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Short Communication

The oncogenic activity of the Src family kinase Hck requires the cooperative action of the plasma membrane- and lysosome-associated isoforms

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ABSTRACT

Hck is a phagocyte specific proto-oncogene of the Src family expressed as two isoforms, p59Hck and p61Hck. It plays a critical role in Bcr/Abl-chronic myeloid leukaemia and is able to transform fibroblasts *in vitro*. However, the tumourigenic activity of Hck and the respective oncogenic functions of Hck isoforms have not been examined. Tet-Off fibroblasts expressing constitutively active mutants of p59Hck and p61Hck together or individually were used. In contrast to cells expressing p59Hck^{ca} or p61Hck^{ca} alone, cells expressing both isoforms were transformed *in vitro* and induced tumour formation in 90% of nude mice within 2 weeks. This is the first demonstration of (i) the tumourigenic activity of Hck in mice, (ii) the cooperative action of the two Hck isoforms *in vitro* and *in vivo*. To our knowledge, this is the first example of a transforming activity 'split' in two requisite isoforms.

1. Introduction

Src family protein-tyrosine kinases (SFKs) are known to mediate mitogenesis, differentiation, survival, migration, and adhesion.[1] In contrast to the ubiquitous expression pattern of Src, Yes, and Fyn, the expression of Lck, Blk, Lyn, Fgr and Hck is restricted to hematopoietic cells.[2,3]

Like other Src-family kinases, the proto-oncogene Hck harbours an *in vitro* transforming activity in murine fibroblasts[4] upon constitutive activation either by mutation of the negative regulatory tyrosine residue in its carboxy-terminal region to phenylalanine,[1] or by the HIV protein Nef that is a well known ligand of its SH3 domain.[5] To date, spontaneous mutation of this proto-oncogene has not been described in human cancers. Constitutive activation of Hck by direct interaction with the oncogene Bcr/Abl is required for the establishment of leucocyte transformation during chronic myeloid leukaemia (CML).[6–8] Actually, inhibitors of Src-PTKs block the

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transformation process in Bcr/Abl-expressing cells and, in myeloid cells, transformation by Bcr/Abl is suppressed in Hck-defective cells.[6,9] The recent demonstration of a Bcr/Abl-independent, SFKs-dependent form of resistance to imatinib, a specific Bcr/Abl inhibitor used to treat CML patients,[10] has further supported the central role of SFKs in this leukaemia, which are therefore considered as rational targets for anti-CML therapy either alone or in combination with Bcr/Abl inhibitors.[11]

Hck is specifically expressed in myeloid cells as two isoforms of molecular weights 59 kDa (p59Hck) and 61 kDa (p61Hck) generated by alternative initiations of translation of a single mRNA.[12] The isoforms only differ by 21 residues at the N-terminal end, and by their differential addressing, p59Hck being mainly associated to the plasma membrane and p61Hck to the lysosome membrane.[13,14] Expression of the constitutively activated variant of p61Hck (p61Hck^{ca}) induces the *de novo* formation of podosome rosettes in a lysosome-dependent manner,[15] whereas p59Hck^{ca} triggers the formation of plasma membrane protrusions.[16] Thus, although very similar, p59Hck and p61Hck are differentially located and trigger distinct phenotypes when activated.

Despite the growing pharmacological interest of Hck, its oncogenic activity has been very poorly documented since its discovery 20 years ago. The aim of our work is to investigate the tumourigenic ability of Hck, which has not been investigated yet, and to examine the respective contribution of p59Hck and p61Hck in the transformation process *in vitro* and *in vivo*. Therefore, we used murine MEF3T3 Tet-Off fibroblasts that stably and conditionally expressed constitutively active isoforms of the kinase. This cellular model has the advantage of avoiding long-lasting- as well as over-expression of the oncogenic Hck mutant. We herein report that the combined expression of p59Hck and p61Hck is necessary for the full exercise of its transforming and tumourigenic activities.

2. Materials and methods

2.1. Antibodies

Antibodies directed against Hck and GFP (used at final dilution 1:200 and 1:100, respectively) were purchased from Santa Cruz Biotechnology (Tebu-bio, Le Perray-en-Yvelines, France) and Abcam (ab6556, Abcam, Paris, France). Texas-Red Phalloidin was from Molecular Probes (Leiden, The Netherlands).

2.2. Constructs, cells and cultures

Constitutively active (HckY501F) mutants of human p59Hck^{ca}, p61Hck^{ca} and p59/61Hck^{ca} have been previously described [15–17]. For doxycyline regulatable oncogene expression, we used mouse embryonic fibroblast 3T3 (MEF-3T3) Tet-Off cell line (Clontech) that were grown, transfected and cloned as described.[15,17] Hck expression was optimal in doxycycline-free medium after 7 days. Several clones were selected: MEF-p59, MEF-p61 and MEF-p59/61 clones A and B, expressing p59Hck^{ca}-GFP, p61Hck^{ca}-GFP and both p59 and p61Hck^{ca}-GFP respectively. Hck negative-MEF-3T3 Tet-Off (Parental-MEF) was used as a negative control in transformation experiments *in vitro*.

2.3. SDS-PAGE and immunoblot analysis

Equal amounts of total cell lysates in Laemmli buffer were separated by SDS-PAGE, transferred to nitrocellulose membranes that were probed with polyclonal rabbit anti-Hck antibodies (0.4 μg/ml) and revealed by an enhanced chemiluminescence system (Immobilon^{TM} Western, Millipore corporation, Billerica, U.S.A.) as described.[17]

2.4. Direct fluorescence and F-actin staining

Cells were plated on glass coverslips for 24 h, fixed and processed as described for F-actin staining using Texas-Red phalloidin.[18] F-actin labelling and direct Hck-GFP fluorescence were detected with a Leica DM-RB fluorescence microscope or a Leica TCS-SP2 confocal scanning microscope.

2.5. In vitro transformation assays

Proliferation curves. Growth curves were generated over 2 weeks for Hck-positive and Hck-negative MEF3T3 stable clones as previously described [19]. Proliferation rate is expressed as the doubling time in days.

Adhesion capacity. Adhesion defect of transformed cells was assessed by direct light microscopy observations of cell detachment once they reached confluency after plating on glass coverslips.

Dependence on serum growth factors. Cell proliferation in culture medium containing 10% FCS was compared to proliferation in 0.5% FCS and measured by counting the cells every day.

Focus forming activity. 5×10^4 cells were seeded on fibrinogen-coated glass coverslips in 24 well-plates. Foci were observed at 10 and 20 days of culture.[19]

Anchorage-independent proliferation. Colony formation in soft agar was followed for 18 days as described.[4] Briefly, 10^4 cells were seeded in DMEM containing 10% FCS and 0.35% w/v low-melting temperature (LMP) agarose. This suspension was sandwiched between layers of DMEM containing 0.7% w/v LMP agarose. Colonies were photographed by phase contrast and fluorescence microscopy and counted.

2.6. In vivo tumourigenicity assay

10^6 cells were inoculated subcutaneously into the flank of 6-week-old male athymic SWISS nude mice (Charles River, France). Tumour development was assessed every week until the tumour was removed for histopathological analysis or until 4 months post-injection for negative mice. Tumours were measured every 5 days from day 21 to day 37. To study the development of metastasis in nude mice, primary tumours were induced as described above and after 2 to 4 weeks (depending on the size of the tumour), primary tumours were removed and mice follow-up for metastatic development was undertaken.

2.7. Histopathological analysis and immunohistochemistry

Subcutaneous tumours were fixed in 10% v/v neutral buffered formalin and embedded in paraffin. Sections were stained