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Original Article

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

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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

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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.

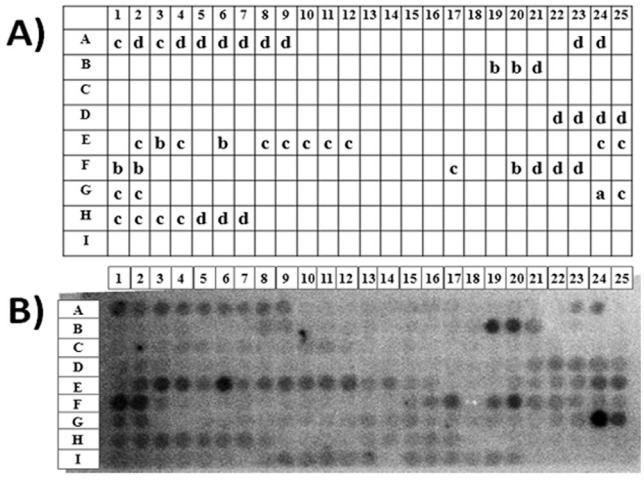

Fig. 1. Illustrative representation of the intensities of each peptide spot (A) and a phosphorimager scan of the peptide array paper (B).

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

Table 1
The sequence of peptides according to their positions on the peptide array together with the predicted p-sites and their intensities.

Spot	Intensity	Sequence	Predicted p-sites
A1	B	1 MNP**T**KKPRL**S**PMQP**S**VFEILN 21	T4, S10, S15
A2	C	5 KKPRL**S**PMQP**S**VFEILN**D**PEL 25	S10, S15, D22
A3	C	16 VFEILN**D**PEL**Y**SQ**H**C**HS**LRE**T** 36	D22, Y26, S27, H29, H31, S32
A4	C	17 FEILN**D**PEL**Y**SQ**H**C**HS**LRE**T**L 37	D22, Y26, S27, H29, H31, S32, T36
A5	C	19 ILN**D**PEL**Y**SQ**H**CH**S**LRE**T**LL**D 39**	D22, Y26, S27, H29, H31, S32, T36,
A6	C	21 N**D**PEL**Y**SQ**H**CH**S**LRE**T**LL**DH**F 41	Y26, S27, H29, H31, S32, T36, D39, H40
A7	C	**22 D**PEL**Y**SQ**H**C**HS**LRE**T**LL**DH**FN 42	Y26, S27, H29, H31, S32, T36, D39, H40
A8	C	26 **Y**SQ**H**C**HS**LRE**T**LL**DH**FN**H**QA**T 46**	H29, H31, S32, T36, D39, H40, H43
A9	C	30 C**HS**LRE**T**LL**D**HFN**H**QA**T**LI**DT 50**	T36, D39, H40, H43, T46, D49
A23	D	95 N**D**PEILKVKI**T**IN**T**MM**D**QLQ**T 115**	T105, T108, D111
A24	C	98 EILKVKI**T**IN**T**MM**D**QLQ**T**FAN 118	T105, T108, D111, T115
B8	D	169 V**T**RAVAKG**DLS**RKINV**H**AQGE 189	D177, S179, H185
B9	D	175 KG**DLS**RKINV**H**AQGEILQLQR 195	S179, H185
B19	B	261 V**TT**AVAKG**DLS**KKV**TAD**CKGE 281	D269, S271, T275, D277
B20	B	265 VAKG**DLS**KKV**TAD**CKGEIL**D**L 285	D269, S271, T275, D277, D284
B21	C	278 CKGEIL**D**LKLTINQMV**D**RLQN 298	D284, T288, D294
C4	E	339 MA**T**NL**T**NQVR**S**IA**T**V**TT**AVA**H** 359	T344, S349, T352, T354, T355
C5	E	342 NL**T**NQVR**S**IA**T**V**TT**AVA**HGD**L 362	S349, T352, T354, T355, H359
C10	E	359 **HGDLS**QKI**D**G**H**PKGEILQLKN 379	S363, D367, H369
C11	E	370 PKGEILQLKN**T**INKMV**DS**LQL 390	T380, D386, S387
D6	null	499 SAKSEFLANMSHEIRTPLNGI 519	
D7	null	504 FLANM**SH**EI**R**TPLNGIIGM**T**Q 524	
D20	E	569 VEQI**D**F**S**LRG**T**VFGALK**T**LAV 589	D573, S575, T579, T586
D21	D	576 LRG**T**VFGALK**T**LAVKAIEKNL 596	T579, T586
D22	D	589 VKAIEKNL**DLT**YQC**DSS**FP**D**N 609	D597, T599, Y600, D603, S604, S605, D608
D23	D	590 KAIEKNL**DLT**YQC**DSS**FP**D**NL 610	D597, T599, Y600, D603, S604, S605, D608
D24	D	594 KNL**DLT**YQC**DSS**FP**D**NLIG**DS 614**	D597, T599, Y600, D603, S604, S605, D608, D613
D25	D	595 NL**DLT**YQC**DSS**FP**D**NLIG**DS**F 615	D597, T599, Y600, D603, S604, S605, D608, D613, S614
E2	C	622 LNLAGNAIKF**T**KEGKV**S**V**S**VK 642	T632, S638, S640
E3	B	628 AIKF**T**KEGKV**S**V**S**VKK**SD**KMV 648	T632, S638, S640, S644, D645
E4	C	630 KF**T**KEGKV**S**V**S**VKK**SD**KMVL**D** 650	T632, S638, S640, S644, D645, D650
E5	D	634 EGKV**S**V**S**VKK**SD**KMVL**DS**KLL 654	S638, S640, S644, D645, D650
E6	B	641 VKK**SD**KMVL**DS**KLLLEVCV**SD** 661	S644, D645, D650, S651, S660, D661
E7	E	**650 DS**KLLLEVCV**SDT**GIGIEK**D**K 670	S660, D661, T662, D669
E8	D	652 KLLLEVCV**SDT**GIGIEK**D**KLG 672	S660, D661, T662, D669
E9	C	667 EK**D**KLGLIF**DT**FCQA**DGSTT**R 687	D669, D676, T677, D682, S684, T685, T686
E10	C	674 IF**DT**FCQA**DGSTT**RKFGG**T**GL 694	D676, T677, D682, S684, T685, T692
E11	C	675 F**DT**FCQA**DGSTT**RKFGG**T**GLG 696	D676, T677, D682, S684, T685, T692
E12	C	676 **DT**FCQA**DGSTT**RKFGG**T**GLGL 696	D676, T677, D682, S684, T685, T692
E13	E	682 **DGSTT**RKFGG**T**GLGL**SIS**KQL 702	S684, T685, T692, S697, S699
E14	D	687 RKFGG**T**GLGL**SIS**KQLI**H**LMG 707	T692, S697, S699, H704
E15	E	689 FGG**T**GLGL**SIS**KQLI**H**LMGGE 709	T692, S697, S699, H704
E16	E	694 LGL**SIS**KQLI**H**LMGGEIWVT**S** 714	T692, S697, S699, H704
E20	E	708 GEIWV**TS**EY**GSGS**NF**Y**F**T**VCV 728	T713, S714, Y716, S718, S720, Y723, T725
E21	D	710 IWV**TS**EY**GSGS**NF**Y**F**T**VCV**S**P 730	T713, S714, Y716, S718, S720, Y723, T725
E22	D	713 **TS**EY**GSGS**NF**Y**F**T**VCV**S**PSNI 733	S720, Y723, T725, S729, S731
E23	D	715 E**YGSGS**NF**Y**F**T**VCV**S**PS**N**IR**Y** 735	Y723, T725, S729, S731
E24	C	719 G**S**NF**Y**F**T**VCV**S**PS**N**IR**Y**T**R**Q**T** 739	Y723, T725, S729, S731, Y735, T736
E25	C	721 NF**YFT**VCV**S**PS**N**IR**YT**RQ**TEQ 741**	Y723, T725, S729, S731, Y735, Y736, T739
F1	B	725 **T**VCV**S**PS**N**IR**YT**RQ**T**EQLLPF 745	S729, S731, Y735, Y736, T739
F2	B	726 VCV**S**PS**N**IR**YT**RQ**T**EQLLPF**S** 746	S729, S731, Y735, Y736, T739
F3	E	729 **S**PS**N**IR**YT**RQ**T**EQLLPF**SSH**Y 749	Y735, Y736, T739, S746, S747, H748
F15	E	790 PVK**YD**IIMI**DS**IEIAKKLRLL 810	Y793, D794, D799, S800
F16	D	801 IEIAKKLRLL**S**EVK**Y**IPLVLV 821	S811, Y815
F17	C	805 KKLRLL**S**EVK**Y**IPLVLV**HHS**I 825	S811, Y815, H822, H823, S824
F19	C	813 VK**Y**IPLVLV**HHS**IPQLNMRVC 833	Y815, H822, H823, S824
F20	B	814 K**Y**IPLVLV**HHS**IPQLNMRVCI 834	H822, H823, S824
F21	D	829 NMRVCI**D**LGI**SSY**ANTPC**SIT** 849	D835, S839, S840, Y841, S847
F22	D	830 MRVCI**D**LGI**SSY**ANTPC**SIT**D 850	D835, S839, S840, Y841, S847, T849
F23	D	831 RVCI**D**LGI**SSY**ANTPC**SIT**D**L** 851	D835, S839, S840, Y841, S847, T849, D850
F24	D	834 IDLGI**SSY**ANTPC**SIT**D**LAS**A 854	D835, S839, S840, Y841, S847, T849, D850, S853
F25	D	837 GI**SSY**ANTPC**SIT**D**LAS**AIIP 857	S840, Y841, S847, T849, D850, S853
G1	C	839 **SSY**ANTPC**SIT**D**LAS**AIIPAL 859	S847, T844, T849, S853
G2	C	843 N**TPCSIT**D**LAS**AIIPALE**SRS** 863	S847, T849, D850, S853, S861
G13	E	914 KRNK**YD**VVLM**D**VQMPVMGGFE 934	Y918, D919, D924
G14	E	926 QMPVMGGFEA**T**EKIRQWEKK**S** 946	T936
G15	D	936 **T**EKIRQWEKK**S**NPI**DS**L**T**FR**T** 956	S946, D950, S951, T953
G16	E	941 QWEKK**S**NPI**DS**L**T**FR**T**PIIAL 961	S946, D950, S951, T953, T956
G17	E	943 EKK**S**NPI**DS**L**T**FR**T**PIIAL**T**A 963	S946, D950, S951, T953, T956, T962
G22	D	971 EK**S**LAKGM**DDY**V**S**KPLKPKLL 991	D979, D980, Y981, S983
G23	D	973 **S**LAKGM**DDY**V**S**KPLKPKLLMQ 993	D979, D980, Y981, S983
G24	A	984 KPLKPKLLMQ**T**INKCI**H**NINQ 1004	T994, H1000
G25	C	990 LLMQ**T**INKCI**H**NINQLKEL**S**R 1010	T994, H1000, S1009
H1	C	999 I**H**NINQLKEL**S**RN**S**RG**SD**FAK 1019	S1009, S1012, S1015, D1016

(*continued on next page*)

Table 1 (*continued*)

Spot	Intensity	Sequence	Predicted p-sites
H2	C	1002 INQLKEL**S**RN**S**RG**SD**FAKKM**T** 1022	S1009, S1012, S1015, D1016
H3	D	1005 LKEL**S**RN**S**RG**SD**FAKKM**T**RN**T** 1025	S1009, S1012, S1015, D1016, T1022
H4	D	1012 **S**RG**SD**FAKKM**T**RN**T**PG**STT**RQ 1032	S1015, D1016, T1022, T1025, S1028, T1029, T1030
H5	D	1015 **SD**FAKKM**T**RN**T**PG**STT**RQG**SD** 1035	T1022, T1025, S1029, T1030, T1031, S1034
H6	D	1018 AKKM**T**RN**T**PG**STT**RQG**SD**EG**S 1038**	T1022, T1025, S1029, T1030, T1031, S1034, D1035
H7	D	1019 KKM**T**RN**T**PG**STT**RQG**SD**EG**S**V 1039	T1022, T1025, S1029, T1030, T1031, S1034, D1035, S1038
H8	E	1020 KM**T**RN**T**PGS**TT**RQG**SD**EG**S**VK 1040	T1025, S1029, T1030, T1031, S1034, D1035, S1038
H13	E	1047 PRQG**S**VEGGG**TSS**RPVQRR**S**A 1067	S1051, T1057, S1058, S1059, S1066
H14	E	1048 RQG**S**VEGGG**TSS**RPVQRR**S**AR 1068	S1051, T1057, S1058, S1059, S1066
H15	D	1049 QG**S**VEGGG**TSS**RPVQRR**S**ARE 1069	T1057, S1058, S1059, S1066
H16	D	1056 G**TSS**RPVQRR**S**AREG**S**I**TT**I**S** 1076	S1059, S1066, S1071, T1073, T1074
H17	D	1061 PVQRR**S**AREG**S**I**TT**I**S**EQIDR 1081	S1066, S1071, T1073, T1074, S1076
H21	null	498 NSAKSEFLANMSHEIRTPLNG 518	
H22	null	499 SAKSEFLANMSHEIRTPLNGI 519	
H23	null	504 FLANMSHEIRTPLNGIIGMTQ 524	
I1	E	499 **S**AK**S**EFLANM**S(**Q)EIR**T**PLNGI 519	S502, S509, T51
I2	E	504 FLANM**S(**Q)EIR**T**PLNGIIGM**T**Q 524	S509, T514, T523
I3	E	492 EAAELAN(A)AK(A)EFLANM(A)**H**EI 512	H510
I4	E	498 N(A)AK(A)EFLANM(A)**H**EIR(A)PLNG 518	H510
I5	E	499 (A)AK(A)EFLANM(A)**H**EIR(A)PLNGI 519	H510
I8	null	908 EA**Y**EAIKRNK**YD**VVLM**D**VQMP 928	
I9	D	914 KRNK**YD**VVLM**D**VQMPVMGGFE 934	Y918, D919, D924
I10	D	908 EA**Y**EAIKRNK**YD**VVLM(N)VQMP 928	Y918, D919
I11	D	914 KRNK**YD**VVLM(N)VQMPVMGGFE 934	Y918, D919
I12	E	908 EA**Y**EAIKRNK**Y**(N)VVLM**D**VQMP 928	Y910, Y918, D924
I13	E	914 KRNK**Y**(N)VVLM**D**VQMPVMGGFE 934	Y918, D924
I15	D	1056 G**TSS**RPVQRR**S**AREG**S**I**TT**I**S** 1076	S1059, S1066, S1071, T1073, T1074
I16	D	1061 PVQRR**S**AREG**S**I**TT**I**S**EQI**D**R 1081	S1066, S1071, T1073, T1074, S1076
I17	D	1056 G**TSS**RPVQRR**S**AREG(A)I**TT**I**S** 1076	S1059, S1066, T1073, T1074
I18	E	1061 PVQRR**S**AREG(A)I**TT**I**S**EQI**D**R 1081	S1066, S1071, T1073, T1074, S1076
I19	d	1056 G(AAA)RPVQRR(A)AREG**S**I(AA)I(A) 1076	S1071
I20	D	1061 PVQRR(A)AREG**S**I(AA)I(A)EQI**D**R 1081	S1071, D1080

For illustration, all the phosphoaccepting residues (S, T, Y, H, D) in each peptide sequence were adjusted to bold format.
The phosphoaccepting residues between brackets indicate that they are non phosphorylable amino acid that are substitution for possible p-sites in one of the previous peptides.

[17].

In this study, we utilized the peptide array technique for identification of the probable potential p-sites in CaNik1p. The principle of the phosphorylation of different peptide spots in the peptide array after incubation with CaNik1p, in the presence of radioactive ATP, relies mainly on the trans-phosphorylation property of CaNik1p. When the radioactive ATP binds to the ATP binding domain of CaNik1p, the radioactive phosphate group is then transferred to a p-site of a peptide spot as long as such p-site is preceded or followed by the minimum number of amino acids required to maintain the conserved protein kinase recognition sequence [24–26].

The well known phosphoaccepting residues H510 and D924 either showed null or weak intensities. This indicates that the peptide array protocol utilized in this study was not suitable for detection of His and Asp p-sites. However, this could be related to the acid liability of the phosphorylated His and Asp residues [28–30]. Lapek et al. (2010) demonstrated that only buffer systems at pH 5–6 successfully maintained the presence of the phosphate group on His [28], while pH of the incubation buffer utilized in this study was 6.9. Based on this result, we decided to reject the Asp and His residues from the possible p-sites that are present Table 1, and to consider only Ser, Thr, and Tyr as potential probable p-sites in this study (Table 2).

The highest intensity (a) was corresponding to T994 (peptide spot G24). Although the detection of the phosphorylatable Ser/Thr/Tyr residue in a HK of TCS is not common, previous researchers have detected Ser phosphorylation in HKs, e.g., DegS in *B. subtilis* [31] and the amoebic HK DokA [32].

In conclusion, our work provides a semi-quantitative prediction of the possible p-sites of the CaNik1p. Further studies will be done to confirm the predicted p-sites, particularly T994, that contribute in CaNik1p phosphorylation via site directed mutagenesis followed by investigation of kinase activity. Finally, the protocol of this study could be used in the prediction of the possible Ser, Thr, and Tyr phosphoaccepting residues in newly identified kinases.

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Table 2
Summary of the considered p-sites arranged according to their intensities. P-sites possessing different intensities in various peptide spots are raised as superscript format.

a	T994[c]
b	T4, S271, T275, S10[c], S15[c], T632[c], S638[c,d], S640[c,d], S644[c,d], S651, S660[d,e], S729[c,d], S731[c,d], Y735[c,e], S736[c,e], T739[c,e], S824[c]
c	Y26, S27, S32, T36, T46, T115, T105[d], T108[d], T677, T684[e], T685[e], T686[e], T692[d,e], Y732[d,e], T725[d,e], S811[d], Y815[d], S847[d], T849[d], S853[d], S861[d], S1009[d], S1012[d], S1015[d]
d	S179, T599, Y600, S604, S605, S614, T579[e], T586[e], S839, S840, Y841, S946[e], S951[e], T953[e], T662[e], S697[e], S699[e], T713[e], S714[e], Y716[e], Y718[e], S720[d], Y918[e], Y981, S983, T1022, T1025[e], S1029[e], T1030[e], T1031[e], S1034[e], S1038[e], T1057[e], S1058[e], S1059[e], S1066[e], S1071[e], T1073[e],T1074[e], S1076[e]
e	T344, S349, T352, T354, T355, S363, T380, S387, S502, S509, T514, T523, S575, S746, S747,Y793, S800,Y910, T936, T956, T962, S1051

Conflict of interest

All authors must declare financial/commercial conflicts of interest. Even if there is none, this should be stated in a separate paragraph following the acknowledgements section. This is a mandatory requirement for all types of articles.

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