Hck Is Activated by Opsonized Zymosan and A23187 in Distinct Subcellular Fractions of Human Granulocytes*

(Received for publication, June 25, 1996, and in revised form, September 2, 1996)

Tyrosine phosphorylation is involved in the regulation of all major neutrophil responses: respiratory burst, degranulation, and phagocytosis (Ref. 1, and references therein). The extracellular ligands that initiate this tyrosine phosphorylation all appear to bind to receptors without intrinsic protein-tyrosine kinase (PTK) activity, so that the responsible PTKs must be of the non-receptor type (1).

Hck (hematopoietic cell kinase) is a non-receptor PTK of the Src family which is almost exclusively found in myeloid cells, predominantly in neutrophils (2, 3). Two isoforms of Hck exist, proteins of 59 and 61 kDa in humans, that are generated by alternative translation (4, 5). p59hck is exclusively membrane-bound, whereas p61hck can be membrane-bound or cytosolic (4–6). Hck is involved in the regulation of FcRIIB-mediated neutrophil activation (7) and FeγRL- and FeγRII-linked monocytic activation (8–10), in the production of tumor necrosis factor by macrophages (11), and in the signaling of lipopolysaccharide and urokinase receptors in monocytes (12, 13). Under certain conditions, phagocytosis is reduced in macrophages of Hck-deficient mice (14). Furthermore, Hck was shown to have overlapping roles with other Src family PTKs, i.e. with Src in osteoclast functioning (15) and with Fgr in integrin-dependent neutrophil activation (16). These results indicate that Hck is involved in many signal transduction pathways of myeloid cells, and that it can serve several functions even within a cell type.

It is conceivable that an enzyme such as Hck could serve more than one function within a cell due to its sequestration into several cellular compartments. We have shown that Hck is mainly localized on the membrane of azurophil granules in human neutrophils, but that it is also present in a granule-free membrane fraction and in the cytosol (6). During phagocytosis, Hck translocates with the azurophil granules to the phagosomal membrane (6) which is the major site for granule fusion in the course of this process (17). Furthermore, 20% of p59hck are localized on caveolae in monocytes (5), and finally, a partial association of Hck with the plasma membrane is suggested by its implication in FcγR signaling (7–10). Therefore, Hck is a multicompartmental enzyme, but at present it is not known whether the different subcellular fractions of the kinase are involved in different signal transduction pathways. In this study, we identified stimuli that activate Hck in granulocytes. We tested whether these stimuli can activate selectively Hck in different subcellular fractions, and we searched for a link between the subcellular localization of the activated Hck forms and the function of the respective cellular response.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—All-trans-retinoic acid was dissolved and stored at 10 μM in ethanol at −80 °C. Zymosan A yeast particles were opsonized (covered with complement factors and immunoglobulins) with human serum as described (18). PMA was prepared at 1 mg/ml in dimethyl sulfoxide and stored at −80 °C. Forskyl-methionyl-leucyl-phenylalanine (FMLP) was solubilized in methanol at 1 μM, then diluted with H2O to 1 mM, and kept at −20 °C. The calcium ionophore A23187 was stored at 10 μM in dimethyl sulfoxide at −20 °C. All these reagents were from Sigma. Sodium vanadate (from Amersham) was prepared as reported (19) and stored in the dark at 4 °C for maximally 2 months. Polyclonal rabbit anti-Hck antiserum or affinity-purified anti-Hck IgG (from Santa Cruz Biotechnology) were used as described (6).

Cells—Human promyelocytic NB4 cells were cultured as described (1). For differentiation into neutrophil-like cells, they were maintained in the presence of 1 μM all-trans-retinoic acid for 5 days, unless otherwise indicated (20). Nonadherent cells were used for experiments. Human neutrophils from healthy donors were isolated by dextran sedimentation and centrifugation through Ficoll-Hypaque as reported (21), and were reuspended in minimal essential medium buffered with 20 mM Hepes, pH 7.4.

Cell Activation—Before cell activation, neutrophils were incubated for 5 min at 4 °C with 1 μM diisopropyl fluorophosphate and then

*This study was funded in part by grants from the Ligue Nationale contre le Cancer, Association pour la Recherche sur la Polyarthrite, and the Association Recherche contre le Cancer, Association pour la Recherche sur le Cancer, Association Recherche sur la Polyarthrite, et Partage. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Supported by a scholarship from the Boehringer Ingelheim Foundation.

§To whom correspondence should be addressed. Tel.: 33-5-61-17-59-77; Fax: 33-5-61-17-59-94.

The abbreviations used are: PTK, protein-tyrosine kinase; FcγR, receptor for the Fc region of IgG; FMLP, formyl-methionyl-leucyl-phenylalanine; Hck, p59/61hck, hematopoietic cell kinase; O2, serum-opsonized zymosan; PMA, phorbol myristate acetate; Pipes, 1,4-piperazine diethanesulfonic acid; GTPγS, guanosine 5′-O-(thio)triphosphate; PAGE, polyacrylamide gel electrophoresis.

Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 272, No. 1, Issue of January 3, pp. 102–109, 1997

Subcellular Fractions of Human Granulocytes*

From the Institut de Pharmacologie et de Biologie Structurale, CNRS, UPR 9062, 205 route de Narbonne, 31077 Toulouse Cedex, France

Heidi Welch‡ and Isabelle Maridonneau-Parini§

This paper is available on line at http://www-jbc.stanford.edu/jbc/
Hck in Granulocyte Activation

103

washed once. For activation, NB4 cells or neutrophils were taken up at 1 × 10^7 cells/ml in minimal essential medium buffered with 20 mM Hepes, pH 7.4, prewarmed for 20 min at 37 °C, and then stimulated for various time points with either 4.5 mg/ml serum-saponized zymosan (OSO, 5 μg/ml FMLP, 100 ng/ml PMA or 5 μM A23187 (final concentrations). Activated cells were terminated by transferring the tubes to a melting ice bath and, in Hck experiments, by immediate addition of 5 volumes of ice-cold Hepes-buffered minimal essential medium containing 1 mM EDTA. The cells were then sedimented at 300 × g for 10 min at 4 °C, and either whole cell lysates were prepared or the cells were fractionated as described below. For all experiments, cell activation was monitored in parallel by measurement of the NADPH oxidase and degranulation activities.

Superoxide Production and Degranulation—NADPH oxidase activity was determined as the superoxide dismutase-inhibitable reduction of cytochrome c by discontinuous measurement as described (22), using a double-beam Uvikon 930 spectrophotometer (Kontron, France). Superoxide production was calculated from the increase in absorbance at 550 nm with an extinction coefficient of 21.1 mM^-1 cm^-1. Degranulation of azurophil NB4 cell granules was measured as β-glucuronidase release. Cells were activated at 5 × 10^6 cells/ml and then sedimented. The supernatant containing secreted β-glucuronidase was frozen in the assay. The cell pellets were resuspended in 8 mM Na$_2$PO$_4$/KH$_2$PO$_4$, 1% Triton X-100, 3 mM KCl, 0.5 M NaCl, pH 7.4, passed several times through a 25-gauge needle, and solubilized overnight at 4 °C. Unsolubilized material was removed at 11,000 × g for 45 min. The solubilized pellet and cell supernatant were incubated with 1 mg/ml 4-nitrophenyl-β-D-glucopyranosiduronic acid (Merk) as substrate for β-glucuronidase in 0.1 M acetate buffer at pH 4.5 for 18 h at room temperature in the dark. Color development was achieved by addition of 1 volume of 2 N NaOH, and the samples were measured in a Uvikon 930 spectrophotometer at 405 nm.

Cell Fractionation—NB4 cells were fractionated by differential centrifugation essentially as described previously (6, 23). The cells (2.5 × 10^6/ml) were cavitatated in a nitrogen bomb at 350 p.s.i. for 4 min at 4 °C in the following buffer: 100 mM KCl, 3 mM NaCl, 10 mM Pipes, pH 7.3, 3.5 mM MgCl$_2$, 1000 IU/mg aprotinin, 1 mM EDTA, 5 mM EGTA, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM diisopropyl fluorophosphate, 30 μM calpeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate. In time course experiments the cells were sonicated instead of cavitatated with a Branson tip sonifier with 20 strokes at output control 1, duty cycle 20, in a melting-ice bath. Each cell disruption method left approximately 5–10% unbroken cells. The nuclei, cell debris, and resting intact cells were then sedimented at 300 × g for 10 min. For in vitro phagocytosis experiments, this fraction also contained all the OZ (either free, enclosed in phagosomes, or bound to plasma membrane receptors). The 300 × g supernatant was free of OZ particles, as determined microscopically. A fraction enriched in azurophil granules was then obtained by centrifugation at 11,000 × g for 10 min, followed by separation of the granule-free membranes and the cytisol at 200,000 × g for 45 min. Marker proteins, human leukocyte antigen class I (plasma membrane marker), and myeloperoxidase (azurophil granule marker), were assayed by enzyme-linked immunosorbent assay (6).

Solubilization and Immunoprecipitation of Hck, and in Vitro PTK Activity Assay—Hck was solubilized from NB4 cells or cell fractions with a buffer containing 2% Nonidet P-40 (1), then immunoprecipitated and assayed for its in vitro PTK activity in the presence of acid-treated enolase as exogenous substrate, 10 mM MnCl$_2$, 10 mM MgATP, and 10 μCi of [γ-32P]ATP (6000 mCi/mmol), as described (1, 6). In the kinase assay, both Hck autophosphorylation and phosphorylation of the exogenous substrate enolase by Hck were measured. During cell stimulation, these activities changed in parallel, but under "Results" only phosphorylation of enolase by Hck is shown. The relative specific activity of Hck was calculated dividing the PTK activity by the relative quantity of Hck in a sample. The relative quantity of Hck protein was estimated by Western blotting of immunoprecipitates that were done with affinity-purified anti-Hck IgG covalently coupled to CNBr-activated Sepharose beads (from Pharmacia Biotech Inc.) according to the manufacturers instructions, in parallel to the measurement of PTK activity. For solubilization and immunoprecipitation of Hck from human neutrophils, 200,000 × g supernatant of a sonicate of non-differentiated NB4 cells was used (approx. 1 mg of protein/ml) instead of water in all buffers, in order to compete out the proteolysis of Hck by azurophil-granule-associated protease(s).

Expression of Hck, Acquisition of NADPH Oxidase Activity, and Degranulation during NB4 Cell Differentiation

In non-differentiated NB4 cells, Hck was undetectable by Western blotting (Fig. 1A) or in vitro PTK activity assays of whole cell lysates (Fig. 1B). Upon differentiation of NB4 cells with 1 μM all-trans-retinoic acid (20), Hck became expressed, reaching a plateau of expression at day 5. The acquisition of elementary features of mature granulocytes, a functional NADPH oxidase activity (Fig. 1C), and the capability to mobilize their azurophil granules (Fig. 1D), was also investigated. These cellular responses appeared in parallel to Hck expression. Acquisition of the third major granulocyte response, phagocytosis, during retinoic acid differentiation of NB4 cells has been reported earlier (24). Therefore, in all further experiments, NB4 cells were differentiated for 5 days with 1 μM all-trans-retinoic acid in order to obtain neutrophil-like cells.

Hck is Mainly Localized in the Secretory Granule Fraction of NB4 Cells, and Different Forms Are Found in Different Fractions

When neutrophils are disrupted and the post-nuclear supernatant is fractionated by differential centrifugation, Hck is mainly found in the secretory granule fraction; it is also present in granule-free membranes and the cytosol (6). To further validate the NB4 cell model, we verified that the distribution of Hck in differentiated NB4 cells was similar to that in neutrophils. The secretory granule fraction (G), which contained nearly all the azurophil-granule marker (myeloperoxidase; Fig. 2A), also contained most of Hck (Fig. 2, B and C). Since this NB4 cell fraction G was contaminated with plasma membrane marker (human leukocyte antigen class I), it was defined as a granule-enriched fraction. The non-granular membranes (M) and cytosol (C) were essentially free of contaminating markers (Fig. 2A) and contained small amounts of Hck (Fig. 2, B and C). Quantification of the Western blot in Fig. 2B by densitometric scanning showed that 55% of the total cellular Hck was present in the granule-enriched fraction, 12% in the granule-free membranes, and 14% in the cytosol. Some Hck (18%) was also present in the nuclear pellet, corresponding to the remaining intact cells and contaminating granules. In addition, the partitioning of the different Hck isoforms between the subcellular fraction was similar in NB4 cells and neutrophils (6). The granule-enriched fraction contained more p61$^{hck}$ than p59$^{hck}$ whereas in the situation was inverse in the granule-free membranes, and the cytosol contained exclusively soluble p61$^{hck}$ (Fig. 2B). Importantly, the protease(s) that degrades the granular form of Hck in neutrophils was either not present, or not active, in NB4 cells. This cell line constitutes therefore an adequate cell line for our study.
OZ, A23187, fMLP, and PMA Differentially Activate NADPH Oxidase and Degranulation in NB4 Cells

Before studying Hck activation, we screened various stimuli that are widely used in neutrophil activation for their efficiency to stimulate retinoic acid-differentiated NB4 cells. The cells were stimulated for various periods of time with the receptor agonists OZ or fMLP, or with stimuli that by-pass membrane receptors, PMA or A23187. The efficiency of these agents to stimulate the NADPH oxidase response was of the following order: PMA > OZ > fMLP > A23187 (Fig. 3A). For azurophil granule exocytosis, it was OZ > PMA > A23187, while fMLP did not stimulate degranulation (Fig. 3B). PMA was quite a good secretory stimulus, like in differentiated HL60 neutrophil-like cells (25), although it does not induce secretion of azurophil granules in human neutrophils. OZ is, in addition, a particulate stimulus that is phagocytosed by the cells. Therefore, using these stimuli, which can elicit either one or more of the cellular responses, we were able to test whether Hck could be involved in the signal transduction pathways that they activate.

OZ and A23187, but Not PMA, Activate Hck in NB4 Cells and Human Neutrophils

OZ and A23187, but not PMA, activated Hck in retinoic acid-differentiated NB4 cells (Fig. 4). Activation of Hck was 1.7-fold over the basal activity of the kinase, it occurred within seconds of adding the stimuli to the cells, and it was transient, being back to basal at 30 min (see also Fig. 6). The Hck response to OZ was biphasic, with a first sharp peak at 0.5 min, and a plateau-like high activity between 5 and 10 min of stimulation. A23187-induced Hck activation resembled somewhat the OZ response. In NB4 cells, Hck was also activated by fMLP, in a weaker (1.4-fold) and more transient manner (back to basal at 7.5 min).

We studied whether the activation of Hck by these stimuli was similar in human neutrophils. As in NB4 cells, OZ and A23187 activated Hck in neutrophils, with similar efficiency, whereas PMA did not significantly affect it (Fig. 5). A difference between the two cell types was that the weak activation of Hck by fMLP was not found in neutrophils. Now that we had identified stimuli that activate Hck, we could examine next whether the different subcellular fractions of Hck might be activated differentially.

Activation of Hck in Subcellular Fractions of NB4 Cells

OZ Activates Hck in the Granule- and the OZ-containing Fractions—Stimulation of human neutrophils with OZ provokes translocation of Hck with the azurophil granules to the OZ-containing phagosomes (6). Therefore, in experiments with OZ, a fourth NB4 cell fraction, the OZ-containing fraction, was recovered.

Hck activation was strongest (2–3-fold) in the granule-enriched and in the OZ-containing fractions (Fig. 6). Activation in the OZ-containing fraction was biphasic with a first peak between 0.5 and 1.5 min and a second one at 5 min. Activation in the granule-enriched fraction occurred between 5 and 10 min of stimulation. As approximately 55% of the total Hck activity per cell is localized in the granule-enriched fraction (see Fig. 2), activation in this fraction accounted for most of the effect seen with OZ in whole cell lysates. The cytosolic form of Hck was also weakly activated, but Hck in the granule-free membrane fraction was not activated at all. These results show that the different forms of Hck, in their respective subcellular compartments, can be activated selectively during OZ signal transduction.

We tested whether activation of Hck by OZ was due to translocation of Hck protein into the respective subcellular fractions. Translocation was monitored by Western blotting (Fig. 7) and quantified by densitometric scanning of the blots. As expected, Hck translocated progressively into the OZ-containing fraction during cell stimulation (panel O, 2.5-fold at 30 min compared to zero time) whereas it decreased concomitantly in the granule-enriched fraction (panel G, 0.6-fold at 30 min). Hck also accumulated in the granule-free membrane fraction and weakly in the cytosol (panel M, 2.4-fold, and panel C, 1.3-fold at 30 min). Western blots of whole cell lysates (panel L) are shown to demonstrate that the overall amount of Hck in the cell did not change during the incubation period. Both isoforms...
of Hck, p59\textsuperscript{hck} and p61\textsuperscript{hck}, translocated between the membrane fractions, and apparently with similar kinetics. The slight accumulation of p61\textsuperscript{hck} in the cytosol suggests that a small amount of the membrane-bound form was converted to the soluble form of p61\textsuperscript{hck}, whereas activation of Hck protein accumulated approximately 1.5-fold in the granule-enriched fraction, whereas it decreased in the granule-free membranes to half of the level in resting cells (Fig. 7). The amount of Hck in the cytosol did not change in A23187-stimulated cells during the incubation period.

**fMLP Has a Weak Effect on Hck in the Granule-enriched Fraction and the Cytosol, and PMA Does Not Activate Hck in Any Fraction**—Finally, we checked whether the weak effect of fMLP, and the lack of effect of PMA, on Hck activity in the whole cell lysates of NB4 cells, might be due to simultaneous activation and inhibition of Hck in different fractions. This was not the case. When the cells were stimulated with fMLP, Hck was activated 1.4-fold in the granule-enriched fraction and in the cytosol (Fig. 6), yielding a similar profile to that seen in whole cell lysates. PMA did not affect the activity of any Hck fraction (Fig. 6). Furthermore, neither fMLP nor PMA caused translocation of Hck between the fractions (maximal changes were 0.9- and 1.2-fold of the controls after 7.5 min of stimulation with fMLP, or after 30 min of stimulation with PMA, respectively) (Fig. 7).

**Specific Activity of Hck in Fractions of Stimulated NB4 Cells**—For calculation of the relative specific activity, Hck protein was quantitated by Western blotting after its immunoprecipitation from fractions showing peaks of activation and from the respective zero time controls, i.e. the quantity of Hck was determined under the same conditions as the kinase activity. This method of Hck quantification gave similar results to the data obtained from the Western blots shown in Fig. 7 that were performed without prior immunoprecipitation (Table I). In the case of NB4 cell stimulation with OZ, Hck activation in the granule-enriched fraction was due to an approximately 5-fold increase in specific activity, whereas activation in the cytosol was caused by accumulation of Hck protein. Activation in the...
OZ-containing fraction was a combination of translocation and increase in specific activity. On the contrary, when the cells were stimulated with A23187, the increase in relative specific Hck activity was moderate in the granule-enriched fraction (1.7-fold), while it was 3-fold in the granule-free membrane fraction. Stimulation with fMLP caused a weak increase in relative specific Hck activity in both the granule-enriched fraction and the cytosol.

**DISCUSSION**

Src family PTKs are regulators of a great variety of signal transduction events. Several of them, particularly Hck, are multicompartmental enzymes. This has given rise to the hypothesis that the sequestration of these kinases into several compartments might govern their involvement in distinct pathways. Our study shows that OZ and A23187, which produce similar activation profiles of Hck in whole cells, activate distinct subcellular fractions of Hck.

In this study, we have compared the particulate stimulus OZ with three soluble stimuli. They elicited to various degrees the granulocyte responses of NADPH oxidase and degranulation and, in addition, OZ induced phagocytosis. We have demonstrated that OZ and A23187 activate Hck in NB4 cells and human neutrophils. Hck activation was rapid and transient, consistent with a role of the kinase in early OZ and A23187 signaling. The level of activation was similar in both cell types, and it was comparable to those reported for the activation of Hck in FcyR-dependent signaling (9), or for other Src family PTKs, like Fgr in integrin-mediated neutrophil activation (26), or Lyn in fMLP signaling (27). Signaling by the particulate stimulus OZ is partially mediated by receptors for the opsonic factors, FcyRs and complement receptors (a subgroup of integrins), and Hck is known to be one of the PTKs that mediate FcyR and integrin signaling (7–10, 16). Hck activation by A23187, in the presence of 1.5 mM calcium in the extracellular medium, is in line with two other reports that have shown an implication of Src in calcium-related signaling (28, 29).

fMLP and PMA are two other potent activators of granulocyte responses, and signaling by both stimuli involves tyrosine phosphorylation (see Ref. 1, and references therein). Recently, Lyn has been identified as one of the PTKs activated by fLMP in neutrophils (27, 30). In our study, Hck was only weakly activated by fMLP in NB4 cells, and not in neutrophils, while Lyn was activated 2.5-fold under the same conditions (data not shown). Thus, it is unlikely that Hck could play a key role in fMLP signaling. PMA did not activate Hck at any time up to 30 min. Together, our findings suggest that Hck is involved in OZ and A23187-induced pathways leading to early granulocyte responses, but not in PMA-generated signals.

It has recently been proposed that Hck could be activated subsequently to the respiratory burst response, by NADPH oxidase-derived superoxide anions (31). This was based on the observations that O_{2} can stimulate tyrosine phosphorylation (31, 32) and that both NADPH oxidase and Hck activities increased in GTPγS-stimulated permeabilized neutrophils (31). We show here in intact cells, that there is no apparent correlation between increases in Hck and NADPH oxidase activities. Indeed, PMA, by far the most powerful stimulus for O_{2} production in neutrophils and NB4 cells, did not activate Hck.

Having identified stimuli that activate Hck in whole cells,
next we tested whether different subcellular fractions of Hck can be activated selectively. OZ strongly activated Hck in the granule- and OZ-containing fractions, weakly in the cytosol, and not at all in the granule-free membranes. While activation in the granule-enriched fraction was due to a strong increase in its specific activity, Hck activation in the OZ-containing and cytosolic fractions was rather caused by translocation of the kinase into these compartments. Accumulation of Hck in the OZ-containing fraction was consistent with the phagosome–granule fusion events that accompany phagocytosis (17), and translocation to the cytosolic fraction upon cell stimulation has been reported earlier for Src in platelet-derived growth factor-stimulated platelets (33). The finding that Hck was not activated in the granule-free membrane fraction was unexpected, since this fraction contains the plasma membrane, and therefore FcR IIIB and integrins. As OZ particles sediment at 300 × g, the receptors bound to OZ might co-sediment during this centrifugation step; it is therefore possible that part of the Hck activation in the OZ-containing fraction results from plasma-membrane receptor triggering.

A23187 also selectively activated Hck in certain subcellular compartments, in the granule-enriched and granule-free membrane fractions, but not in the cytosol. Increase in the specific kinase activity was stronger in the granule-free fraction than in the granule-enriched fraction. This could be the result of potential differences between the calcium sensitivity of the p59hck and p61hck isoforms which are predominant in the granule-free and the granule-enriched fraction, respectively. This hypothesis is currently under investigation in the laboratory. Thus, the effects of OZ and A23187 on Hck activity are complex, as they involve translocation of the kinase and selective
Hck in Granulocyte Activation

Quantification of Hck was performed by two methods for each stimulus. For each method, fractions were chosen from separate experiments of those shown in Fig. 6. One method gave the data set shown in Fig. 7. For the second method, samples were chosen from time points with peaks of Hck activation. Hck was immunoprecipitated from these samples with Sepharose-coupled antibody, prior to SDS-PAGE, Western blotting, autoradiography, and densitometric scanning of the Hck bands as described under “Experimental Procedures.” Relative specific Hck activity was calculated, dividing the mean activity by the quantity of immunoprecipitated Hck (determined by method two) in the respective samples.

| Stimulus Fraction Time | Quantity method 1 | Quantity method 2 | Total activity | Specific activity |
|------------------------|-------------------|-------------------|----------------|-----------------|
|                        | X-fold over basal |                   |                |                 |
| OZ                     | 5                 | 1.07              | 1.41          | 2.32 ± 0.96     | 1.65            |
| G                      | 10                | 0.39              | 0.47          | 2.51 ± 1.02     | 5.34            |
| C                      | 5                 | 1.14              | 1.73          | 1.50 ± 0.25     | 0.87            |
| A23187                 | 5                 | 1.66              | 1.36          | 2.32 ± 0.30     | 1.71            |
| M                      | 1                 | 0.82              | 0.58          | 1.78 ± 0.44     | 3.07            |
| IMLP                   | 1                 | 1.07              | 1.08          | 1.47 ± 0.14     | 1.36            |
| C                      | 2                 | 0.93              | 1.15          | 1.45 ± 0.23     | 1.26            |

It will be important to determine whether the activation is due to one or both isoforms of Hck, p59

activation in distinct subcellular fractions.

The cell fractionation approach permitted us to show that although the profiles of Hck activation by OZ and A23187 were quite similar in whole cell lysates, the stimuli affected different subcellular fractions. OZ augmented the specific Hck activity in the granule-enriched fraction by 5-fold, while A23187 produced a stronger increase in specific Hck activity in the granule-free membrane fraction (3-fold) than in the granule-enriched membranes (1.7-fold). Therefore, comparison of the effects of OZ and A23187 on Hck by subcellular fractionation has demonstrated that different stimuli can activate distinct fractions of Hck.

It is impossible to distinguish between the isoforms in the kinase assay and to correlate them to the two bands quantified by Western blotting. Isoform-specific antibodies would be necessary to investigate this question further, but such antibodies are currently not available.

Src family PTK activation in selected subcellular compartments has been shown once before in our laboratory. The specific activity of the Src family PTK Lck increases 1.6-fold upon internalization of the kinase to endosomal structures in CD2-triggered T lymphocytes (34). Other reports have shown activation of several Src family PTKs in Triton X-100-insoluble fractions (7, 35, 36). It seems therefore that selective activation of Src family PTKs in distinct subcellular compartments, together with translocation events, are general behavior of these kinases.

Since the localization of proteins is crucial for their functional role, we have attempted to establish a correlation between the subcellular fractions in which Hck is activated, and the cell responses elicited by a given stimulus. We have shown here that, upon cell stimulation with OZ, which is a potent inducer of azurophil granule mobilization, Hck activation was strongest in the granule-enriched fraction. Together with our observations that Hck is mainly localized in the granular fraction and translocates with the azurophil granules toward the phagosomal membrane during OZ-induced phagocytosis in neutrophils and HL60 cells (6), this report suggests that Hck could regulate phagocytosis-linked degranulation. Since A23187 also activates Hck in the granule-enriched fraction, although with a weaker increase in specific activity, the putative role of Hck in the secretory process seems not be restricted to secretion of azurophil granules toward phagosomes, but to be more general. Furthermore, on the signal transduction pathways leading to degranulation, Hck should be either situated upstream of protein kinase C or in an independent pathway. This can be deduced from our results showing that PMA, although a good stimulus for degranulation in NB4 cells, does not involve Hck in its signaling mechanisms.

Other investigations of the subcellular localization of Src family PTKs have revealed that, apart from Hck, Fgr and Src are also present on secretory vesicles (6, 37–40). Therefore, it has long been suggested that these kinases could play a role in the secretory process. However, before this study, it has never been attempted to measure PTK activities in secretory compartments isolated from cells undergoing secretion. Our results show that subcellular fractionation could be a valuable tool to correlate PTK activation to cellular responses.

In conclusion, this work shows that Hck can be differentially activated in its various subcellular compartments. This could give the kinase access to several discrete sets of effectors and substrates, thus enabling it to serve more than one function within a cell. We propose that one of the functions of Hck could be the regulation of degranulation.

Acknowledgments—We gratefully acknowledge Michel Lanotte for the gift of the NB4 cell line and Honore Mazarguil for the preparation of immunogen anti-Hck peptide.

REFERENCES
1. Welch, H., Mauran, C., and Maridonneau-Parini, I. (1996) Methods: A Companion to Methods in Enzymology 9, 607–618
2. Ziegler, S. F., Marth, J. D., Lewis, D. B., and Perlmutter, R. M. (1987) Mol. Cell. Biol. 7, 2276–2285
3. Quintrell, N., Lebe, 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
4. Lock, P., Ralph, S., Stanley, E., Boulet, I., Ramsay, R., and Dunn, A. R. (1991) Mol. Cell. Biol. 11, 4363–4370
5. Robbins, S., Quintrell, N. A., and Bishop, J. M. (1995) Mol. Cell. Biol. 15, 3507–3515
6. Mo¨hn, H., Le Cabec, V., Fischer, S., and Maridonneau-Parini, I. (1995) Biochem. J. 309, 657–665
7. Zhou, M., Lublin, D. M., Link, D. C., and Brown, E. J. (1995) J. Biol. Chem. 270, 13553–13560
8. Ghazizadeh, S., Bolen, J. B., and Fleit, H. B. (1994) J. Biol. Chem. 269, 8878–8884
9. Wang, A. V. T., Scholl, P. R., and Geha, R. S. (1994) J. Exp. Med. 180, 1165–1170
10. Durden, D. L., Kim, H. M., Calore, B., and Liu, Y. (1995) J. Immunol. 154, 1039–1047
11. English, B. K., Ihle, J. N., Myracle, A., and Yi, T. (1995) J. Exp. Med. 178, 1017–1022
12. Stefanova, I., Corcoran, M. L., Horak, E. M., Wahl, M. L., Bolen, J. B., and Horak, I. D. (1995) J. Biol. Chem. 270, 20725–20728
13. Resnati, M., Guttinger, M., Valeamunicosa, S., Sidienie, T., Blasi, F., and Fazioli, F. (1996) EMBO J. 15, 1572–1582
14. Lowell, C. A., Soriano, P., and Varmus, H. E. (1994) Gene Dev. 8, 378–387
15. Lowell, C. A., Niwa, M., Soriano, P., and Varmus, H. E. (1996) Blood 87, 1780–1792
16. Lowell, C. A., Fumagalli, L., and Bertos, G. (1996) J. Cell. Biochem. 63, 895–910
17. Fanger, M., Henson, J. E., Fittschen, C., Bratton, D. L., and Biches, D. H. (1992) In Inflammation. Basic Principles and Clinical Correlates (Gallin, J. I., Goldstein, I. M., and Snyderman, R., eds.) pp. 411–439, Raven Press, New York
18. Maridonneau-Parini, I., Tringale, S. M., and Tauber, A. I. (1986) J. Immunol. 137, 2952–2959
19. Krypta, R. M., Hemming, A., and Courtneidge, S. (1988) EMBO J. 7, 3851–3854
20. Lanotte, M., Martin-Touvenin, V., Najman, S., Balerini, P., Valensi, F., and Berger, R. (1991) Blood 77, 1080–1086
21. Maridonneau-Parini, I., and de Gunzburg, J. (1992) J. Biol. Chem. 267, 6396–6402
22. Le Cabec, V., and Maridonneau-Parini, I. (1995) J. Biol. Chem. 270, 2067–2073
23. Le Cabec, V., and Maridonneau-Parini, I. (1994) Biochem. J. 303, 481–487
24. Testa, U., Grgini, F., Barberi, T., Fagioli, M., Maceiulli, R., Ferrucci, P. F., Seripa, D., Camagna, A., Alcalay, M., Pellicci, P. G., and Peschle, C. (1994) Cancer Res. 54, 4508–4518
25. Blackwell, G. J., Bonser, R. W., Dawson, J., and Garland, L. G. (1985) Biochem.
26. Berton, G., Fumagalli, L., Laudanna, C., and Sorio, C. (1994) J. Cell Biol. **126**, 1111–1121.
27. Ptasznik, A., Traynor-Kaplan, A., and Bokoch, G. M. (1995) J. Biol. Chem. **270**, 19969–19973.
28. Rusanescu, G., Qi, H., Thomas, S. M., Brugge, J. S., and Helegous, S. (1995) Neuron **15**, 1415–1425.
29. Zhao, Y., Sudol, M., Hanafusa, H., and Krueger, J. (1992) Proc. Natl. Acad. Sci. U. S. A. **89**, 8298–8302.
30. Gaudry, M., Gilbert, C., Barabé, F., Poujol, P. E., and Naccache, P. H. (1995) Blood **86**, 3567–3574.
31. Brumell, J. H., Burkhardt, A. L., Bolen, J. B., and Grinstein, S. (1996) J. Biol. Chem. **271**, 1455–1461.
32. Fialkow, L., Chan, C. K., Grinstein, S., and Downey, G. P. (1993) J. Biol. Chem. **268**, 17131–17137.
33. Walker, F., deBlaiquiere, J., and Burgess, A. W. (1993) J. Biol. Chem. **268**, 19552–19558.
34. Marie-Cardine, A., Fischer, S., Gorvel, J.-P., and Maridonneau-Parini, I. (1996) J. Biol. Chem. **271**, 20734–20739.
35. Pumiglia, K. M., and Feinstein, M. B. (1993) Biochem. J. **294**, 253–260.
36. Clark, E. A., and Brugge, J. S. (1992) Mol. Cell. Biol. **13**, 1863–1871.
37. Grandori, C., and Hanafusa, H. (1988) J. Cell Biol. **107**, 2125–2135.
38. Rendu, F., Lebret, M., Del-Castillo, S., Fagard, R., Levy-Toledano, S., and Fischer, S. (1989) Blood **83**, 1545–1551.
39. Linstedt, A. D., Vetter, M. L., Bishop, J. M., and Kelly, R. B. (1992) J. Cell Biol. **117**, 1077–1084.
40. Gutkind, J. S., and Robbins, K. C. (1989) Proc. Natl. Acad. Sci. U. S. A. **86**, 8783–8787.
Hck Is Activated by Opsonized Zymosan and A23187 in Distinct Subcellular Fractions of Human Granulocytes
Heidi Welch and Isabelle Maridonneau-Parini

J. Biol. Chem. 1997, 272:102-109.
doi: 10.1074/jbc.272.1.102

Access the most updated version of this article at http://www.jbc.org/content/272/1/102

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 34 of which can be accessed free at
http://www.jbc.org/content/272/1/102.full.html#ref-list-1