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In Silico Studies of Potential Phosphoresidues in the Human Nucleophosmin/B23: Its Kinases and Related Biological Processes

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ABSTRACT

Human nucleophosmin/B23 is a phosphoprotein involved in ribosome biogenesis, centrosome duplication, cancer, and apoptosis. Its function, localization, and mobility within cells, are highly regulated by phosphorylation events. Up to 21 phosphosites of B23 have been experimentally verified even though the corresponding kinase is known only for seven of them. In this work, we predict the phosphorylation sites in human B23 using six kinase-specific servers (KinasePhos 2.0, PredPhospho, NetPhosK 1.0, PKC Scan, pkaPS, and MetaPredPS) plus DISPHOS 1.3, which is not kinase specific. The results were integrated with information regarding 3D structure and residue conservation of B23, as well as cellular localizations, cellular processes, signaling pathways and protein–protein interaction networks involving both B23 and each predicted kinase. Thus, all 40 potential phosphosites of B23 were predicted with significant score (>0.50) as substrates of at least one of 38 kinases. Thirteen of these residues are newly proposed showing high susceptibility of phosphorylation considering their solvent accessibility. Our results also suggest that the enzymes CDKs, PKC, CK2, PLK1, and PKA could phosphorylate B23 at higher number of sites than those previously reported. Furthermore, PDK, GSK3, ATM, MAPK, PKB, and CHK1 could mediate multisite phosphorylation of B23, although they have not been verified as kinases for this protein. Finally, we suggest that B23 phosphorylation is related to cellular processes such as apoptosis, cell survival, cell proliferation, and response to DNA damage stimulus, in which these kinases are involved. These predictions could contribute to a better understanding, as well as addressing further experimental studies, of B23 phosphorylation. J. Cell. Biochem. 113: 2364–2374, 2012.

KEY WORDS: PHOSPHORYLATION; PREDICTION; KINASES; PROTEIN–PROTEIN INTERACTIONS; CELLULAR LOCALIZATION; SIGNALING; PATHWAY; CONSERVATION

The identification of phosphorylation sites in proteins is crucial to understand the role of this event in several cellular processes. Experimental methods used for this purpose are expensive and time consuming, therefore in silico prediction of phosphorylation sites is an useful alternative [Miller and Blom, 2009]. Some of the predictors already developed are non-specific (e.g., DISPHOS) while others are kinase-specific prediction programs, such as KinasePhos, PredPhospho, NetPhosK, etc. (Table I). However, it is difficult for users to determine which programs are more reliable, considering the kind of problems they have. In this scenario, Wan et al. [2008] have proposed a meta-predicting strategy, MetapredPS that combines the strengths of the individual predictors and has a higher performance.

The phosphoprotein B23, also called numatrin or nucleophosmin (NPM1) is mainly a nucleolar phosphoprotein that plays a central role in ribosome biogenesis, centrosome duplication, cell cycle progression, cell survival, apoptosis, and cancer [Tawfic et al., 1995; Okuda et al., 2000; Okuwaki et al., 2002]. The cellular distribution of B23, which translocates between the nucleus and the cytoplasm, seems to be closely related with phospho/dephosphorylation events

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TABLE I. Description of the Seven Servers Used to Predict the Potential Phosphorylation Sites in Protein B23

Server	Description	Web site	References
DISPHOS version 1.3	A non kinase-specific phosphorylation site predictor that uses position-specific amino acid frequencies refined with disorder information to perform generic phosphorylation site predictions, with a predictive accuracy reaching 76% for serine, 81% for threonine, and 83% for tyrosine	http://core.ist.temple.edu/pred/pred/predict	Iakoucheva et al. [2004]
KinasePhos version 2.0	Kinase-specific predictor, mainly based on Hidden Markov Model (HMM) that exhibits a predictive accuracy of 91%. This server currently covers 58 groups of kinases 32 S/T kinases and 25 Y kinases	http://KinasePhos2.mbc.nctu.edu.tw	Wong et al. [2007]
PredPhospho	Kinase-specific predictor, mainly based on Standard Vector Model (SVM) and Neural Network algorithms. The accuracy of the predictions ranged from 83% to 95% at the kinase family level, and 76–91% at the kinase group level. This server covers four kinase groups (AGC, CAMK, CMGC, and TK) and four kinase families (CDK, CK2, PKA, and PKC)	http://www.nih.go.kr/predphospho/proteo/html/inc_PredPhospho.htm	Kim et al. [2004b]
NetPhosK	Kinase-specific predictor, mainly based on Neural Network algorithms. Currently NetPhosK covers the following kinases: PKA, PKC, PKG, CKII, Cdc2, CaM-II, ATM, DNA PK, Cdk5, p38 MAPK, GSK3, CKI, PKB, RSK, INSR, EGFR, and Src	http://www.cbs.dtu.dk/services/NetPhosK/	Blom et al. [2004]
PKC Scan	A kinase-specific predictor that uses positional scanning of biotinylated oriented peptide libraries to predict the potential PKC phosphosites in the proteins with high specificity and sensitivity (80–90%)	http://mpr.nci.nih.gov/pkc_scan	Fujii et al. [2004]
pkaPS	A kinase-specific predictor based on a heuristic method with a scoring function implemented that predicts PKA phosphorylation sites with the best sensitivity (~96%) and specificity (~94%)	http://mendel.imp.ac.at/sat/pkaPS	Neuberger et al. [2007]
MetaPredPS	A generalized weighted voting meta-predicting strategy for the phosphorylation site predicting problem for the four major families of S/T kinases: CDK, CK2, PKA, and PKC. This meta-predictor exhibits a higher predictive accuracy (91%) than the individual predictors from which it is constructed: GPS, NetPhosK, PPSP, KinasePhos 2.0, PredPhospo, and Scansite	http://metapred.umn.edu/MetaPredPS	Wan et al. [2008]

[Yun et al., 2003]. The already known kinases involved in these modifications are the complex cyclin-dependent kinase (CDK)1/cyclin B that promotes dissociation of B23 from the nucleolus at the beginning of mitosis [Peter et al., 1990]. Also, the complex CDK2/cyclin E promotes the dissociation of B23 from the centrosome at the mid-late G1 phase, thus regulating the duplication of this structure [Okuda et al., 2000]. The association of B23 with unduplicated centrioles, during late S and G2 phase, is probably prevented by CDK2/cyclin A-mediated phosphorylation [Tokuyama et al., 2001]. Likewise, phosphorylation through the complexes CDK4/cyclin D and CDK6/viral cyclin V regulates the centrosome cycle [Adon et al., 2010] and latency of oncogenic Kaposi's sarcoma herpesvirus [Sarek et al., 2010], respectively. Moreover, casein kinase 2 (CK2)-mediated phosphorylation contributes to B23 dissociation from the nucleolus during the interphase [Negi and Olson, 2006]. The polo-like kinase 1 (PLK1) also phosphorylates B23, thereby displaying its regulatory role in mitosis [Zhang et al., 2004]. In addition, protein kinase C (PKC), a very mobile enzyme [Liu, 2010], has been reported to phosphorylate nuclear B23 in vitro, although the relevance of this modification in vivo still remains unclear [Beckmann et al., 1992]. Other kinases verified through in vitro assays are the never in mitosis gene a (NIMA)-related kinase 2 (Nek2a) and the cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) [Chan et al., 1990; Yao et al., 2004]. Two other enzymes, the check point kinase 1 (Chk1) and protein kinase B (PKB), are known to interact with B23 but have not been proved to phosphorylate it [Lee et al., 2008; Chen et al., 2009]. Finally, the anaplastic lymphoma kinase (AKL) has also been associated to B23 since both molecules have appeared fused, forming the aberrant protein B23-ALK in lymphomas [Fujimoto et al., 1996], but there are no evidences of phosphorylation of B23 by ALK in normal cells.

Current knowledge on B23 phosphorylation is still limited despite its relevance in cellular control. Of the 40 serine (S), threonine (T), and tyrosine (Y) residues in the sequence of B23, only 21 residues have been experimentally verified (see UniProt code: P06748, PHOSIDA code: IPI00549248 and Phospho.ELM code: P06748). Most of them have been drawn from high throughput experimental studies using mass spectrometry; but this information is typically not kinase-specific [Miller and Blom, 2009]. Therefore, the specific effector kinase has been reported only for seven of the B23 experimentally verified phosphosites. In this study, we performed a meta-predictive analysis aimed to predict kinase-specific phosphorylation sites in human B23. Results from the servers were integrated with structural and functional information of B23 and the predicted kinases. Based on this analysis we identified 13 new potential phosphosites that are exposed in B23 structure and up to 14 potential phosphorylation sites for PKA that lacks site-specific reports in this protein. We suggest that well-known kinases for B23

may phosphorylate this protein at a higher number of sites than those previously reported. Finally, our analysis proposed new kinases that are likely to phosphorylate B23 as they are functionally related to this protein.

MATERIALS AND METHODS

PREDICTION OF THE PHOSPHORYLATION SITES IN B23

The sequence of protein B23/NMP1 was extracted from the UniProt database (http://www.uniprot.org) (UniProt code: P06748). The annotations of experimental verified phosphorylation sites were extracted from the scientific literature and public databases such as UniProt, PHOSIDA [Gnad et al., 2007] (http://www.phosida.de/) and Phospho.ELM 8.1 (http://phospho.elm.eu.org/).

Prediction of phosphorylation sites was performed using seven prediction servers (Table I). All prediction scores were mostly on a scale of 0.00–1.00, with a cutoff value of 0.50. The score data sets outside this scale were normalized by dividing their scores by the highest one obtained in the corresponding data set.

The value that gather the servers performance per predicted residue (SPV) was calculated as the sum of the scores predicted by different servers, regardless of the kinase predicted. If a server predicted the same residue with different kinases, the highest prediction score was selected. The tendency that a kinase phosphorylates B23 was designated as HSK that represents the highest score obtained for a kinase, and it is independent of the predicted site. Similarly, the susceptibility that a site undergoes phosphorylation was referred as HSS and represents the highest score obtained for a potential phosphosite, regardless of the kinase. The propensity of multisite phosphorylation of each kinase (PMP) was calculated as the sum of the prediction scores for all sites predicted for the kinase. In the case of identical kinase-specific prediction by different servers, the highest prediction score was selected. The phosphosite's promiscuity index was defined as its propensity of phosphorylation by several kinases. This index was calculated as the sum of the scores obtained for every kinase. The highest prediction score was selected in case of simultaneous prediction of the same kinase.

ANALYSIS OF THE SOLVENT ACCESSIBILITY OF PHOSPHORESIDUES PREDICTED IN B23

The accessibility of the potential phosphoresidues located at N- and C-terminal domains (segments 15–118 and 250–294, respectively) was determined by WhatIF [Vriend, 1990] (http://swift.cmbi.ru.nl/servers/html/index.html). For this purpose, the 3D structures of the decameric N-terminal domain (PDB code: 2P1B) and the C-terminal domain (PDB code: 2VXD) of B23, were used. Residues in intrinsically disordered regions (IDes: residues 1–14 and 119–249) [Lee et al., 2007; Grummitt et al., 2008] were considered fully exposed with relative solvent accessibility of 5. We examined the monomer–monomer interactions described for the experimental structure of B23 (PDBsum code: 2P1B) at the database PDBsum (http://www.ebi.ac.uk/pdbsum). To simplify this analysis, we considered only monomers A and B and assumed the same interactions between the remaining monomers (PDBsum: 2P1B). We also analyzed the accessibility in the monomer A previously separated from the other monomers (B–J), using WhatIF. The relative accessibility of a residue is referred as the percentage of its accessibility that is retained when the protein changes from the unfolded to the folded state. Such percentages were standardized from 0 to 9, where 0 = buried, 9 = exposed, as follows: 0% was allocated to 0, and (0–100]% went to (0–9] [Hooft et al., 1996]. The cutoff values for accessibility were the average accessibility for phosphoresidues in proteins with solved 3D structure, that is, 4.28 for phosphoserines (pS), 3.88 for phosphothreonines (pT), and 2.96 for phosphotyrosines (pY) [Durek et al., 2009].

The missing loops in every monomer of the B23 structure (PDB code: 2P1B) were modeled using as template a complete model of B23 generated by the meta-server Robetta [Kim et al., 2004a] (http://robetta.bakerlab.org). The superposition and segments exchange between these structures were performed through the program WhatIF. The visual analysis of the structures was carried out using VMD [Humphrey et al., 1996].

ANALYSIS OF THE CONSERVATION OF PHOSPHORESIDUES PREDICTED IN B23

Protein sequences homologous to human B23 were selected from the NCBI's non-redundant database using the blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Multiple and profile alignments were carried out with the CLUSTALX program version 1.83 [Thompson et al., 1997]. In both cases, the default parameters were used. Meaningless sequences were filtered by eliminating sequences of nucleoplasmin, aberrant nucleophosmin fusion protein, and putative nucleophosmin isoforms with incomplete sequences and/or gaps that could disrupt the function of B23. Finally, 39 nucleophosmin sequences were selected from four groups of animals (fish, amphibian, mammals, and birds) with more than 44% sequence identity to the human sequence. Finally, we performed a profile alignment using structural information of the human nucleophosmin (Supplementary Fig. S2).

ANALYSIS OF THE INTERACTIONS BETWEEN B23 AND THE PREDICTED KINASES

The interactions or associations between the predicted kinases and B23 were defined as direct or indirect. Direct interactions comprised the experimentally determined physical contacts, including phosphorylation [von Mering et al., 2005]. Indirect interactions considered the subcellular localization shared by B23 and each predicted kinase, as well as the cellular processes and signaling pathways involving their genes [von Mering et al., 2005]. Also the protein–protein interaction networks in which B23 and each kinase are connected by shared components [Gagneur et al., 2004]. The first neighborhood was used as network cutoff, except when second or third neighbors were co-localized. The databases used were the String interaction database version 8.2 (http://string-db.org/), Genecards (http://www.genecards.org), Expasy (www.expasy.ch/sprot/), UniProt, Genatlas (http://www.dsi.univ-paris5.fr/genatlas/), EntrezGene (http://www.ncbi.nlm.nih.gov/sites/entrez), and KEGG Pathway Database (http://www.genome.jp/kegg/pathway.html). The cellular process (GO:0009987) and signaling pathway (GO:0023033) terms were assigned according to the Gene Ontology

database (http://www.geneontology.org). All percentages of kinases were calculated from the total number of predicted kinases (i.e., 35).

The propensity (*P*) of a given kinase to phosphorylate B23 considered the overall interactions between both proteins. A unitary value was assigned for every interaction and *P* was finally calculated as the sum of interactions divided by the highest value obtained for an experimentally confirmed kinase.

STATISTICAL ANALYSIS

Analyses were performed using the GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA). To compare the predicted residues with or without experimental evidence of phosphorylation, the non-parametric Mann Whitney *U*-test was carried out. The differences between the serine, threonine, and tyrosine residues were analyzed by the Kruskal–Wallis test. The Spearman's rank correlation coefficient (r) was used to determine dependency between two quantitative variables.

RESULTS

ALL PREDICTED PHOSPHORYLATION SITES IN B23 ARE POTENTIAL SUBSTRATES OF 35 TYPES OF KINASES

As a result of our meta-predictive strategy (Table I), the 40 S, T, and Y residues of B23 were predicted with significant score (>0.50) as potential phosphorylation sites. Most of these potential phosphoresidues were predicted by several programs, although tyrosines were only predicted by KinasePhos 2.0 (see SPV in Supplementary Table SII and Fig. S1). Twenty-one of these predicted phosphoresidues (12 pS, 8 pT, and 1 pY) have been experimentally verified (UniProt code: P06748, PHOSIDA code: IPI00549248, and Phospho.ELM code: P06748).

We also predicted 38 kinases involved in the phosphorylation of B23, a number that was reduced to 35 because CDK1, CDK2, and CDK5 were grouped as CDK and Mitogen-Activated Protein Kinases (MAPK1 and P38MAPK) were grouped as MAPK (Supplementary Fig. S1 and Table SIII). Only five of them have been described for B23 [Chan et al., 1990; Peter et al., 1990; Beckmann et al., 1992; Okuda et al., 2000; Zhang et al., 2004] and showed high HSK values ranging from 0.84 to 1.00 [CK2 (1.00), PKC (1.00), PKA (1.00), CDK (0.98) and PLK1 (0.84)]. Similar HSK values were scored for the enzymes ATM (0.98), PKG (0.97), Aurora (0.93), CK1 (0.89), and PDK (0.88) that have not been reported as kinases for B23 (Supplementary Table SII).

MOST OF THE PREDICTED KINASES HAVE THE POTENTIAL TO PHOSPHORYLATE B23 AT MULTIPLE SITES

The PMP analysis showed that most of the predicted kinases could phosphorylate B23 on multiple sites, with the exception of ALK (Fig. 1) (Supplementary Fig. S1, Table SII). Overall, S/T kinases showed higher PMP values than Y kinases (Fig. 1). The enzymes ATM and PKC obtained the highest PMP (23.45 and 17.28, respectively) and very high HSK values (≥0.98) (Supplementary Table SII, Fig. S1). This is interesting considering that ATM-mediated phosphorylation of B23 has not been previously described.

A second group of kinases showed PMP values ranging from 13.04 to 13.96 and included the enzymes PKB, RSK, STK4, CHK1, PKG, and CDK (Fig. 1). Our approach correctly predicted T199, T219, T234, and T237 as potential phosphosites and CDK as the predominant kinase involved in their phosphorylation (Supplementary Table SII). The third group of enzymes included more specific S/T kinases, meaning they were predicted in fewer sites of B23. These enzymes (PKA, Aurora, CK1, CK2, PLK1, MAPK, PDK, GRK, IKK, and GSK3) showed PMP values from 3.11 to 10.15. Their HSK were high (≥0.79), except for GRK (0.55). Considering that PKA and CK2/PLK1 phosphorylate B23 in vitro and in vivo, respectively [Chan et al., 1990; Zhang et al., 2004], we chose the lower PMP observed in these enzymes ($PMP_{PLK1} = 7.29$) as significance cutoff. Analysis of this group revealed that the CK2 was accurately predicted as kinase for

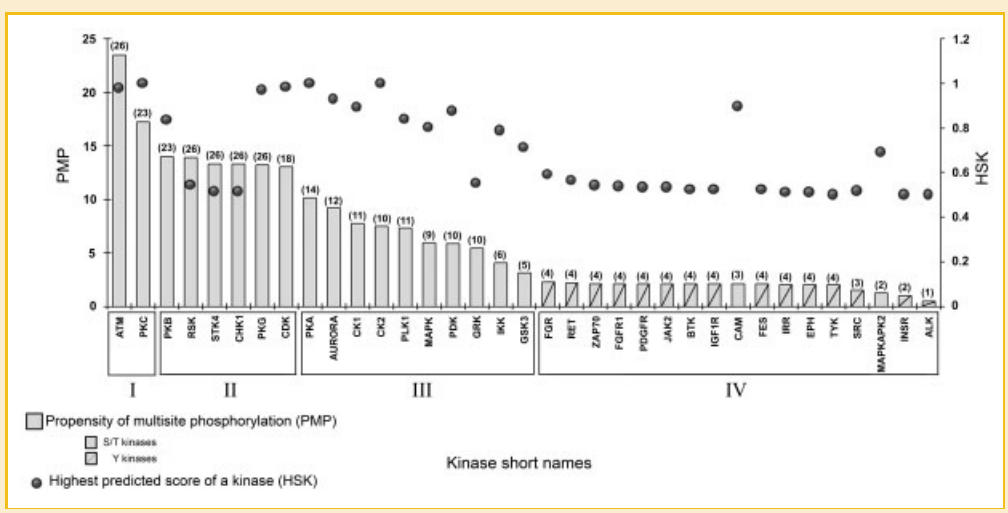

Fig. 1. Propensity of kinases to phosphorylate human nucleophosmin/B23 in multiple sites. The propensity (left Y axis) was calculated as the sum of the prediction scores corresponding to all sites for each predicted kinase. The right Y axis shows the highest scores predicted per kinase (HSK). The number of sites predicted for each kinase is within brackets. The squares enclose groups of serine/threonine (S/T) and tyrosine (Y) kinases according to their PMP values.

the well-known CK2-phosphosite S125 [Chan et al., 1990], whereas PLK1 was not for the well-known PLK1-phosphosite S4 [Zhang et al., 2004] (Supplementary Table SII, Fig. S1). This last residue was predicted only by four servers with low prediction scores and with CK2 as the effective kinase (Supplementary Table SII). We realized that although KinasePhos 2.0 was the only server able to predict PLK1 (Table I), it did not predict S4 as phosphosite. This server neither predicted S293 at the C-terminal of the protein, whereas this residue was identified by other three servers (NetPhosK, PredPhospho, and pkaPS). As KinasePhos 2.0 analyzes -4 to $+4$ positions flanking the phosphorylation residue (position 0), the underprediction of S4 and S293 could be related with their proximity to the N- and C-terminus of B23, respectively. This prompted new predictions after in silico insertion of three alanines at N- or C-terminus of this protein. As a result, both modified sites ($A_{-3}A_{-2}A_{-1}MEDS_4MDMD$ and $QWRKS_{293}LA_{+1}A_{+2}A_{+3}$) were predicted by KinasePhos 2.0 as potential targets of 13 and 9 kinases, respectively (Supplementary Table SII). Besides, PLK1 was among the kinases predicted for the modified site at S4.

Finally, the fourth and last group of predicted kinases did not showed significant values of PMP (Fig. 1). It comprised mainly Y kinases (FGR, RET, ZAP70, FGFR1, PDGFR, JAK2, BTK, IGF1R, CaM, FES, IRR, EPH, TYK, SRC, MAPKAPK2, INSR, and ALK), as well as the S/T kinases CaM and MAPKAPK2.

SOME POTENTIAL PHOSPHORESIDUES ARE EXPOSED ONLY IN THE MONOMERIC STATE OF B23

While assessing the susceptibility of phosphorylation at specific site (see HSS in Materials and Methods Section) we observed that all experimentally verified phosphoresidues of B23 showed HSS values ranging from 0.54 (Y67) to 1.00 (S227, S217, and S125) (Supplementary Table SII). Therefore, the lowest HSS value among those phosphoresidues (0.54) was taken as cutoff. All the predicted phosphorylation sites lacking experimental evidence, showed HSS above this cutoff HSS value. Analysis of solvent accessibility showed that 17 experimentally verified phosphosites are exposed in the decameric structure of B23. Eleven of them are located in IDes of this protein and the remaining six are in globular domains (Fig. 2A). Other four experimentally confirmed phosphosites (at T75, T78, S88, and S106) showed low solvent accessibility or are buried (Fig. 2A). Therefore, we explored whether they are masked by residues of the adjacent monomers (see Materials and Methods Section). We observed that they are involved in monomer-monomer interactions, but without the context of the oligomeric structure their solvent accessibility values are higher than 4.17 (Fig. 2). In contrast, S106, which is not involved in monomer–monomer interactions in the decamer [Lee et al., 2007], also showed low solvent accessibility in the monomer (Fig. 2B,C). This analysis showed that 10 newly predicted phosphosites are exposed in the decameric structure of B23 (Fig. 2A).

MOST OF THE B23 PREDICTED PHOSPHORESIDUES ARE PROMISCUOUS AND CONSERVED AMONG NUCLEOPHOSMINS

Considering our predictions, each of 40 predicted phosphosites of B23 is a potential substrate of several kinases. The median promiscuity value was 5.90, which means 3–14 kinases predicted per site (Supplementary Table SII). A multiple sequence alignment of nucleophosmin-related proteins showed that all potential phosphoresidues have a median conservation score of 84% (Supplementary Fig. S2). The median of the promiscuity index/conservation score for phosphoresidues were 3.36/90.50% (pT), 6.44/78.00% (pS), and 6.26/84.00% (pY). The values for non-phosphoresidues were 2.78/83.00% (non-pT), 6.53/84.00% (non-pS), and 7.38/76.00% (non-pY). As observed, no significant differences in terms of conservation median were found between them. Besides, T residues were significantly less promiscuous than S and Y residues, which showed no differences between them.

Some residues with low median promiscuity indexes (pT, non-pT, and non-pS) were highly conserved while the less conserved residues (pY, pS, and non-pY) were among the most promiscuous ones. However, we did not found a significant negative linear relationship ($r = -0.771429$, $P = 0.072397$) between these two variables. Unexpectedly, pS residues showed a lower conservation index than non-pS.

PREDICTED KINASES CLOSELY ASSOCIATED WITH THE BIOLOGICAL FUNCTION OF B23

Potential kinases with a closer relationship with the biological function and cell localization of B23. It is already known that B23 is substrate of several kinases during the different cell cycle phases (Supplementary Table SI). An extensive review of the subcellular localization of the kinases here predicted (Supplementary Table SIII) showed that 74.3% of them are located in the nucleus (N) while 68.6% were found in the cytoplasm (C). The predicted kinases located in both compartments were mostly S/T-, while Y kinases were preferentially located at the plasma membrane (PM) (Fig. 3A). Most of these kinases are mobile proteins that translocate among the nucleus, the cytoplasm and the PM, and only a few of them are exclusively located in one of these structures (Fig. 3B). As observed in Table II, the predicted kinases inside the nucleus are preferentially confined to the nucleoplasm (Np) (34.3%). Moreover, the cytoplasmic kinases move mostly among the cytosol (37.1%) and other structures, although 17.1% are not associated with any particular structure. Overall, predicted kinases are mostly S/T kinases that localize, coincidently with B23, in the nucleus and/or the cytoplasm.

The activation of cellular processes and signaling pathways involving B23 and its potential kinases, as well as their coincidence in protein networks, could induce the phosphorylation. Analysis of the published data (Supplementary Table SIII) also revealed that B23 and the predicted kinases share 18 cellular processes (Fig. 4A). The most representative shared processes were apoptosis, negative regulation of cell death, cell proliferation, regulation of transcription, and cell cycle process. As shown in Figure 4, S/T kinases were markedly predominant over Y kinases not only in the cellular processes (Fig. 4A), but also in the signaling pathways (Fig. 4B) shared with B23. The most represented signaling pathways comprised the phosphoinositide 3-kinase (PI3K) cascade that, contrasting with the other pathways, involved higher percentages of Y- than S/T kinases. Besides, the PKB signaling cascade and the I-kappaB kinase/NF-kappaB cascade, as well as the Nerve growth

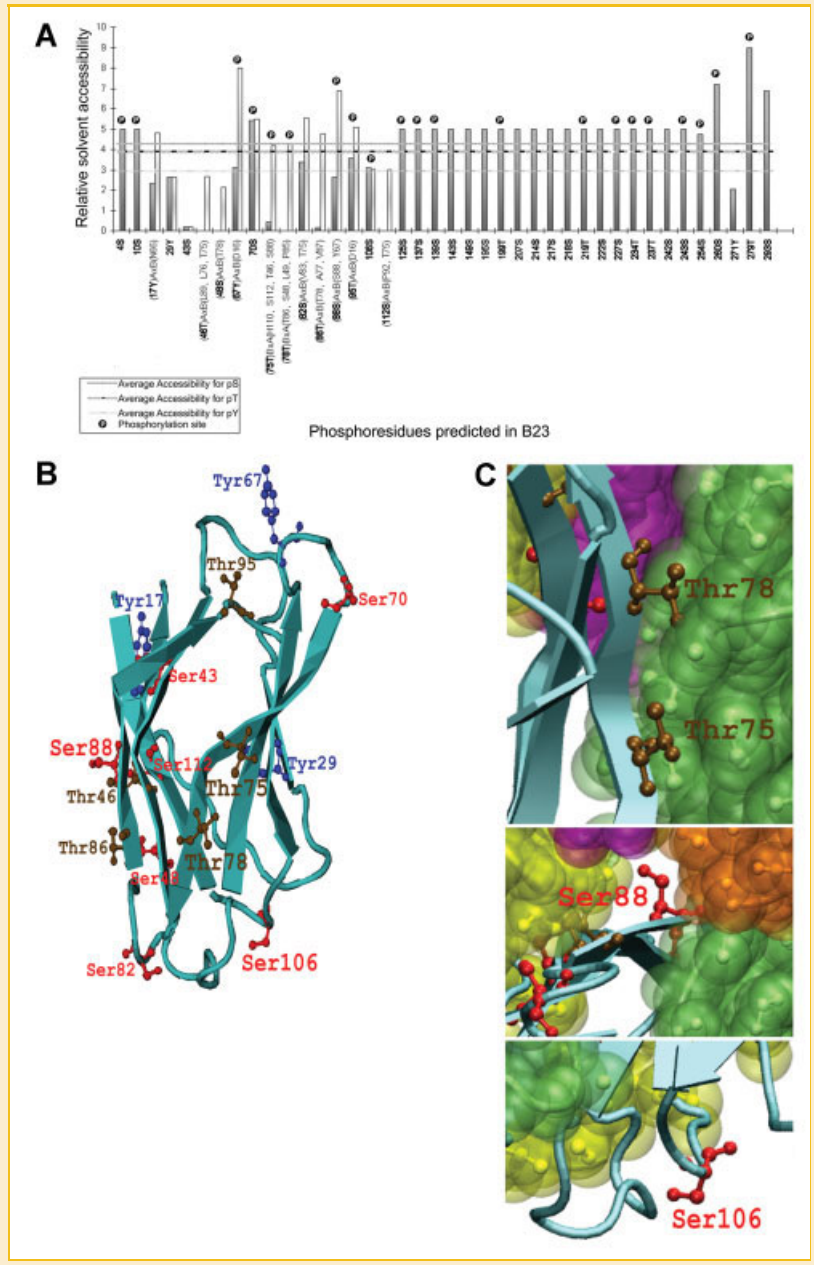

Fig. 2. Accessibility of phosphoresidues predicted in human nucleophosmin/B23. A: Each relative solvent accessibility percentage was standardized from 0 (buried) to 9 (100% or exposed) [Hooft et al., 1996]. A residue is considered accessible when its accessibility is similar or higher than 4.28 for pS, 3.88 for pT, and 2.96 for pY. Dark bars: accessibility of predicted residues in a monomer included in the B23 decamer (PDB code: 2P1B). White bars: accessibility of predicted residues in a monomer separated from the decamer. Residues involved in monomer A–monomer B interactions are shown within brackets. B: Phosphorylatable residues at the core domain of a single monomer. C: Buried phosphoresidues at the decameric structure of B23. Residues Thr75, Thr78, and Ser88 are localized in the interface between the monomers and show low accessibility. The residue Ser106 is not at such interface, and therefore its accessibility is not affected by the adjacent monomer. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

factor (NGF) receptor signaling pathway. Finally, the S/T kinases PKG and CaM as well as the Y kinases INSR and ZAP70 were not involved in any cellular process or signaling pathway common to B23. Therefore, they are not likely to mediate phosphorylation of this protein. Finally, 60% of the predicted kinases are connected with B23 in protein–protein interaction networks (Supplementary Table SIII). In these networks, the S/T kinases (45.7% of the total) were also predominant over the Y kinases (14.3%).

Overview of the number of interactions between B23 and the predicted kinases. Based on all analyzed interactions between B23 and the predicted kinases, we aimed to identify those more likely to phosphorylate B23 (Fig. 5). The S/T kinases showed markedly higher P scores (see Materials and Methods Section), than the Y kinases (Supplementary Table SIV). The lower P value observed for the experimentally verified kinases of B23 was 0.62 (CK2, see Supplementary Table SIV). Thus, this value was taken as the

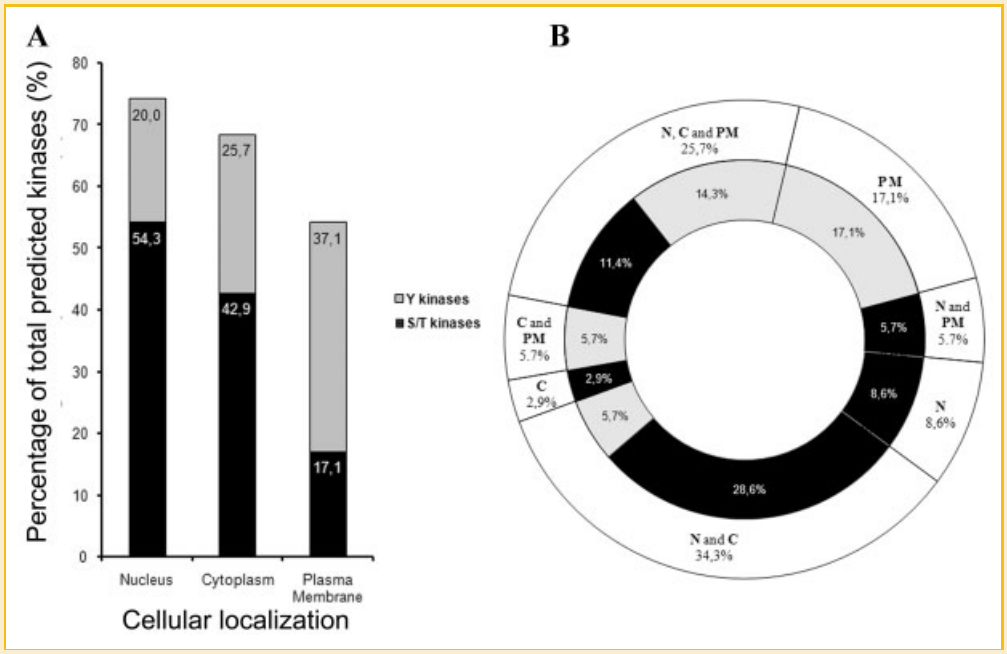

Fig. 3. Cellular localization of the kinases predicted for human nucleophosmin/B23. Data are based in published data and information at databases (see Materials and Methods Section). A: Predicted S/T and Y kinases that are located at the nucleus (N), cytoplasm (C), and plasma membrane (PM). B: Outer ring: Simultaneous localization of kinases among N, C, and PM. Inner ring: percentages of S/T (black) and Y kinases (gray). The percentages are based on the total number of predicted kinases (i.e., 35).

significance cutoff for this parameter (Fig. 5). Surprisingly, six kinases that have not been reported in phosphorylation events of B23 showed P scores within this threshold (Fig. 5, Supplementary Table SIV).

DISCUSSION

Experimental data on B23 phosphorylation is still limited, even though this event is crucial in the biology of this protein (Supplementary Table SI). An interesting fact is the presence of 21 phosphorylation sites in this protein (see UniProt code: P06748, PHOSIDA code: IPI00549248 and Phospho.ELM code: P06748), whereas the average number for human proteins at PHOSIDA database is only three phosphosites. On the other hand, the effector kinase remains unknown for 14 of these verified phosphosites (see Supplementary Table SII). In order to fill this knowledge gap we have used a meta-prediction approach. As a result, we correctly predicted five well-known kinases of B23 (CK2, PKC, PKA, CDK, and PLK1) and proposed 30 new kinases (Supplementary Table SII). We also suggested that all serine, threonine and tyrosine residues of B23 (40 in total) are potential phosphoacceptors. Serines were the most predicted residue, which agrees with the experimental evidence and their higher abundance in B23 (26 residues). The lower susceptibility of phosphorylation was found for tyrosines, being predicted only by KinasePhos 2.0 server. In this sense, prediction of Y67, which has been reported as phosphorylated [Dephoure et al., 2008], demonstrates the advantage of our meta-predictive strategy over a particular predictor.

Our results also showed that ATM, PKB, RSK, STK4, CHK1, PKG, Aurora, and CK1, not reported as kinases of B23, are very likely to phosphorylate this protein in multiple sites. Moreover, CDK, CK2, PKA, PKC, and PLK1, already reported as kinases of B23, could phosphorylate more sites than those previously informed. Although multiple phosphorylation sites is a common feature of the nucleoplasmin family members, such as NPM3, NPM2, and B23/NPM1 [Okuwaki et al., 2002; Zhang et al., 2004; Eirin-Lopez et al., 2006], only a few sites are known for the same kinase. In B23, some authors have demonstrated multiple phosphorylations at T199,

TABLE II. Subcellular Localization of the Kinases Predicted to Phosphorylate B23

Cellular structures	Subcellular localization of kinases	Percentage of predicted kinases (%)
Nucleus	Nucleus	31.4
	Nucleolus	2.9
	Nucleolus and nuclear matrix	2.9
	Nuclear matrix and Nucleoplasm	2.9
	Nucleoplasm	34.3
Cytoplasm	Cytoplasm	11.4
	Cytosol	17.1
	Cytosol and centrioles	5.7
	Cytosol and spindle	5.7
	Cytosol and mitochondrion	5.7
	Cytosol and Golgi apparatus (Golgi)	2.9
	Centrioles and spindle	5.7
	Spindle and ribosomes	2.9
	Endothelial reticulum (ER) and Golgi	2.9
	Golgi apparatus	5.7
	Centrioles, spindle, ER, and Golgi	2.9
Plasma membrane	Plasma membrane	54.3

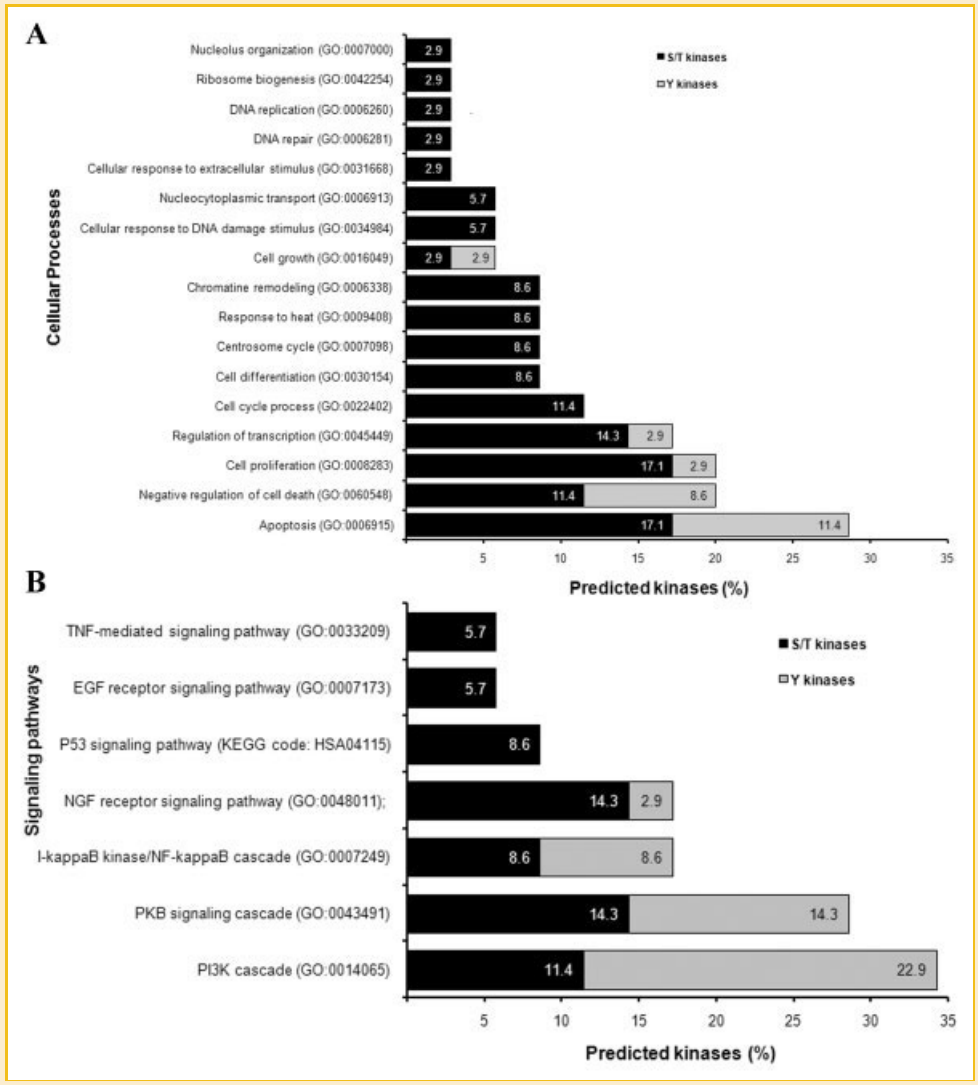

Fig. 4. Cellular processes and signaling pathways shared by human nucleophosmin/B23 and the predicted kinases. Numbers within bars show the percentages of predicted serine/threonine (S/T) and tyrosine (Y) kinases involved, as well as B23, in (A) cellular processes and (B) signaling pathways common to B23. These percentages are based on the total number of predicted kinases (i.e., 35). The GO codes were assigned according to the Gene Ontology database (http://www.geneontology.org).

T219, T234, and T237 through the enzymes CDK1 and CDK2 [Peter et al., 1990; Okuwaki et al., 2002; Negi and Olson, 2006]. As observed in Table I, these residues as well as CDK as the effective kinase, were correctly predicted by our approach. Other authors have also suggested Nek2A-mediated multisite phosphorylation at S4, S70, and S254 [Falini et al., 2007; Okuwaki, 2008]. However, even when in vitro assays confirmed Nek2A-mediated phosphorylation of B23 [Yao et al., 2004], there is not enough experimental evidences supporting these assertions (Xuebiao Yao, personal communication). This enzyme was not predicted by the strategy used here, which could be related to the non-inclusion of NEK enzymes in the programs used. During the course of this research, Xue et al. [2011] published an improved version of GSP 2.1 (http://gps.biocuckoo.org). This new version is able to predict a higher number of kinases, including the NEK family (NEK2, NEK6, and NEK9) and its use in our meta-predictive strategy could contribute to further studies.

Moreover, it is known that PKA-mediated in vitro phosphorylation of B23 occurs at sites other than S125, although the precise positions are still unknown [Chan et al., 1990]. We accurately predicted that S125 is not a substrate of PKA and we proposed 14 potential phosphosites for this enzyme. The previous evidence of in vitro PKC-mediated phosphorylation at S227 was also correctly predicted (Supplementary Table SII). Besides, the terminal residues S4 and S193 were recognized by 13 (including PLK1) and 11 kinases, respectively.

Nowadays, many authors have demonstrated that the incorporation of structural information of phosphorylation sites into the predictors can improve their performance [Via et al., 2011]. Previous studies have suggested that phosphosites are typically exposed and

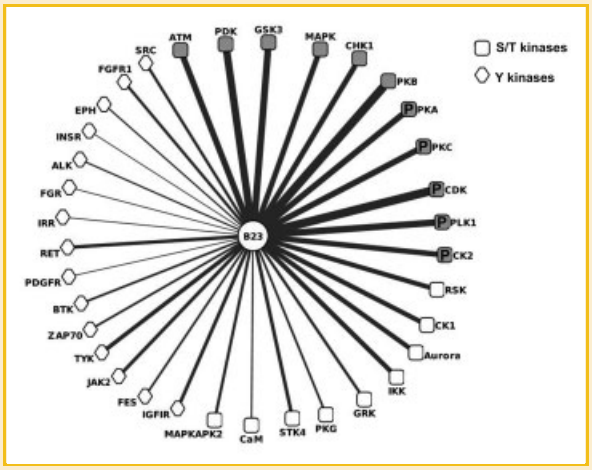

Fig. 5. Overview of interactions between human nucleophosmin/B23 and the predicted kinases. The edge thickness is proportional to the number of interactions. The different types of interactions analyzed are detailed in Materials and Methods Section. Gray nodes represent kinases with a number of interactions higher than or equal to those of CK2, the kinase with the lowest number of interaction (8) but experimentally verified in phosphorylation events of B23 (see Supplementary Table SIV). P: denotes kinases with experimental evidence of phosphorylation.

localized at IDes regions of the proteins [Iakoucheva et al., 2004]. However, with the lately augmented number of available protein three-dimensional structures, it has been demonstrated a relevance of the 3D context around the phosphoacceptor residues for recognition by a kinase [Jimenez et al., 2007; Durek et al., 2009]. As previous structural studies of phosphorylation sites [Gnad et al., 2007; Jimenez et al., 2007; Durek et al., 2009], most of the verified phosphoresidues in B23 (17 of 21) were accessible to the solvent. The majority of them (11) are located at IDes regions of B23, which agrees with Iakoucheva et al. [2004]. The remaining six were found at N- and C-terminal globular domains of B23, which is also consistent with previous structural analysis of protein phosphosites [Jimenez et al., 2007]. The unexpected low accessibility of the experimentally verified residues T75, T78, and S88 at the decameric structure of B23, was found to be related with their masking by others residues of the adjacent monomers. This finding suggests that phosphorylation of the experimentally confirmed phosphoacceptors Y67, T75, T78, S88, and T95, involved in monomer–monomer contacts, could destabilize the decamer. It also suggests that the predicted residues Y17, S82, and T86 that are exposed at B23 monomeric structure could undergo phosphorylation although they are not experimentally confirmed. Besides, their dephosphorylation may be required for oligomerization of B23. This hypothesis explains previously described transitions between monomeric and oligomeric states of B23 during apoptosis [Sautkina et al., 2006] and cell cycle phases [Chou and Yung, 1995]. Anyhow, the level of phosphorylation of these residues must be low considering its occurrence only in the monomeric state of the protein. Based on this accessibility analysis, 13 newly predicted phosphoresidues (10 residues at IDes regions and 3 residues at nucleus domain) that are exposed in the structure of B23 emerged as very likely to undergo modification.

Although further experimental studies are needed, the in silico study here shown is the first approach suggesting promiscuous phosphorylation of B23. We propose that the predicted kinases could share up to 14 potential phosphorylation sites in B23, which is even higher than the number (11) reported for prokaryotic proteins [Prisic et al., 2010]. Tyrosines (non-pY and pY) resulted among the most promiscuous residues, consistent with previous reports describing Y kinases as more promiscuous enzymes than S/T kinases [Brunati et al., 1989; Jia, 2008]. The conservation of predicted phosphoresidues among the nucleophosmin-related sequences was high (median conservation of 84%) and comparable with those at other proteins [Eirin-Lopez et al., 2006; Ramamoorthy et al., 2010]. The correlation analysis between conservation of these residues and their promiscuity showed that the most conserved ones (pT, non-pT, and non-pS) may be targeted by a small group of selected kinases. Conversely, residues less conserved ones (pS and non-pY) were also the most promiscuous. Thus, their modification, through a wide spectrum of kinases, could be a requisite to allow proper function of B23 in the cell.

Also, we observed a higher conservation of T compared to S and Y that contrasts with the higher conservation of Y reported for vertebrate proteins [Gnad et al., 2007]. The higher conservation of pT in the nucleophosmin family reveals a strong functional constraint. This is supported by previous findings showing that phosphorylation of the highly conserved threonines T199, T219, T234, and T237 (Supplementary Fig. S2) is essential for the mobility, localization, and function of B23 [Okuda et al., 2000; Negi and Olson, 2006]. The lower detected conservation of pS compared to non-pS in the nucleophosmin family, differs from that revealed for other proteins in vertebrates, showing higher conservation of pS [Gnad et al., 2007]. In agreement with these authors, we also observed a higher conservation of pT over non-pT. The divergent findings here described could be related with mutations from serine to threonine detected in nucleophosmin sequences (Supplementary Fig. S2). This is consistent with previous studies suggesting that ortholog peptides are not affected by this mutation [Blom et al., 2004; Miller and Blom, 2009].

Given the high number of kinases predicted, we tried to identify those connected with the biology of B23. Most of the predicted kinases (89.9%) coincide with B23 at nucleus and/or cytoplasm. They share with this protein a higher number of signaling pathways than cellular processes, which is reasonable considering that signaling pathways usually involve several cellular processes [Klipp and Liebermeister, 2006]. These predicted kinases are mostly S/T kinases, which agree with experimental evidences describing 20 S/T phosphoresidues in B23. It also agrees with the S/T kinase-mediated phosphorylation reported for this protein during apoptosis [Tawfic et al., 1995], negative regulation of cell death or cell survival [Lee et al., 2008], cell cycle [Okuwaki et al., 2002; Zhang et al., 2004], and centrosome cycle process [Okuda et al., 2000]. Despite these findings, the Y kinases were predominant in the PI3K cascade, the most represented pathway. This is related to the essential role of Y kinases during the reception and transduction of the signal in the PI3K cascade [Cantley, 2002]. Overall, we suggest that the activation

of shared cellular processes and signaling pathways alters the phosphorylation state of B23 through the predicted kinases, probably mediated by S/T- rather than Y kinases. Finally, 60% of predicted kinases, mostly S/T kinases, are connected with B23 in protein–protein interaction networks. In these networks such kinases could get close enough to target phosphorylation motifs on B23 that can oligomerize into a decamer [Chan and Chan, 1995], exhibiting a large surface to interact with cellular partners.

There are evidences showing that motif-based predictions of phosphorylation sites improve with analysis of the network context of kinases and phosphoproteins [Linding et al., 2008]. From a cellular perspective, the different types of analyzed interactions such as subcellular localization, cellular processes, signaling pathways, and protein–protein interaction networks shared between B23 and the predicted kinases, should simultaneously prompt the phosphorylation event. The analysis of data regarding these interactions showed that S/T kinases are more associated than Y kinases with the functionality of B23 in the cell. Some predicted kinases, not reported for B23, emerged as closely related to this protein (PKB, PDK, GSK3, ATM, CHK1, and MAPK). Only CHK1 and PKB are known to interact physically with B23 [Lee et al., 2008; Chen et al., 2009], but they have not been confirmed as kinases of this protein. We suggest that these six kinases could phosphorylate B23 in vivo, although this assumption should be experimentally verified.

In summary, we studied all potential phosphorylation sites in B23 through a meta-predictive strategy involving seven public servers. This approach takes advantage of the individual programs used since it combines servers-based motif predictions with diverse biological information already published in several databases. Thereby, we proposed new sites that are exposed in B23 and therefore, have a high susceptibility of phosphorylation. We found new potential kinases that are significantly associated with this protein functionality and could phosphorylate it in vivo. New potential phosphosites were also predicted for the enzyme PKA, for which the specific phosphosites are still unknown. Other kinases reported for B23 such as PKC, CDK, CK2, and PLK1, could phosphorylate this protein in new additional phosphorylation sites from those previously described. Furthermore, all the predicted sites could undergo promiscuous phosphorylation; hence additional kinases may target functionally important phosphosites in B23. The threonines showed the highest conservation among the phosphorylatable residues in nucleophosmin sequences, caused by mutations from serine to threonine. The predictive results here shown, not only confirmed many previous experimental evidences, but also provide new information concerning B23 phosphorylation. These results contribute to fill the information gap about this event. They also could contribute to address further experimental studies of this protein and its related kinases.

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REFERENCES

Adon AM, Zeng X, Harrison MK, Sannem S, Kiyokawa H, Kaldis P, Saavedra HI. 2010. Cdk2 and Cdk4 regulate the centrosome cycle and are critical mediators of centrosome amplification in p53-null cells. Mol Cell Biol 30:694–710.

Beckmann R, Buchner K, Jungblut PR, Eckerskorn C, Weise C, Hilbert R, Hucho F. 1992. Nuclear substrates of protein kinase C. Eur J Biochem 210: 45–51.

Blom N, Sicheritz-Ponten T, Gupta R, Gammeltoft S, Brunak S. 2004. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics 4:1633–1649.

Brunati A, Marchiori F, Ruzza P, Calderan A, Borin G, Pinna L. 1989. Phosphorylation of small peptides by spleen TPK-IIA, a tyrosine protein kinase stimulated by polylysine and by high ionic strength. FEBS Lett 254: 145–149.

Cantley LC. 2002. The phosphoinositide 3-kinase pathway. Science 296: 1655–1657.

Chan PK, Chan FY. 1995. Nucleophosmin/B23 (NPM) oligomer is a major and stable entity in HeLa cells. Biochim Biophys Acta 1262:37–42.

Chan PK, Liu QR, Durban E. 1990. The major phosphorylation site of nucleophosmin (B23) is phosphorylated by a nuclear kinase II. Biochem J 270:549–552.

Chen S, Maya-Mendoza A, Zeng K, Tang CW, Sims PF, Loric J, Jackson DA. 2009. Interaction with checkpoint kinase 1 modulates the recruitment of nucleophosmin to chromatin. J Proteome Res 8:4693–4704.

Chou YH, Yung BY. 1995. Cell cycle phase-dependent changes of localization and oligomerization states of nucleophosmin/B23. Biochem Biophys Res Commun 217:313–325.

Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ, Gygi SP. 2008. A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci USA 105:10762–10767.

Durek P, Schudoma C, Weckwerth W, Selbig J, Walther D. 2009. Detection and characterization of 3D-signature phosphorylation site motifs and their contribution towards improved phosphorylation site prediction in proteins. BMC Bioinformatics 10:117.

Eirin-Lopez JM, Frehlick LJ, Ausio J. 2006. Long-term evolution and functional diversification in the members of the nucleophosmin/nucleoplasmin family of nuclear chaperones. Genetics 173:1835–1850.

Falini B, Nicoletti I, Bolli N, Martelli MP, Liso A, Gorello P, Mandelli F, Mecucci C, Martelli MF. 2007. Translocations and mutations involving the nucleophosmin (NPM1) gene in lymphomas and leukemias. Haematologica 92:519–532.

Fujii K, Zhu G, Liu Y, Hallam J, Chen L, Herrero J, Shaw S. 2004. Kinase peptide specificity: Improved determination and relevance to protein phosphorylation. Proc Natl Acad Sci USA 101:13744–13749.

Fujimoto J, Shiota M, Iwahara T, Seki N, Satoh H, Mori S, Yamamoto T. 1996. Characterization of the transforming activity of p80, a hyperphosphorylated protein in a Ki-1 lymphoma cell line with chromosomal translocation t(2;5). Proc Natl Acad Sci USA 93:4181–4186.

Gagneur J, Krause R, Bouwmeester T, Casari G. 2004. Modular decomposition of protein–protein interaction networks. Genome Biol 5:R57.

Gnad F, Ren S, Cox J, Olsen JV, Macek B, Oroshi M, Mann M. 2007. PHOSIDA (phosphorylation site database): Management, structural and evolutionary investigation, and prediction of phosphosites. Genome Biol 8:R250.

Grummitt CG, Townsley FM, Johnson CM, Warren AJ, Bycroft M. 2008. Structural consequences of nucleophosmin mutations in acute myeloid leukemia. J Biol Chem 283:23326–23332.

Hooft RW, Sander C, Scharf M, Vriend G. 1996. The PDBFINDER database: A summary of PDB, DSSP and HSSP information with added value. Comput Appl Biosci 12:525–529.

Humphrey V, Dalke A, Schulten K. 1996. VMB—Visual molecular dynamics. J Mol Graph 14(1):33–38.

Iakoucheva LM, Radivojac P, Brown CJ, O'Connor TR, Sikes JG, Obradovic Z, Dunker AK. 2004. The importance of intrinsic disorder for protein phosphorylation. Nucleic Acids Res 32:1037–1049.

Jia Y. 2008. Current status of HTRF® technology in kinase assays. Expert Opin Drug Discov 3:1461–1474.

Jimenez JL, Hegemann B, Hutchins JR, Peters JM, Durbin R. 2007. A systematic comparative and structural analysis of protein phosphorylation sites based on the mtcPTM database. Genome Biol 8:R90.

Kim DE, Chivian D, Baker D. 2004. Protein structure prediction and analysis using the Robetta server. Nucleic Acids Res 32:W526–W531.

Kim JH, Lee J, Oh B, Kimm K, Koh I. 2004. Prediction of phosphorylation sites using SVMs. Bioinformatics 20:3179–3184.

Klipp E, Liebermeister W. 2006. Mathematical modeling of intracellular signaling pathways. BMC Neurosci 7(Suppl 1):S10.

Lee HH, Kim HS, Kang JY, Lee BI, Ha JY, Yoon HJ, Lim SO, Jung G, Suh SW. 2007. Crystal structure of human nucleophosmin-core reveals plasticity of the pentamer–pentamer interface. Proteins 69:672–678.

Lee SB, Xuan Nguyen TL, Choi JW, Lee KH, Cho SW, Liu Z, Ye K, Bae SS, Ahn JY. 2008. Nuclear Akt interacts with B23/NPM and protects it from proteolytic cleavage, enhancing cell survival. Proc Natl Acad Sci USA 105:16584–16589.

Linding R, Jensen LJ, Pasculescu A, Olhovsky M, Colwill K, Bork P, Yaffe MB, Pawson T. 2008. NetworKIN: A resource for exploring cellular phosphorylation networks. Nucleic Acids Res 36:D695–D699.

Liu JP. 2010. Protein kinase C and its substrates. Mol Cell Endocrinol 116: 1–29.

Miller M, Blom N. 2009. Kinase-specific prediction of protein phosphorylation sites. In: Clifton NJ, editor. Phospho-proteomics: Methods and protocols. Lyngby, Denmark, Clifton, NJ: Technical University of Denmark, Center for Biological Sequence Analysis. pp 299–310.

Negi SS, Olson MO. 2006. Effects of interphase and mitotic phosphorylation on the mobility and location of nucleolar protein B23. J Cell Sci 119:3676–3685.

Neuberger G, Schneider G, Eisenhaber F. 2007. pkaPS: Prediction of protein kinase A phosphorylation sites with the simplified kinase-substrate binding model. Biol Direct 2:1.

Okuda M, Horn HF, Tarapore P, Tokuyama Y, Smulian AG, Chan PK, Knudsen ES, Hofmann IA, Snyder JD, Bove KE, Fukasawa K. 2000. Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell 103:127–140.

Okuwaki M. 2008. The structure and functions of NPM1/Nucleophsmin/B23, a multifunctional nucleolar acidic protein. J Biochem 143:441–448.

Okuwaki M, Tsujimoto M, Nagata K. 2002. The RNA binding activity of a ribosome biogenesis factor, nucleophosmin/B23, is modulated by phosphorylation with a cell cycle-dependent kinase and by association with its subtype. Mol Biol Cell 13:2016–2030.

Peter M, Nakagawa J, Doree M, Labbe JC, Nigg EA. 1990. Identification of major nucleolar proteins as candidate mitotic substrates of cdc2 kinase. Cell 60:791–801.

Prisic S, Dankwa S, Schwartz D, Chou MF, Locasale JW, Kang CM, Bemis G, Church GM, Steen H, Husson RN. 2010. Extensive phosphorylation with overlapping specificity by Mycobacterium tuberculosis serine/threonine protein kinases. Proc Natl Acad Sci USA 107:7521–7526.

Ramamoorthy K, Potala S, Verma R. 2010. Insilco analysis of functionally important residues in folate receptors. Bioinformation 2:157–162.

Sarek G, Jarviluoma A, Moore HM, Tojkander S, Vartia S, Biberfeld P, Laiho M, Ojala PM. 2010. Nucleophosmin phosphorylation by v-cyclin-CDK6 controls KSHV latency. PLoS Pathog 6:e1000818.

Sautkina EN, Potapenko NA, Vladimirova NM. 2006. State of nucleolar proteins B23/nucleophosmin and UBF in HeLa cells during apoptosis induced by tumor necrosis factor. Biochemistry (Mosc) 71:634–643.

Tawfic S, Olson MO, Ahmed K. 1995. Role of protein phosphorylation in post-translational regulation of protein B23 during programmed cell death in the prostate gland. J Biol Chem 270:21009–21015.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882.

Tokuyama Y, Horn HF, Kawamura K, Tarapore P, Fukasawa K. 2001. Specific phosphorylation of nucleophosmin on Thr(199) by cyclin-dependent kinase 2-cyclin E and its role in centrosome duplication. J Biol Chem 276:21529–21537.

Via A, Diella F, Gibson TJ, Helmer-Citterich M. 2011. From sequence to structural analysis in protein phosphorylation motifs. Front Biosci 16:1261–1275.

von Mering C, Jensen LJ, Snel B, Hooper SD, Krupp M, Foglierini M, Jouffre N, Huynen MA, Bork P. 2005. STRING: Known and predicted protein–protein associations, integrated and transferred across organisms. Nucleic Acids Res 33:D433–D437.

Vriend G. 1990. WHAT IF: A molecular modeling and drug design program. J Mol Graph 8(52–6):29.

Wan J, Kang S, Tang C, Yan J, Ren Y, Liu J, Gao X, Banerjee A, Ellis LB, Li T. 2008. Meta-prediction of phosphorylation sites with weighted voting and restricted grid search parameter selection. Nucleic Acids Res 36:e22.

Wong YH, Lee TY, Liang HK, Huang CM, Wang TY, Yang YH, Chu CH, Huang HD, Ko MT, Hwang JK, 2007. KinasePhos 2.0: A web server for identifying protein kinase-specific phosphorylation sites based on sequences and coupling patterns. Nucleic Acids Res 35:W588–W594.

Xue Y, Liu Z, Cao J, Ma Q, Gao X, Wang Q, Jin C, Zhou Y, Wen L, Ren J. 2011. GPS 2.1: Enhanced prediction of kinase-specific phosphorylation sites with an algorithm of motif length selection. Protein Eng Des Sel 24:255–260.

Yao J, Fu C, Ding X, Guo Z, Zenreski A, Chen Y, Ahmed K, Liao J, Dou Z, Yao X. 2004. Nek2A kinase regulates the localization of numatrin to centrosome in mitosis. FEBS Lett 575:112–118.

Yun JP, Chew EC, Liew CT, Chan JY, Jin ML, Ding MX, Fai YH, Li HK, Liang XM, Wu QL. 2003. Nucleophosmin/B23 is a proliferate shuttle protein associated with nuclear matrix. J Cell Biochem 90:1140–1148.

Zhang H, Shi X, Paddon H, Hampong M, Dai W, Pelech S. 2004. B23/nucleophosmin serine 4 phosphorylation mediates mitotic functions of polo-like kinase 1. J Biol Chem 279:35726–35734.