in which it is normally expressed (see Fig. 3). Transfection of these various cell lines with p61Hckca did not result in the formation of plasma membrane protrusions, suggesting that each isoform may play a specific role.

The involvement of Src PTKs in regulating the actin cytoskeleton has previously been suggested by the observations that (i) a constitutively active variant of the viral Src, v-Src, induced lamellipodia and invadopodia and phosphorylation of several actin-binding proteins (2), and (ii) c-Src is involved in the actin-dependent internalization of Shigellae and Neisseria meningitidis by epithelial and endothelial cells, respectively (45, 46). We report here that expression of a constitutive active form of p59Hck is sufficient to regulate plasticity of the cortical actin cytoskeleton, which triggers the formation of membrane protrusions. This effect is strictly dependent on (i) the kinase activity of the enzyme, (ii) its association with the plasma membrane, (iii) the presence of the SH2 domain, and (iv) the activity of both Cdc42 and Rac GTPases activity.

FIG. 5. p61Hck is present at focal adhesion sites in a kinase-dispensable manner. p61Hckca (A–C) and p61Hckdn (D–F) expressed as GFP fusion proteins colocalized with vinculin as shown in Fig. 4 for the corresponding variants of p59Hck. Association with focal adhesions is not isoform-specific. Scale bar (C), 4 μm; scale bar (F), 2 μm.

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revealed by TRITC-conjugated secondary antibodies (H and K). Colocalization of p59Hckca (M–O) with vinculin (N and O) is shown. Green fluorescence revealing Hck localization (A, D, G, J, and M) and red fluorescence F-actin (B and E) or vinculin (H, K, and N) are shown in gray tones. Merged images are shown in color plates in C, F, I, L, and O. Scale bar, 10 μm.

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Cdc42 and Rac are implicated in the formation of pseudopodia during FcγR-mediated phagocytosis (15). Src PTKs are also involved in FcγR signaling. On one hand, they mediate the phosphorylation of FcγRIIIa ITAM domain, leading to Syk kinase activation; on the other hand, Src PTKs regulate the formation of actin cups around IgG-coated particles, which is delayed in macrophages from Hck, Fgr, and Lyn triple knockout mice (12, 47, 48). Whereas Fgr has already been shown to inhibit phagocytosis (49), the relative contribution of the two other major Src-PTKs expressed in phagocytes in FcγRIIIa-mediated phagocytosis remains unclear. Hck and Lyn are physically linked with the FcγRIIIa receptor (50, 51) and could therefore regulate the phagocytic process in concert, possibly by indirectly acting on the GTPases Cdc42 and Rac. Here we show that expression of a dominant negative form of p59Hck inhibited FcγRIIIa-mediated phagocytosis (15). In addition, we show that, in professional phagocytes, p59Hck acts via the Cdc42/Rac pathway to signal to the actin cytoskeleton. In J774 cells, coexpression of PAKCRIB, a PAK fragment that binds specifically to GTP-bound Cdc42 and Rac, abolished p59Hck-induced membrane protrusions clearly indicating that Hck-induced reorganization of the actin cytoskeleton is dependent upon the activity of endogenous Rac and Cdc42.

![Diagram](A)  
**Fig. 6.** p59Hck SH2 domain is necessary for the kinase to trigger formation of membrane protrusions. **A**, schematic representation of p59Hck deletion mutants. **B**, immunoblotting of cell lysates expressing one of the different p59Hck constructs in fusion with GFP. Hck was revealed with anti-Hck antibodies. Extracts from neutrophils showed the endogenous expression of Hck (p61Hck(*)) and p59Hck(**)). Cells transfected by Hck constructs showed a specific and prominent signal at the predicted molecular weight. C, the SH2 domain is necessary for protrusion formation. Cells expressing the indicated deletion mutants were observed by direct fluorescence. p59(ΔSH2-ΔSH3)Hckca (A), p59(ΔSH2)Hckca (B), and p59(ΔSH3)Hckca (C) promoted formation of membrane protrusions, while the two forms of p59Hckca lacking the SH2 domain were associated with the plasma membrane but failed to trigger membrane protrusion. Scale bar, 10 μm.

![Diagram](C)  
**Fig. 7.** Expression of a dominant negative form of p59Hck blocks phagocytosis mediated by FcγRIIa. HeLa cells expressing the indicated constructions were incubated with IgG-opsonized zymozan (100 particles/cell) for 3 h. Cells were washed and fixed, and human FcγRIIa was detected using a monoclonal anti-FcγRIIa antibody revealed by TRITC-conjugated secondary antibodies. Intracellular particles were revealed by differential fluorescence staining that allowed us to distinguish between intra- and extracellular particles. Cells positive for both constructs were counted, and the percentage of cells having ingested at least one particle was calculated. The data are expressed as the percentage of phagocytosis compared with control values (100%, FcγRIIa + GFP). The values are the mean ± S.E. of three separate experiments with a rate of phagocytosis of 29 ± 3.6% for the control. ***, p < 0.01 when compared with control calculated with paired Student's t test.
Actin rearrangements are also necessary for cell migration, another critical function of phagocytes. Interestingly, neutrophils and macrophages from Hck/Fgr knockout animals show a strong defect in adhesion and migration (39, 40). Furthermore, it has been shown that expression of a kinase inactive form of Hck increases the adhesion process (22). In this study, we report that p59Hck is found on focal adhesions. This localization does not require its kinase activity or its plasma membrane attachment and is not isoform-specific, since p61Hckα and its dominant negative variant are also present at focal adhesion sites. Similarly, the constitutively active variant v-Src and the kinase-inactive v-Src are associated with focal adhesions (52). The role of Src in these structures is to regulate their turnover to decrease the cell adhesion, thus facilitating cell movements, a role that could also be played by Hck (23). In addition to adhesion, cell motility requires the extension of the leading edge, through mechanisms reminiscent of pseudopod formation during FcyR-mediated phagocytosis (53). The presence of p59Hck on focal adhesions, together with its ability to induce plasma membrane protrusions, strongly suggests that this particular isoform could also play a role in cell migration, a hypothesis that is presently under study in the laboratory.

The strict requirement of the SH2 domain for induction of membrane protrusions implicates an unidentified tyrosine-phosphorylated p59Hck partner in that process. Interestingly, Cbl and Vav, two substrates of Hck (54, 55), comprise several phosphorylatable tyrosines and are known to regulate plasticity of the actin cytoskeleton during FcγR-mediated phagocytosis (47, 56). Vav proteins are exchange factors that catalyze GDP/GTP exchange, resulting in activation of small GTPases of the Rho subfamily (35, 57, 58). The possible involvement of Vav or Cbl in the signaling pathway between Hck and Cdc42/Rac is currently under study in the laboratory. In macrophages, expression of p59Hck was sufficient to trigger the formation of membrane protrusions to a similar extent as the constitutively active variant. One possible explanation could be that Hck finds its regular substrates more easily in these cells than in HeLa cells. For example, Vav1 expression is restricted to hematopoietic cells and is phosphorylated by Hck during macrophage activation (55). The ubiquitous Vav2 that shows 63% sequence similarity with Vav1 could replace it in the signaling pathway of Hck in HeLa cells. The other explanation could be that p59Hck overexpression mimics p59Hckα because its negative regulation is overwhelmed.

In this report, we present direct evidence that the Src family member p59Hck is able to control the formation of actin-rich protrusions. This effect is strictly dependent on its kinase activity and its plasma membrane association and not mimicked by p61Hck. The p59Hck signaling pathway involves Cdc42 and Rac, and a dominant negative form of p59Hck inhibits the FcγRIIa-dependent phagocytosis. We propose that a specific function of p59Hck is to act downstream of plasma membrane receptors to promote the Cdc42/Rac-dependent actin reorganization that occurs at the early steps of phagocytosis or during cell motility.

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