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Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages

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SUMMARY

Integrin-mediated adhesion of monocytes and macrophages initiates a signal transduction pathway that leads to actin cytoskeletal reorganization, cell migration and immunologic activation. This signaling pathway is critically dependent on tyrosine kinases. To investigate the role of the Src-family of tyrosine kinases in integrin signal transduction, we have examined the adhesive properties of macrophages isolated from *hck*$^{-/-}$*fgr*$^{-/-}$ double knockout mice which lack two of the three predominant Src-family kinases expressed in myeloid cells. Previous examination of polymorphonuclear leukocytes from these animals indicated that these kinases were critical in initiating the actin cytoskeletal rearrangements that lead to respiratory burst and granule secretion following integrin ligation. Double mutant peritoneal exudate macrophages demonstrated markedly reduced tyrosine phosphorylation responses compared to wild-type cells following plating on fibronectin, collagen or vitronectin-coated surfaces. Tyrosine phosphorylation of several actin-associated proteins (cortactin, paxillin, and tensin), as well as the Syk and Pyk2 tyrosine kinases, were all significantly reduced in double mutant cells. The subcellular localization of focal-adhesion associated proteins was also dramatically altered in mutant macrophages cultured on fibronectin-coated surfaces. In wild-type cells, filamentous actin, paxillin, and talin were concentrated along leading edges of the plasma membrane, suggesting that these proteins contribute to cellular polarization during migration in culture. Double mutant cells failed to show the polarized subcellular localization of these proteins. Likewise, double mutant macrophages failed to form normal filopodia under standard culture conditions. Together, these signaling and cytoskeletal defects may contribute to the reduced motility observed in in vitro assays. These data provide biochemical and morphological evidence that the Src-family kinases Hck and Fgr are required for normal integrin-mediated signal transduction in murine macrophages.

Key words: Src-family tyrosine kinase, Integrin signaling, Cell motility

INTRODUCTION

Macrophages are attracted to sites of injury and infection by chemotactic and cell activating factors. During this migration, macrophages interact with various extracellular matrix (ECM) proteins in the subendothelial basement membrane as well as with counter-receptors present on vascular endothelial cells and connective tissue cells (Butcher, 1991; Osborn, 1990; Zimmerman et al., 1992). These cell-cell and cell-ECM interactions are mediated by the integrin family of cell surface receptors (Hynes, 1992). In macrophages, the primary adhesive interactions with endothelial cells and ECM components occurs through the α4/β1, α5/β1, αv/β5, αLβ2, and αMβ2 integrins (Chuluyan and Issekutz, 1993; De Nichilo and Yamada, 1996; Meerschaert and Furie, 1994; Weber et al., 1996). The β1 integrins recognize the counter receptor VCAM-1 present on endothelial cells and the ECM proteins fibronectin, collagen and vitronectin, while the β2 integrins primarily recognize endothelial expressed ICAM-1. Blockade of β1 or β2 interactions inhibits binding and extravasation of monocytes into inflammatory sites (Chuluyan et al., 1995; Issekutz, 1995; Meerschaert and Furie, 1995).

Besides establishing a physical link between cells and ECM, integrins also serve as signal transducers that regulate aspects of cell growth, differentiation and motility (Assoian and Zhu, 1997; Clark and Brugge, 1995; Damsky and Werb, 1992; Kumar, 1998; Lafrenie and Yamada, 1996). In human and murine monocytes/macrophages, integrin cross-linking by fibronectin induces the expression of multiple inflammatory mediator genes including the cytokines IL-1β, TNF-α, IL-8, the procoagulant tissue factor protein, as well as causing the activation of transcription of factors such as NF-κB, c-*fos*, and

c-*jun* (Fan and Edgington, 1993; Juliano and Haskill, 1993; McGilvray et al., 1997; Shaw et al., 1990). The majority of these functional responses have been observed following crosslinking of surface β1 integrins. Cross-linking of leukocyte integrins by ECM molecules also leads to rearrangement of the actin cytoskeleton, cell spreading and migration (Miyamoto et al., 1995; Yamada and Geiger, 1997). Inhibition of actin cytoskeletal reorganization with cytochalasin D blocks most, but not all, functional responses elicited by integrin crosslinking in macrophages and other leukocytes (Lin et al., 1995; Zhou and Brown, 1993).

In monocytes/macrophages, as well as other cells, tyrosine phosphorylation events are critical in initiating the integrin-mediated signaling (Freedman et al., 1993; Fuortes et al., 1993; Kornberg et al., 1991; Lin et al., 1994; Romer et al., 1994). Three classes of tyrosine kinases have been implicated as the major signal transducers in the integrin pathway, the FAK-family kinases, pp125^{FAK} and Pyk2, the Src-family kinases, and p72^{Syk} (Syk) (Kaplan et al., 1995; Li et al., 1996; Lin et al., 1995; Parsons and Parsons, 1997; Schaller and Parsons, 1994). There is abundant evidence demonstrating that FAK localizes to focal contacts and becomes enzymatically activated in cells spreading on ECM-coated surfaces; FAK may also phosphorylate a variety of actin associated proteins, such as paxillin and tensin (Parsons, 1996; Richardson and Parsons, 1996). Fibroblasts from *fak*$^{-/-}$ embryos have increased numbers of focal adhesions, resulting in reduced cellular motility (Ilic et al., 1995, 1996). However, FAK is weakly expressed in monocytes/macrophages (Choi et al., 1993; De Nichilo and Yamada, 1996; Lin et al., 1994; Roach et al., 1997), suggesting that its function may be carried out by Pyk2, which is highly expressed in these cells (Hatch et al., 1998). Indeed, Pyk2 and Src have been shown to compensate for FAK deficiency in fibroblasts (Sieg et al., 1998). Syk has been implicated in integrin signaling as it becomes rapidly tyrosine phosphorylated in human monocytes spreading over fibronectin-coated surfaces and in human neutrophils adherent to fibrinogen (Lin et al., 1995; Yan et al., 1997). ZAP-70, the Syk homolog expressed in lymphocytes, is required for integrin-dependent motility in T-cells (Soede et al., 1998).

The role of Src-family tyrosine kinases in regulating cell adhesion in monocytes/macrophages has not been intensively studied; however, there is significant evidence from fibroblasts and other leukocytes that these kinases are critically involved in integrin-mediated signaling pathways. In spreading fibroblasts, p60$^{c\text{-}src}$ (Src) is complexed with FAK in a fashion that may directly regulate Src kinase activity (Schaller et al., 1994; Schlaepfer et al., 1994). Moreover, fibroblasts isolated from *src*$^{-/-}$ mice show defects in spreading on fibronectin-coated surfaces and impaired phosphorylation of cytoskeletal associated proteins such as p130^{cas} (Kaplan et al., 1995; Schlaepfer et al., 1997; Vuori et al., 1996). In osteoclasts, Src associates with microtubules following adhesion to ECM proteins such as vitronectin; the lack of this complex may explain the impaired ruffled border formation and altered cytoskeletal structure observed in *src*$^{-/-}$ osteoclasts (Abu-Amer et al., 1997; Schwartzberg et al., 1997).

The major Src-family kinases expressed in neutrophils, monocytes, and macrophages are p59/61^{hck} (Hck), p58$^{c\text{-}fgr}$ (Fgr) and p53/56^{lyn} (Lyn) (Tsygankov and Bolen, 1993). During neutrophil adhesion to fibrinogen-coated surfaces, these kinases become activated and associate with the cytoskeleton (Berton et al., 1994, 1996; Yan et al., 1995). Neutrophils isolated from *hck*$^{-/-}$*fgr*$^{-/-}$ double mutant mice are defective in ECM-adhesion induced respiratory burst and granule secretion, providing direct evidence that these kinases are required for normal integrin signaling in these cells (Lowell et al., 1996; Mocsai et al., 1999). Double mutant neutrophils also demonstrate retarded migration into the liver during systemic endotoxemia which may contribute to the reduced tissue damage observed in these animals following lipopolysaccharide adminstration (Lowell and Berton, 1998).

To directly assess the role of Hck and Fgr in integrin-mediated signaling, we examined adhesion-dependent tyrosine phosphorylation events in peritoneal exudate macrophages (PEMs) and bone-marrow derived macrophages (BMDMs) from *hck*$^{-/-}$, *fgr*$^{-/-}$ or *hck*$^{-/-}$*fgr*$^{-/-}$ mutant mice. Double mutant *hck*$^{-/-}$*fgr*$^{-/-}$ macrophages manifested very poor tyrosine phosphorylation events normally elicited by plating on ECM-coated surfaces, an abnormal morphology with reduced numbers of filopodia and reduced motility in migration assays. These data implicate the Src-family kinases Hck and Fgr in regulating critical events in integrin signaling and actin-cytoskeletal organization in primary murine macrophages.

MATERIALS AND METHODS

Antibodies

Rabbit antisera containing anti-Hck and anti-Fgr antibodies were raised by immunization with Hck-glutathionine S-transferase (GST) and Fgr-GST fusion proteins. Regions coding for the unique domain of each kinase (Hck amino acids 3-60; Fgr amino acids 3-67) were cloned into the pGEX-2t GST expression vector and GST fusion proteins were produced in *E. coli* as described (Grieco et al., 1992). Antisera were affinity purified by chromatography over columns containing the GST fusion protein covalently coupled to Affigel (Bio-Rad) and eluted with sequential glycine (pH 3.0) and triethylamine (pH 11.5) treatments as described (Harlow and Lane, 1988).

Mouse monoclonal antibodies reactive with paxillin, tensin, FAK, Syk, and actin filament associated protein (AFAP), were obtained from Transduction Laboratories (Lexington, KY). Anti-Pyk2 (N terminus) was from Santa Cruz Biotechnology (Santa Cruz, CA) and from D. Schlaepfer (Scripps Institute, La Jolla, CA). Anti-vinculin (VIN-11-5) antibody and anti-talin antibody were obtained from Sigma (St Louis, MO). Biotin-conjugated rat anti-mouse CD49d (α4 integrin) antibody, biotin-conjugated rat anti-mouse CD49e (RMFR5 or 5H10 clone, α5 integrin) antibody, hamster anti-mouse/rat CD29 (Ha2/5 clone, β1 subunit) monoclonal antibodies were obtained from Pharmingen (San Diego, CA). Lissamine rhodamine (LRSC)-conjugated affinity pure donkey anti-rat IgG (H+L) was obtained from Jackson ImmunoResearch Labs (West Grove, PA). Goat F(ab′)$_2$ anti-hamster IgG (H+L), rabbit F(ab′)$_2$ anti-rat IgG (H+L), goat anti-rabbit IgG rhodamine conjugate, and goat anti-mouse IgG fluorescein conjugate were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Anti-p80/85 (Cortactin), anti pp125^{FAK} (BC3) rabbit polyclonal antibody, and anti-p130^{cas} rabbit polyclonal antibody were the generous gifts of Dr J. Thomas Parsons (Department of Microbiology, Univ. of Virginia). Affinity-isolated anti-mouse horseradish peroxidase conjugate was obtained from CalTag Laboratories (South San Francisco, CA). Anti-phosphotyrosine mAb 4G10 was obtained from UBI Products (Lake Placid, New York). Antisera were used at 1 μg/ml for immunoblotting and 1 μg per 250 μg of protein extract for immunoprecipitation. For immunofluorescence, primary antibodies were used at a dilution of

1:25-1:100 while secondary antibodies were used at a dilution of 1:100.

Cell culture

PEMs were obtained by intraperitoneal injection of 2 ml of 2% thioglycolate medium (Gibco) followed by peritoneal lavage with PBS/0.2% BSA 3-5 days later. The resulting cells were then resuspended in DMEM without serum and maintained at 37°C in polypropylene culture tubes, with rocking for 1-2 hours. Subsequently, cells were washed in fresh DMEM and resuspended to a concentration of 2.5×10^6/ml in fresh DMEM/no serum. Cells were then plated on dishes pre-coated with an ECM protein as described below.

BMDMs were isolated from mouse femurs and tibias. Bone marrow was flushed from these bones into 50 ml Falcon tubes with PBS/2% FBS, followed by disaggregation by pipeting through an 18 g needle attached to a 20 ml syringe. Cells were centrifuged and RBCs were lysed by resuspension in 5 ml of NH_4Cl lysis solution (Lowell et al., 1996). After 5 minutes, the cells were centrifuged, resuspended in α-MEM/10% FBS, and plated in 150 cm^2 flasks supplemented with 20% murine L-cell conditioned medium (LCM). After 2 days, the nonadherent cells were isolated and cultured in α-MEM/10% FBS/20% LCM on standard tissue culture dishes until sub-confluent monolayers of adherent macrophages were obtained.

Preparation of ECM-coated plates

Mouse type I collagen, vitronectin, and fibronectin were obtained from Collaborative Biomedical Products (Bedford, MA). All ECM proteins were diluted to a concentration of 10 μg/ml in PBS. 5 ml of the diluted proteins were added to standard tissue culture plates and incubated for 1-2 hours at 37°C or overnight at 4°C. ECM proteins were then removed, the plates were rinsed twice with PBS then blocked by incubation with PBS/2% BSA for 1 hour at 37°C, following which plates were rinsed with PBS and used for adhesion assays. Glass coverslips placed in six-well tissue culture dishes were coated similarly.

Cell lysate preparation and immunoblotting

5×10^6 PEMs suspended in 2 ml of DMEM/no serum were plated on ECM-coated 10 cm dishes. Following 15, 30, and 45 minutes of incubation at 37°C, adherent cells were washed once with ice-cold PBS and solubilized in 500 μl of ice-cold lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, 10 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 1 mM Na_3VO_4, 1 mM PMSF, and 1 μg/ml each of leupeptin/pepstatin/aprotinin). Dishes were then rocked for 10 minutes at 4°C. Resulting lysates were collected with a cell scraper and cleared by centrifugation for 15 minutes at 4°C. Protein concentration was determined by Bio-Rad protein assay. Samples (50 μg) were electrophoresed on a 10% SDS-polyacrylamide gel (SDS-PAGE) and electrotransferred to nitrocellulose for 8 hours at 30 V as previously described (Harlow and Lane, 1988). Equal loading and transfer of proteins in all samples was confirmed by Ponceau S (Sigma) staining of nitrocellulose filters. Filters were blocked with TBS (Tris-buffered saline: 150 mM NaCl, 25 mM Tris pH 7.5) + 0.2% Tween + 2% BSA for 1 hour at room temperature. After one wash with TBS + 0.2% Tween, filters were incubated with primary antibody for 1 hour at room temperature. Following another two washes, filters were incubated with horseradish peroxidase-conjugated anti-mouse antibody (Boehringer) in TBS, 0.2% BSA, and 0.2% Tween 20 for 1 hour at room temperature. The Enhanced Chemiluminescence detection system (Amersham) was used to develop the filters.

Immunoprecipitation and autophosphorylation assay

250 μg of protein extracts were incubated with 1-2 μg of anti-Hck or anti-Fgr antisera for 1-2 hours at 4°C. Immediately afterwards, the extracts were mixed with Protein A-coupled Sepharose beads (Sigma) for 30-60 minutes at 4°C and washed with lysis buffer (+$NaVO_3$, –protease inhibitors). If immunoblotting was to be done, the immunoprecipiates were subsequently resuspended in Laemmli buffer (Harlow and Lane, 1988), boiled for 5 minutes, and loaded on SDS-PAGE gels.

If an in vitro kinase assay was to be done, the immunoprecipitates were washed twice in kinase buffer (20 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 0.5% Triton X-100, 10 mM $MnCl_2$, 0.1 mM $NaVO_3$) and incubated with kinase buffer + 1 μCi of [γ-^{32}P]ATP + 1 μM ATP for 15 minutes. Autophosphorylation was terminated by washing the beads in lysis buffer. Samples were then resuspended in Laemmli buffer and electrophoresed on a 10% SDS-PAGE gel.

Flow cytometry

1×10^6 PEMs were stained with the appropriate monoclonal antibody for 1 hour at 4°C, washed once, stained with secondary reagents (FITC- or PE-conjugated strepavidin or goat anti-rat; Boehringer Mannheim Biochemicals, Indianapolis, IN), washed twice, and resuspended in 1 ml PBS/0.2% FBS + 1 μg/ml propidium iodide. Viable cells were analyzed with FACScan (Becton Dickinson, Mountain View, CA). Background staining was assessed by omitting the primary antibody.

Integrin cross-linking

Equal numbers of PEMs suspended in DMEM were aliquoted into Eppendorf tubes. As appropriate, each tube was incubated with anti-α5 or anti-β1 mAb (5 μg/ml) or no primary antibody for 45 minutes on ice. The samples were then washed with cold DMEM and incubated with DMEM in the presence or absence of secondary $F(ab')_2$ antibody (25 μg/ml) at 37°C for the time periods indicated. Cells were centrifuged and lysed directly in the Eppendorf using the protocol described above.

Cell microscopy

BMDMs used for phase microscopy were obtained by cultivating bone marrow cells in RPMI/15% FBS supplemented with 10% LCM in gelatin-coated dishes as described (Lin and Gordon, 1979). After 4 days of culture, phase microscopy photographs of wild-type and mutant adherent macrophages were taken at ×40. PEMs (elicited by injection of 1% casein rather than thioglycolate) were plated in DMEM/5% FBS plates and photographed 48 hours later.

Immunofluorescence

Coverslips were heat sterilized, placed in 6 well plates (Falcon), and coated with 100 μg/ml fibronectin (Sigma) for 1-2 hours at 37°C or overnight at 4°C. After two PBS washes, 1×10^5 BMDMs from sub-confluent cultures were re-plated on the coverslips and allowed to spread for 45 minutes or cultured (in α-MEM/10% FCS/20% LCM) for 4 days. Prior to fixing, cells cultured for 4 days were incubated in α-MEM/no serum for 3 hours. Serum deprivation for 3 hours did not effect viability of wild-type or mutant cells; however, culture in serum-free medium beyond 24 hours resulted in progressive cell death (data not shown). The samples were washed twice with PBS and fixed in freshly prepared 2% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were then permeabilized in 0.1% Triton X-100 for 4 minutes at room temperature. Following two more PBS washes, the coverslips were blocked with 2% BSA in PBS for 1 hour. Slips were incubated in primary Ab/PBS/2% BSA solution for 45 minutes at room temperature, washed twice, incubated with secondary Ab dilution for 30 minutes, washed in PBS and water, and mounted on glycerol 3% n-propylgallate. Slides were then visualized by immunofluorescence microscopy. Actin was stained with 1:100 dilution of rhodamine phalloidin obtained from Molecular Probes (Eugene, OR). Paxillin, tensin, vinculin, and talin were used at a dilution of 1:100. The secondary antibody used was fluorescein-conjugated goat anti-mouse IgG (Biosource) used at a dilution of 1:100.

In vitro migration assays

Cell migration was assessed using a culture model in which a portion of a confluent monolayer of BMDMs was denuded of cells by scraping with a pipette tip then the number of cells migrating into the open space was assesed microscopically. This 'wound healing' assay has been used previously to assess cell migration in other cell types (Tamura et al., 1998) BMDMs were cultured in 24-well plates until confluent, then the monolayers were wounded by scratching with the tip of a pipette and photomicrographs were taken immediatedly and at various intervals thereafter to assess cell migration. The number of cells migrating into the open space was counted microscopically. Migration was also conducted on bacterial (non-tissue culture) plates, which revealed much faster kinetics than on tissue culture plates.

Migration was also assessed using modified Boyden chemotaxis chambers. Cells were incubated in the upper well of 6-well dishes containing a tissue culture insert with 8 μm pores through a polyvinylpyrrolidone-free polycarbonate membrane filter (Bio-Coat; Becton Dickinson, San Jose, CA), coated with fibronectin (at 10 μg/ml for 1 hour at 37°C), that separated the upper and lower wells (Falk et al., 1980). After incubation for 0, 6, 12, 18, and 24 hours at 37°C, nonmigrating cells on the upper surface of the membrane were removed by scraping using cotton tipped swabs and macrophages on the bottom surface of the membrane were stained with Diff-Quick stain then counted microscopically. Similar migration experiments were conducted with Matrigel-coated filters (Bio-coat, a complex of type IV collagen, laminin, and other basement membrane proteins). Matrigel filter inserts were rehydrated in DMEM for 1-2 hours at 37°C before the cell suspensions were added. Migration was conducted for the time periods indicated and migrated cells on the under surface of the filter were counted microscopically.

RESULTS

Integrin clustering activates Hck and Fgr

To determine if the enzymatic activity of Hck and Fgr were effected in macrophages spreading on ECM-coated surfaces, we examined the tyrosine kinase activity of these proteins by immunocomplex auto-kinase assay. Adherence of PEMs to fibronectin produced a rapid 3- to 4-fold activation of Hck and Fgr kinase activity, compared to non-adherent cells, which diminished slightly over the 45 minutes time course of the assay (Fig. 1A and B). This time course is similar to that previously reported for Fgr and Lyn activation in adherent neutrophils as well as Hck and Lyn activation in macrophages spreading on plastic (Berton et al., 1994; Roach et al., 1997; Yan et al., 1995).

To confirm that activation of Hck and Fgr in fibronectin-adherent macrophages was due to signals elicited by integrin clustering (versus other adhesion receptors) we directly crosslinked integrin subunits on PEMs in suspension using anti-α5 and β1 mAbs. As shown in Fig. 1C-F, crosslinking of α5 and β1 integrin subunits (using primary anti-integrin mAbs followed by secondary anti-mAb treatment) resulted in a level of enzymatic activation of Hck and Fgr similar to that seen in cells spreading on fibronectin-coated surfaces.

Cell-surface expression of integrin subunits is normal in *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs

In order to objectively compare the integrin signaling events in macrophages from wild-type and mutant mice, we first examined the expression levels of various integrin subunits on these cells by flow cytometry. FACS analysis revealed equivalent levels of expression of the α4, α5, αM, β1, β2, and β3 integrin subunits in wild-type and *hck*$^{-/-}$*fgr*$^{-/-}$ mutant PEMs (Fig. 2). Relative to the control (secondary stain alone) cells, PEMs showed highest expression of αMβ2, while the α4β1 and α5β1 dimers were intermediate and β3 expression was low. Expression of these integrin subunits, as well as α6, αL, αX, αv and β5, have been previously reported on human macrophages (De Nichilo and Burns, 1993).

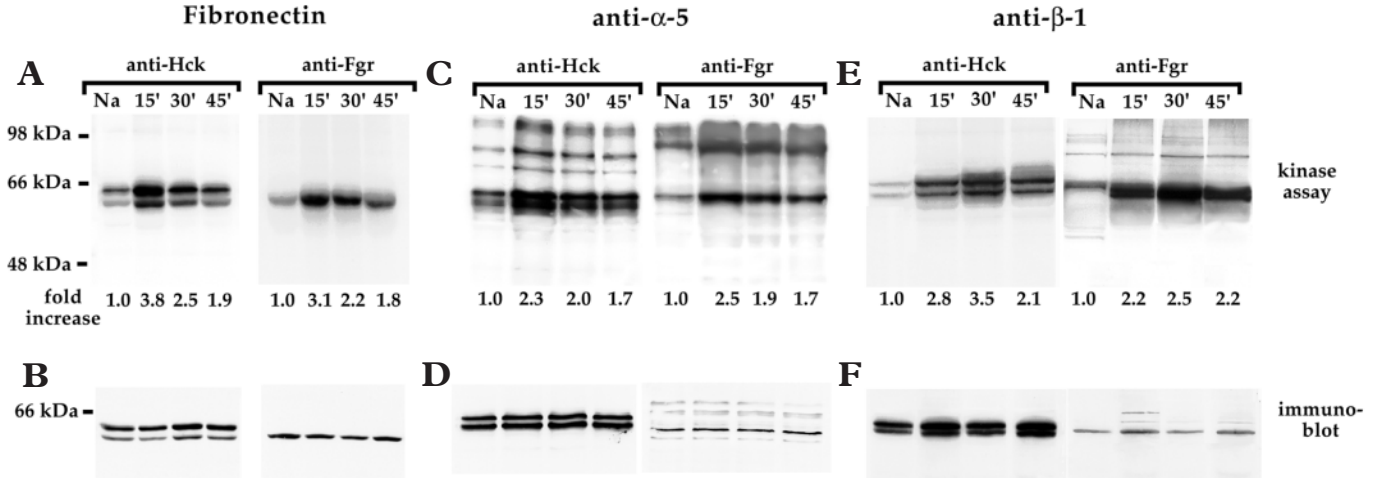

Fig. 1. Hck and Fgr are activated in PEMs adhering to fibronectin or following crosslinking of α5 or β1 integrins. (A) Wild-type PEMs were plated for 0 (Na=non-adherent), 15, 30, and 45 minutes on fibronectin-coated dishes, adherent cells were washed and lysed. Protein extracts were immunoprecipitated with anti-Hck and anti-Fgr antibodies and an in vitro kinase assay was conducted. Autophosphorylated Hck and Fgr were resolved on a 10% SDS-PAGE followed by autoradiography and quantitation by phosphoimager analysis. (B) 30 μg of each lysate was resolved on a 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with the anti-Hck and anti-Fgr antibodies to confirm equal p59/61^{hck} and p58^{c-fgr} protein levels in all samples. (C and E) Wild-type PEMs (5×10^6/sample) were incubated in suspension with anti-α5 or anti-β1 mAbs (5 μg/ml) for 45 minutes at 4°C, followed by treatment with secondary antibodies ($F(ab')_2$ anti-rat IgG or $F(ab')_2$ anti-hamster IgG at 25 μg/ml) for the indicated times at 37°C. Cells were lysed and protein extracts were immunoprecipitated with anti-Hck or anti-Fgr then subjected to autokinase assay. (D and F) Immunoblots of same lysates.

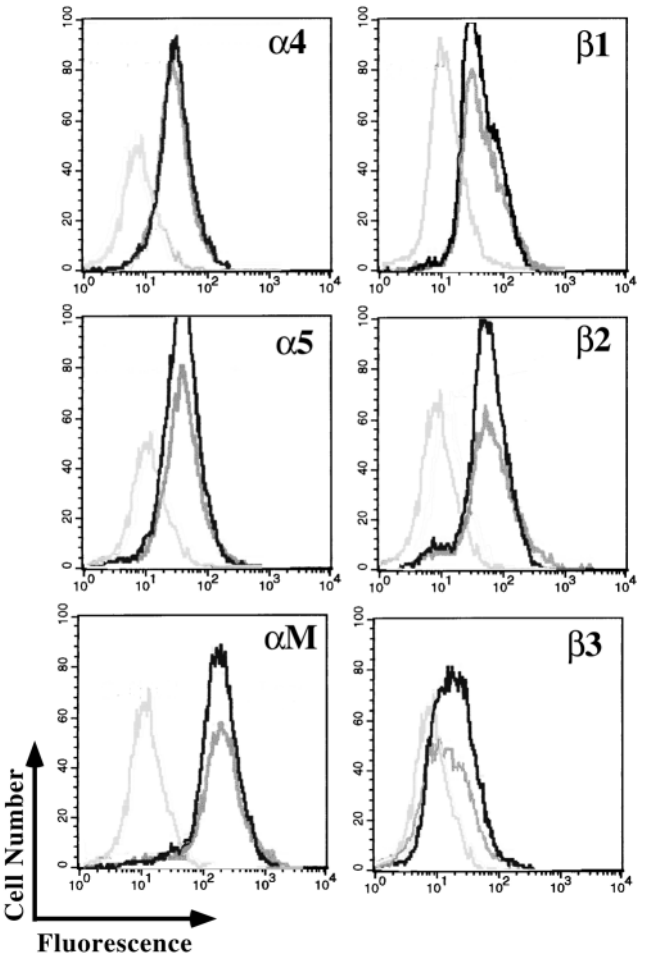

Fig. 2. *Hck*$^{-/-}$*fgr*$^{-/-}$ PEMs express normal levels of integrin subunits α4, α5, αM, β1, β2 and β3. PEMs (10^6/sample) taken from wild-type and mutant mice were stained with biotinylated anti-α4, α5, αM, β1, β2 and β3 monoclonal antibodies for 1 hour at 4°C. Cells were washed and incubated with FITC-conjugated strepavidin and then analyzed by flow cytometry. Black lines indicate wild-type cells, dark grey represents *hck*$^{-/-}$*fgr*$^{-/-}$ cells, and non-specific staining using FITC-streptavidin alone is shown as the light grey plot.

Impaired tyrosine phosphorylation responses in *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs adherent to ECM proteins

To determine if the absence of the Hck and Fgr tyrosine kinases resulted in impaired integrin signaling we examined total protein tyrosine phosphorylation levels in wild-type and *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs plated on several ECM-coated surfaces. Wild-type cells plated on fibronectin and vitronectin showed dramatic increases in tyrosine phosphorylation (Fig. 3A) while less substantial changes were observed following plating on collagen type I coated surfaces (to which cells were less adherent). Double mutant *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs showed substantially reduced induction of tyrosine phosphorylation compared to wild-type cells, especially in cells adherent to fibronectin. Large differences in overall tyrosine phosphorylation between wild-type and mutant cells were also observed in cells plated on tissue culture plastic alone (data not shown). In contrast, single mutant *hck*$^{-/-}$ or *fgr*$^{-/-}$ PEMs produced completely normal tyrosine phosphorylation responses following binding and spreading on fibronectin-coated plates (Fig. 3B). Hence, similar to previously reported results with PMNs (Lowell et al., 1996) the defects observed in integrin-signaling required the absence of at least two of the three major Src-family kinases in these cells.

hck$^{-/-}$*fgr*$^{-/-}$ PEMs have defective tyrosine phosphorylation responses following mAb-induced crosslinking of α5 and β1 integrin subunits

To confirm that differences in overall tyrosine phosphorylation between wild-type and mutant macrophages were due to integrin-specific signaling defects we crosslinked integrin subunits directly with anti-α5 and β1 mAbs as described above. As shown in Fig. 4, in solution crosslinking of α5 and β1 integrin subunits produced tyrosine phosphorylation responses in wild-type PEMs but only very weak responses were observed in mutant PEMs. Some tyrosine phosphorylation was observed with primary antibody crosslinking alone, although this was clearly enhanced following supra-crosslinking with secondary antibodies. Tyrosine phosphorylation responses were, in general, much less robust using antibody crosslinking in suspended cells, especially with the anti-α4 mAb available to us, than that observed following adhesion and spreading on ECM protein coated surfaces (not shown). Nevertheless, we conclude that following either binding and spreading on ECM protein-coated surfaces or with direct crosslinking of integrin subunits, the *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs manifest severely impaired tyrosine phosphorylation responses.

Impaired tyrosine phosphorylation of cytoskeletal associated proteins, Pyk2 and Syk in *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs spreading on fibronectin.

Several cytoskeletal associated proteins are known to be complexed with Src-family kinases and/or become tyrosine phosphorylated following cell adhesion (Yamada and Geiger, 1997). To determine whether these proteins were effected in *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs, we carried out a series of immunoprecipitation/immunoblotting experiments. As shown in Fig. 5, significant differences in the levels of tyrosine phosphorylation of paxillin, tensin, cortactin, Pyk2 and Syk were observed in *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs compared to wild-type cells following 45 minutes of adhesion to fibronectin-coated surfaces. In all cases, tyrosine phosphorylation of these substrates was not completely abolished, however, it was significantly reduced. There were no detectable changes in the overall expression levels of these proteins in wild-type versus mutant PEMs. In contrast to the above molecules, we observed no significant differences in tyrosine phosphorylation of vinculin between mutant and wild-type cells. Expression of p130^{cas}, which has been described as a major tyrosine phosphorylated protein in adherent fibroblasts (Harte et al., 1996; Vuori et al., 1996; Vuori and Ruoslahti, 1995), appeared to be low in PEMs and we were unable to reach a conclusion concerning its phosphorylation status in adhering cells. In agreement with previous reports (Choi et al., 1993; De Nichilo and Yamada, 1996; Lin et al., 1994; Roach et al., 1997), we found that FAK expression was very weak in PEMs and BMDMs from both wild-type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice, hence we were unable to observe changes in the tyrosine phosphorylation status or enzymatic activity of FAK in adherent cells (F. Meng

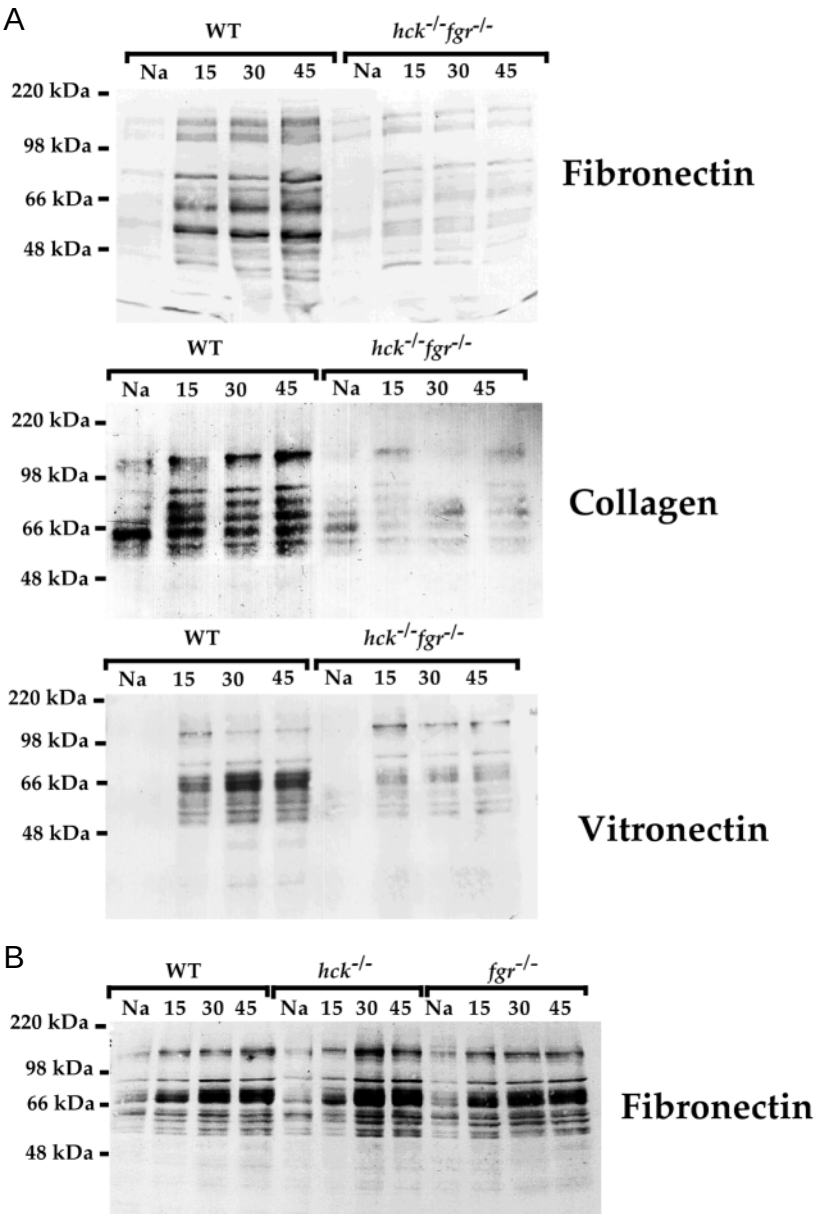

Fig. 3. Double mutant PEMs show impaired tyrosine phosphorylation responses following plating on ECM-coated surfaces. (A) Wild-type (WT) and *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs were plated for 0 (Na=non-adherent), 15, 30, and 45 minutes on fibronectin, collagen type 1, or vitronectin-coated dishes; adherent cells were washed, lysed and protein extracts made. 50 μg of each sample was electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose then immunoblotted with anti-phosphotyrosine antibody. Equal loading and transfer of total protein lysates for all samples was confirmed by Ponceau S staining of nitrocellulose filters. (B) Wild-type and single mutant *hck*$^{-/-}$ or *fgr*$^{-/-}$ PEMs were plated on fibronectin as above and cell lysates were immunoblotted with anti-phosphotyrosine antibody.

and C. A. Lowell, unpublished observation). We conclude that the defective β1 integrin-dependent tyrosine phosphorylation responses in *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs results in impaired phosphorylation of a subset of focal adhesion associated proteins.

hck$^{-/-}$*fgr*$^{-/-}$ BMDMs and casein-elicited PEMs show altered morphology with reduced numbers of filopodia

In the course of these experiments we observed that *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs and BMDMs cultured under a variety of conditions showed an altered morphology compared to wild-type macrophages. An example of such a difference is shown in Fig. 6. A significant finding was that *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs showed reduced numbers of filopodia compared to wild-type cells (A and B). In contrast, we observed no significant morphologic differences between wild-type and single mutant BMDMs (not shown). Overall, *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs showed an average 3-fold reduction in the number of filopodia, with a corresponding increase in membrane structures consistent with lamellopodia, compared to wild-type, *hck*$^{-/-}$ or *fgr*$^{-/-}$ single mutant cells. Even more striking morphological differences were observed in casein-elicited PEMs. In fact, double mutant PEMs maintained in DMEM/5% FBS did not form filopodial extensions and were observed to remain rounded (Fig. 6C and D show cellular morphology at 96 hours of culture but the same differences were seen at earlier time points). Additionally, Biogel-elicited PEMs showed similar alterations in morphology (not shown), while the morphology of wild-type and mutant thioglycolate-elicited cells was altered due to the presence of large amounts of intracellular thioglycolate.

Altered actin cytoskeleton and sub-cellular localization of focal adhesion-associated proteins in *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs

To determine if the morphological abnormalities and impaired tyrosine phosphorylation events observed in the *hck*$^{-/-}$*fgr*$^{-/-}$ macrophages would correlate with an altered subcellular localization of cytoskeletal associated proteins, we carried out a series of immunofluorescent studies of macrophages plated and cultured for varying times on fibronectin-coated surfaces. At short periods following plating, mutant cells showed a consistent delay in spreading and instead maintained a rounded morphology (Fig. 7, upper panels). After several days of culture mutant cells were fully spread but showed a markedly abnormal actin cytoskeletal structure revealed by staining with phalloidin. F-actin filaments were best observed in wild-type cells along the leading edges of cell migration, while in mutant cells displayed large numbers of microspikes, reminiscent of the appearance of fibroblasts from *fak*$^{-/-}$ mutant mice (Ilic et al., 1995), distributed around the cell borders and disorganized bundles of F-actin. In contrast, double mutant BMDMs had a normal appearing tubulin filamentous network. Similar to the F-actin, both paxillin and talin appeared to be concentrated at the leading edges of cell migration in wild-type cells while these proteins were randomly distributed around the cell borders in the *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs. Tensin was observed to be concentrated at specific focal points over the entire cell body

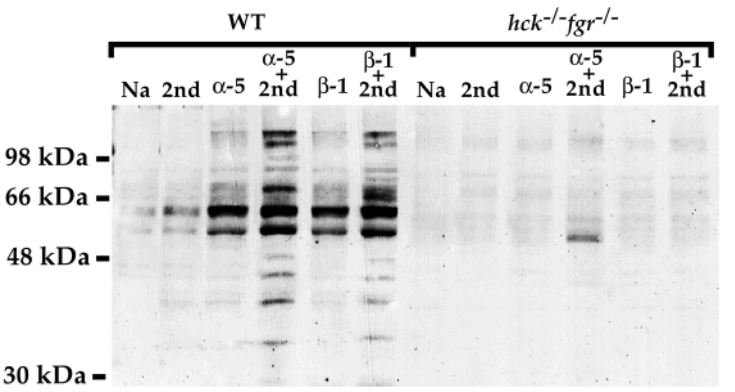

Fig. 4. *Hck*$^{-/-}$*fgr*$^{-/-}$ PEMs have impaired tyrosine phosphorylation responses following crosslinking of α5 and β1 integrin subunits. Wild-type (WT) and *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs (5×10^6/sample) were incubated in suspension with anti-α5 or anti-β1 mAbs (5 μg/ml) for 45 minutes at 4°C. Some samples were further incubated with the appropriate secondary antibodies (F(ab′)$_2$ anti-rat IgG or F(ab′)$_2$ anti-hamster IgG at 25 μg/ml) for 15 minutes at 37°C. Cells were also treated with secondary antibodies alone (45 minutes at 4°C) as a control. Cells were then lysed and the resulting protein extracts (50 μg) were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with anti-phosphotyrosine antibody. Equal loading and transfer of total protein lysates for all samples was confirmed by Ponceau S staining of nitrocellulose filters. All primary and secondary antibodies were non-murine in origin, so that they would not interfere with the phosphotyrosine immunoblots. Na=non-adherent – non-Ab treated. 2nd=secondary antibody crosslinking.

in wild-type macrophages but was found diffusely throughout the cytoplasm in *hck*$^{-/-}$*fgr*$^{-/-}$ cells. The localization of other cytoskeletal regulatory proteins such as vinculin, integrin linked kinase (Hannigan et al., 1996), β1 and β2 integrin subunits as well as Pyk2 showed no difference between mutant and wild-type cells (not shown). Staining for the ER-associated protein calreticulin also showed no differences, while specific immunofluorescent staining of cortactin was not achieved. We conclude that double mutant *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs have an altered actin cytoskeletal structure with impaired localization of paxillin, talin, and tensin.

hck$^{-/-}$*fgr*$^{-/-}$ macrophages have impaired migration

To determine whether the deficit in filopodia and the altered actin cytoskeleton in double mutant cells affected the ability of these cells to migrate in culture, we used an in vitro wound healing assay. In this assay, confluent monolayers were scratched with a pipette tip and cell migration into the empty areas of the culture was monitored microscopically. As shown in Fig. 8, *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs demonstrated clearly retarded migration in this assay. Direct quantitation revealed a 2- to 3-fold difference in the rate of migration between wild-type and mutant macrophages (Fig. 9A). Differences in the rate of cell growth could not account for the inability of *hck*$^{-/-}$*fgr*$^{-/-}$ macrophages to fill in the empty spaces in the wound assay, as mutant cells and wild-type cells showed equal growth kinetics in dose-response curves to M-CSF and GM-CSF stimulation (R. Holmes et al., not shown).

Cell migration was also assessed using 2 chamber Boyden assays. In this assay, cell migration through 8 μm pores, on fibronectin-coated membranes or through Matrigel, was determined at various times after plating. As shown in Fig. 9B and C, double mutant thioglycolate-PEMs demonstrated a clear delay, but not an absolute block, in migration in these assays. We conclude that the absence of Hck and Fgr results in significantly reduced migration in both BMDMs and PEMs.

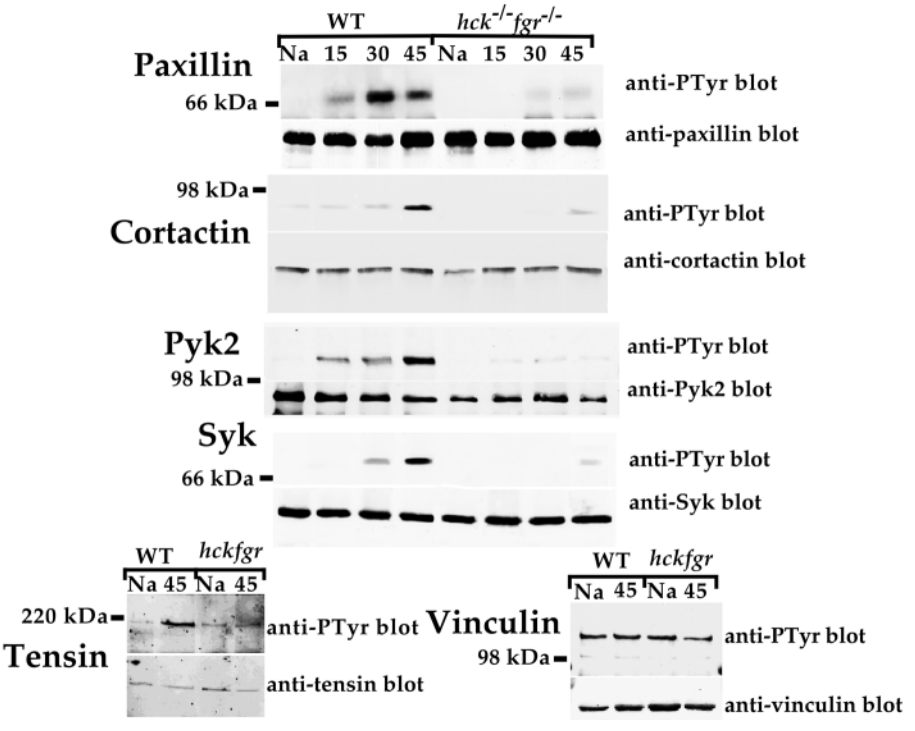

Fig. 5. *Hck*$^{-/-}$*fgr*$^{-/-}$ macrophages showed impaired tyrosine phosphorylation of cytoskeletal associated proteins following adhesion to fibronectin. Wild-type (WT) and *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs were plated for 0 (Na=non-adherent), 15, 30, and 45 minutes on fibronectin-coated dishes, adherent cells were washed, lysed and protein extracts made. 250 μg of each cell lysate was used for immunoprecipitation with antibodies against the indicated proteins, followed by electrophoresis on 8% SDS-PAGE, then transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibody. To confirm equal precipitation of all proteins, blots were stripped and re-blotted with the same antibody used for immunoprecipitation (except for Pyk 2 which was immunoprecipitated with N-terminal specific antisera and re-probed with rabbit polyclonal sera).

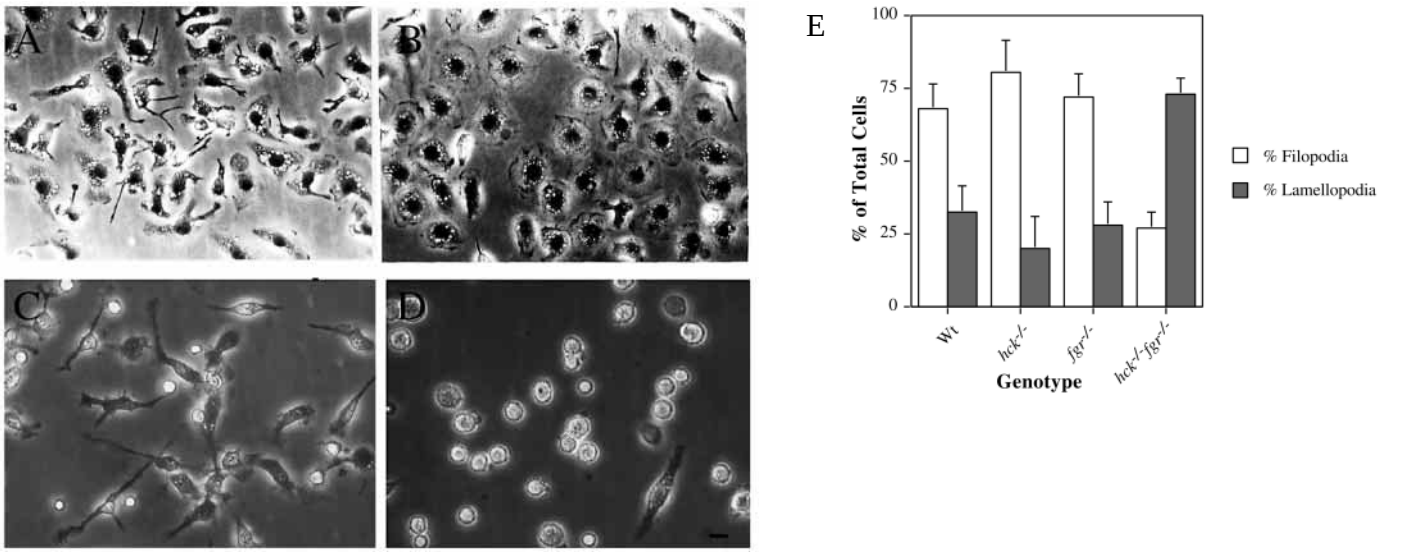

Fig. 6. PEMs and BMDMs from *hck*$^{-/-}$*fgr*$^{-/-}$ mice have altered morphology and reduced filopodia. BMDMs from (A) wild-type or (B) *hck*$^{-/-}$*fgr*$^{-/-}$ mice were maintained for 4 days in DMEM/15% FBS/10% LCM in gelatin-coated dishes then photographed under phase microscopy. Casein-elicited PEMs from (C) wild-type or (D) *hck*$^{-/-}$*fgr*$^{-/-}$ animals were cultured in DMEM/5% FBS for 48 hours and then photographed. Bar, 10 μm. (E) BMDMs from wild-type, *hck*$^{-/-}$ single mutant, *fgr*$^{-/-}$ single mutant, and *hck*$^{-/-}$*fgr*$^{-/-}$ double mutant mice were cultured on gelatin-coated plates for 4 days, following which the number of cells with filopodia or lamellopodia were counted. For each genotype, 20 different fields were counted and the results averaged. Results are combined from multiple experiments. Error bars indicate the standard deviation for each condition.

DISCUSSION

When integrins bind to ECM proteins, they physically link the ECM on the outside of the cell with the actin cytoskeleton on the inside of the cell (Clark and Brugge, 1995). In addition, adhesion-dependent signaling pathways that regulate the actin cytoskeleton are also required for many of the functional responses elicited by integrin-mediated cell adhesion. In this work, we demonstrate that the Src-family kinases, Hck and Fgr, are central to integrin-dependent signaling in macrophages. In the absence of both these kinases, macrophages are unable to initiate normal tyrosine

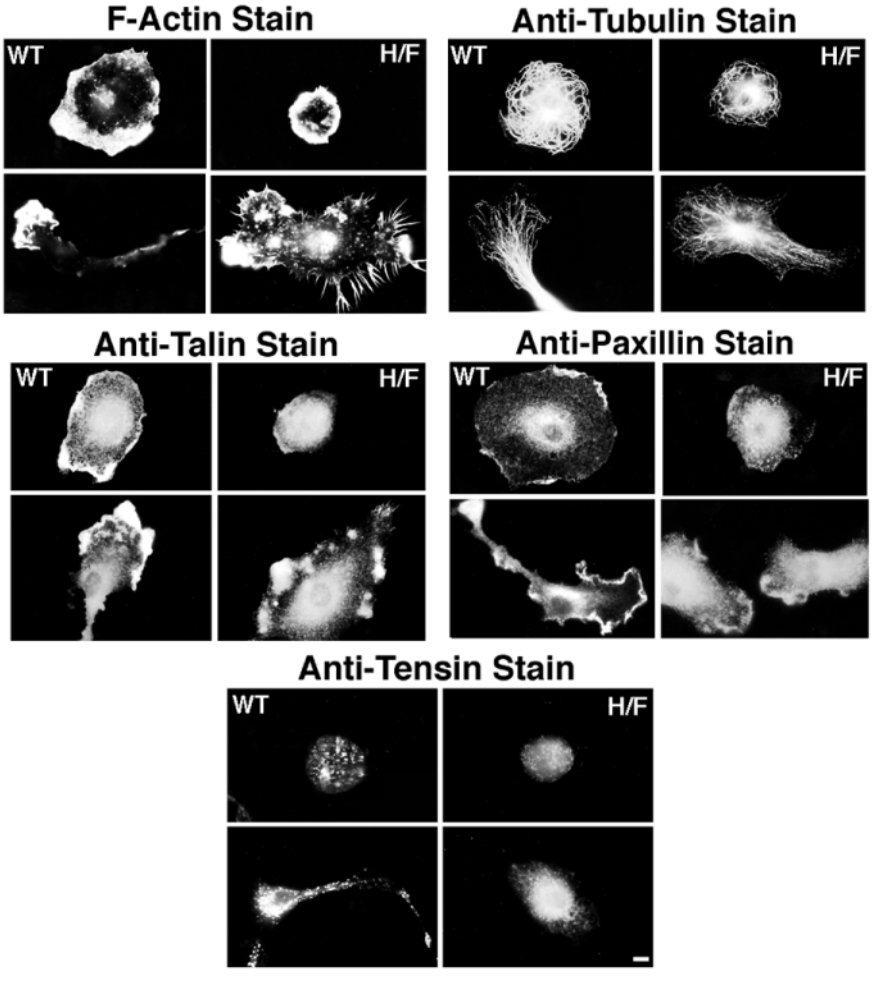

Fig. 7. *Hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs show abnormal subcellular localization of focal adhesion associated proteins and an abnormal actin cytoskeleton. Wild-type (WT) and *hck*$^{-/-}$*fgr*$^{-/-}$ (H/F) macrophages were plated on fibronectin-coated coverslips and cultured for 45 minutes or 4 days, then fixed in 2% paraformaldehyde and processed for immunofluorescent staining with the antibodies shown. Cells cultured for 4 days were serum starved for 3 hours prior to fixing. The photomicrographs in the upper panels show cells plated for 45 minutes, the lower pictures show cells after 96 hours of culture. For F-actin staining, rhodamine-conjugated phalloidin was used; for other proteins primary and secondary antibodies were used as described in Materials and Methods. Bar, 3 μm.

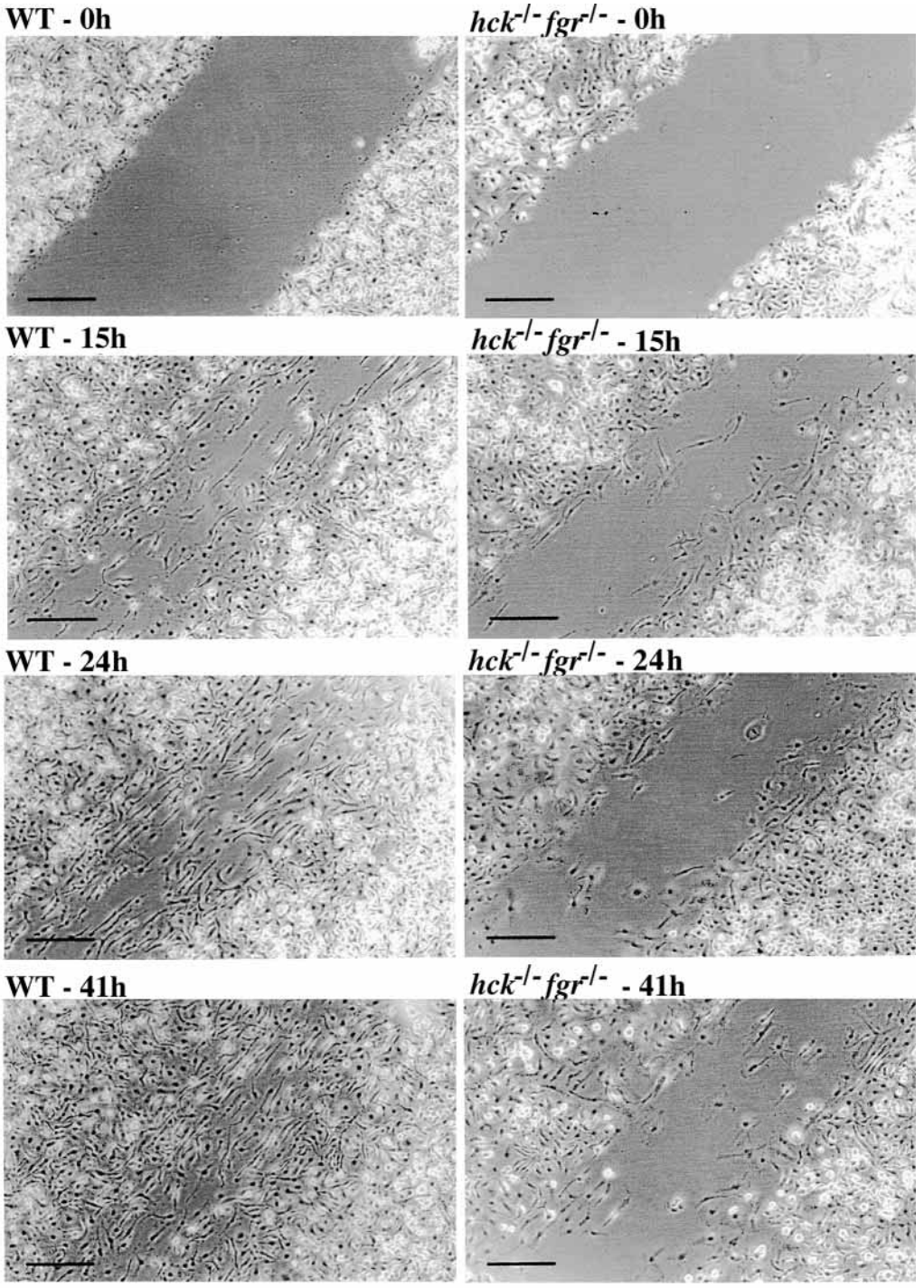

Fig. 8. *Hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs demonstrate reduced migration in an in vitro wound healing assay. BMDMs were cultured in 24-well plates in DMEM/15% FBS/10% LCM for 10 days until confluent. Macrophage monolayers were wounded by scratching with the tip of a pipette. Photomicrographs were taken immediatedly and at the indicated times after wounding. Bars, 50 μm. Representative of 3 independent experiments.

phosphorylation responses after integrin cross-linking and demonstrate an altered morphology, impaired cytoskeletal structure and retarded in vitro migration. In contrast, deficiencies of Hck or Fgr alone do not result in these signaling or functional defects, suggesting that these kinases serve overlapping or redundant roles in the integrin signaling pathway.

We chose to use peritoneal exudate macrophages for most of the signaling experiments in this work because these cells were readily isolated in the non-adherent state and they demonstrated very robust tyrosine phosphorylation responses following adhesion and spreading. In similar signaling experiments with BMDMs, in which the cells were detached from tissue culture plates and then re-plated, tyrosine phosphorylation responses were not as strong as those observed with PEMs, and suspended cells showed higher background phosphorylation. However, BMDMs were clearly superior for morphologic studies, revealing defects in cytoskeletal structure that were consistent with the signaling experiments done using PEMs. Therefore, it is likely, though not formally proved, that under correct culture conditions (such as lack of significant prior adhesion) *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs would manifest the same adhesion-dependent signaling defects observed in PEMs and that *hck*$^{-/-}$*fgr*$^{-/-}$ PEMs (especially cells elicited by compounds other that thioglycolate) would show the same morphological abnormalities observed in the BMDMs. In other words, we have no reason to suspect that the defects observed in the mutant cells are specific to one particular macrophage type.

To begin to assess which intracellular proteins were not appropriately phosphorylated in the *hck*$^{-/-}$*fgr*$^{-/-}$ macrophages, we examined a number of focal adhesion associated proteins that had previously been shown to be tyrosine phosphorylated following integrin-mediated adhesion (Yamada and Geiger, 1997). Of the proteins we tested, clear impairments in paxillin, tensin, and cortactin tyrosine phosphorylation were observed in mutant cells. Likewise, phosphorylation of both Pyk2 and Syk were dramatically decreased in double mutant cells. This is not unexpected, as both of these kinases are felt to be activated subsequent to Src-family kinase activation; Pyk2 is a

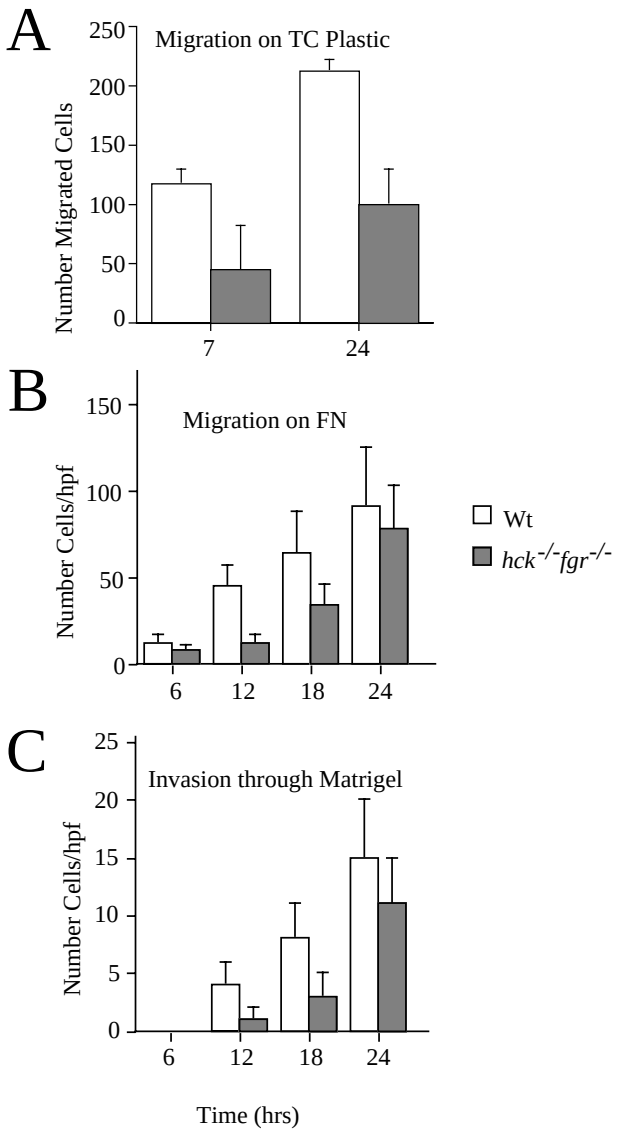

Fig. 9. *Hck*$^{-/-}$*fgr*$^{-/-}$ macrophages manifest reduced migration in vitro. (A) Quantitation of the in vitro wound healing assay shown above. The number of cells that had migrated into the open area of the plate were counted directly and data averaged from three separate experiments which had the 7 and 24 hour time points in common. Error bars represent ± s.d. (B and C) Wild-type and *hck*$^{-/-}$*fgr*$^{-/-}$ mutant PEMs were incubated in the top well of a 2-well modified Boyden chamber at 37°C for the indicated time periods. The membrane separating the upper and lower wells was studded with 8 μm pores and coated with fibronectin (10 μg/ml; B) or Matrigel (C). After incubation, the number of cells which migrated through the pores and became adherent to the underside of the filter were counted. Two filters were used for each time experiment and data shown are averages of 10 separate fields per time point (± s.d.). Data is representative of 4 independent experiments.

direct substrate of Src-family kinases (Duong et al., 1998; Li et al., 1999), while activation of Syk is believed to occur following association with membrane proteins which are in turn phosphorylated by Src-family kinases (Chan et al., 1994; Qian and Weiss, 1997). As both Pyk2 and Syk have been implicated as potential signal transducers following integrin-mediated adhesion (Astier et al., 1997; Lin et al., 1995), it is possible that the altered cytoskeletal structure observed in *hck*$^{-/-}$*fgr*$^{-/-}$ mutant cells was due to retarded activation of Pyk2 and/or Syk. Ultimately, the roles of Pyk2 and Syk in cell adhesion, signaling and migration will be determined by studies of *pyk2*$^{-/-}$ and *syk*$^{-/-}$ macrophages.

The general subcellular localization of filamentous actin, paxillin and talin we observed in wild-type macrophages was unlike the classical focal adhesion-plaque association seen in fibroblasts. Given that monocytes/macrophages are highly motile cells designed to enter sites of infection and injury, it is not unexpected that these cells must have a differently organized focal adhesion structure than fibroblasts. However, in double mutant cells the polarized localization of these proteins was clearly impaired which very likely contributes to the impaired filopodia formation and migratory capacity we observed in in vitro assays. Similarly, deficiency of all the major Src-family tyrosine kinases found in fibroblasts also significantly reduces cell migration (Klinghoffer et al., 1999).

The exact biochemical mechanism by which integrin crosslinking leads to activation of Src-family kinases remains to be determined. In lymphocytes, the tyrosine phosphatase CD45 is the principle regulator of Src-family kinase activity, by its ability to de-phosphorylate the regulatory tyrosine at the C-terminus of Src-family members (Neel, 1997). This may not be the case in macrophages, as Hck and Lyn activity appear to be elevated in *CD45*$^{-/-}$ macrophages (Roach et al., 1997), however, it is clear that activation of Src family kinases by other phosphatases such as PTP-1α leads to a hyper-adhesive phenotype in epithelial cells (Harder et al., 1998). Likewise, expression of a dominant-negative mutant of the non-receptor tyrosine phosphatase PTP-1B in fibroblasts has been shown to lead to reduction in Src kinase activity, reduced adhesion, and altered cytoskeletal structure (Arregui et al., 1998). Thus it is possible that integrin clustering in macrophages leads to activation of Hck, Fgr, and Lyn through the action of tyrosine phosphatases.

These data, combined with our previous observations in neutrophils (Lowell et al., 1996; Mocsai et al., 1999), support the model that the Src-family kinases present in myeloid cells function to regulate integrin mediated signal transduction that affects the actin cytoskeletal structure. It is important to note that this appears to be the only signaling pathway that is severly disrupted in these cells; LPS (Meng and Lowell, 1997), FcγR (Crowley et al., 1997), cytokine, and growth factor signal transduction are all grossly normal. Double mutant macrophages also show no defects in cytokine secretion, tumor cell killing activity or bactericidal function (Lowell et al., 1994). Hence, it is very unlikely that the cytoskeletal and morphological alterations we have observed in these cells are secondary to general defects in viability or growth properties. However, impaired myeloid cell adhesion should affect the immune responses in *hck*$^{-/-}$*fgr*$^{-/-}$ mice. Indeed, it is possible that the previously described susceptibility of these mice to *Listeria monocytogenes* infection may be explained by defects in neutrophil or macrophage migration to sites of infection. Further studies examining myeloid cell adhesion in mutant mice using in vivo models is clearly warranted.

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