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An ultrasensitive high-throughput electrochemiluminescence immunoassay for the Cdc42-associated protein tyrosine kinase ACK1

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Abstract

Several drugs inhibiting protein kinases have been launched successfully, demonstrating the attractiveness of protein kinases as therapeutic targets. Functional genomics research within both academia and industry has led to the identification of many more kinases as potential drug targets. Although a number of well-known formats are used for measuring protein kinase activity, some less well-characterized protein kinases identified through functional genomics present particular challenges for existing assay formats when there is limited knowledge of the endogenous substrates or activation mechanisms for these novel kinase targets. This is especially the case when a very sensitive assay is required to differentiate often highly potent inhibitors developed by late-stage medicinal chemistry programs. ACK1 is a non-receptor tyrosine kinase that has been shown to be involved in tumorigenesis and metastasis. Here we describe the development of an extremely sensitive high-throughput assay for ACK1 capable of detecting 240 fmol per well of the kinase reaction product employing a BV-tag-based electrochemiluminescence assay. This assay is universally applicable to protein tyrosine kinases using a BV-tag-labeled monoclonal antibody against phosphotyrosine. Furthermore, this assay can be extended to the evaluation of Ser/Thr kinases in those cases where an antibody recognizing the phospho-product is available.

Keywords: High-throughput immunoassay; BV-tag; Electrochemiluminescence assay; Cdc42-associated protein tyrosine kinase; ACK1; K_i; IC_{50}; Ultrasensitive

Protein kinases have been shown to play very important roles in many signal transduction pathways. Pharmaceutical and biotechnology companies have invested extensively in the discovery and development of small molecule inhibitors targeting protein kinases for various human diseases such as cancer and inflammation [1,2]. For example, deregulation due to chromosomal translocation of the non-receptor protein kinase Abl leads to chronic myelogenic leukemia (CML).[1] The Abl inhibitor Gleevec (STI571, Novartis) was the first U.S. Food and Drug Administra-

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[1] *Abbreviations used:* CML, chronic myelogenic leukemia; FDA, U.S. Food and Drug Administration; EGFR, epidermal growth factor receptor; ACK1, activated Cdc42-associated kinase 1; SH3, Src homology 3 domain; CRIB, Cdc42/Rac interactive binding domain; PR, proline-rich domain; cDNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay; DELFIA, dissociation and enhancement lanthanide fluoroimmunoassay; CV, coefficient of variance; HTRF, homogeneous time-resolved fluorescence; SPA, scintillation proximity assay; BV ECL, BV-tag-based electrochemiluminescence; mAb, monoclonal antibody; tACK1, truncated ACK1; BV-tag, ruthenium tri-bipyridine; MOI, multiplicity of infection; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CSK, C-terminal Src kinase.

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tion (FDA)-approved small molecule drug targeting protein kinases. Subsequently the FDA approved two epidermal growth factor receptor (EGFR) kinase inhibitors, Tarceva (OSI774, OSI Pharmaceuticals/Genentech) and Iressa (ZD1839, AstraZeneca) for the treatment of non-small cell lung cancer. Recently, the number of FDA-approved kinase inhibitors grew significantly and now includes the RAF, VEGFR-2, and PDFR-β inhibitor Nexavar (sorafenib, codeveloped by Onyx and Bayer) and the multiple kinase inhibitors Sutent (SU11248, Pfizer) and Sprycel (dasatinib, Bristol–Myers Squibb). These successes demonstrate that protein kinases are attractive drug targets. Furthermore, sequencing of the human genome revealed that more than 500 protein kinases were encoded, making this gene family one of the largest in the genome [3]. Together, these discoveries have continued to fuel great interest in protein kinases as small molecule drug targets across the pharmaceutical and biotechnology industries, and many more kinase inhibitors are in clinical development [1,2].

Activated Cdc42-associated kinase 1 (ACK1) is a non-receptor tyrosine kinase. It initially was identified as a binder to the GTP-bound Cdc42 with consequent inhibition of GTPase activity and, hence, was suggested as an effector of Cdc42 [4]. ACK1 contains an N-terminal tyrosine kinase domain, followed by an Src homology 3 domain (SH3), a Cdc42/Rac interactive binding domain (CRIB), and a proline-rich domain (PR) (Fig. 1A). An ACK isoform termed ACK2 was identified in a bovine complementary DNA (cDNA) library [5], but according to the literature and database searches, other species, including mouse and human, have only one ACK gene and protein (ACK1). Several reports presented evidence for an important role of ACK in the transduction of Ras/cdc42 signals in several cellular processes such as stimulating the activities of guanine nucleotide exchange factors Ras-GRF1 [6] and Dbl [7], cell spreading and motility [8–10], v-Ha-Ras-induced transformation [11], receptor degradation [12,13], and cell survival [14,15]. Recently, we reported that the ACK1 gene is amplified in several types of tumors and that ACK1 is involved in the process of metastasis in vitro and in vivo. In cancer cell lines of epithelial origin, its overexpression enhances cellular motility, invasiveness, and the ability to metastasize to the lung, resulting in increased mortality [16]. Therefore, ACK1 is a very interesting target for small molecule inhibitors for various oncology indications.

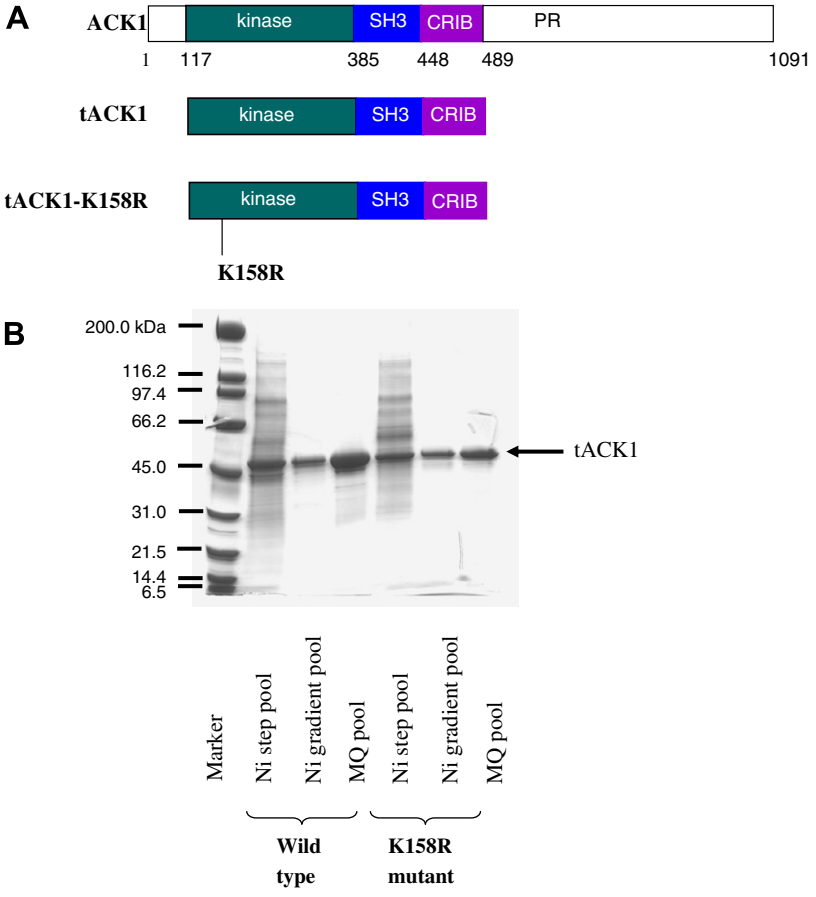

Fig. 1. Expression and purification of recombinant ACK1. (A) Schematic diagram of ACK1 constructs: tACK1 and tACK1–K158R. Numbers below the diagram indicate ACK1 amino acid residues. (B) Purified recombinant ACK1 proteins. Aliquots of purified recombinant ACK1 proteins imidazole step eluted from Ni^{2+} column, imidazole gradient eluted from Ni^{2+} column, or salt gradient eluted from Mono Q (MQ) column were loaded and separated by 5 to 15% gradient SDS–PAGE and stained with Coomassie blue. The tACK1 band is indicated by the arrow. Marker: Bio-Rad broad range protein size marker.