The *src*-family protein-tyrosine kinase p59^{hck} is located on the secretory granules in human neutrophils and translocates towards the phagosome during cell activation

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The *src*-family protein-tyrosine kinase p59^{hck} is mainly expressed in neutrophils; however, its functional role in these cells is unknown. Several other *src*-family members are localized on secretory vesicles and have been proposed to regulate intracellular traffic. We have established here the subcellular localization of p59^{hck} in human neutrophils. Immunoblotting of subcellular fractions showed that approx. 60% of the p59^{hck} per cell is localized on the secretory granules; the other 40% is distributed equally between non-granular membranes and the cytosol. Immunofluorescence of neutrophils and HL60 cells suggests that the p59^{hck}-positive granules are azurophil granules. Granular

p59^{hck} is highly susceptible to degradation by an azurophil-granule proteinase. Different forms of p59^{hck} occur in the three subcellular compartments: a 61 kDa form is mainly found in the granules, a 59 kDa form is predominant in the non-granular membranes, whereas cytosolic p59^{hck} migrates as a doublet at 63 kDa. During the process of phagocytosis-linked degranulation, induced by serum-opsonized zymosan in neutrophils or HL60 cells, granular p59^{hck} translocates towards the phagosome. The subcellular localization of p59^{hck} suggests that the enzyme could be involved in the regulation of the degranulation process.

INTRODUCTION

p59^{hck} (haematopoietic cell kinase; Hck) is a member of the *src* family of non-receptor protein-tyrosine kinases (PTKs) [1–3] which is principally expressed in the myeloid cell lineage, especially in neutrophils [1]. Expression of *hck* is low in immature myeloid cells and increases as the cells differentiate [1,2,4,5]. In mature macrophages, *hck* RNA is further increased at late stages of cell priming [6] or activation [7]. The expression pattern suggests that Hck exerts its functional role in lineage-typical responses of mature myeloid cells. Manipulations of *hck* expression support this notion. Macrophages from homozygous *hck*-knockout mice carry out phagocytosis with reduced efficiency [8], and *hck* antisense RNA affects the production of tumour necrosis factor in murine macrophages [9]. Few data are available about the functional importance of the enzymic PTK activity of Hck. p59^{hck-F501}, which is constitutively activated by mutation of the regulatory site Tyr-501, can cause transfected fibroblasts to undergo transformation [10]. Stimulation of monocytic cells with lipopolysaccharide or anti-FcγRII induces a transient activation of Hck [11,12]. Furthermore, the PTK activity of murine Hck increases slightly during anti-IgM stimulation of B-lymphoma cells, indicating that the enzyme may be involved in Ig-mediated signal transduction [13]. Finally, murine Hck is activated during leukaemia inhibitory factor/interleukin-6 receptor stimulation in embryonic stem cells, showing that it participates in interleukin-6 receptor signalling [14]. Taken together, the studies indicate that Hck may be involved in several different signal transduction pathways. However, at present nothing is known about the functional role of Hck in the neutrophil.

The location of proteins is closely related to their functional role. The subcellular localization of the PTK p60^{c-src} has been studied extensively. In secretory cells that express high levels of the kinase, such as platelets and neuroendocrine cells, a fraction

of p60^{c-src} is associated with membranes of exocytic vesicles [15–18]. In fibroblasts, the enzyme is primarily localized on endosomal membranes [19–21]. The association of p60^{c-src} with endocytic or exocytic vesicles suggests that the kinase might play a role in the regulation of intracellular traffic. Other members of the *src* family are also partially located on intracellular vesicles. p56^{lck}, which is associated with the plasma membrane in resting T cells, is found on endosomes in CD2-stimulated cells [22,23]. p55^{fer}, the other *src*-family PTK besides Hck which is principally expressed in myeloid cells, co-localizes with markers of the specific granules in human neutrophils and translocates towards the plasma membrane during cell activation [24]. This study was the first indication that a PTK could be involved in the regulation of exocytosis in human neutrophils. In support of this suggestion, the PTK inhibitors genistein and herbimycin A were recently shown to diminish granule exocytosis in neutrophils [25].

Upon neutrophil stimulation, exocytosis of each of the three secretory granule populations, namely the specific, azurophil and gelatinase granules, occurs independently (for review, see [26]), suggesting the presence of distinct regulatory proteins on the surface of each granule subtype [27,28]. However, no suitable protein has been identified to date at the surface of azurophil granules. The high expression of Hck in mature human neutrophils, together with the potential involvement of *src*-family PTKs in intracellular traffic, led us to investigate the subcellular localization of Hck in resting and zymosan-activated human neutrophils.

EXPERIMENTAL

Materials

Antibodies

Rabbit anti-Hck antiserum was generated against a peptide

Abbreviations used: Hck, p59^{hck} (haematopoietic cell kinase); PTK, protein-tyrosine kinase; mbHck, non-granular membrane-bound Hck; cytHck, cytosolic Hck; grHck, granular membrane-bound Hck; HLA, histocompatibility locus antigen.

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corresponding to the N-terminal amino acid residues 38–52 that are unique to Hck [1,2]; this peptide was coupled to ovalbumin. The antiserum was affinity-purified by binding to the immobilized immunogen peptide [29]. Affinity-purified immunoglobulin directed against the N-terminal amino acids 8–37 of Hck was obtained from Santa Cruz Biotechnology, Inc. (Tebu, France). A serum directed against azurophil granules was generated by immunizing rabbits with intrinsic azurophil-granule membrane proteins which had been prepared as described [27].

Recombinant Hck

Hck cDNA, obtained from R. Perlmutter (Howard Hughes Medical Institute, Seattle, WA, U.S.A.), was subcloned in a pGEX 2T vector and expressed in *Escherichia coli* as a glutathione *S*-transferase–Hck fusion protein [30].

Cell isolation and cell culture

Human polymorphonuclear leukocytes

Neutrophils from healthy donors were isolated by Dextran sedimentation and centrifugation through Ficoll–Hypaque as described [31], and were resuspended in Hepes-buffered minimal essential medium.

HL60 and NB4 cells

HL60 and NB4 cells were grown in suspension in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY, U.S.A.), 1% L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere [32].

Neutrophil fractionation by differential centrifugation

Cells were fractionated essentially as described [33]. Briefly, neutrophils were resuspended at 10⁸ cells/ml in relaxation buffer (100 mM KCl, 3 mM NaCl, 10 mM Pipes, 3.5 mM MgCl₂, pH 7.2), which routinely contained the following proteinase inhibitors: 1000 units/ml aprotinin, 1 mM EDTA, 5 mM EGTA, 10 µg/ml leupeptin, 2 µg/ml pepstatin, 1 mM di-isopropyl fluorophosphate, 30 µM calpeptin and 1 mM PMSF, as well as 1 mM sodium vanadate. The cells were cavitated in a nitrogen bomb for 4 min at 2588 kPa (375 lb/in²). Using this method, 10% of the cells remained intact. Debris, nuclei and remaining cells were sedimented for 10 min at 250 *g*. The granules were sedimented from the post-nuclear supernatant at 15000 *g* for 10 min at 4 °C. The cytosol was separated from the post-granular membranes by centrifugation at 100000 *g* for 45 min at 4 °C. Fractions for immunoblotting were solubilized in boiling Laemmli buffer [34]. Fractions for immunoprecipitation were treated as detailed below. Fractions for ELISA assays were solubilized in the presence of 1% Triton X-100 overnight at 4 °C [35].

Subcellular fractionation of neutrophils on a discontinuous Percoll gradient

Fractionation on a Percoll gradient was performed as previously described [32,36]. Briefly, neutrophils were disrupted and debris was removed as described above. The post-nuclear supernatant was centrifuged on a discontinuous isotonic Percoll gradient at 48000 *g* for 20 min. Four fractions, corresponding to the cytosol, the non-granular membranes, the specific/gelatinase granules and the azurophil granules were recovered, and the Percoll was

removed by centrifugation at 250000 *g* for 90 min. All steps were performed at 4 °C. Fractions were resuspended as described above.

Solubilization, immunoprecipitation and PTK activity assay of Hck

Hck was solubilized to completion by resuspending the membrane pellets obtained during cell fractionation at 10⁸ cell equivalents/ml in ice-cold solubilization buffer (1% Nonidet P40 in TBS with routine proteinase inhibitors and sodium vanadate, at pH 7.4) and incubation in a melting-ice bath for 30 min. Solubilization was terminated by sedimenting the debris at 50000 *g* for 30 min at 4 °C. Before immunoprecipitation, 1 vol. of 2× solubilization buffer was added to 1 vol. of the cytosolic fraction. All fractions were cleared of proteins that bind non-specifically to protein A–Sepharose (Sigma) by incubation with the beads (15 mg/ml final concentration) for 30 min. After removal of the beads, anti-Hck antiserum was added (1:500 final dilution) to the fractions for 60 min. For precipitation, Protein A–Sepharose beads were added in excess (30 mg/ml final concentration), and the fractions were again incubated for 60 min. All incubations were performed in a melting-ice bath under gentle agitation. After immunoprecipitation, the beads were washed twice in EDTA-free solubilization buffer and twice in the reaction buffer utilized in the subsequent PTK activity assay. PTK activity was assayed with acid-denatured rabbit-muscle enolase (Boehringer Mannheim) as an exogenous non-specific substrate [22,37]. Hck immunoprecipitates were assayed at 10⁷ cell equivalents per sample, in a final volume of 50 µl. The washed beads were resuspended in 40 µl of ice-cold reaction buffer containing 100 mM Pipes, 10 mM MnCl₂, 1000 units/ml aprotinin, 0.5 mM PMSF and 100 µM vanadate, at pH 7.2. The incubation was carried out at 30 °C for 4 min, after the addition of 10 µl per tube of a cocktail containing 10 µCi of [³²P]ATP

Figure 1 Characterization of a polyclonal rabbit anti-Hck antiserum by immunoblotting (a) and immunoprecipitation (b)

(a) Proteins from non-granular neutrophil membranes (lanes 1–3 and 6; 50 µg/lane), *E. coli* lysate containing recombinant glutathione *S*-transferase–Hck (lane 4; 300 µg) or a Jurkat T-cell (J77) lysate (lane 5; 300 µg) were separated by SDS/PAGE (8% gel), transferred to nitrocellulose and immunoblotted with the antibodies listed below as detailed in the Experimental section. (b) Solubilized non-granular neutrophil membranes (lanes 1–3; 10 µg/lane), *E. coli* lysate containing glutathione *S*-transferase (GST)–Hck (lane 4; 300 µg), or Jurkat T-cell (J77) lysate (lane 5; 300 µg) were immunoprecipitated with the antibodies listed below, and subjected to a PTK-activity assay in the presence of acid-denatured enolase. Antibodies: lanes 1, 4, 5 and 6, anti-Hck antiserum; lanes 2, preimmunization anti-Hck serum; lanes 3, anti-Hck serum after antibody depletion with the peptide antigen. One experiment representative of three is shown.

Figure 2 Localization and activity of Hck in subcellular neutrophil fractions obtained by differential centrifugation

(a) Immunoblot of Hck in neutrophil whole-cell lysate and subcellular fractions obtained by differential centrifugation as detailed in the Experimental section. Proteins from 1×10^7 cell equivalents of whole-cell lysate (L), granules (G), non-granular membranes (M) or cytosol (C) were separated by SDS/PAGE (8% gel) and blotted with anti-Hck antiserum. (b) ELISA of marker enzymes for: plasma membrane, HLA class I (HLA); azurophil granules, myeloperoxidase (MPO); specific granules, lactoferrin (LF). (c) Hck PTK-activity assays with 10^7 cell equivalents of neutrophil whole-cell lysate (L), granules (G), non-granular membranes (M) or cytosol (C). Fractions were solubilized, immunoprecipitated with anti-Hck antiserum and then assayed for PTK activity *in vitro* as described in the Experimental section, in either the absence (left panel) or the presence (right panel) of 500 $\mu\text{g}/\text{ml}$ of a 200 000 g supernatant of promyelocytic NB4 cell sonicate to 'compete out' the proteolysis of Hck. The upper panels show the PTK activity and the lower panels show the corresponding quantity of Hck protein on the immunoblot after solubilization. Lane B shows the absence of Hck PTK activity and protein in the 200 000 g supernatant of the NB4 cell sonicate. One experiment representative of five is shown.

(6000 Ci/mmol), 50 μM MgATP and 500 $\mu\text{g}/\text{ml}$ acid-treated enolase. The reaction was terminated by boiling the samples for 5 min in $3 \times$ Laemmli buffer. Proteins were separated by SDS/PAGE (8% gels). Dried gels were exposed to autoradiography, and labelled bands were excised and quantified by β -counting.

Immunoblotting, marker enzymes and protein determination

For immunoblotting, proteins were separated by SDS/PAGE (8% gels), transferred to nitrocellulose and blotted as previously described [32]. Histocompatibility locus antigen (HLA) class I (plasma membrane), myeloperoxidase (azurophil granules) and lactoferrin (specific granules) were assayed by ELISA with antibodies kindly donated by N. Borregaard [28,35] (State University Hospital, Copenhagen, Denmark). Protein concentrations were determined using the Bio-Rad assay with BSA for standard curves as described by the supplier.

Indirect immunofluorescence

Human neutrophils were allowed to adhere for 5 min at 37 °C on to sterilized glass coverslips in 24-well tissue-culture plates as

previously described [27]. For immunolocalization of Hck in HL60 cells, the cells were differentiated with 20 ng/ml phorbol myristate acetate for 3 days on glass coverslips [32]. In some experiments, neutrophils or HL60 cells were stimulated with 4.5 mg/ml zymosan particles that had been opsonized in human serum [31]. Cells were fixed and permeabilized by plunging the glass coverslips into methanol at -20°C for 6 min, washed in PBS/0.1% Tween for 5 min and then exposed to primary and secondary antibodies (affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit IgG; KPL, Gaithersburg, MD, U.S.A.) [27].

RESULTS

The antiserum is specific for Hck

For our study of Hck in the neutrophil, we generated a rabbit anti-Hck antiserum and characterized it by immunoblots (Figure 1a) and immunoprecipitation experiments (Figure 1b) with non-granular neutrophil membranes. On immunoblotting, the antiserum labelled two bands, a major one of 59 kDa and a minor one of 61 kDa (Figure 1a, lane 1). The same bands were recognized by the affinity-purified antibodies (results not shown),

Figure 3 PTK activities of grHck, mbHck and cytHck

(a) ATP-dependence of grHck, mbHck and cytHck for autophosphorylation and enolase phosphorylation. Solubilized neutrophil granules, non-granular membranes or cytosol (10^7 cell equivalents/lane) were immunoprecipitated with anti-Hck antiserum and incubated in the presence of enolase with increasing concentrations of ATP at 30 °C for 4 min. The specific radioactivity of the ATP was kept constant at 80 Ci/mol. The grHck activity was exposed for autoradiography for 2 h and the mbHck and cytHck activities were exposed for 5 h (all at room temperature). (b) Time-dependence of the autophosphorylation (left panel) and enolase phosphorylation (right panel) activities of grHck, mbHck and cytHck (symbols as in a). Solubilized neutrophil granules, non-granular membranes and cytosol (10^7 cell equivalents/lane) were immunoprecipitated with anti-Hck antiserum and incubated in the presence of 10 μ M ATP (including 10 μ Ci of [³²P]ATP) at 30 °C for the indicated periods of time. One experiment representative of three is shown.

and by commercially available rabbit anti-Hck antibodies, which reacted in addition with an unidentified band at 92 kDa (Figure 1a, lane 6). Our antiserum also recognized recombinant glutathione *S*-transferase-Hck (Figures 1a and 1b, lanes 4), and it immunoprecipitated enzymically active Hck (Figure 1b, lanes 1 and 4). The serum did not cross-react with the other *src*-family PTKs that are expressed in neutrophils or Jurkat T cells, namely p55^{src}, p60^{src}, p53/56^{lyn} and p56^{lck} (Figures 1a and 1b, lanes 5). Therefore the different bands labelled or immunoprecipitated by the anti-Hck antiserum corresponded to genuine forms of non-

granular membrane-bound Hck (mbHck) with distinct electrophoretic mobilities.

Subcellular localization of Hck in fractions of human neutrophils

As a first approach to the subcellular localization of Hck, neutrophils were separated into three fractions by differential centrifugation: a fraction containing the three granule populations (azurophil, specific, and gelatinase granules), a fraction consisting of the non-granular membranes, and a cytosolic fraction. The extent of cross-contamination between the fractions was monitored with the marker enzymes HLA class I (plasma membrane), myeloperoxidase (azurophil granules), and lactoferrin (specific granules), and was found to be low (Figure 2b).

On immunoblotting, Hck was present in all three fractions, but mainly in the granule fraction (Figure 2a). Quantification of immunoblots by γ -radiation counting showed that approx. 60 % of the Hck is present in the granule fraction, 20 % in the non-granular membranes and 20 % in the cytosol. The same distribution was found when blots were hybridized with the commercial anti-Hck antibodies. Therefore Hck is mainly localized on the secretory granules in human neutrophils.

The granular Hck (grHck) signal on the immunoblot (Figure 2a, lane G) could only be observed when the granules had been quickly resuspended in boiling Laemmli buffer after their sedimentation and then boiled for 10 min, otherwise the protein was degraded (Figure 2c, lower left panel). This demonstrates that proteinases contained in the granule fraction can efficiently degrade grHck.

Both grHck (Figure 2a, lane G) and mbHck (Fig. 2a, lane M) migrated as two bands of 59 and 61 kDa. However, the 61 kDa form was mainly found in the granule fraction, whereas the 59 kDa form predominated in the non-granular membranes. Cytosolic Hck (cytHck) appeared as a doublet at 63 kDa (Figure 2a, lane C).

Immunoprecipitates of mbHck and cytHck were enzymically active, i.e. they underwent autophosphorylation and catalysed phosphorylation of the exogenous substrate enolase (Figure 2c). Four bands with apparent molecular masses of 59, 60, 61 and 63 kDa could be distinguished as autophosphorylation forms of mbHck (Figure 2c, lane M, and Figure 3a). The autophosphorylation forms of cytHck migrated with slightly lower electrophoretic mobilities (Figure 2c, lane C, and Figure 3a). We verified that the labelled bands were indeed phosphorylated on tyrosine residues by alkaline treatment of the gels [38] (results not shown). The enzymic PTK activities of mbHck and cytHck were in good agreement with the protein quantities found on Western blot. In contrast, the activity of grHck was at first difficult to detect, since the granular protein was proteolysed under standard immunoprecipitation conditions (Figure 2c, lower left panel, lane G). However, the grHck protein and its enzymic activity could be protected from proteolysis by addition of excess exogenous protein during solubilization and immunoprecipitation (Figure 2c, right panel, lane G). To achieve maximal competition by a large variety of soluble exogenous proteins, the 200000 *g* supernatant (500 μ g of protein/ml) of a sonicate of promyelocytic NB4 cells, which do not by themselves express Hck (Figure 2c, right panel, lane B), was included in the solubilization buffer. Comparison of the quantities of grHck on immunoblot before (Figure 2a) and after (Figure 2c, right panel) solubilization showed that by this means the proteolysis could be overcome completely. As a result, grHck activity per cell was 3-fold higher than mbHck or cytHck activity, in accordance with the quantities of protein found on immunoblot. The auto-

Figure 4 Susceptibility of Hck to proteolysis

(a) Hck immunoblot of neutrophil fractions obtained by Percoll-gradient centrifugation as detailed in the Experimental section. Proteins from 1×10^7 cell equivalents of azurophil granules (AG), specific granules (SG), non-granular membranes (M), or cytosol (C) were blotted. (b) Marker enzymes for plasma membrane (HLA-class I; HLA), azurophil granules (myeloperoxidase; MPO) and specific granules (lactoferrin; LF) were assayed by ELISA. One experiment representative of ten is shown. (c) Digestion assay. Fractions were obtained by Percoll-gradient centrifugation and resuspended in 0.34 M sucrose, 10 mM Pipes, pH 7.2. Increasing amounts of azurophil granules (AG) or specific/gelatinase granules (SG) were added to quantitatively constant aliquots of the Hck-positive non-granular membrane fraction (50 μ g/sample), followed 1 min after mixing of the two fractions by addition of 2 vol. of hot $2 \times$ Laemmli sample buffer and boiling for 10 min. Concentrations of AG and SG were 2, 5, 20 and 50 μ g/sample in lanes 2, 3, 4 and 5 respectively; 50 μ g corresponds to 2×10^7 cell equivalents of AG and 3×10^7 equivalents of SG. Proteins were separated by SDS/PAGE (8% gel) and blotted with anti-Hck antiserum. One experiment representative of seven is shown.

phosphorylation forms of grHck migrated quite distinctly from the mbHck and cytHck forms (Figure 2c, right panel, lane C, and Figure 3a).

Characterization of the different Hck activities

In order to further characterize the different forms of Hck found in the subcellular fractions, first the ATP-concentration-dependences of grHck (Figure 3a, top panel), mbHck (centre panel) and cytHck (bottom panel) activity were determined. For the determination of the properties of grHck activity, the 200000 g supernatant of a NB4 cell sonicate was included in the procedure, as described above. The K_m for ATP for the autophosphorylation of mbHck or cytHck was estimated to be 0.3 μ M, and the K_m for grHck autophosphorylation was 0.6 μ M. The K_m for ATP for enolase phosphorylation by mbHck or cytHck was 0.8 μ M, and for phosphorylation by grHck it was 2.2 μ M.

All Hck forms exhibited very similar kinetics of autophosphorylation (Figure 3b, left panel) and enolase phosphorylation (Figure 3b, right panel).

Hck is susceptible to degradation by an azurophil-granule-associated proteolytic activity

After finding that Hck is mainly localized on the secretory granules, we examined on which population of the secretory granules grHck was present. To do this, the azurophil granules were separated from the specific and gelatinase granules by an isotonic Percoll gradient [26]. However, grHck was not detectable in any of the granule fractions by immunoblotting (Figure 4a, lanes AG and SG). Although a maximum of 1 % of the granules

were disrupted during this fractionation procedure (Figure 4b, lane C), this was apparently sufficient for the complete degradation of grHck. We undertook extensive unsuccessful trials to inhibit this proteolysis, in addition to the routinely used proteinase inhibitors. Even the addition of the exogenous cytosolic proteins that protected the kinase during immunoprecipitation yielded no improvement. We further tried to shorten the Percoll procedure by plunging the fractions into methanol at -80°C immediately after the gradient step, but without success.

To find out in which of the granule populations the Hck-degrading proteinase(s) was located, increasing amounts of either azurophil or specific/gelatinase granules obtained by Percoll centrifugation were combined with aliquots of the Hck-positive non-granular membrane fraction. Immunoblotting showed that the proteolytic activity which can most efficiently degrade mbHck is associated primarily with the azurophil granules (Figure 4c, M + AG), and to a lesser extent with the specific/gelatinase granules (Figure 4c, M + SG). The Hck-degrading activity in the specific granule fraction could stem from the presence of contaminating azurophil granules (Figure 4b).

Detection of Hck on the azurophil granules by immunofluorescence

The efficient proteolysis of Hck did not allow the identification of the Hck-hosting granule population by biochemical methods. Therefore the subcellular localization of Hck was further investigated by indirect immunofluorescence, using a rapid cell-fixation method [28] in order to minimize proteolysis. When neutrophils were labelled with our affinity-purified anti-Hck

Figure 5 Subcellular localization of Hck by indirect immunofluorescence of neutrophils and HL60 cells

Immunofluorescence micrographs of human neutrophils (**a–c**, and insert of **e**) and differentiated myeloid HL60 cells (**d**, **e**) that were quick-fixed in methanol and exposed to the antibodies listed below, and then to fluorescent anti-rabbit antibodies as described in the Experimental section. Antibodies: (**a**) and (**d**), affinity-purified anti-Hck antibodies; (**b**) rabbit antiserum against intrinsic azurophil-granule membrane proteins; (**c**) affinity-purified rabbit antibody against rap1, a specific granule and plasma membrane protein; (**e**) fluorescent anti-rabbit antibody alone. One experiment representative of three is shown. The bar represents 10 μm .

serum, an intense staining of granules was revealed, showing again that Hck is mainly localized on the secretory granules (Figure 5a). To exclude potential non-specific binding of the antibodies to the granules, parallel experiments were performed with antibodies that had been preincubated with excess immunizing peptide. In these cases, the granules were not stained and the signal did not differ from that in cells that were only incubated with the second fluorescent antibody (Figure 5e), showing that the granular staining was indeed due to labelling of Hck. Similar images as with our antibodies were obtained with the affinity-purified commercial anti-Hck antibodies (results not shown) and with an antiserum directed against intrinsic

azurophil-granule proteins (Figure 5b). For comparison, the specific granules, labelled with affinity-purified anti-rap1 antibodies, appeared slightly different from the azurophil granules (Figure 5c). The size of specific and gelatinase granules is about half that of azurophil granules [26], suggesting that the Hck-positive granules are azurophil granules. In support of this notion, additional immunofluorescence experiments were carried out with the human myeloid cell line HL60, which possesses the azurophil granule population exclusively [39]. An intense granular labelling was obtained in the HL60 cells (Figure 5d), suggesting that grHck is indeed located on the azurophil granules. It is noteworthy that, on immunofluorescence of neutrophils or

Figure 6 Translocation of Hck towards the phagosome in phagocytosing neutrophils and HL60 cells

Immunofluorescence micrographs of neutrophils (**a**, **b**) and differentiated HL60 cells (**c–e**) that were stimulated with serum-opsonized zymosan for 30 min at 37 °C to perform phagocytosis prior to quick-fixation of the cells. Staining of Hck was performed as described in the legend to Figure 5. (**a**) and (**c**) focus on the Hck-positive secretory granules. (**b**), (**d**) and (**e**), focus on the phagosomes that had been formed around the engulfed zymosan particles. One experiment representative of three is shown. The bar represents 10 μ m.

HL60 cells, no Hck was found on the plasma membranes. For comparison, the plasma membrane was partially stained by antibodies against rap1 which, in addition to its localization on specific granules, also co-fractionates with plasma-membrane markers (Figure 5c) [27].

Translocation of Hck during phagocytosis

Next, we investigated whether the subcellular localization of Hck changed with the activation state of the cells. Azurophil granules are mobilized when the cells are stimulated to perform phagocytosis [40]. Immunofluorescence experiments were performed with phagocytosing neutrophils (Figures 6a and 6b) and HL60 cells (Figures 6c–6e). Following stimulation with serum-opsonized zymosan for 30 min, neutrophils and HL60 cells had

changed their morphology (Figures 6a and 6c) when compared with control cells (Figures 5a and 5d). Activated cells were partially depleted of Hck-positive granules in the cytoplasm; this can easily be observed in HL60 cells (Figure 6c). When the microscope was focused on the phagosomes which were formed around the engulfed zymosan particles (Figures 6b, 6d and 6e), a strong Hck fluorescence signal was observed in the region of the phagosomal membrane. This probably reflected the translocation of azurophil granules towards the phagosome. The location of grHck in the phagosomal area was observed 5 min after the addition of the zymosan particles to the neutrophils (results not shown). It should be noted that at no time point of cell activation could Hck be detected by immunofluorescence in any compartment other than the granules and the phagosomal area.

DISCUSSION

Hck is predominantly expressed in neutrophils [1], but its functional role has not yet been investigated in these cells. Here we investigated the subcellular localization of Hck in human neutrophils. Hck is mainly associated with the secretory granules, most probably the azurophil population, suggesting that these granules could be the major site of action for the physiological functions of Hck in human neutrophils.

Immunoblotting showed that Hck is mainly localized on the secretory granules, but is also present on non-granular membranes and in the cytosol. In addition, neutrophils contain several forms of Hck. Both grHck and mbHck have apparent molecular masses of 59 and 61 kDa, the 61 kDa form being mainly found in the granules and the 59 kDa form predominating in the non-granular membranes, whereas cytHck migrates as a doublet at 63 kDa. A previous report showed that hyperphosphorylation of *src*-family PTKs can cause shifts in their electrophoretic mobilities [41]. However, we found that mbHck and cytHck retained their electrophoretic mobilities after dephosphorylation by alkaline phosphatase, suggesting that at least the 59 and 63 kDa forms are distinct isoforms of Hck (results not shown). Unfortunately, during incubation of granules with alkaline phosphatase, grHck was degraded, thus preventing a similar characterization. Hck forms of 59 and 61 kDa have been described previously in whole-cell lysates of HL60 cells [5]. It was suggested that these forms are generated by the use of alternative translation start codons, in analogy to the origin of the 56 and 59 kDa forms of murine Hck [42]. The human 63 kDa cytHck has not been described before. The electrophoretic mobilities of the autophosphorylation forms in the PTK-activity assay suggest the existence of additional cytHck forms, smaller than 63 kDa, which were below the detection limit of the immunoblots. Further studies are needed to elucidate the molecular properties of the various forms of human Hck.

The enzymic activity of Hck (ATP-dependence and kinetics) is similar to that reported for p60^{src} [43]. As in the case of p60^{src}, the K_m values for ATP for the autophosphorylation forms of Hck are lower than the K_m for ATP for enolase phosphorylation [43]. In addition, the K_m for ATP of grHck is slightly higher than those of mbHck and cytHck.

grHck is highly susceptible to degradation by an azurophil-granule-associated proteolytic activity. The fact that grHck is located on a compartment which serves as a proteinase store suggests that the degradation might be a non-specific phenomenon. Alternatively, it could be a means of down-regulating the PTK activity of Hck, as was recently shown for p60^{c-src}, which is hydrolysed by the ubiquitous proteinase calpain in human platelets [44]. Calpain is a cytosolic proteinase which can be inhibited by calpeptin and millimolar concentrations of EGTA. The Hck-degrading proteinase cannot be inhibited by either 150 μ M calpeptin or 5 mM EGTA, indicating that Hck is not a substrate for calpain. Further, the Hck-degrading enzyme is probably not a serine proteinase, since we employed very efficient inhibitors against these enzymes, e.g. di-isopropyl fluorophosphate. For a continuation of the study of grHck by biochemical methods, successful inhibition of this proteolysis would be a prerequisite.

To identify the Hck-carrying granule population, immunofluorescence experiments were performed with cells that had been quick-fixed in methanol at -20°C [28] in order to minimize the proteolysis of Hck. The granular Hck signal which was observed in neutrophils was similar to the staining of azurophil granules, suggesting that the Hck-positive granules might be the azurophil population. However, the size difference between

specific and azurophil granules in the neutrophil did not allow an unambiguous identification of the Hck-positive granule population. Nevertheless, in HL60 cells, which only possess azurophil granules, a granular staining was also observed, further supporting the hypothesis that Hck is localized on the azurophil granules. Finally, it has recently been demonstrated that, while specific granules can fuse directly with the plasma membrane, azurophil granule secretion occurs via phagosomal or endosomal organelles [45]. Our observation that phagocytosis of opsonized zymosan was accompanied by depletion of the cytoplasm from Hck-positive granules, intense Hck staining at the phagosome membrane and the absence of plasma membrane labelling is in favour of the presence of Hck on the azurophil rather than the specific granules.

Only two other proteins have so far been found to be associated with the membranes of azurophil granules [26]: CD63, a neutrophil homologue of platelet granulophysin, and CD68, a macrophage-associated antigen. Interestingly, in platelets, CD63 co-localizes with p60^{c-src} on the dense granules, and they redistribute together during platelet activation [46,47]. Furthermore, CD63 is homologous to synaptophysin, which co-localizes with p60^{c-src} on synaptic vesicles and is a substrate for p60^{c-src} [48]. It is therefore conceivable that neutrophil granulophysin could be a substrate for Hck.

On immunoblotting, Hck was present in the non-granular membrane fraction, which contains the plasma membrane, endosomal vesicles, endoplasmic reticulum, Golgi and non-granular secretory vesicles [26,27]. Reports on myeloid, B lymphoma and embryonic stem cell lines [12–14] showed that at least a fraction of Hck is associated with plasma-membrane receptors. In addition, p60^{c-src} and p55^{c-fgr} are also partially associated with the plasma membrane in secretory cells [18,24]. However, the plasma membrane was not stained by anti-Hck antibodies in our immunofluorescence experiments, suggesting that the bulk of non-granular mbHck is localized on a dispersed intracellular membrane compartment. The labelling of such an intracellular membrane compartment may be difficult to detect under the intense granular Hck fluorescence signal. This does not of course exclude the possibility that a small proportion of mbHck, below the detection limit of immunofluorescence microscopes, is associated with the plasma membrane of neutrophils. A similar situation was reported for the *src*-family PTK p59^{lyn}, which was not detectable at the plasma membrane of T cells although several studies had demonstrated a plasma membrane receptor association [49].

The predominant localization of Hck on neutrophil granules, together with the observation that it translocates towards the phagosome during phagocytosis, suggests that this enzyme might be involved in regulating the process of phagocytosis-linked degranulation. Interestingly, a reduced particle uptake by macrophages was observed in *hck*-knockout mice [8]. p55^{c-fgr}, a *src*-family PTK which, like Hck, is mainly expressed in myeloid cells, is localized on the specific granules in human neutrophils and translocates towards the plasma membrane during cell activation. It was thus suggested that p55^{c-fgr} could be involved in regulating the degranulation of specific granules [24]. Here we have identified the first candidate protein that could be a regulator for the mobilization of azurophil granules. Therefore Hck and p55^{c-fgr} might be involved in the signal transduction events leading to the differential mobilization of their respective granule populations during the activation of human neutrophils.

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REFERENCES

- 1 Ziegler, S. F., Marth, J. D., Lewis, D. B. and Perlmutter, R. M. (1987) *Mol. Cell. Biol.* **7**, 2276–2285
- 2 Quintrell, N., Lebo, R., Varmus, H., Bishop, J. M., Pettenati, M. J., Le Beau, M. M., Diaz, M. O. and Rowley, J. D. (1987) *Mol. Cell. Biol.* **7**, 2267–2275
- 3 Holtzman, D. A., Cook, W. D. and Dunn, A. R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8325–8329
- 4 Willman, C. F., Stewart, C. C., Longacre, T. L., Head, D. R., Habbersett, R., Ziegler, S. F. and Perlmutter, R. M. (1991) *Blood* **77**, 726–734
- 5 Lichtenberg, U., Quintrell, N. and Bishop, J. M. (1992) *Oncogene* **7**, 849–858
- 6 Boulet, I., Ralph, S., Stanley, E., Lock, P., Dunn, A. R., Green, S. P. and Phillips, W. A. (1992) *Oncogene* **7**, 703–710
- 7 Ziegler, S. F., Wilson, C. B. and Perlmutter, R. M. (1988) *J. Exp. Med.* **168**, 1801–1810
- 8 Lowell, C. A., Soriano, P. and Varmus, H. E. (1994) *Genes Dev.* **8**, 387–398
- 9 English, B. K., Ihle, J. N., Myracle, A. and Yi, T. L. (1993) *J. Exp. Med.* **178**, 1017–1022
- 10 Ziegler, S. F., Levin, S. D. and Perlmutter, R. M. (1989) *Mol. Cell. Biol.* **9**, 2724–2727
- 11 Stefanova, I., Corcoran, M. L., Horak, E. M., Wahl, L. M., Bolen, J. B. and Horak, I. D. (1993) *J. Biol. Chem.* **268**, 20725–20728
- 12 Ghazizadeh, S., Bolen, J. B. and Fleit, H. B. (1994) *J. Biol. Chem.* **269**, 8878–8884
- 13 Li, Z.-H., Mahajan, S., Prendergast, M. M., Fargnoli, J., Zhu, X., Klages, S., Adam, D., Schieven, G. L., Blake, J., Bolen, J. B. and Burkhardt, A. L. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1536–1544
- 14 Ernst, M., Gearing, D. P. and Dunn, A. R. (1994) *EMBO J.* **13**, 1574–1584
- 15 Grandori, C. and Hanafusa, H. (1988) *J. Cell Biol.* **107**, 2125–2135
- 16 Rendu, F., Labret, M., Danielian, S., Fagard, R., Levy-Toledano, S. and Fischer, S. (1989) *Blood* **73**, 1545–1551
- 17 Linstedt, A. D., Vetter, M. L., Bishop, J. M. and Kelly, R. B. (1992) *J. Cell Biol.* **117**, 1077–1084
- 18 Anand, R., Wilkinson, J. M. and Kellie, S. (1993) *Oncogene* **8**, 3013–3020
- 19 David-Pfeuty, T. and Nouvian-Dooche, Y. (1992) *J. Cell Biol.* **118**, 3097–3116
- 20 Kaplan, K. B., Swedlow, J. R., Varmus, H. E. and Morgan, D. O. (1992) *J. Cell Biol.* **118**, 321–333
- 21 Redmond, T., Brott, B. K., Jove, R. and Welsh, M. J. (1992) *Cell Growth Differ.* **3**, 567–576
- 22 Marie-Cardine, A., Maridonneau-Parini, I., Ferrer, M., Danielian, S., Rothhut, B., Fagard, R., Dautry-Varsat, A. and Fischer, S. (1992) *J. Immunol.* **148**, 3879–3884
- 23 Marie-Cardine, A., Maridonneau-Parini, I. and Fischer, S. (1994) *Eur. J. Immunol.* **24**, 1255–1261
- 24 Gutkind, J. S. and Robbins, K. C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8783–8787
- 25 Asahi, M., Taniguchi, T., Hashimoto, E., Inazu, T., Maeda, H. and Yamamura, H. (1993) *J. Biol. Chem.* **268**, 23334–23338
- 26 Borregaard, N., Lollike, K., Kjedsen, L., Sengeløv, H., Bastholm, L., Nielsen, M. and Bainton, D. (1993) *Eur. J. Haematol.* **51**, 187–198
- 27 Maridonneau-Parini, I. and de Gunzburg, J. (1992) *J. Biol. Chem.* **267**, 6396–6402
- 28 Le Cabec, V. and Maridonneau-Parini, I. (1994) *Biochem. J.* **303**, 481–488
- 29 Smith, D. E. and Fisher, P. A. (1984) *J. Cell Biol.* **99**, 20–28
- 30 Smith, D. B. and Johnson, K. S. (1988) *Gene* **67**, 31–40
- 31 Maridonneau-Parini, I., Tringale, S. M. and Tauber, A. I. (1986) *J. Immunol.* **137**, 2925–2929
- 32 Maridonneau-Parini, I., Yang, C.-Z., Bornens, M. and Goud, B. (1991) *J. Clin. Invest.* **87**, 901–907
- 33 Le Cabec, V., Möhn, H., Gacon, G. and Maridonneau-Parini, I. (1994) *Biochem. Biophys. Res. Commun.* **198**, 1216–1224
- 34 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 35 Bjerrum, O. W. and Borregaard, N. (1990) *Scand. J. Immunol.* **31**, 305–313
- 36 Borregaard, N., Heiple, J. M., Simons, E. R. and Clark, R. A. (1983) *J. Cell Biol.* **97**, 52–61
- 37 Cooper, J. A., Esch, F. S., Taylor, S. S. and Hunter, T. (1984) *J. Biol. Chem.* **259**, 7835–7841
- 38 Beeman, K. and Hunter, T. (1978) *J. Virol.* **28**, 551–556
- 39 Harris, P. and Ralph, P. (1985) *J. Leukocyte Biol.* **37**, 407–422
- 40 Henson, P. M., Henson, J. E., Fittschen, C., Kimani, G., Bratton, D. L. and Riches, D. W. H. (1988) in *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., Goldstein, I. M. and Snyderman, R., eds.), pp. 363–390, Raven Press, New York
- 41 Danielian, S., Fagard, R., Alcover, A., Acuto, O. and Fisher, S. (1989) *Eur. J. Immunol.* **19**, 2183–2189
- 42 Lock, P., Ralph, S., Stanley, E., Boulet, I., Ramsay, R. and Dunn, A. R. (1991) *Mol. Cell. Biol.* **11**, 4363–4370
- 43 Feder, D. and Bishop, J. M. (1990) *J. Biol. Chem.* **265**, 8205–8211
- 44 Oda, A., Druker, B. J., Ariyoshi, H., Smith, M. and Salzman, E. W. (1993) *J. Biol. Chem.* **268**, 12603–12608
- 45 Fittschen, C. and Henson, P. M. (1994) *J. Clin. Invest.* **93**, 247–255
- 46 Nishibori, M., Cham, B., McNicol, A., Shalev, A., Jain, N. and Gerrard, J. M. (1993) *J. Clin. Invest.* **91**, 1775–1782
- 47 Rendu, F., Cramer, E. M., Tenza, D. and Fischer, S. (1993) *Platelets* **4**, 167–173
- 48 Barnekow, A., Jahn, R. and Scharlt, M. (1990) *Oncogene* **5**, 1019–1024
- 49 Ley, S. C., Marsh, M., Bebbington, C. R., Proudfoot, K. and Jordan, P. (1994) *J. Cell Biol.* **125**, 639–649