Bulletin of Faculty of Pharmacy, Cairo University 56 (2018) 68–72

Contents lists available at ScienceDirect

Bulletin of Faculty of Pharmacy, Cairo University

journal homepage: www.elsevier.com/locate/bfopcu

Original Article

Identification of possible Ser/Thr/Tyr phosphorylation sites in the fungal histidine kinase CaNik1p by peptide array technique

Mohammed El-Mowafy*, Ursula Bilitewski

Compound Profiling und Screening, Helmholtz Centre for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany

ARTICLE INFO

Keywords:
Histidine kinase
CaNik1p
Peptide array

ABSTRACT

CaNik1p is a histidine kinase (HK) that is present in *Candida albicans*. It was found to be a target for antifungal activities on the hyperosmotic glycerol pathway. The protein has two well-known phosphorylation sites (P-sites); His510 and Asp924, that were found to be crucial for maintaining the fungicidal sensitivity. Our previous work showed that the double mutated protein, in H510 and D924, was still possessing kinase activity. In this study, we aimed to identify additional possible P-sites in this HK. Therefore, we constructed a peptide array that covers the full length protein. Incubation of the purified His-tagged CaNik1p with the peptide array in the presence of radioactive ATP [γ-^{32}P] revealed the possible P-sites in each peptide. We classified the peptides according to their intensities. Peptides bearing His510 and D924 showed either null or very weak intensities. The highest intensity was corresponding to the peptide containing the amino acid T994, while lower intensities were related mainly to serine and threonine residues and to lower extent to tyrosine amino acid. We could show for the first time the detection of additional P-sites in CaNik1p that might contribute in the signalling pathways of *C. albicans*. Moreover, the protocol used in this study allows for direct focusing and prediction of the possible Ser, Thr, and Tyr phosphoaccepting residues in the newly discovered kinases.

1. Introduction

CaNik1p (1081 amino acids) is a histidine kinase (HK) that is present in the opportunistic fungus *Candida albicans* [1]. It is a part of the fungal two component system (TCS) [2,3]. The TCSs are usually used by both bacteria [4] and fungi [5–7] for adaptation to external stimuli, and expression of virulence factors.

In *C. albicans*, CaNik1p was found to play an essential role in hyphal formation (virulence factor) [3,5]. It was also found to be a target for antifungals e.g. fludioxonil and pyrrolnitrin, which act on the hyperosmotic glycerol pathway [8]. Additionally, it was proven to transfer the antifungal sensitivity to the *Saccharomyces cerevisiae* after heterologous expression in this yeast [9,10]. This indicates that it will be a promising target for development new antifungals.

CaNik1p is characterized by the presence of an ATP-binding domain called HATPase_c domain, as well as two conserved phosphoaccepting domains: HisKA, and REC domains, which contain the phosphoaccepting residues H510 and D924 respectively [2]. Being a hybrid HK and a part of the TCS, the CaNik1p undergoes dimerization and the phosphate group is transferred from the HATPase_c bound-ATP in one molecule to His510 and then Asp924 of another molecule via trans-autophosphorylation mechanism [11].The phosphate group is subsequently transferred to a downstream phosphotransfer protein then to a response regulator protein, which in turn activates a typical eukaryotic signal transduction module (mitogen activated protein kinase) [12]. The phosphorylation sites (H510, and D924) were confirmed to be crucial for maintaining the fungicidal sensitivity via mutational analysis [10]. However, a recent work in our group showed that the heterologously expressed mutated CaNik1p(H510Q, D924N) was still possessing *in vitro* kinase activity [13]. This indicates that additional phosphorylation site (p-site) is still present and may play a role in the downstream cascades. The possibility of the phosphorylation of residues other than H510 and Asp924 in CaNik1p was investigated by LC-MS/MS peptide analysis and only Ser1071 was identified as being phosphorylated with a probability of more than 99.1% [13]. Conventional methods for identification of p-sites involve incorporation radiolabeled ATP into cellular proteins [14–16].The radioactive phosphorylated proteins can then be detected by subsequent two-dimensional gel electrophoresis or high-performance liquid chromatography. The p-sites can be determined by proteolytic digestion of the radiolabeled protein, separation and detection of phosphorylated peptides via two-dimensional peptide mapping, followed by peptide

Peer review under responsibility of Faculty of Pharmacy, Cairo University.
* Corresponding author: Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, 60 Elgomhoria street, 35516, Mansoura, Egypt.
E-mail address: seven@mans.edu.eg (M. El-Mowafy).

https://doi.org/10.1016/j.bfopcu.2018.03.007
Received 9 November 2017; Received in revised form 21 March 2018; Accepted 30 March 2018
Available online 08 April 2018

sequencing by Edman degradation [17]. These techniques are tedious and require considerable amounts of the phosphorylated protein [17]. Recently, mass spectrometry is now used for investigation of protein phosphorylation [18]. However, such technique is still hindered by the complexity of the processing and analysis of proteomic data [19,20]. Peptide array technique was also used in several studies for investigation of probable p-sites [21–23].

Our main aim in this work was to identify additional possible p-sites in this HK via the peptide array technique.

2. Materials and methods

2.1. Purification of the his-tagged CaNiK1p

The his-tagged CaNiK1p [10] was expressed in the transformed *S. cerevisiae* strain BWG1-7a followed by further purification of the his-tagged protein from the cell lysate using Protino® Ni-NTA agarose beads (Macherey-Nagel) as previously described [9]. The purified protein was stored at −80 °C till further use.

2.2. Design and synthesis of the peptide array

The peptide array was constructed in the department of chemical biology, HZI as previously mentioned in Ref. [21]. The peptide array could be divided into 25 columns (1–25) and 8 rows (A–I) [Fig. 1a], which include a library of 216 short peptides covering the whole protein length (Table 1). Each peptide (21 amino acids) was synthesized at a distinct site (spot) on a cellulose porous membrane [24]. For optimum interaction of the kinase with the conserved protein kinase recognition sequence, the minimal number of amino acids before the P-site was found to be three or one while that following the P-site was four or one [24–26]. Therefore, overlapping between sequences of the synthesized peptides was considered to avoid false negative prediction of the p-sites that are present at the N- and C-termini of the peptides and not preceded or followed by the minimum number of amino acids required to maintain the conserved protein kinase recognition sequence.

Additional controls were included in the peptide array. The peptide spots H18, H19, I7, I14 and I21-I25 were left empty without spotting. As a control for the amino acid H510: All the possible p-sites, except H510, in the peptides I3-I5 were replaced with the non phosphorylable amino acid alanine, while in the peptides I1 and I2, only H510 was replaced with the non phosphorylable amino acid glutamine. Regarding the residue D924, it was replaced in the peptides I10-11 with the non phosphorylable amino acid asparagine to be used as control in comparison with the peptides of the same sequences (I8 and I9). The possibility of the residue S1071 to be a potential p-site was investigated in the spots H16, H17, and I15-I20, where S1071 was replaced by alanine in the spots I17 and I18, while in the spots I19 and I20, the S and T amino acids (except S1071) were replaced by alanine.

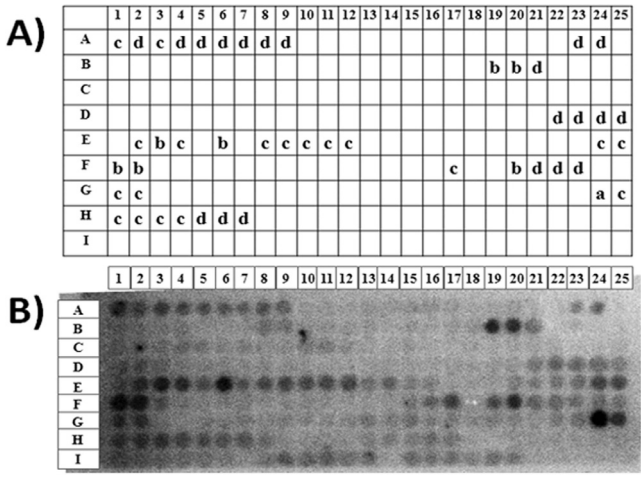

Fig. 1. Illustrative representation of the intensities of each peptide spot (A) and a phosphorimager scan of the peptide array paper (B).

2.3. Incubation of the peptide array with the purified protein

The peptide array cellulose paper was treated prior to incubation with the purified protein as previously described [21]. Briefly, the paper was moistened with ethanol, washed twice with incubation buffer [50 mM MOPS, 200 mM NaCl, 1 mM Mg acetate, 0.4 mM EGTA, 1 mg/ml bovine serum albumin, pH 6.9 (adjusted with 1 N NaOH)] before being incubated with this buffer at 4 °C for an overnight.

The peptide array was then incubated with 18 ml fresh incubation buffer at 30 °C for 2 h. Cold ATP (10 mM, Sigma) and hot ATP (40 µCi of ɣ32P, Hartman Analytic) were added to the incubation buffer and the phosphorylation reaction was initiated by addition of the purified CaNik1p (20 nM). The reaction was incubated at 30 °C for 1 h with gentle shaking.

The buffer was decanted and the reaction was stopped by washing the peptide array with 100 ml 1 M NaCl for 10 times. The paper was dried in air for 1 h before being wrapped in a plastic bag and exposed to a phosphor screen (preflashed) at RT for 4 h. The phosphor screen was scanned via the phosphorimage analyzer BAS2500 to detect the signal intensities from different peptide spots.

3. Results

We divided the signal intensities obtained from different peptide spots of the array in a descending order as follows: a, b, c, d and e followed by prediction of the possible phosphoaccepting residues (T, S, Y, H, D) in these peptides (Fig. 1, Table 1). Peptide spots showing no signals were considered as null. The amino acids that are located immediately N-terminal and C-terminal to the phosphorylation site (p-site) often contribute to a large extent to kinase–substrate recognition. A phosphoaccepting amino acid was predicted as a p-site if it is preceded by at least three amino acids or followed by one amino acid for p-sites at the N-or C-terminal respectively.

The empty spots H18, H19, I7, I14 and I21-I25 showed null intensities (Fig. 1). As shown in Table 1 and Fig. 1, the peptides harboring H510 either showed null intensities in the peptide spots: D6, D7 and H21-23 or very weak intensity (e) as in the peptide spots I3–I5. Additionally, the peptides harboring D924 showed either null intensities in the peptide spots G12 and I8 or weak intensities in the peptide spots G13 (e) and I9 (d) as demonstrated in Table 1. The peptide spots I10 and I11 showed e and d intensities respectively despite that they have the same sequence of G13 and I9 respectively, except that the D924 residue was replaced by asparagine. These results indicate that the protocol utilized in this study was unable to detect the phosphorylation of H510 and D924 residues. The S1071 is not confirmed to be a phosphoaccepting residue in the CaNik1p because of the weak intensity (d) of the peptides harboring this residue (I15–16, I19–20), in addition to obtaining the same intensity (d) from the peptide spots I15 and I17 even after replacement of S1071 with alanine in the spot I17.

The greatest intensity (a) was obtained in the peptide spot G24 which contains the possible p-sites; T994 and H1000.

4. Discussion

Identification of p-sites in HKs is important to understand how signalling networks integrate and relay signals. In case of proteins with multiple p-sites, careful analysis of such sites is an essential step in defining the mechanism of phosphorylation and to determine the nature of the signalling response [27]. Though phosphorylation is observed on a diversity of amino acid residues, the most frequent sites of phosphorylation in eukaryotes arise mainly on Ser, Thr and Tyr residues