Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 372 (2008) 106–115
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

A quantitative peptide array for evaluation of protein kinase activity

Xiaoming Han [a], Syuhei Shigaki [b], Takayuki Yamaji [a], Go Yamanouchi [a], Takeshi Mori [b,c], Takuro Niidome [a,b,c], Yoshiki Katayama [a,b,c,d,*]

[a] Graduate School of System Life Sciences, Kyushu University, Nishi-ku Fukuoka 819-0395, Japan
[b] Department of Applied Chemistry, Faculty of Engineering, Kyushu University, Nishi-ku Fukuoka 819-0395, Japan
[c] Center for Future Chemistry, Kyushu University, Nishi-ku Fukuoka 819-0395, Japan
[d] CREST, Japan Science and Technology (JST), Saitama 332-0012, Japan

Received 21 June 2007
Available online 29 September 2007

Abstract

Peptide array, which is known as an emerging technology, has been developed for identification of protein kinase activity. For this purpose, the ability of quantitative analysis is very important because the absolute change in protein kinase activity is critical for the determination of cellular function. Here we report an original type of peptide array for quantitative evaluation of protein kinase activity by fluorescence imaging. We used the peptide array for the quantitative evaluation of the nonreceptor tyrosine kinase c-Src activity as a model for detecting protein kinase activities. By using positive and negative control peptides, we obtained the actual ratio of tyrosine phosphorylation of substrate peptide not only by purified c-Src but also by c-Src in cell lysate. In addition, the experimental approach provided simple immobilization of peptide. Our sensitive, specific, and high-throughput peptide array can be used for quantitative evaluation of kinase activity and potentially can be applied to drug discovery and screening.

Keywords: Phosphorylation; Peptide array; Protein kinase; Quantitative; High-throughput; Cell lysate

In a living cell, proteins are key players for expressing or controlling cellular functions. Proteins play their role through extremely complex interaction networks, and one of the most important purposes of the protein network is processing extracellular information to form accurate cellular responses. This is called an intracellular signal transduction system and contains many kinds of signal processing enzymes. Among these information processing enzymes, protein kinases that catalyze protein phosphorylation are believed to be the core elements in the network acting as regulatory hubs and have been shown to be correlated with the formation of cellular phenotypes such as cell adhesion, proliferation, and differentiation [1–3]. In fact, the perturbation and aberration of protein kinase activity often are seen in cancer growth, invasion, and metastasis [4–7]. To analyze such important kinase activity or expression, several strategies have been evolved. The most common conventional technologies—scintillation counting using ^{32}P-labeled ATP [8], immunobead assay [9], Western blotting [10], mass spectrometry [11], and enzyme-linked immunosorbent assay (ELISA)[1] [12]—currently are restricted by their low throughput, cumbersome procedures and high cost due to the use of a large quantity of reactant.

* Corresponding author. Fax: +81 92 642 3606.
E-mail address: ykatatcm@mbox.nc.kyushu-u.ac.jp (Y. Katayama).

[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; HTS, high-throughput screening; SPR, surface plasmon resonance; HBTU, 2-(1*H*-Benzotriazole-1-*yl*)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; NMP, *N*-methylpyrrolidone; DMF, dimethylformamide; TFA, trifluoroacetic acid; NGF, nerve growth factor; TBS-T, Tris-buffered saline containing 0.05% Tween 20; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; CHCA, α-cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; MEM, minimum essential medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; D-PBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

0003-2697/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.09.026

During the past few years, the high-throughput screening (HTS) assays for kinase activity monitoring and kinase-related drug screenings have been developed extensively. These HTS assays include homogeneous kinase assays of well plate format based on technologies such as time-resolved-fluorescence resonance energy transfer [13], fluorescence polarization [13], deep quench [14], scintillation proximity [15], and gold nanoparticle-based colorimetric method [16]. Although such homogeneous assays provide simple separation-free detection, optically active compounds and fluorescence-labeled substrates used in the assays sometimes are interfered with by the contents included in a reaction solution, especially when they are applied to component-abundant cell lysate. The other platform of HTS, the chip-based heterogeneous array system, has advantages in a higher density of substrate peptides and a low sample volume. Although the extra steps, such as washing and blocking, are needed in detection protocols, the chip-based assay is principally robust because the washing and blocking steps can reduce the effect of interfering substances included in a reaction solution. As the substrates used in the arrays, peptides are more reliable than comparable proteins [17–19] because they are more resistant to denaturation [20,21] and can be immobilized homogeneously. Despite the promise of peptide arrays, their development has been limited by several factors; immobilization of the peptides to the chemically modified surface is rather time-consuming and cumbersome [22–25], and it is difficult to avoid nonspecific adsorption of many biological components, disturbing the detecting signal and resulting in low sensitivity [26]. Nonspecific adsorption is a serious issue, especially in fluorescence or surface plasmon resonance (SPR) detection [27]. Autoradiography using radioactive ^{32}P for the detection of substrate phosphorylation is another sensitive technique [24,28,29] but presents a potential risk to human health. However, sensitive and quantitative peptide array for the detection of protein kinase activity cannot be designed unless such issues are resolved. Although the previously reported peptide arrays were applied successfully to screen substrates or inhibitors using purified kinases, most of them were not sensitive enough to detect the targeted protein kinase activity in cell lysate containing various disturbing substances. Quantitative detection is one of the most important points for the monitoring of kinase activity because activity changes in many kinases often are very small in signal processing and the extent of activity change from the basal level is critical for cellular function. On the other hand, it is difficult to quantitate phosphorylation with most peptide arrays, or at least their potential for quantitative analysis has not been reported [22,30,31]. To put peptide arrays to wider use for drug screening and other functions, it is imperative to develop a new type of peptide array that can provide rapid immobilization as well as sensitive and quantitative detection while causing little harm to human health.

Here we report the development of a quantitative peptide array for evaluation of protein kinase activity by fluorescence imaging. We used the peptide array for quantitative evaluation of nonreceptor tyrosine kinase c-Src activity as a model system. We used cysteine-terminal peptides of c-Src [32], corresponding to positive and negative control peptides for the quantitative assay. Taking the positive control as 100% and the negative control as 0% phosphorylation, we obtained the actual on-chip phosphorylation ratio for the substrate. With this method, we evaluated c-Src inhibitor efficacy using purified c-Src kinase and also quantitatively detected the drug-responded intracellular c-Src kinase activity in MCF-7 cell lysate. Compared with other counterparts [22–24,27,30,31], our peptide array system provided higher sensitivity and simpler immobilization of peptides. In addition, the peptide array system also eliminated harm to human health by using fluorescence-labeled antibody instead of radioactive ^{32}P detection [22,30,31]. Our sensitive, robust, and high-throughput peptide array can be used for the quantitative evaluation of kinase activity, cell-based drug screening, and diagnosis using tissue lysate.

Materials and methods

Reagents

Amino-terminal glass slide was purchased from Matsunami Glass (Osaka, Japan). Rink amide resin and Fmoc amino acids were purchased from Novabiochem (Darmstadt, Germany). 2-(1*H*-Benzotriazole-1-*yl*)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), piperidine, and *N*-methylpyrrolidone (NMP) were purchased from Watanabe (Hiroshima, Japan). Dimethylformamide (DMF) for peptide synthesis and acetonitrile for HPLC were obtained from Kanto Chemical (Tokyo, Japan). Trifluoroacetic acid (TFA), 1,2-ethanedithiol, and glutaraldehyde were purchased from Wako Pure Chemical (Osaka, Japan), and triisopropylsilane was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). c-Src was obtained from Carna Bioscience (Kobe, Japan). Cy5 anti-phosphotyrosine antibody and SU6656 were purchased from Amersham Biosciences (Chalfont St. Giles, Bucks, UK). Nerve growth factor (NGF) was purchased from Invitrogen (Carlsbad, CA, USA).

Peptide synthesis

The peptides shown in Table 1 were synthesized by an APEX 396 Multiple Peptide Synthesizer (Advanced ChemTech, Louisville, KY, USA) using corresponding Fmoc-amino acids and Rink Amide AM resin for C-terminal amide in peptides. Peptides were detached from the resin using a cleavage solution containing 94% TFA, 2.5% water, 2.5% 1,2-ethanedithiol, and 1% triisopropylsilane. The resin was filtered out, and the filtrate was added to cold diethylether for precipitation of the cleaved peptide. The obtained peptide was purified by reverse-phase liquid chromatography equipped with an electrospray mass spec-