

Expression and Tyrosine Phosphorylation of Crk-Associated Substrate Lymphocyte Type (Cas-L) Protein in Human Neutrophils

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ABSTRACT

Crk-associated substrate lymphocyte type (Cas-L) protein, also known as human enhancer of filamentation 1 (Hef1) or neural precursor cell-expressed, developmentally down-regulated gene 9 (Nedd9), belongs to the Cas family of adapter proteins, which are involved in integrin signaling. Previous reports showed that Cas-L is expressed preferentially in lymphocytes and epithelial cells. Cas-L mediates signals from integrins, T-cell receptors, B-cells receptors, and transforming growth factor beta, leading to cell movement and cell division. Here, we report the expression of Cas-L in neutrophils. Cas-L was tyrosine-phosphorylated when human neutrophils were stimulated by fMLP, tumor necrosis factor-alpha (TNF), or lipopolysaccharide. The tyrosine phosphorylation of Cas-L in fMLP- or TNF-stimulated neutrophils was further enhanced by adhesion of the cells to their substrates. Cas-L was found to be localized at focal adhesions in stimulated neutrophils based on immunofluorescence microscopy. These findings suggest that Cas-L is one of the targets of inflammatory cytokines and is also modulated by cell adhesion process in neutrophils. *J. Cell. Biochem.* 105: 121–128, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: Cas-L; fMLP; Hef1; LIPOPOLYSACCHARIDE; Nedd9; NEUTROPHIL; TUMOR NECROSIS FACTOR ALPHA

The Cas (Crk-associated substrate) family of proteins are docking proteins that mediate integrin-initiated signal transduction pathways [O'Neill et al., 2000; Chodniewicz and Klemke, 2004; Defilippi et al., 2006]. The family consists of p130^{Cas}, Cas-L/Hef1/Nedd9, and Efs/sin (embryonal Fyn substrate/Src-interacting protein). They have characteristic primary structure: an N-terminal SH3 domain, a substrate domain that contains multiple YXXP motifs, an Src binding sequence, and a C-terminal helix–turn–helix domain. The founding member, p130^{Cas}, was originally cloned as a

major tyrosine-phosphorylated protein in *v-crk*-transformed cells [Sakai et al., 1994] and was subsequently shown to mediate integrin signals, actin reorganization, cell migration, anoikis, bacterial infection, and cancer progression [Defilippi et al., 2006].

Cas-L/Hef1/Nedd9 was cloned because it is one of the proteins that are down-regulated during neural development (Nedd9 = neural precursor cell-expressed, developmentally down-regulated gene 9) [Kumar et al., 1992], it is a human protein that elicits filamentous budding in yeast (Hef1 = human enhancer of

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filamentation) [Law et al., 1996], and is a 105-kDa tyrosine-phosphorylated protein in lymphocytes (Cas-L = Crk-associated substrate in lymphocytes) [Minegishi et al., 1996].

In contrast to the ubiquitous expression of p130^{Cas} among adherent cells, Cas-L is preferentially expressed in epithelial cells and lymphocytes. In adherent cells, Cas-L is involved in integrin signaling, transforming growth factor beta (TGF- β) signaling, proteasomal regulation, apoptosis, cell migration, and cell division [Law et al., 1996, 1998; Zheng and McKeown-Longo, 2002; Feng et al., 2004; Pugacheva and Golemis, 2005]. In hematological cells, most studies of Cas-L have focused on the role of Cas-L in lymphocytes. Cas-L is tyrosine-phosphorylated by β 1 integrin stimulation and CD3 stimulation in T cells, leading to interleukin-2 production [Minegishi et al., 1996; Kanda et al., 1997; Iwata et al., 2002]. In B cells, Cas-L is tyrosine phosphorylated by β 1 integrin ligation, BCR signaling, and CXCL12 signaling [Astier et al., 1997a,b; Seo et al., 2005]. Furthermore, Cas-L-deficient mice showed a defect in marginal B-cell development and a cell migration defect in both B and T cells [Seo et al., 2005, 2006].

Neutrophils are components of natural immune system and form the first line defence against invading bacteria and fungi. Various stimuli including chemokines, inflammatory cytokines, and toll like receptor ligands activate neutrophils. Especially, fMLP, TNF- α , and lipopolysaccharide elicit the activation of many signaling pathways leading to the effector functions of neutrophils. Focal adhesion proteins such as paxillin, focal adhesion kinase (FAK), and PYK2, are already reported to be phosphorylated and to play important roles in stimulated neutrophils [Graham et al., 1994; Fuortes et al., 1999; Ryu et al., 2000]. However, the expression of Cas-L in neutrophils has only been suggested in a few microarray analyses [Subrahmanyan et al., 2001; Theilgaard-Monch et al., 2005] and Cas family proteins in neutrophils have not been analyzed further. Therefore, we examined the expression of Cas-L protein in neutrophils and the tyrosine phosphorylation of Cas-L in response to fMLP, TNF- α , or lipopolysaccharide.

MATERIALS AND METHODS

REAGENTS

Ficoll and the enhanced chemiluminescence (ECL) Western blotting system were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Conray was purchased from Mallinckrodt (St. Louis, MO). Rabbit polyclonal antibodies against p38 MAP kinase, Tyr⁴⁰¹-phosphorylated p130^{Cas}, and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody against p130^{Cas} was purchased from BD-Transduction Laboratories (San Jose, CA). Mouse monoclonal antibody against Hef1/Cas-L was purchased from Immquest (Cleveland, UK). Goat anti-mouse IgG antibody conjugated with alkaline phosphatase was purchased from Invitrogen (Carlsbad, CA). Phospho-specific antibodies to Cas (α Cas-pYDVP, α Cas-pYDpYV) were generated as described previously [Huang et al., 2006]. Anti-CD18 (L130) antibody and normal mouse IgG1 κ were purchased from BD-Pharmingen (San Jose, CA).

AlexaFluor 546-conjugated phalloidin was purchased from Invitrogen.

PREPARATION OF NEUTROPHILS

Human neutrophils and mononuclear cells (PBMC) were prepared from cells of healthy adult donors using dextran sedimentation, centrifugation with Conray-Ficoll, and hypotonic lysis of contaminated erythrocytes as described previously [Suzuki et al., 1999]. Neutrophil fractions contained >95% neutrophils. PBMC fractions contained 75–85% lymphocytes, 15–25% monocytes, and <1% neutrophils. Lymphocytes were further purified from mononuclear cells by centrifugal elutriation in a Hitachi SRR6Y elutriation rotor (Hitachi, Tokyo, Japan) [Yuo et al., 1992]. Cells were suspended in Hanks balanced salt solution (HBSS) containing 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid (HEPES) at pH 7.4.

WESTERN BLOTTING

Cells were suspended in HBSS containing 10 mM HEPES (pH 7.4) and treated with fMLP for various times at 37°C. The reactions were terminated by adding 10% by volume of trichloroacetic acid (Wako, Tokyo). After 1 h of incubation on ice, the cells were centrifuged and the pellet was washed twice with 10 mM dithiothreitol/acetone. The pellet was resuspended in 1.3 \times sample buffer (4% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol, and a trace amount of bromophenol blue dye in 125 mM Tris aminomethane hydrochloride, pH 6.8), sonicated, heated at 100°C for 5 min, and frozen at -20°C until use. Samples were subjected to 10% SDS gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane in a buffer containing 100 mM Tris, 192 mM glycine, and 20% methanol at 2 mA/cm² for 1.5 h at 25°C. Residual binding sites on the membrane were blocked by incubating the membrane in Tris-buffered saline (pH 7.6) containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) and 5% nonfat dried milk for 2 h at 25°C. The blots were washed in Tris-buffered saline containing 0.1% Tween 20 (TBST) and then incubated with the appropriate antibody overnight at 4°C. After three washings with TBST, the membrane was incubated with anti-rabbit immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase or alkaline phosphatase, and the antibody complexes were visualized using the ECL detection system (Amersham) or NBT/BCIP (Promega) as directed by the manufacturer. All the experiments were repeated to confirm the reproducibility.

IMMUNOFLUORESCENCE MICROSCOPY

Actin organization was analyzed using confocal laser scanning microscopy. Neutrophils (5×10^6 ml⁻¹) suspended in HBSS were treated with fMLP on FCS-coated glass cover slips at 37°C for 5 min. After incubation, cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). Cells were blocked with 5% bovine serum albumin in PBS at 4°C. Cells were then incubated with α Cas-pYDVP or α Cas-pYDpYV at room temperature for 3 h. Cells were washed three times with PBS and incubated with AlexaFluor 546-conjugated phalloidin (0.2 U ml⁻¹) and AlexaFluor 488-conjugated goat anti-rabbit

antibody (Molecular Probes) in the dark at room temperature for 30 min. Fluorescence images were photographed using a confocal laser scanning microscope (Zeiss LSM 510, Welwyn, Garden City, UK).

RESULTS

Cas-L IS EXPRESSED IN HUMAN NEUTROPHILS

We examined the expression of Cas-L in human neutrophils using the monoclonal antibody from BD-Transduction Laboratories that reacts with both p130^{Cas} and Cas-L (Fig. 1A). After the preparation of cells, the cells were fixed with trichloroacetic acid [Kobayashi et al., 2002] to prevent the artificial degradation of proteins by highly active proteases in neutrophils during the lysis procedure [Kato et al., 2004]. This was done because we could not detect bands of Cas family proteins after the neutrophils were lysed in the RIPA buffer with protease inhibitors (PMSF and leupeptin) nor directly in the 1.3× sample buffer (data not shown).

K562 cells expressed p130^{Cas}, but not Cas-L as previously noted [Minegishi et al., 1996] (Fig. 1A, lane 1). Lymphocytes and mononuclear cells expressed Cas-L as a doublet of p105Cas-L and p115Cas-L and expressed a trace amount of p130^{Cas} (Fig. 1A, lanes 2,3). In contrast, neutrophils only expressed only p105Cas-L, and the expression of p130^{Cas} was not detected (Fig. 1A, lane 4). We also used Cas-L/Hef1-specific monoclonal antibody from Immunoquest, which does not cross-react with p130^{Cas}. This antibody did not detect p130^{Cas} in K562 cells (Fig. 1B, lane 1), but did detect bands corresponding p105Cas-L and p115Cas-L (Fig. 1B, lanes 2–4). Thus, human neutrophils express p105Cas-L, but do not express p115Cas-L nor p130^{Cas}.

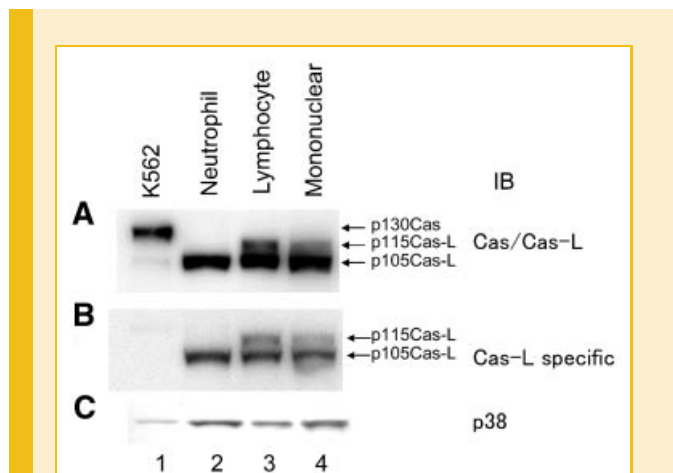


Fig. 1. Expression of Cas-L in human neutrophils. Lysates from K562 cells, human neutrophils, human lymphocytes, and human mononuclear cells were electrophoresed and transferred to nitrocellulose membrane. A: Immunoblotting with anti-Cas/Cas-L antibody (BD Transduction) that reacts with both p130Cas and Cas-L. B: Immunoblotting with Cas-L-specific antibody (Immunoquest). C: Immunoblotting with anti-p38 antibody.

TYROSINE PHOSPHORYLATION OF Cas-L IN fMLP-STIMULATED NEUTROPHILS

We examined the tyrosine phosphorylation of Cas-L in human neutrophils stimulated by fMLP (fMLF), a bacteria-derived chemokine for neutrophils. Because Cas family proteins including Cas-L, are implicated in the adhesion process [Nojima et al., 1995; Minegishi et al., 1996], we examined the tyrosine phosphorylation of Cas-L in the adhesion or in suspensions of neutrophils. We used phospho-specific anti-Cas antibodies because immunoprecipitation was not possible after the fixation of cells using trichloroacetic acid (TCA). Three types of anti-phospho-Cas antibody were used. The anti-pY410 phospho-Cas antibody (Cell Signaling) detects the phosphorylated YAXP sequence found in both p130^{Cas} and Cas-L. The α Cas-pYDXP antibody [Huang et al., 2006] was raised against the pYDXP sequences, which are the binding consensus sequences for the Crk SH2 domain [Songyang et al., 1993; Sakai et al., 1994] and exist multiply in the substrate domain of Cas family proteins. The α Cas-pYDpYV antibody was raised against the pYDpYV sequence, which is conserved in p130^{Cas} and Cas-L proteins and is the binding site for the Src SH2 domain [Nakamoto et al., 1996; Tachibana et al., 1997].

When neutrophils were allowed to adhere to an FBS-coated dish for 30 min and were then stimulated by fMLP, the tyrosine phosphorylation of Cas-L detected by anti-pYAXP was prominent 1 min after fMLP stimulation, peaked from 5 to 20 min and declined after 40 min (Fig. 2A), although the time course varied somewhat among donor individuals (data not shown). The α Cas-pYDpYV antibody similarly detected phosphorylation of Cas-L. In contrast, phosphorylation detected by the α Cas-pYDpYV antibody peaked 1 min after fMLP stimulation and began to decline after 5 min (Fig. 2A).

When we stimulated neutrophils in suspension, the tyrosine phosphorylation of Cas-L was detected 1 min after stimulation, declining rapidly thereafter (Fig. 2A), and the phosphorylation was not as prominent as found for adhesion, indicating that the signal from adhesion enhanced the persistence and extent of Cas-L phosphorylation.

The tyrosine phosphorylation of Cas-L was dependent on the concentrations of fMLP in both the suspension and adhesion conditions (Fig. 2B). The tyrosine phosphorylation of Cas-L in suspension increased with the dosage of fMLP up to 10^{-5} M (Fig. 2B). In contrast, the tyrosine phosphorylation of Cas-L in adhesion increased up to the concentration of 10^{-6} M and further increase was not detected at 10^{-5} M (Fig. 2B).

We also examined the effect of neutrophil adhesion to various extracellular matrix proteins on the fMLP-induced tyrosine phosphorylation of Cas-L (Fig. 2C). Neutrophils express LFA-1, Mac-1, $\alpha 4\beta 1$ integrin, and $\alpha 2\beta 1$ integrins. FBS contains ligands for LFA-1, Mac-1, and $\alpha 4\beta 1$ integrins, but fibrinogen can only serve as a ligand for Mac-1, and fibronectin serves as a ligand for Mac-1 and $\alpha 4\beta 1$ integrins [Heit et al., 2005]. In contrast, type I collagen serves as a ligand for LFA-1 and $\alpha 2\beta 1$ integrins [Garnotel et al., 1995]. Fibrinogen and fibronectin supported the fMLP-induced tyrosine phosphorylation of Cas-L as well as did FBS, but the tyrosine phosphorylation on collagen was weaker (Fig. 2C).

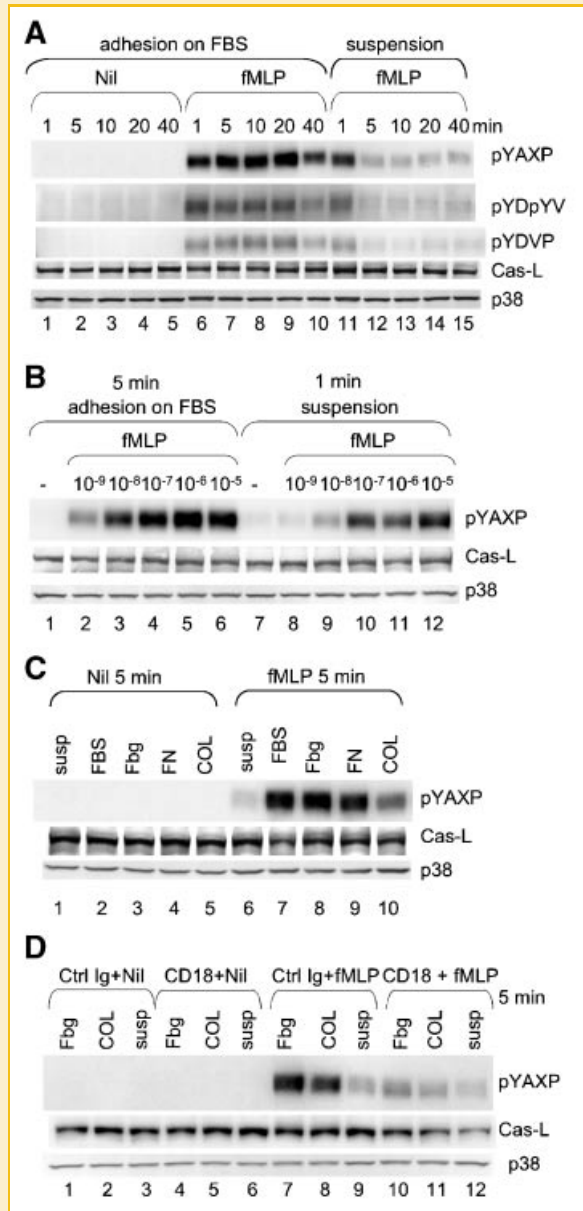


Fig. 2. Tyrosine phosphorylation of Cas-L in fMLP-stimulated neutrophils. **A:** Human neutrophils either adherent to an FBS-coated dish or in suspension were stimulated with 10^{-7} M fMLP at 37°C for the indicated times. **B:** Neutrophils adherent to the FBS-coated dish were stimulated for 5 min and neutrophils in suspension were stimulated for 1 min with the indicated concentrations of fMLP at 37°C . **C:** Neutrophils in suspension or adherent to the dishes coated with the indicated substrates were stimulated with 10^{-7} M fMLP at 37°C for 5 min. **D:** Neutrophils in suspension or adherent to the dishes coated with fibrinogen or collagen were stimulated with 10^{-7} M fMLP at 37°C for 5 min in the presence of either anti-CD18 or control murine IgG1 κ . The tyrosine phosphorylation and expression of Cas-L was analyzed by immunoblotting using antibodies against the phosphorylated form of Cas and against total Cas. The expression of p38 was examined as a loading control. Cell lysates equivalent to 2.5×10^5 cells were loaded onto each lane.

To further clarify the mechanisms of the tyrosine phosphorylation of Cas-L, we inhibited $\beta 2$ integrin using anti-CD18 blocking antibody. Blocking $\beta 2$ integrin reduced the tyrosine phospho-

rylation of Cas-L in fMLP-stimulated neutrophils on fibrinogen and on collagen to the level comparable to that in suspension (Fig. 2D).

TYROSINE PHOSPHORYLATION OF Cas-L IN TNF- α -STIMULATED NEUTROPHILS

We investigated the tyrosine phosphorylation of Cas-L in human neutrophils stimulated with tumor necrosis factor- α (TNF- α). Isolated neutrophils were allowed to adhere to an FBS-coated dish and were stimulated with 10 U ml^{-1} TNF- α . Neutrophils were fixed with TCA at each sampling time and were then analyzed. Tyrosine phosphorylation of Cas-L detected by the anti-pYAXP antibody (anti-p-Cas-L antibody) was observed 10 min after stimulation, peaked after 20 min, and persisted for more than 90 min (Fig. 3A). We then examined the dose-response of tyrosine phosphorylation of Cas-L induced by TNF- α -stimulation. The phosphorylation was most prominent at a concentration of 10 U ml^{-1} TNF- α and declined when neutrophils were stimulated with 100 U ml^{-1} or at $1,000 \text{ U ml}^{-1}$ of TNF- α (Fig. 3B). The tyrosine phosphorylation of

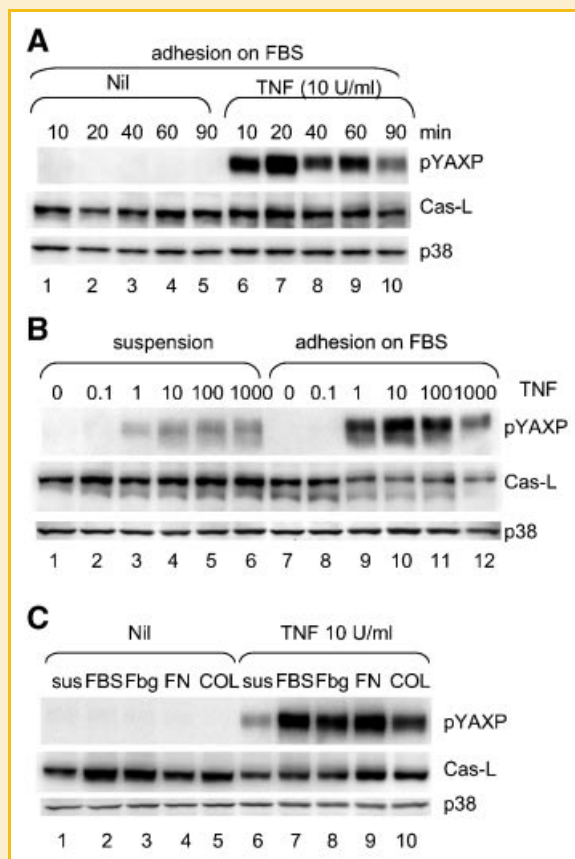


Fig. 3. Tyrosine phosphorylation of Cas-L in TNF- α -stimulated neutrophils. **A:** Human neutrophils adherent to an FBS-coated dish were stimulated with 10 U ml^{-1} TNF- α at 37°C for the indicated times. **B:** Neutrophils in suspension or adherent to an FBS-coated dish were stimulated with the indicated concentrations of TNF- α at 37°C for 20 min. **C:** Neutrophils in suspension or adherent to the dishes coated with the indicated substrates were stimulated with 10 U ml^{-1} TNF- α at 37°C for 20 min.

suspension (Fig. 3B). We also examined TNF- α -induced tyrosine phosphorylation of Cas-L when neutrophils were placed on various extracellular matrix proteins. Fibrinogen and fibronectin supported the TNF-induced tyrosine phosphorylation of Cas-L as well as did FBS, but tyrosine phosphorylation on collagen was weaker (Fig. 3C).

TYROSINE PHOSPHORYLATION OF Cas-L IN LPS-STIMULATED NEUTROPHILS

We examined whether Cas-L is also tyrosine phosphorylated by stimulation with lipopolysaccharide (LPS), a Toll-like receptor ligand. The tyrosine phosphorylation of Cas-L was detected after 40 min of stimulation with $1 \mu\text{g ml}^{-1}$ LPS and declined thereafter (Fig. 4A). The tyrosine phosphorylation of Cas-L was weaker when neutrophils were stimulated with 100 ng ml^{-1} LPS (Fig. 3E), but the phosphorylation of Cas-L did not increase even when neutrophils were stimulated with $>1 \mu\text{g ml}^{-1}$ LPS (Fig. 4B). In contrast to the phosphorylation in response to fMLP and TNF- α , LPS-induced phosphorylation of Cas-L was prominent in suspension and was not enhanced when neutrophils were allowed to adhere on an FBS-coated dish (Fig. 4B). However, neutrophils that adhered to the extracellular matrix proteins, fibronectin and collagen showed slightly reduced tyrosine phosphorylation in response to LPS stimulation (Fig. 4C).

PHOSPHORYLATED Cas-L IS LOCALIZED TO FOCAL ADHESIONS IN NEUTROPHILS

We investigated the localization of phosphorylated Cas-L in LPS- or fMLP-stimulated adherent neutrophils (Fig. 5D–L). Unstimulated neutrophils kept in suspension were used as controls (Fig. 5A–C). $\alpha\text{Cas-pYDXP}$ or $\alpha\text{Cas-pYVpYD}$ was used to visualize the localization of phosphorylated Cas-L in neutrophils. The phosphorylated Cas-L in the LPS- or fMLP-stimulated neutrophils stained in a dot-like pattern at the edge of adhesion sites (Fig. 5E,H,K) and co-localized with vinculin, a focal adhesion marker (Fig. 5D), or with F-actin (Fig. 5G). However, when the leading edge was formed from a gross actin bundle, Cas-L was localized in dot-like structures both under the leading edge and in the tail region, and did not colocalize with actin bundle. This suggests that the localization of Cas-L was not affected by the formation of a leading edge (Fig. 5J–L). Phosphorylated Cas-L was not observed in unstimulated neutrophils (Fig. 5A–C).

DISCUSSION

We demonstrated that: neutrophils expressed p105Cas-L, but not p115Cas-L nor p130^{Cas}; Cas-L was tyrosine-phosphorylated in response to fMLP, TNF- α , and LPS in neutrophils; tyrosine phosphorylation of Cas-L in neutrophils was enhanced by adhesion; and tyrosine-phosphorylated Cas-L was localized to focal adhesions in neutrophils.

The roles of Cas-L in lymphocytes have been investigated extensively [Minegishi et al., 1996; Kanda et al., 1997, 1999; Tachibana et al., 1997; Astier et al., 1997b; Ohashi et al., 1998, 1999; Kamiguchi et al., 1999; Hogg et al., 2003; Iwata et al., 2005; Seo et al., 2005], but the expression of Cas-L in phagocytes has only been

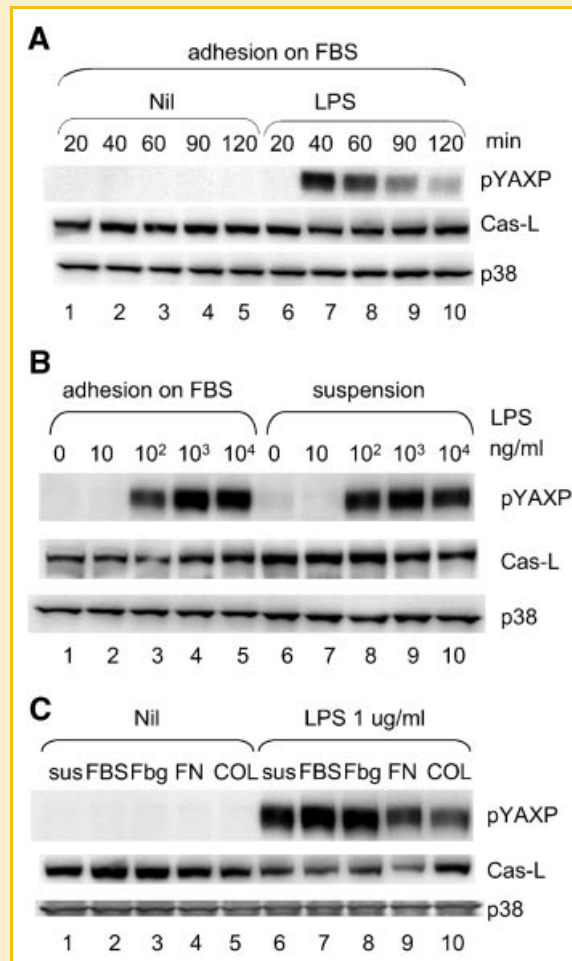


Fig. 4. Tyrosine phosphorylation of Cas-L in LPS-stimulated neutrophils. A: Human neutrophils adherent to an FBS-coated dish were stimulated with $1 \mu\text{g ml}^{-1}$ LPS at 37°C for the indicated times. B: Neutrophils in suspension or adherent to the FBS-coated dish were stimulated with the indicated concentrations of LPS at 37°C for 40 min. C: Neutrophils in suspension or adherent to the dishes coated with the indicated substrates were stimulated with $1 \mu\text{g ml}^{-1}$ LPS at 37°C for 40 min.

reported in monocyte-lineage cells (osteoclasts) [Zhang et al., 2002]. In one microarray analysis [Subrahmanyam et al., 2001], mRNA for Cas-L was up-regulated when neutrophils were exposed to bacteria. In another microarray analysis [Theilgaard-Monch et al., 2005], mRNA for Cas-L increased with the differentiation of granulocytes. However, the expression of Cas-L at the protein level has not previously been reported. Cas-L is vulnerable to proteases as already reported for other cell types [Law et al., 2000; O'Neill and Golemis, 2001; Nourry et al., 2004; Zheng and McKeown-Longo, 2006] and neutrophils are rich in proteases, which often cause the artificial degradation of proteins during the preparation of cell lysates [Kato et al., 2004]. We could not detect the expression of Cas-L when neutrophils were lysed with RIPA buffer nor with $1.3\times$ sample buffer. Instead, we detected Cas-L only after neutrophils were fixed with trichloroacetic acid (TCA) to inactivate proteases (Fig. 1). This may be one reason why Cas-L has not previously been reported in neutrophils at the protein level. Fixation with trichloroacetic acid is

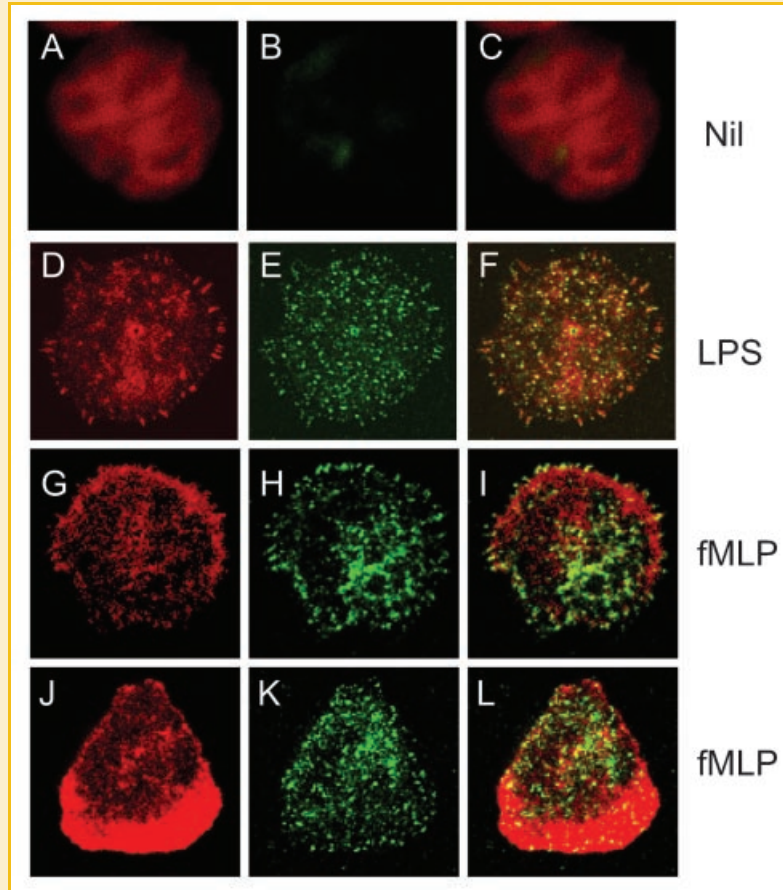


Fig. 5. Subcellular localization of phosphorylated Cas-L in neutrophils. Unstimulated human neutrophils were kept in suspension (A–C). Human neutrophils adherent to a fibrinogen-coated dish were stimulated with $1 \mu\text{g ml}^{-1}$ LPS for 40 min (D–F). Human neutrophils adherent to an FBS-coated dish were stimulated with 10^{-7} M fMLP for 5 min (G–I). Cells were fixed with 4% paraformaldehyde and were stained with anti-pYDpYV (B,E,H) or anti-pYDXP(K), together with anti-vinculin (D) or phalloidin (A,G,J).

a powerful method for the prevention of protein degradation. In contrast to the lysis buffers that release proteases by the destruction of the protease-rich granules in neutrophils, TCA fixes the protease-rich granules without destroying their structure. However, TCA fixation prevents the examination of protein binding by co-immunoprecipitation. Cas-L has been reported to bind Crk or Crk-L to transduce signals to downstream effectors [Minegishi et al., 1996; Manie et al., 1997; Sattler et al., 1997; Astier et al., 1997b; Ohashi et al., 1999]. Therefore, it is possible that Crk or Crk-L could act as downstream adaptor molecules from Cas-L in neutrophils.

Cas-L is reportedly tyrosine-phosphorylated in response to $\beta 1$ integrin, T-cell receptors, B-cell receptors, calcitonin, and TGF $\beta 1$ [Minegishi et al., 1996; Kanda et al., 1997, 1999; Tachibana et al., 1997; Astier et al., 1997a,b; Kamiguchi et al., 1999; Zhang et al., 1999; Zheng and McKeown-Longo, 2002]. Here, we described the tyrosine phosphorylation of Cas-L in response to fMLP, TNF- α , and LPS in neutrophils. The receptors for fMLP are seven transmembrane G-protein-coupled receptors. The tyrosine phosphorylation of Cas-L in response to G-protein-coupled receptor stimulation has been reported with calcitonin [Zhang et al., 1999]. The response to CXCL12, which is mediated by CXCR4, another G-protein-coupled receptor, was impaired in Cas-L-deficient lymphocytes [Seo et al.,

2005], and the tyrosine phosphorylation of Cas-L in response to CXCL12 is reported in T cells [Regelmann et al., 2006]. Although TNF- α was reported to induce the degradation of Cas-L in epithelial cells [Law et al., 2000], our results provide the first evidence of the tyrosine phosphorylation of Cas-L by cytokines. Focal adhesion proteins such as paxillin, PYK2, and FAK are involved in toll-like receptor signaling [Williams and Ridley, 2000; Zeisel et al., 2005]. However, the involvement of Cas family members in the innate immune system has not previously been reported. LPS-induced tyrosine phosphorylation of Cas-L in neutrophils, together with the chemokine- or cytokine-induced tyrosine phosphorylation of Cas-L, suggests that Cas-L would be one of the targets of inflammatory stimuli in neutrophils.

The tyrosine phosphorylation of Cas-L in neutrophils was greatly enhanced or prolonged by adhesion, especially when neutrophils were stimulated with fMLP or TNF- α (Figs. 2 and 3). Although the weak fMLP-induced phosphorylation in suspension was not dependent on $\beta 2$ integrins, the adhesion-induced enhancement of tyrosine phosphorylation in fMLP stimulation was mediated by $\beta 2$ integrins (Fig. 2D). Furthermore, the tyrosine-phosphorylated Cas-L was localized to focal adhesions in neutrophils (Fig. 5). Therefore, the tyrosine phosphorylation of Cas-L appears to be involved in cell

adhesion and cytoskeletal change, as already reported for other cell types such as lymphocytes and epithelial cells [Law et al., 1996; Minegishi et al., 1996]. The tyrosine phosphorylations of Cas-L were variable depending on extracellular matrix proteins (Figs. 2C, 3C, and 4C), suggesting the involvement of specific integrins in the phosphorylation process. However, the Cas-L phosphorylation in fMLP and TNF stimulation is also observed in suspension, suggesting that the phosphorylation occurs before integrin stimulation and that the integrin signaling enhance this phosphorylation. In contrast, LPS-mediated tyrosine phosphorylation of Cas-L is independent on adhesion and is delayed. Toll like receptors may produce signals that are delayed and are independent on integrins in neutrophils. Another possible explanation may be the existence of small numbers of contaminating monocytes in neutrophil preparation [Sabroe et al., 2004]. Those monocytes may produce multiple cytokines that cause neutrophil activation without adhesion. This might also explain the delay of phosphorylation of Cas-L by LPS. In any case, LPS-induced phosphorylation of Cas-L in neutrophils reflects the physiological phenomenon, because neutrophils in vivo are not pure and are influenced by neighboring cells.

In this study, we used human neutrophils to investigate the expression and tyrosine phosphorylation of Cas-L. However, there are no specific inhibitors of Cas-L; thus, it is difficult to investigate the role of Cas-L in human neutrophils. Neutrophils express p105Cas-L and not p115Cas-L (Fig. 1). p115Cas-L is a Ser/Thr phosphorylated form of Cas-L [Law et al., 1998]. The depletion of p115Cas-L impairs cell migration in T cells and p105Cas-L could not substitute the loss of p115Cas-L [Regelmann et al., 2006]. Therefore, it is estimated that p105Cas-L present in neutrophils and p115/p105Cas-L present in lymphocytes might play different roles in cell migration and in other biological functions. In fact, the investigation of neutrophils from Cas-L deficient mice showed that Cas-L deficient neutrophils migrated to fMLP faster than wildtype neutrophils in Boyden chamber assay (Seo et al., in preparation). This result is in contrast to the fact that Cas-L deficient lymphocytes are less responsive to CXCL-12, CXCL-13, and CCL21 [Seo et al., 2005], suggesting the differential roles of p115Cas-L and p105Cas-L.

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REFERENCES

Astier A, Manie SN, Avraham H, Hirai H, Law SF, Zhang Y, Golemis EA, Fu Y, Druker BJ, Haghayeghi N, Freedman AS, Avraham S. 1997a. The related adhesion focal tyrosine kinase differentially phosphorylates p130Cas and the Cas-like protein, p105HEF1. *J Biol Chem* 272:19719–19724.

Astier A, Manie SN, Law SF, Canty T, Haghayeghi N, Druker BJ, Salgia R, Golemis EA, Freedman AS. 1997b. Association of the Cas-like molecule HEF1 with CrkL following integrin and antigen receptor signaling in human B-cells: Potential relevance to neoplastic lymphohematopoietic cells. *Leuk Lymphoma* 28:65–72.

Chodniewicz D, Klemke RL. 2004. Regulation of integrin-mediated cellular responses through assembly of a CAS/Crk scaffold. *Biochim Biophys Acta* 1692:63–76.

Defilippi P, Di Stefano P, Cabodi S. 2006. p130Cas: A versatile scaffold in signaling networks. *Trends Cell Biol* 16:257–263.

Feng L, Guedes S, Wang T. 2004. Atrophin-1-interacting protein 4/human Itch is a ubiquitin E3 ligase for human enhancer of filamentation 1 in transforming growth factor-beta signaling pathways. *J Biol Chem* 279:29681–29690.

Fuortes M, Melchior M, Han H, Lyon GJ, Nathan C. 1999. Role of the tyrosine kinase pyk2 in the integrin-dependent activation of human neutrophils by TNF. *J Clin Invest* 104:327–335.

Garnotel R, Monboisse JC, Randoux A, Haye B, Borel JP. 1995. The binding of type I collagen to lymphocyte function-associated antigen (LFA) 1 integrin triggers the respiratory burst of human polymorphonuclear neutrophils. Role of calcium signaling and tyrosine phosphorylation of LFA 1. *J Biol Chem* 270:27495–27503.

Graham IL, Anderson DC, Holers VM, Brown EJ. 1994. Complement receptor 3 (CR3, Mac-1, integrin alpha M beta 2, CD11b/CD18) is required for tyrosine phosphorylation of paxillin in adherent and nonadherent neutrophils. *J Cell Biol* 127:1139–1147.

Heit B, Colarusso P, Kubus P. 2005. Fundamentally different roles for LFA-1, Mac-1 and alpha4-integrin in neutrophil chemotaxis. *J Cell Sci* 118:5205–5220.

Hogg N, Laschinger M, Giles K, McDowall A. 2003. T-cell integrins: More than just sticking points. *J Cell Sci* 116:4695–4705.

Huang J, Sakai R, Furuichi T. 2006. The docking protein Cas links tyrosine phosphorylation signaling to elongation of cerebellar granule cell axons. *Mol Biol Cell* 17:3187–3196.

Iwata S, Kobayashi H, Miyake-Nishijima R, Sasaki T, Souta-Kuribara A, Nori M, Hosono O, Kawasaki H, Tanaka H, Morimoto C. 2002. Distinctive signaling pathways through CD82 and beta 1 integrins in human T cells. *Eur J Immunol* 32:1328–1337.

Iwata S, Souta-Kuribara A, Yamakawa A, Sasaki T, Shimizu T, Hosono O, Kawasaki H, Tanaka H, Dang NH, Watanabe T, Arima N, Morimoto C. 2005. HTLV-I Tax induces and associates with Crk-associated substrate lymphocyte type (Cas-L). *Oncogene* 24:1262–1271.

Kamiguchi K, Tachibana K, Iwata S, Ohashi Y, Morimoto C. 1999. Cas-L is required for beta 1 integrin-mediated costimulation in human T cells. *J Immunol* 163:563–568.

Kanda H, Mimura T, Morino N, Hamasaki K, Nakamoto T, Hirai H, Morimoto C, Yazaki Y, Nojima Y. 1997. Ligation of the T cell antigen receptor induces tyrosine phosphorylation of p105CasL, a member of the p130Cas-related docking protein family, and its subsequent binding to the Src homology 2 domain of c-Crk. *Eur J Immunol* 27:2113–2117.

Kanda H, Mimura T, Hamasaki K, Yamamoto K, Yazaki Y, Hirai H, Nojima Y. 1999. Fyn and Lck tyrosine kinases regulate tyrosine phosphorylation of p105CasL, a member of the p130Cas docking protein family, in T-cell receptor-mediated signalling. *Immunology* 97:56–61.

Kato T, Sakamoto E, Kutsuna H, Kimura-Eto A, Hato F, Kitagawa S. 2004. Proteolytic conversion of STAT3alpha to STAT3gamma in human neutrophils: Role of granule-derived serine proteases. *J Biol Chem* 279:31076–31080.

Kobayashi S, Yamashita K, Takeoka T, Ohtsuki T, Suzuki Y, Takahashi R, Yamamoto K, Kaufmann SH, Uchiyama T, Sasada M, Takahashi A. 2002. Calpain-mediated X-linked inhibitor of apoptosis degradation in neutrophil apoptosis and its impairment in chronic neutrophilic leukemia. *J Biol Chem* 277:33968–33977.

Kumar S, Tomooka Y, Noda M. 1992. Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochem Biophys Res Commun* 185:1155–1161.

- Law SF, Estojak J, Wang B, Mysliwiec T, Kruh G, Golemis EA. 1996. Human enhancer of filamentation 1, a novel p130cas-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in *Saccharomyces cerevisiae*. *Mol Cell Biol* 16:3327–3337.
- Law SF, Zhang YZ, Klein-Szanto AJ, Golemis EA. 1998. Cell cycle-regulated processing of HEF1 to multiple protein forms differentially targeted to multiple subcellular compartments. *Mol Cell Biol* 18:3540–3551.
- Law SF, O'Neill GM, Fashena SJ, Einarson MB, Golemis EA. 2000. The docking protein HEF1 is an apoptotic mediator at focal adhesion sites. *Mol Cell Biol* 20:5184–5195.
- Manie SN, Beck AR, Astier A, Law SF, Canty T, Hirai H, Druker BJ, Avraham H, Haghayeghi N, Sattler M, Salgia R, Griffin JD, Golemis EA, Freedman AS. 1997. Involvement of p130(Cas) and p105(HEF1), a novel Cas-like docking protein, in a cytoskeleton-dependent signaling pathway initiated by ligation of integrin or antigen receptor on human B cells. *J Biol Chem* 272:4230–4236.
- Minegishi M, Tachibana K, Sato T, Iwata S, Nojima Y, Morimoto C. 1996. Structure and function of Cas-L, a 105-kD Crk-associated substrate-related protein that is involved in beta 1 integrin-mediated signaling in lymphocytes. *J Exp Med* 184:1365–1375.
- Nakamoto T, Sakai R, Ozawa K, Yazaki Y, Hirai H. 1996. Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase. *J Biol Chem* 271:8959–8965.
- Nojima Y, Morino N, Mimura T, Hamasaki K, Furuya H, Sakai R, Sato T, Tachibana K, Morimoto C, Yazaki Y, Hirai H. 1995. Integrin-mediated cell adhesion promotes tyrosine phosphorylation of p130Cas, a Src homology 3-containing molecule having multiple Src homology 2-binding motifs. *J Biol Chem* 270:15398–15402.
- Nourry C, Maksumova L, Pang M, Liu X, Wang T. 2004. Direct interaction between Smad3, APC10, CDH1 and HEF1 in proteasomal degradation of HEF1. *BMC Cell Biol* 5:20.
- Ohashi Y, Tachibana K, Kamiguchi K, Fujita H, Morimoto C. 1998. T cell receptor-mediated tyrosine phosphorylation of Cas-L, a 105-kDa Crk-associated substrate-related protein, and its association of Crk and C3G. *J Biol Chem* 273:6446–6451.
- Ohashi Y, Iwata S, Kamiguchi K, Morimoto C. 1999. Tyrosine phosphorylation of Crk-associated substrate lymphocyte-type is a critical element in TCR- and beta 1 integrin-induced T lymphocyte migration. *J Immunol* 163:3727–3734.
- O'Neill GM, Golemis EA. 2001. Proteolysis of the docking protein HEF1 and implications for focal adhesion dynamics. *Mol Cell Biol* 21:5094–5108.
- O'Neill GM, Fashena SJ, Golemis EA. 2000. Integrin signalling: A new Cas(t) of characters enters the stage. *Trends Cell Biol* 10:111–119.
- Pugacheva EN, Golemis EA. 2005. The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome. *Nat Cell Biol* 7:937–946.
- Regelmann AG, Danzl NM, Wanjalla C, Alexandropoulos K. 2006. The hematopoietic isoform of Cas-Hef1-associated signal transducer regulates chemokine-induced inside-out signaling and T cell trafficking. *Immunity* 25:907–918.
- Ryu H, Lee JH, Kim KS, Jeong SM, Kim PH, Chung HT. 2000. Regulation of neutrophil adhesion by pituitary growth hormone accompanies tyrosine phosphorylation of Jak2, p125FAK, and paxillin. *J Immunol* 165:2116–2123.
- Sabroe I, Prince LR, Dower SK, Walmsley SR, Chilvers ER, Whyte MKB. 2004. What can we learn from highly purified neutrophils? *Biochem Soc Trans* 32:468–469.
- Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Mano H, Yazaki Y, Hirai H. 1994. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO J* 13:3748–3756.
- Sattler M, Salgia R, Shrikhande G, Verma S, Uemura N, Law SF, Golemis EA, Griffin JD. 1997. Differential signaling after beta1 integrin ligation is mediated through binding of CRKL to p120(CBL) and p110(HEF1). *J Biol Chem* 272:14320–14326.
- Seo S, Asai T, Saito T, Suzuki T, Morishita Y, Nakamoto T, Ichikawa M, Yamamoto G, Kawazu M, Yamagata T, Sakai R, Mitani K, Ogawa S, Kurokawa M, Chiba S, Hirai H. 2005. Crk-associated substrate lymphocyte type is required for lymphocyte trafficking and marginal zone B cell maintenance. *J Immunol* 175:3492–3501.
- Seo S, Ichikawa M, Kurokawa M. 2006. Structure and function of Cas-L and integrin-mediated signaling. *Crit Rev Immunol* 26:391–406.
- Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BJ, Birge RB, Fajardo JE, Chou MM, Hanafusa H, Schaffhausen B, Cantley LC. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell* 72:767–778.
- Subrahmanyam YV, Yamaga S, Prashar Y, Lee HH, Hoe NP, Kluger Y, Gerstein M, Goguen JD, Newburger PE, Weissman SM. 2001. RNA expression patterns change dramatically in human neutrophils exposed to bacteria. *Blood* 97:2457–2468.
- Suzuki K, Hino M, Hato F, Tatsumi N, Kitagawa S. 1999. Cytokine-specific activation of distinct mitogen-activated protein kinase subtype cascades in human neutrophils stimulated by granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-alpha. *Blood* 93:341–349.
- Tachibana K, Urano T, Fujita H, Ohashi Y, Kamiguchi K, Iwata S, Hirai H, Morimoto C. 1997. Tyrosine phosphorylation of Crk-associated substrates by focal adhesion kinase. A putative mechanism for the integrin-mediated tyrosine phosphorylation of Crk-associated substrates. *J Biol Chem* 272:29083–29090.
- Theilgaard-Monch K, Jacobsen LC, Borup R, Rasmussen T, Bjerregaard MD, Nielsen FC, Cowland JB, Borregaard N. 2005. The transcriptional program of terminal granulocytic differentiation. *Blood* 105:1785–1796.
- Williams LM, Ridley AJ. 2000. Lipopolysaccharide induces actin reorganization and tyrosine phosphorylation of Pyk2 and paxillin in monocytes and macrophages. *J Immunol* 164:2028–2036.
- Yuo A, Kitagawa S, Motoyoshi K, Azuma E, Saito M, Takaku F. 1992. Rapid priming of human monocytes by human hematopoietic growth factors: Granulocyte-macrophage colony-stimulating factor (CSF), macrophage-CSF, and interleukin-3 selectively enhance superoxide release triggered by receptor-mediated agonists. *Blood* 79:1553–1557.
- Zeisel MB, Druet VA, Sibilica J, Klein JP, Quesniaux V, Wachsmann D. 2005. Cross talk between MyD88 and focal adhesion kinase pathways. *J Immunol* 174:7393–7397.
- Zhang Z, Hernandez-Lagunas L, Horne WC, Baron R. 1999. Cytoskeleton-dependent tyrosine phosphorylation of the p130(Cas) family member HEF1 downstream of the G protein-coupled calcitonin receptor. Calcitonin induces the association of HEF1, paxillin, and focal adhesion kinase. *J Biol Chem* 274:25093–25098.
- Zhang Z, Neff L, Bothwell AL, Baron R, Horne WC. 2002. Calcitonin induces dephosphorylation of Pyk2 and phosphorylation of focal adhesion kinase in osteoclasts. *Bone* 31:359–365.
- Zheng M, McKeown-Longo PJ. 2002. Regulation of HEF1 expression and phosphorylation by TGF-beta 1 and cell adhesion. *J Biol Chem* 277:39599–39608.
- Zheng M, McKeown-Longo PJ. 2006. Cell adhesion regulates Ser/Thr phosphorylation and proteasomal degradation of HEF1. *J Cell Sci* 119:96–103.