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Multi-PK antibodies: Powerful analytical tools to explore the protein kinase world

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ABSTRACT

Diverse biological events are regulated through protein phosphorylation mediated by protein kinases. Some of these protein kinases are known to be involved in the pathogenesis of various diseases. Although 518 protein kinase genes were identified in the human genome, it remains unclear how many and what kind of protein kinases are expressed and activated in cells and tissues under varying situations. To investigate cellular signaling by protein kinases, we developed monoclonal antibodies, designated as Multi-PK antibodies, that can recognize multiple protein kinases in various biological species. These Multi-PK antibodies can be used to profile the kinases expressed in cells and tissues, identify the kinases of special interest, and analyze protein kinase expression and phosphorylation state. Here we introduce some applications of Multi-PK antibodies to identify and characterize the protein kinases involved in epigenetics, glucotoxicity in type 2 diabetes, and pathogenesis of ulcerative colitis. In this review, we focus on the recently developed technologies for kinomics studies using the powerful analytical tools of Multi-PK antibodies.

1. Introduction

Protein kinases play important roles in various biological phenomena through the regulation of phosphorylation signaling pathways [1]. Eukaryotic protein kinases make up a large superfamily of homologous proteins, comprising 1.5–2.5% of all gene products [2]. Genome projects have been completed for various species, and as many as 518 protein kinase genes were identified in the human genome [3]. These enzymes are classified into two major groups: Ser/Thr protein kinases and Tyr protein kinases. Although these enzymes have different sizes, isoelectric points, substrate specificities, and regulatory mechanisms, they share a homologous catalytic core. As shown Fig. 1A, the kinase domain, consisting of 250–300 conserved amino acid residues, can be divided into 12 subdomains that contain essential sequences for the structural features required for protein kinase catalytic activities [4].

A variety of protein kinases are known to be involved in biological phenomena such as proliferation, development, differentiation, and apoptosis through protein phosphorylation [2]. To investigate the protein kinases involved in these events, various tools and methods for analysis of cellular kinases have been developed. For expression analyses, Western blotting with protein kinase-specific antibodies is widely used for evaluation of the protein expression levels of protein kinases [5–8], while real-time PCR is used for examination of the mRNA expression levels [9]. Recently, a cDNA microarray was employed for analysis of the entire RNA expression levels of protein kinases [10]. Meanwhile, for activity analyses, protein and peptide substrates are used for *in vitro* kinase assays to determine the protein kinases activities [11–14], and Western blotting analysis with phosphorylation site-specific antibodies is widely used to assess the activation states [5–8]. However, a method that can simultaneously analyze the protein expression and/or activity levels of the entire protein kinases in cells has not been established, because of the lack of specific probes to globally detect multiple protein kinases. It is important to analyze the expression profiles of the entire protein kinases under varying situations to elucidate the cellular signaling mechanisms. Based on these backgrounds, we have produced monoclonal antibodies, designated as Multi-PK antibodies, to analyze a wide variety of protein kinases [15,16]. In this review, we introduce the process for the generation of Multi-PK antibodies, the methods for analysis of protein kinases using Multi-PK antibodies, and the applications of Multi-PK antibodies to explore phosphorylation signaling.

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CDKL5, cyclin-dependent kinase-like 5; CNBr, cyanogen bromide; DCLK, double-cortin like protein kinase; Dnmt1, DNA methyltransferase 1; FAK, focal adhesion kinase; IEF, isoelectric focusing; IPG, immobilized pH gradient; MAPK, mitogen-activated protein kinase; MeCP2, methylated-CpG-binding protein 2

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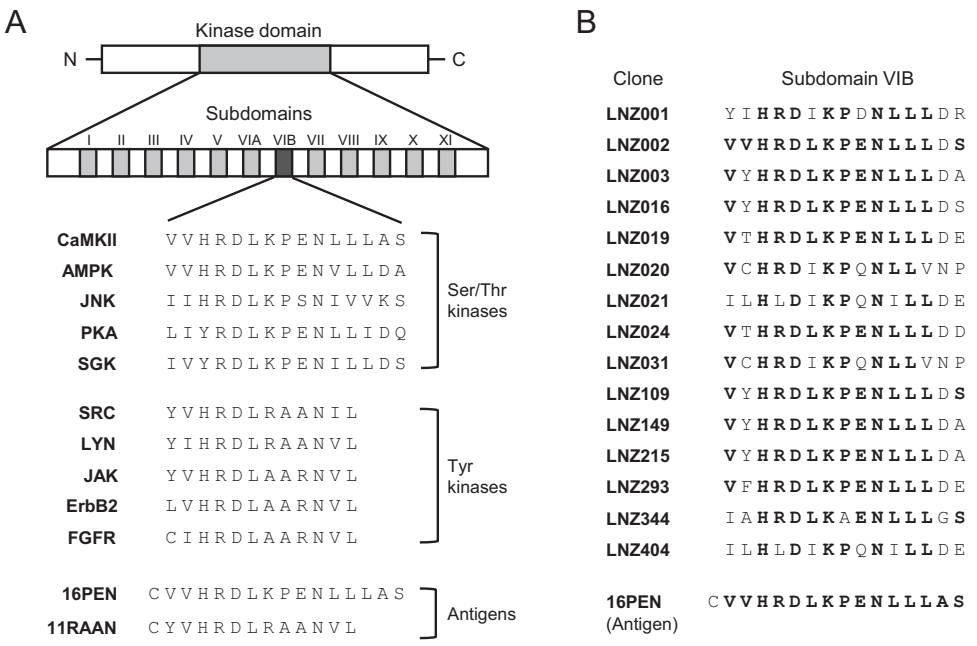

Fig. 1. Amino acid sequences of the subdomain VIB and the peptides used as antigens for the generation of Multi-PK antibodies. (A) Alignment of subdomain VIB sequences in various protein kinases and the peptides used as antigens: 16PEN for M1C and M8C antibody production; 11RAAN for YK34 antibody production. (B) Amino acid sequences of subdomain VIB in Ser/Thr protein kinases in plant. The clones for Ser/Thr kinases were isolated from a cDNA library of *L. japonicus* by expression screening using the M1C/M8C antibodies.

2. Multi-PK antibodies

In general, it is widely recognized that a superior antibody has strict specificity for its specific antigen and does not exhibit nonspecific cross-reactivity. However, we hypothesized that an antibody with broad specificity could be useful for the analysis of diverse protein kinases simultaneously. The main structural feature of the protein kinases families of enzymes is a catalytic core consisting of 12 highly conserved subdomains [4]. While subdomain VIB appears to be the most highly conserved region among the 12 subdomains in many protein kinases, the sequences of this region in Ser/Thr kinases differ somewhat from those in Tyr kinases. The typical subdomain VIB sequences of Ser/Thr kinases are H-R-D-L-K-P-(E/S)-N, while those of Tyr kinase are H-R-D-L-(R/A)-A-(A/R)-N (Fig. 1A) [15,16]. Based on these findings, we synthesized antigenic peptides,16PEN (CVVHRDLKPENLLLAS) and 11RAAN (CYVHRDLRAANVL), corresponding to the subdomain VIB sequences of Ser/Thr kinases and Tyr kinases, respectively, and used these peptides for immunization of BALB/c mice. As a result, we established three hybridoma cell lines (M1C, M8C, YK34) producing monoclonal antibodies, Multi-PK antibodies, with broad cross-reactivities [15,16]. Specifically, the M1C and M8C antibodies recognize Ser/Thr kinases and the YK34 antibody detects Tyr kinases.

2.1. Cross-reactivities of Multi-PK antibodies

To investigate the immunoreactivities of the M1C and M8C antibodies, cDNA expression libraries of the mouse brain [15], *Xenopus laevis* embryo [17], *Lotus japonicus* root nodule [18], basidiomycete mushroom *Coprinopsis cinerea* [19], and zebrafish *Danio rerio* [20] were immunologically screened with the M1C and M8C antibodies. Among the many positive clones obtained using these antibodies, nearly 93% turned out to be Ser/Thr protein kinases. The amino acid sequences of subdomain VIB in the Ser/Thr kinases isolated from *L. japonicus* are shown in Fig. 1B. When Western blot analyses were carried out using the M1C and M8C antibodies, Ser/Thr kinases, such as Ca^{2+}/calmodulin-dependent protein kinase (CaMK) I [21], CaMKII, CaMKIV, CaMK kinase, cAMP-dependent protein kinase [15], cyclin-dependent kinase-like 5 (CDKL5) [22], doublecortin-like protein kinase (DCLK) [23], nuclear dbf2-related kinase [24], Akt, c-Jun N-terminal protein kinase 1, mitogen-activated protein kinase (MAPK), MAPK kinase, and microtubule affinity-regulating kinase, were detected. The results revealed that the M1C and M8C antibodies recognized Ser/Thr protein kinases with subdomain VIB sequences of (H/Y)-(R/L)-D-(L/V/I)-K-(P/A)-(E/D/Q/S)-N.

To examine the cross-reactivity of the YK34 antibody, we employed different SRC Tyr kinase recombinants with various amino acid replacements in subdomain VIB. By Western blotting analysis, we found that the YK34 antibody recognized the amino acid sequences (Y/F/L/C)-(V/I)-H-R-D-L-(R/A)-(A/T)-(A/R)-N [16]. Indeed, Tyr kinases such as SRC, SYK, ABL, LYN [16,25], and focal adhesion kinase (FAK) [26] were detected by the YK34 antibody. Taking these results into consideration, we can speculate that YK34 antibody would recognize more than 75% of Tyr kinases.

Taken together, these findings suggest that Multi-PK antibodies can be powerful tools to detect Ser/Thr kinases and Tyr kinases in proteomics studies focused on a wide range of protein kinases (kinomics).

3. New analytical methods using Multi-PK antibodies

Multi-PK antibodies can be used for Western blotting and immunoprecipitation of various protein kinases [15,16]. Multiple protein kinases can be detected in crude cell extracts from various biological species using Multi-PK antibodies. In this section, we introduce newly developed analytical methods using Multi-PK antibodies: a profiling method for analysis of protein kinase expression in cells and tissues [27]; a method for protein kinase identification by two-dimensional electrophoresis in combination with cyanogens bromide (CNBr) digestion of protein kinases [23]; and an analytical method for intracellular protein kinase expression and phosphorylation state [25].

3.1. Expression profiling of protein kinases using MicroRotofor/SDS-PAGE

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), consisting of isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension, is the most common technique for analysis of cellular proteins [28]. In recent proteomics studies, 2D-PAGE, in which an immobilized pH gradient (IPG) gel is employed for the first IEF, has been conducted in conjunction with mass spectrometric analysis [29,30]. However, it was difficult to detect cellular proteins with molecular masses larger than 100 kDa by Western blotting using Multi-PK antibodies after separation by 2D-PAGE. Therefore, we developed a new profiling method for detection of total proteins