ARTICLE
Journal of Cellular Biochemistry 113:2126–2135 (2012)

Journal of Cellular Biochemistry

Phosphorylation Target Site Specificity for AGC Kinases DMPK E and Lats2

Lieke Gerrits,[1] Hanka Venselaar,[2] Bé Wieringa,[1] Derick G. Wansink,[1] and Wiljan J.A.J. Hendriks[1*]

[1] *Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands*

[2] *Centre for Molecular and Biomolecular Informatics, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands*

ABSTRACT

Serine/threonine kinases of the AGC group are important regulators of cell growth and motility. To examine the candidate substrate profile for two members of this group, DMPK E and Lats2, we performed in vitro kinase assays on peptide arrays. Substrate peptides for both kinases exhibited a predominance of basic residues surrounding the phosphorylation target site. 3D homology modeling of the kinase domains of DMPK E and Lats2 indicated that presence of two negative pockets in the peptide binding groove provides an explanation for the substrate preference. These findings will aid future research toward signaling functions of Lats2 and DMPK E within cells. J. Cell. Biochem. 113: 2126–2135, 2012.

KEY WORDS: AGC KINASES; DMPK; KINASE SUBSTRATE; Lats2; PEPTIDE ARRAY; 3D HOMOLOGY MODELS

Protein kinases catalyze transfer of the terminal phosphate group of ATP to the hydroxyl group on serine, threonine, or tyrosine residues in target proteins. This process is a key mechanism in the regulation of signaling cascades and, hence, in many cellular processes [Ubersax and Ferrell, 2007]. Aberrant phosphorylation can be involved in the etiology of disease [Hunter, 2000] and improving our knowledge about kinases and their substrate specificities would help explain molecular consequences and identify therapeutic targets.

Most kinases are either serine/threonine- or tyrosine-directed, but dual-specificity kinases also exist. One group of kinases with Ser/Thr specificity is known as the AGC (protein kinase A, -G, -C) group [Manning et al., 2002]. Activation of many AGC kinases depends on phosphorylation of two highly conserved motifs within these enzymes, i.e., the activation segment and the hydrophobic motif [Pearce et al., 2010]. Interactions with substrate proteins are largely defined by the substrate peptide binding groove located near the active site [Yang et al., 2002], but other structural domains or binding motifs may also shape substrate affinity and specificity of AGC kinases [Pearce et al., 2010].

The AGC group comprises several families, including NDR (nuclear Dbf2-related) and DMPK (Dystrophia Myotonica Protein Kinase) kinases, of which Lats2 (large tumor suppressor 2) and DMPK E, respectively, are representative members [Manning et al., 2002]. Lats2 has been subject of intense research since its expression was found to be down-regulated in multiple cancer types [Cho et al., 2009; Lee et al., 2009; Voorhoeve et al., 2006]. Lats2 is a component of the mammalian tumor suppressor Salvador–Warts–Hippo pathway [Hergovich and Hemmings, 2009] and participates also in p53 signaling [Aylon et al., 2006; Voorhoeve et al., 2006]. Functional studies suggest roles for Lats2 in cell cycle regulation [Kamikubo et al., 2003; Li et al., 2003; Yabuta et al., 2007], induction of apoptosis [Kamikubo et al., 2003; Ke et al., 2004], centrosome duplication, and maintenance of mitotic fidelity and genomic stability [McPherson et al., 2004]. Despite the many studies on Lats2's function and interaction partners, at present only YAP (yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif), two additional components of the Salvador–Warts–Hippo pathway, have been identified as substrates [Hergovich and Hemmings, 2009].

Additional supporting information may be found in the online version of this article.

Grant sponsor: Radboud University Nijmegen Medical Centre; Grant number: RUNMC 2006-12.

*Correspondence to: Wiljan J.A.J. Hendriks, PhD, Department of Cell Biology (internal code 283), Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: w.hendriks@ncmls.ru.nl

Manuscript Received: 18 October 2011; Manuscript Accepted: 24 January 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 1 February 2012

DOI 10.1002/jcb.24086 • © 2012 Wiley Periodicals, Inc.

DMPK E is one of the kinase isoforms encoded by the *DMPK* gene, which is mutated by an expanded, non-coding (CTG)n repeat in myotonic dystrophy type 1 patients [Sicot et al., 2011]. Alternative splicing of *DMPK* transcripts gives rise to several DMPK isoforms which differ in the presence of a five-amino-acid VSGGG insertion in the kinase domain and in the nature of their C-terminus [Groenen et al., 2000]. Not much is known about DMPK's biological function, but available data point toward roles in ion homeostasis [Benders et al., 1997; Mounsey et al., 2000b; Kaliman et al., 2005; Geering, 2006] and actomyosin dynamics [Jin et al., 2000; Mulders et al., 2011]. Identified substrates include phospholamban (a muscle-specific Ca^{2+} ATPase inhibitor) [Kaliman et al., 2005], phospholemman (a regulator of the Na^{+}/Ca^{2+} exchanger and Na^{+}/K^{+} ATPase) [Mounsey et al., 2000a], myosin phosphatase targeting subunit (MYPT) [Muranyi et al., 2001], serum response factor (SRF) [Iyer et al., 2003], and CUG-binding protein (CUG-BP) [Roberts et al., 1997]. The significance of phosphorylation modification of these target proteins remains largely unknown.

Here, we report on the application of peptide array technology and homology modeling to acquire insight into the substrate specificity profiles of AGC kinases DMPK E and Lats2.

MATERIALS AND METHODS

GENERATION OF EXPRESSION PLASMIDS

Plasmid pEBG-DMPK E, encoding N-terminally GST-tagged DMPK E, was generated by ligation of the *Bgl*II-digested DMPK E cDNA insert from pSG8-DMPK E [Groenen et al., 2000] in-frame into *Bam*HI-digested pEBG vector [Tanaka et al., 1995]. Plasmid pEBG-DMPK E kinase dead (KD; the enzymatically inactive mutant) was created in a similar manner using the cDNA insert from pSG8-DMPK E KD [Wansink et al., 2003].

Plasmid pEYFP-C1-Lats2 CD, encoding YFP fused N-terminally to a truncated Lats2 version representing its C-terminal catalytic domain (CD), was created by cloning a *Bgl*II-digested PCR fragment (spanning position 2209–2780 of mouse Lats2 cDNA, acc. nr. NM_015771) into pEYFP-C1. Primers used were 5′-ATGA-GATCTGCTGGGCTCTGTGAGGCCG-3′ (sense, *Bgl*II site in bold) and 5′-CTTAGATCTAGTACTTGGAATTGTGAGTCC-3′ (antisense, *Bgl*II site in bold, *Sca*I site underlined). Plasmid pSG8-VSV-mLats2 served as template. Mammalian expression plasmid pSG8-VSV-mLats2 had been constructed by subcloning a PCR-generated mouse Lats2 (mLats2) cDNA fragment (nucl. pos. 134-3262 in acc. nr. NM_015771) in-frame into BamHI-digested pSG8-VSV vector [Cuppen et al., 1998]. To complement the pEYFP construct with the remaining 3′-part of Lats2 cDNA, it was linearized with *Sca*I and *Bam*HI and ligated with a *Sca*I/*Bam*HI-digested Lats2 fragment (spanning position 2781 to the 3′ end) from pSG8-VSV-mLats2. Plasmid pEBG-Lats2 CD, encoding an N-terminally GST-tagged version of the Lats2 CD, was constructed from pEYFP-C1-Lats2 CD by releasing the kinase domain insert (spanning position 2209 to the 3′ end, encoding amino acids 592-1042, of mouse Lats2) using *Bam*HI and *Bgl*II and ligating this into vector pEBG [Tanaka et al., 1995]. In parallel, construct pEYFP-C1-Lats2 CD KD (encoding the enzymatically inactive mutant) was generated exploiting the partial Lats2 cDNA insert from pSG8-VSV-mLats2-KD bearing a Lys655Ala mutation. Likewise, pEYFP-C1-Lats2 CD KD enabled the creation of pEBG-Lats2 CD KD via *Bam*HI-*Bgl*II cloning into pEBG.

To generate pGEX-4T-3-MLC2-nm, the nm-MLC2 coding sequence was obtained by *Eco*RI/*Xho*I digestion from expression construct pSG8Δ*Eco*RI-h/m-nm [Gerrits et al., 2012] and ligated into *Eco*RI/*Xho*I-digested pGEX-4T-3. Plasmids pEBG-PTP-PEST WT and pS65T-lamA have been described elsewhere [Broers et al., 1999; Halle et al., 2007].

CELL CULTURE AND TRANSIENT TRANSFECTION

COS-1 cells (ATCC #CRL1650) were cultured in DMEM (Gibco/Invitrogen, Paisley, UK) supplemented with 10% (v/v) FCS at 37°C and 7.5% CO_2. Transfection of COS-1 cells was done using DEAE-Dextran as described [van Ham et al., 2003]. Cells expressing wild type (WT) or KD GST-Lats2 CD were treated with 1 μM okadaic acid for 1 h prior to lysis [Millward et al., 1999].

GST PULL-DOWN

COS-1 cells expressing GST-tagged proteins were washed once with ice-cold PBS and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% NP-40; 25 mM NaF; 1 mM sodium pyrophosphate; 0.1 mM $NaVO_3$; 2 μM microcystin LR (Alexis Biochemicals/Enzo Life Sciences, Farmingdale, NY); 1 mM PMSF and protease inhibitor cocktail (Roche, Basel, Switzerland)) 1–2 days after transfection. Cell debris was pelleted by centrifugation for 30 min at 15,000 rpm. Glutathione Sepharose 4B beads were added to cleared lysates and incubated overnight at 4°C while rotating. Before being used in the kinase assay, beads were washed once in lysis buffer containing 0.5 M NaCl, once in lysis buffer, and finally once in 50 mM Tris-HCl, pH 7.5; 1 mM DTT; 1 mM benzamidine. In case GST-fusion kinases were to be used for a peptide array, proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, diluted in kinase storage buffer (50% glycerol; 1 μM DTT; 1 μM EDTA; 200 μg/μl bovine serum albumin), snap frozen, and stored at −80°C.

PEPTIDE ARRAY AND DATA ANALYSIS

To determine DMPK E and Lats2 CD activity toward a peptide library, PepChip Kinase I arrays (Pepscan PRESTO, Lelystad, The Netherlands) were used according to the manufacturer's instructions. Enzymatically inactive mutants were included to determine background phosphorylation caused by potentially co-purifying endogenous kinases.

In short, the concentration of eluted GST fusion proteins of DMPK E (WT or KD) and Lats2 CD (WT or KD) was determined on a Coomassie Brilliant Blue (CBB) stained SDS gel by comparison with standard BSA concentrations. GST fusion proteins were diluted in a 1:1 mixture of glutathione elution buffer and kinase storage buffer yielding a recombinant protein concentration of 100 ng/μl (Lats2 CD WT or KD) or 150 ng/μl (DMPK E WT or KD). The kinase reaction contained 5 (Lats2 CD WT or KD) or 8 (DMPK E, WT or KD) μg kinase, 1× kinase master mix (50 mM Hepes, pH 7.4; 10 mM $MgCl_2$; 10% glycerol; 0.01 mg/ml BSA; 0.01% Brij-35; 2.5 mM $MnCl_2$; 1 μM microcystin), 1 μM PKA inhibitor peptide (Bachem, Bubendorf, Switzerland), 10 μM ATP, and 300 μCi/ml γ-^{33}P-ATP. Two-thirds of the total reaction volume (60 μl) was applied to the PepChip Kinase I

slide and the remaining one-third was used for SDS–PAGE to confirm kinase input levels. Slides were then coverslipped and incubated for 4 h at 30°C in a humidified chamber. Slides were washed once for 5 min with PBS containing 1% Triton X-100 (PBS-Tx), twice with NaCl-Tx (2 M NaCl; 1% Triton X-100) and three times with water. Finally, slides were air dried and exposed to a phosphor-imager screen (Bio-Rad, Berkeley, CA). Spot intensities were measured with GenePix software (Axon instruments/Molecular Devices, Sunnyvale, CA) and the average intensity of duplicate spots and standard deviation were calculated.

ANTIBODIES

Endogenous Cdc2 was immunoprecipitated (1:100) and detected on western blot (1:1,000) with anti-Cdc2 antibody A17 (Ab18, Abcam, Cambridge, UK). Polyclonal anti-DMPK antibody B79 antibody (1:10,000 [Groenen et al., 2000]) was used to detect GST-DMPK E (WT or KD) on western blot. Polyclonal anti-GST-GFP antiserum [Cuppen et al., 2000] was used to immunoprecipitate GFP-Lamin A (in a 1:250 dilution) and to detect GST-tagged proteins on western blot (1:5,000). To detect GFP-Lamin A on western blot, monoclonal anti-GFP antibody was used (sc-9996, Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:5,000). All primary antibodies for western blotting were diluted in blocking buffer.

IMMUNOPRECIPITATION

Protein immunoprecipitation from cleared lysates of transfected COS-1 cells was performed as described for GST pull-down, but now antibody-coupled protein A beads were used. Polyclonal anti-GFP antiserum or Cdc2 antibody was coupled to beads by overnight rotation at 4°C in PBS. Unbound antibodies were removed from the beads by brief centrifugation and washing at 4°C in PBS, before loaded beads were added to the lysates. After overnight immunoprecipitation, beads were washed five times with PBS containing 1% NP-40 and immediately used in an in vitro kinase assay.

BACTERIAL GST-PROTEIN PRODUCTION AND PURIFICATION

Competent BL21(DE3)pLysS cells were transformed with plasmid pGEX-4T-3-MLC2-nm and subsequently grown to log phase in LB medium at 37°C, induced with 1 mM IPTG, and grown for an additional 3 h. Bacteria were pelleted by centrifugation at 5,000 rpm for 5 min, resuspended in ice-cold PBS and subjected to three freeze-thaw cycles and three sonication steps of 10 s with an interval of 1 min on ice in between steps. Cell debris was pelleted by centrifugation at 9,500 rpm for 15 min and supernatant was incubated with glutathione-Sepharose 4B beads overnight. Subsequently, beads were washed extensively with PBS and GST-fusion proteins were eluted from the beads using 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, snap frozen, and stored at −80°C.

SDS–PAGE AND WESTERN BLOTTING

Protein samples were mixed with 5× SDS–PAGE sample buffer [2.5 M DTT; 25% glycerol; 150 mM Tris-HCl, pH 6.8; 5% (w/v) SDS], boiled for 5 min and subjected to SDS–PAGE. Subsequently, proteins were electro-blotted onto PVDF membrane (Millipore, Billerica, MA). For immunodetection purposes, membranes were blocked with 5% (w/v) non-fat dry milk in TBS-T (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween-20 (Sigma-Aldrich; St. Louis, MO)) for 30–60 min, incubated with primary antibodies overnight at 4°C and then washed three times with TBS-T. Subsequently, the appropriate secondary antibodies—goat anti-mouse or anti-rabbit IRDye® 600 or 800 (LI-COR Biosciences (Lincoln, NE), dilution 1:10,000)—were applied and incubated for 1 h at room temperature. Following three successive washes with TBS-T, detection of fluorescent signals was done on an Odyssey infrared Imaging System (LI-COR Biosciences). For CBB staining of proteins, membranes were incubated with CBB staining solution (0.25% (w/v) CBB; 50% methanol; 10% acetic acid) for 1 min and then destained with 50% methanol.

3D-MODELING

A 3D model of the Lats2 CD was created using the automatic homology modeling script in the YASARA & WHAT IF Twinset [Vriend, 1990; Krieger et al., 2002]. As a template for this model, we used PDB file 1XH9, containing the structure of cAMP-dependent protein kinase catalytic subunit alpha [Breitenlechner et al., 2005]. The 3D structure of DMPK E CD has been solved experimentally and can be found in PDB file 2VD5 [Elkins et al., 2009]. Peptide docking onto DMPK E and Lats2 CDs was based on GSK3 peptide position in PKB (PDB file 1O6K) and optimized using energy minimizations in the YASARA & WHAT IF Twinset.

IN VITRO KINASE ASSAY

In vitro kinase assay was performed as described [Wansink et al., 2003]. Briefly, substrate-coated beads (Cdc2, Lamin A, PTP-PEST) were washed once with lysis buffer containing 0.5 M NaCl, once with lysis buffer, and once with 50 mM Tris-HCl, pH 7.5; 1 mM DTT, 1 mM PMSF; 1 mM benzamidine. Beads were then aliquoted in the desired number of kinase reaction samples and subjected to the final washing step before being added to 20 μl 2× kinase assay buffer (100 mM Tris-HCl, pH 7.5; 20 mM $MgCl_2$; 4 mM $MnCl_2$; 2 mM DTT; 2 μM PKA inhibitor peptide; 2 μM PMSF; 2 μM benzamidine; 4 μM microcystin LR). Likewise, eluted MLC2 substrate was aliquoted in the desired number of kinase reaction samples and diluted in 20 μl 2× kinase assay buffer. Beads containing GST-coupled kinase or the enzymatically inactive mutant were added to the substrate and H_2O was added to a final volume of 40 μl. To determine specific activities, 500 ng eluted GST-fusion kinase was used in an in vitro kinase assay and the final peptide concentration was 30 μM. Reactions were started by adding 5 μl 0.5 mM γ-^{32}P-ATP and continued for 30 min at 30°C. Reactions were stopped by the addition of 5× SDS–PAGE sample buffer and samples were used for SDS–PAGE and western blotting as described above.

RESULTS

PURIFICATION OF DMPK E AND LATS2 PROTEIN

In order to gain insight into DMPK E and Lats2 kinase specificity both kinases need to be obtained in their active, i.e., properly phosphorylated, state. To this end, kinases were expressed in COS-1 cells as GST-fusion proteins and subsequently purified via glutathione beads. Full-length Lats2 protein was found to be very

inefficiently produced and purified, thus an N-terminally truncated version representing the essential CD (referred to as Lats2 CD) was used instead. Localization of ectopically expressed Lats2 CD in the cell matched that of full-length Lats2 (data not shown) and also Lats2 CD depended on okadaic acid treatment of cells to acquire an active state. This indicates that regulatory kinases and phosphatases did not noticeably discriminate between the two forms and supports use of Lats2 CD to identify potential substrates. Next to WT versions of both kinases also enzymatically inactive mutants (KD) were included to be able to monitor any contribution of potentially co-purifying kinases (Fig. 1A).

Prior to determining specificity profiles for Lats2 CD and DMPK E, enzymatic activity of purified proteins was assessed via an in vitro kinase assay in which two peptide substrates were included: (i) Standard NDR substrate peptide CGGGGKKRNRRLSVA [Millward et al., 1998]—also known to be phosphorylated by DMPK E [Wansink et al., 2003]—and (ii) PKA substrate kemptide CGGGGLRRASLG as negative control [Wansink et al., 2003]. Specific activity of Lats2 CD and DMPK E toward the NDR kinase substrate peptide was approximately 0.25 pmol P_i/min/μg kinase (Fig. 1B). Background activity in the DMPK E KD protein preparation was marginal, but some residual activity was detected in the Lats2 CD KD preparation. Kinase activity toward kemptide was insignificant for both kinases. These data confirmed our expectation that purified recombinant WT forms of DMPK E and Lats2 CD are enzymatically active and that DMPK E KD—and to a lesser extent Lats2 CD KD—may serve as specificity controls.

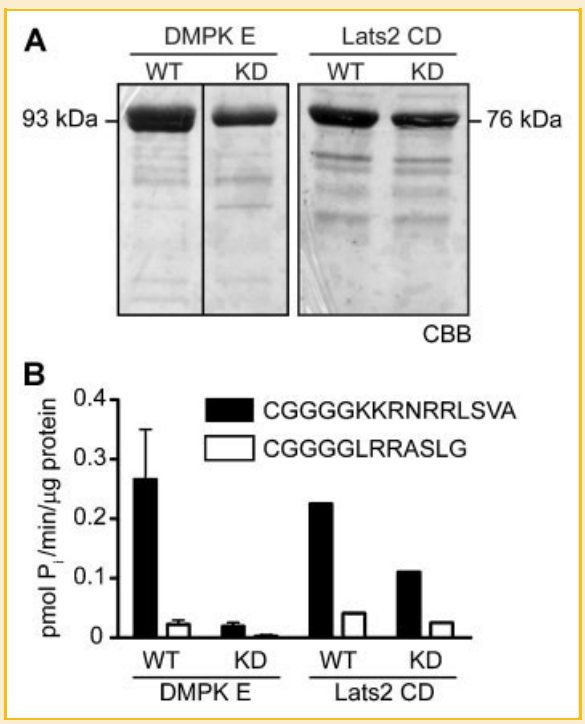

Fig. 1. Purified DMPK E and Lats2 CD are enzymatically active. A: GST fusion proteins of DMPK E and Lats2 CD (i.e., an N-terminally truncated version representing the essential catalytic domain), both in a WT and an enzymatically inactive version (KD), were produced in COS-1 cells and purified via GST pull-down. GST-DMPK E WT, but not KD, appears as a broad doublet band, indicative of autophosphorylation activity [Wansink et al., 2003]. Protein purity and concentration were determined on a CBB-stained SDS gel by comparison with standard BSA concentrations. B: Kinase activity of DMPK E and Lats2 CD toward two different peptides determined in an in vitro kinase assay ($n = 2$ for DMPK E, $n = 1$ for Lats2 CD).

PEPTIDE ARRAY SCREENING

Purified recombinant kinases were subsequently used to phosphorylate peptides on an arrayed library. Most of these peptides represent genuine in vivo phosphorylation targets (www.phosphosite.org). The array glass surface, onto which two duplicate sets of 1,152 different peptide sequences were printed, was incubated with purified kinase in the presence of γ-^{33}P-ATP (Suppl. Fig. 1). Resulting ^{33}P incorporation was quantified using a phosphorimager device. Incubation with DMPK E WT resulted in several distinct spots representing phosphorylated peptides, while incubation with DMPK E KD revealed only minor background phosphorylation (Fig. 2A). On the array incubated with Lats2 CD WT much more peptides were labeled. Also, the slide incubated with Lats2 CD KD revealed a considerable number of radiolabeled spots (Fig. 2B). These data imply that the Lats2 kinase domain, outside the context of the full-length protein, displays broader substrate specificity than DMPK E. We cannot exclude that some of these spots reflect contributions of kinases that co-purified with Lats2 CD.

To extract a cogent set of substrate peptides from these array data, we applied three selection criteria. First, the average signal intensity for a substrate peptide should match at least 5% of the highest average intensity of duplicate spots obtained for the WT kinase. Second, to exclude peptide sequences with high background phosphorylation levels in the KD arrays, average labeling intensity for duplicate spots should be at least twice as high on the WT array as on the KD array. Third, to exclude poorly reproducible signals, the standard deviation of duplicate spots on the WT array should not exceed the average signal for those spots. Peptides that matched all three criteria were manually checked to exclude influences from strong radiation of neighboring spots or non-specific smears on the array surface. This resulted in a list of 47 candidate DMPK E substrate peptides (Suppl. Table I). For Lats2 CD 243 peptides met the criteria and the top 40 sequences are listed in Suppl. Table II.

DMPK E AND LATS2 PREFER BASIC RESIDUES FLANKING THEIR TARGET PHOSPHORYLATION SITE

Almost all peptides phosphorylated by DMPK E contained a Ser or Thr phosphoacceptor site next to a Lys or Arg at position −2 and frequently also a Lys/Arg at positions −1, −3, or −4 (Suppl. Table I). This consensus indicated a preference of DMPK E activity for Ser/Thr residues just C-terminal of basic residues (Fig. 3A). Besides, Lys/Arg residues were often present at positions +1 to +4 as well. Hydrophobic residues were encountered at position +1.

Also the vast majority of peptides in the top list of candidate Lats2 CD substrates (Suppl. Table II) contained at least one Lys/Arg residue at position −2 and −3 (Fig. 3B) but compared to DMPK E, positively charged residues were less frequent at position −1. As for DMPK E, Lys/Arg residues were also observed at positions +2 to +4 in Lats2 substrate peptides. In contrast to DMPK E substrates, hydrophobic

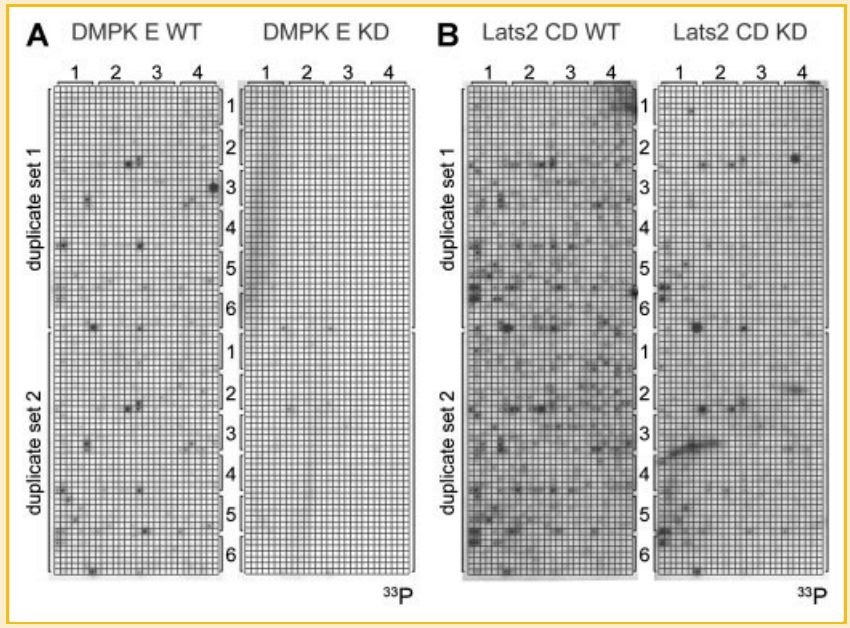

Fig. 2. Testing kinase specificity for DMPK E and Lats2 using a peptide array. The grid of the peptide array carrying two duplicate sets of 1152 kinase substrate peptides was overlaid with phosphorimager results of in vitro phosphorylation with DMPK E WT or KD (A), or Lats2 CD WT or KD (B).

residues were encountered at position −1 and +3 in Lats2 substrates.

We used homology modeling to relate our observations regarding DMPK E and Lats2 preferences for peptide sequences containing Lys/Arg residues at positions −1 to −3 (Fig. 3) to 3D structures of the respective kinase domains [Krieger et al., 2002]. The structure for the DMPK E kinase domain has been solved (PDB file 2VD5; [Elkins et al., 2009]). PDB file 1XH9, containing cAMP-dependent protein kinase catalytic subunit alpha [Breitenlechner et al., 2005] was used as a template to model the Lats2 CD. Due to high sequence identity between the kinase domains of DMPK E and Lats2, the published active site of DMPK E [Elkins et al., 2009] could be used to predict location of the active site and hence the peptide binding groove of Lats2.

Two negative pockets exist in the region where the N-terminus of the target peptide is expected to bind (Fig. 4). The region that should harbor the C-terminus of the peptide, a hydrophobic pocket, is also present. We subsequently docked preferred target peptides into the 3D structures of the CDs. As shown in Figure 4 B,C and E,F, the positively charged residues in docked peptides nicely fill the negative pockets on the CDs. This provides an explanation for the experimental observation that peptides with Lys/Arg residues N-terminal of the target Ser/Thr site are favored by DMPK E and Lats2.

COMPARISON OF HIGHLY SIMILAR DMPK E SUBSTRATE PEPTIDE SEQUENCES

The peptide array data provided a list of potential substrates for the kinases under study (Suppl. Tables I and II). Use of the full-length DMPK E protein in the kinase assay resulted in a more refined phosphorylation pattern than obtained with the Lats2 CD. We therefore decided to use DMPK E to further validate our experimental findings and theoretical predictions. We noted that a difference of one or two amino acids within a peptide sequence could lead to marked differences in phosphorylation activities. Therefore, clusters of peptides with highly similar sequences were selected to extract information on exclusion principles that guide DMPK E target sequence selectivity (Fig. 5).

Data from the cluster depicted in Figure 5A suggest that DMPK E has a preference for Thr over Ser. This finding may appear to contradict the consensus sequence depicted in Figure 3A, but one should bear in mind that the latter is based on a 4–5 times overrepresentation of Ser relative to Thr phosphoacceptor sites in the peptide collection on the array. Data from cluster 5A also suggest that, at least in the context of this peptide, a tyrosine at the C-terminus is favored over alanine. Other comparisons reveal that the presence of a polar residue at position +1 negatively influences phosphorylation by DMPK E (Fig. 5B). Panel 5C underscores the importance of a basic residue at −2: It explains why only the C-terminal one of the two Thr residues in the two upper peptides is phosphorylated by DMPK E. Additional positive charges N-terminal of the target site further boost phosphorylation by DMPK E. One may argue that the target site in the shorter peptide is simply less accessible for the kinase (Fig. 5C) but this is not likely as the second peptide in cluster A contains even less amino acids N-terminal to the phosphorylation site. Figure 5D shows that the N-terminal Ser residue in YRKSSLKSR is phosphorylated by DMPK E, although also the adjacent Ser is positioned relative to a positively charged amino acid at position −2. The C-terminal Ser in this peptide is not phosphorylated by DMPK E, underscoring the −2 Arg/Lys requirement (Fig. 5D).

In some peptides multiple possible phosphorylation target sites that fit the −2 Arg/Lys rule are present. For instance, in the first peptide in cluster E a Ser as well as a Thr residue (indicated by dashed boxes) is preceded by a positively charged residue at position −2.

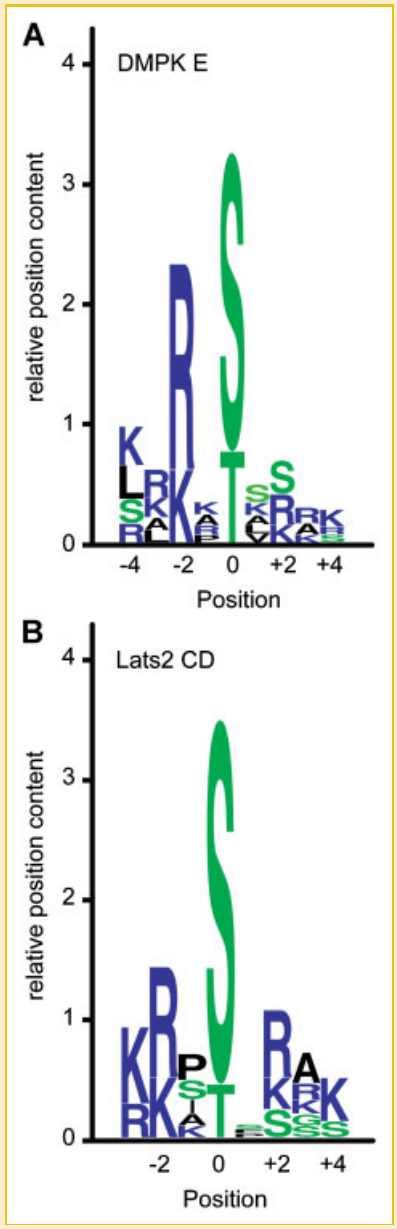

Fig. 3. Consensus phosphorylation target sequence of (A) DMPK E and (B) Lats2, generated using the LOGO algorithm (http://weblogo.berkeley.edu/). The phosphoacceptor site is located at position 0, residues positioned N-terminal (−) or C-terminal (+) of the phosphorylated residue are indicated. The y-axis represents the relative position content. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

The second peptide in this cluster is not phosphorylated (Fig. 5E) but it is impossible to distinguish between two likely causes. The presence of a negatively charged glutamate in the proximity of the N-terminal Ser may well hinder its phosphorylation. Alternatively, in case the Thr residue represented the DMPK E phosphorylation site, its sheer absence in the second peptide explains the lack of signal. Peptides in cluster F also contain multiple possible phosphorylation target sites, two of which contain an Arg residue at position −2 (Fig. 5F). Since three residues N-terminally of the phospho-target site suffices for phosphorylation (Fig. 5A,B) and the middle Ser residue in all cluster F peptides is preceded by an Arg at position −2, it comes as a surprise that the RRSSSVGYI peptide is not phosphorylated. This points to the most N-terminally located Ser residue as the most likely target site, whose phosphorylation in the third peptide may be hampered by insufficient N-terminal residues. In agreement with the results of cluster D, the presence of an extra positive charge at −4 of the phosphorylation site had no significant influence on phosphorylation levels (compare first and second peptide in cluster F).

The only phosphorylated substrate that did not match the DMPK E target site consensus is the first peptide shown in cluster G, which does not carry a positively charged residue at −2 (Fig. 5G). Instead, five negatively charged residues surround the candidate serines. This finding suggests that in addition to the known peptide binding groove of DMPK E, other regions may contribute to peptide recognition as well.

IN VITRO TESTING OF FULL-LENGTH CANDIDATE SUBSTRATES FOR DMPK E

To investigate if data obtained with the array can be extrapolated to full-length substrate proteins, the activity of DMPK E against four proposed candidates (Suppl. Table I) was tested. Availability of proper antibodies allowed immunoprecipitation of endogenous Cdc2 from COS-1 cells. Furthermore, the non-muscle isoform of MLC2 was produced as a GST fusion protein in bacteria, purified and released from glutathione beads. In addition, Lamin A-GFP and GST-tagged PTP-PEST were produced and purified from COS-1 cells with immunoprecipitation and GST pull-down, respectively. The four candidate proteins were tested in in vitro kinase assays in the presence of purified DMPK E WT or KD. Protein input levels were verified by western blotting or CBB staining, while phosphorylation was measured using a phosphorimager. Autophosphorylation of DMPK E served as a control for assay conditions. DMPK E-specific phosphorylation of candidate substrate Cdc2, if at all present, did not reach a level above background as observed in the KD samples (Fig. 6A). The same holds for recombinant Lamin A and PTP-PEST proteins. Recombinant MLC2 protein, on the contrary, displayed significant specific phosphate incorporation upon incubation with DMPK E WT (Fig. 6B).

DISCUSSION

In this study we demonstrate that AGC kinases DMPK E and Lats2 have a similar, yet distinct, target consensus sequence. Both kinases prefer to phosphorylate peptides in which the target residue is surrounded by positively charged amino acids. Preference for this type of sequences is reflected by the presence of two negative pockets in the peptide binding groove that were identified by analysis of 3D models of the kinase domains involved.

The phosphorylation consensus sequences that were delineated from Suppl. Tables I and II are in line with reported consensus target sequences for other AGC kinases [Millward et al., 1998; Turner et al., 2002; Mah et al., 2005; Hao et al., 2008]. Comparison of DMPK E and Lats2 phosphorylation profiles in parallel array screens aids the

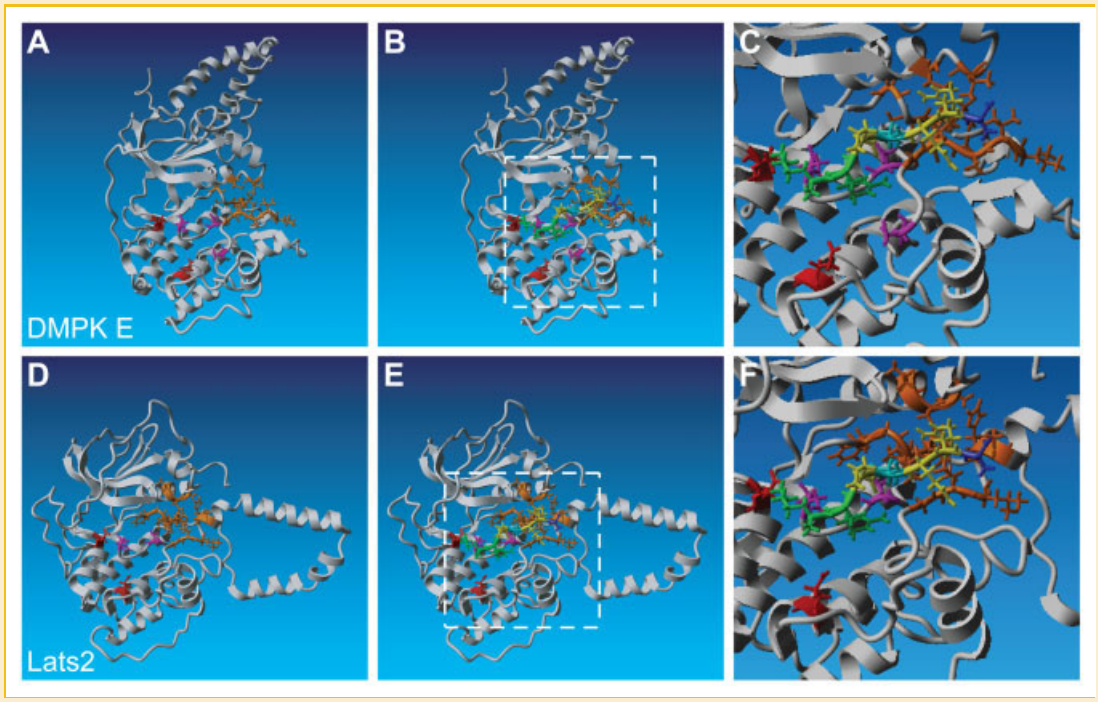

Fig. 4. Three-dimensional model of DMPK E and Lats2 catalytic domains. A: 3D structure of DMPK E (PDB 2VD5) with negatively charged residues of the two negative pockets indicated in red and magenta and the hydrophobic residues of the hydrophobic pocket in orange. B: DMPK E in complex with peptide KRPTQRA. C: Close-up of the area indicated with the white dashed box shown in (B), representing the peptide binding-groove of DMPK E. D: 3D model of the Lats2 catalytic domain (created by homology modeling) with negatively charged residues of the two negative pockets indicated in red and magenta and the hydrophobic residues of the hydrophobic pocket in orange. E: Lats2 catalytic domain in complex with peptide KRPTQRA. F: Close-up of the area indicated with the white dashed box shown in (E), representing the peptide binding-groove of Lats2. Positively charged residues in the peptide that fit in the negative pockets of the kinases are indicated in green, hydrophobic residues are indicated in blue, and the phosphoacceptor residue is indicated in cyan. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

assessment of substrate specificities for these two AGC kinases. Both share a preference for positively charged residues at positions −2 and/or −3 and additional positive charges further N-terminal. In particular, for DMPK E a basic residue at position −2 is highly preferred. The DMPK E consensus sequence described here agrees well with the one discussed in our earlier work [Wansink et al., 2003], although in the present study we could not confirm a preference for Lys/Arg at position −1 concurrently with −3. Another study yielded a consensus sequence with an Arg at position −3, but this sequence was based on phosphorylation analysis of five peptides only [Bush et al., 2000]. All DMPK full-length substrates for which the phosphorylation target sites have been determined indeed display Arg residues at position −2 and −3 (AIRRASTIEMP in phospholamban [Kaliman et al., 2005], KLRRYTTFSKR in SRF [Iyer et al., 2003] and RQSRRSTQGV in MYPT1a [Muranyi et al., 2001]; phospho-acceptor sites in bold). Interestingly, phospholamban was represented on the peptide array by peptides IRRASTIEM and RRASTIEMP but phosphorylation levels did not meet our criteria. The phospholamban target site was identified in the full-length protein [Kaliman et al., 2005] suggesting that DMPK E-mediated phosphorylation of phospholamban may require the full-length protein context. Target requirements for Lats2 appear less discriminative than for DMPK E: At least one basic residue at position −2 or −3. In the Lats2 substrates TAZ and YAP multiple Lats2 target sites were found, each matching the consensus sequence HXRXXS (X represents any amino acid; [Lei et al., 2008; Hergovich and Hemmings, 2009]). Only two peptides that obey this consensus are present on the peptide array: THERSPSPS and HKRKSSQAL, but their phosphorylation levels did not exceed background signal, explaining why the HXRXXS consensus did not emerge from the current screen.

In our study 70% (DMPK E) to 87% (Lats2) of the substrate peptides contain positively charged amino acids at both sides of the phosphorylation target site, even though the array does not seem to be enriched for this type of peptides. In addition, peptide LRRASLRG is a DMPK E substrate, but LRRASLAG not, confirming a preference for positive charges at both sides of the target site. Such a preference has not been described before for any AGC kinase. This observation could point toward the possibility of bidirectional binding of peptides in the peptide binding groove [Chan et al., 1982; Zaliani et al., 1998; Torshin, 2000]. This would also explain why some peptides that only contain positively charged residues downstream of the target site (e.g., GGSVTKKRK) were still phosphorylated by DMPK E and Lats2.

Four candidate substrates that resulted from the peptide array screening were examined for phosphorylation by DMPK E in an in vitro kinase assay. For three of these (Cdc2, PTP-PEST, and Lamin A) phosphorylation levels were not detectably increased by DMPK E. It

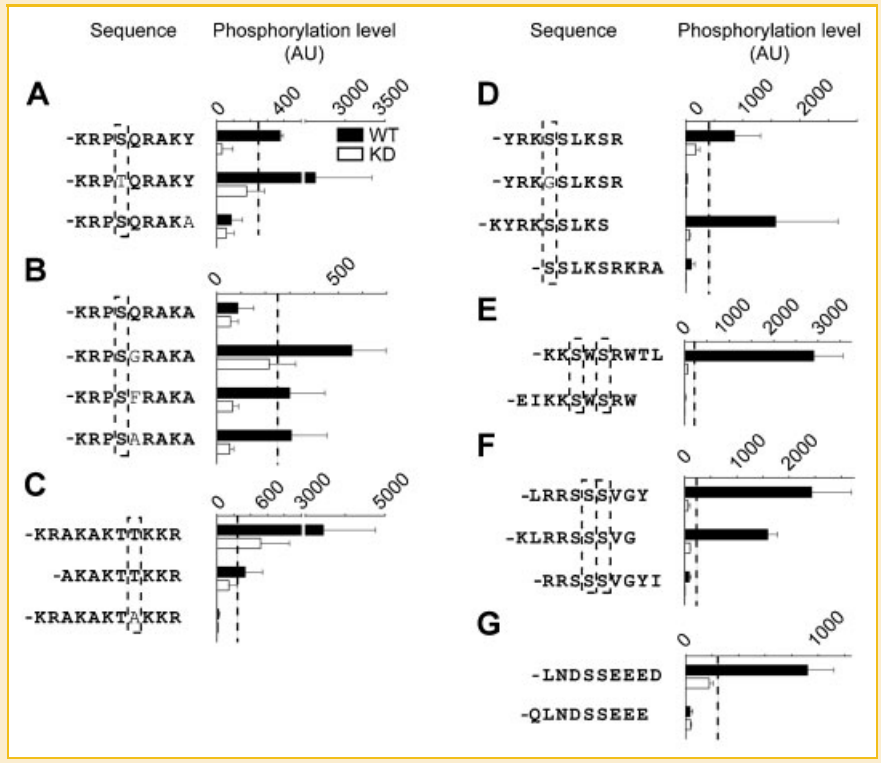

Fig. 5. Analysis of kinase activity toward highly similar peptides grouped in clusters (A–G). Peptide sequences are shown with the common target residue in dashed boxes. In case multiple possible target residues may reside within one peptide, this is indicated with multiple dashed boxes. Only Ser/Thr residues with a Lys/Arg at position −2 were considered possible target sites. The hyphen at the N-terminal end of peptide sequences symbolizes the covalent link to the glass surface of the array. Amino acids in normal font reflect single residue differences with the top sequence in the cluster. Graphs on the right represent average signal intensities (arbitrary units ±SD) for the given peptides measured on the DMPK E WT (black bars) or KD (white bars) arrays (Fig. 2A). A dashed line in the graph indicates the cut-off value for substrate selection (5% of highest average signal on WT array).

is not uncommon that only a limited number of candidate proteins that result from peptide screens actually turn out to be bona fide substrates [Mah et al., 2005]. After all, extrapolation of array data toward full-length target proteins very much depends on the structural context of the corresponding sequence in the substrate. Recently, co-immunoprecipitation of Lamin A with a DMPK isoform that differs from DMPK E was reported [Harmon et al., 2011], but the ability of this isoform to phosphorylate Lamin A was not investigated.

For nm-MLC2 we found convincing evidence that DMPK E was able to phosphorylate the full-length protein. On the PepChip Kinase I array the MLC2 protein was represented by several peptides spanning the position 9 threonine residue for which phosphorylation has been reported [Bengur et al., 1987]. Thr18 and Ser19 are the best-studied MLC2 phosphoacceptor sites [Vicente-Manzanares et al., 2009] but these were not represented in the peptide collection. We investigated whether phosphorylation of full-length MLC2 by DMPK E did occur on this Thr9. Substitution of Thr9 by alanine indeed resulted in a 50% reduction of MLC2 phosphorylation levels, confirming that MLC2 Thr9 is a DMPK E target site but also indicating that phosphorylation at positions Thr18/Ser19 is probably mediated by DMPK E as well (L.G. and D.G.W.; unpublished data).

One of the limitations of peptide array technology is that only a restricted number of proteins can be represented. There could be many more proteins out there that serve as substrates and contain sequences resembling consensus DMPK E and Lats2 target sites. We explored this by performing a motif search in a database of phosphorylated peptides (at phospho.elm.eu.org) which yielded an overwhelming number of possible candidates. To be effective, such an approach would require the delineation of a much more detailed consensus target sequence, for instance, by screening additional custom-made peptide clusters. In addition, structural information on substrate candidates is required to incorporate surface accessibility of the identified peptide sequences in such search algorithms. (Semi-)quantitative phosphoproteomics may therefore represent a more feasible way of expanding and refining the list of DMPK E and Lats2 substrates to provide additional insight into their biological function.

In summary, our findings provide insight into the identity of possible Lats2 and DMPK E substrate proteins and a basis to search for other candidate substrate proteins. More research will be necessary to investigate the phosphorylation capacity of DMPK E and Lats2 toward these proteins. Knowledge on their phosphorylation site preferences will aid in understanding via which molecular pathways Lats2 and DMPK E may exert their activity.

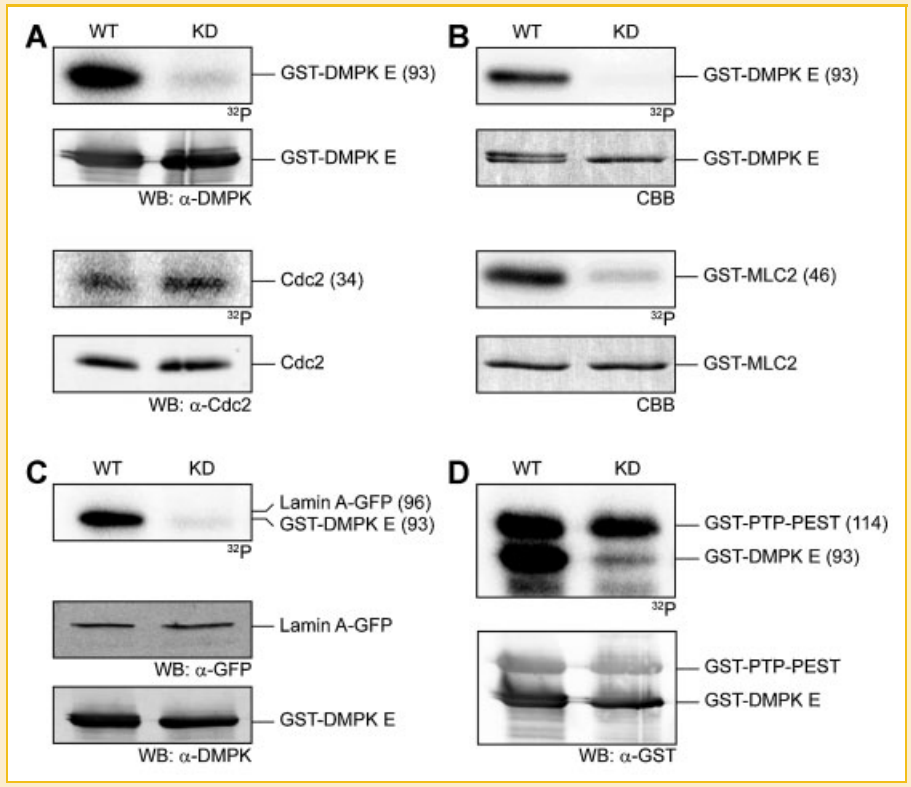

Fig. 6. Testing full-length candidate protein substrates in an in vitro DMPK E kinase assay. Cdc2 (A), nm-MLC2 (B), Lamin A (C), and PTP-PEST (D) were isolated and incubated with either DMPK E WT (WT) or KD (KD) in an in vitro kinase assay. Panels show phosphorylation of DMPK E (i.e., autophosphorylation) and candidate proteins (^{32}P). Protein input levels were determined by western blotting (WB) or Coomassie Brilliant Blue staining (CBB). Numerals between brackets indicate the predicted molecular weight in kDa.

ACKNOWLEDGMENTS

GST-PTP-PEST constructs were a kind gift from Dr. Michel Tremblay (Goodman Cancer Centre, Montreal, Quebec, Canada) and GFP-Lamin A constructs were kindly provided by Drs. Guillaume van Eys and Frans Ramaekers (University of Maastricht, Maastricht, The Netherlands). This work was funded by grant RUNMC 2006-12 from the Radboud University Nijmegen Medical Centre.

REFERENCES

Aylon Y, Michael D, Shmueli A, Yabuta N, Nojima H, Oren M. 2006. A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. Genes Dev 20:2687–2700.

Benders AA, Groenen PJ, Oerlemans FT, Veerkamp JH, Wieringa B. 1997. Myotonic dystrophy protein kinase is involved in the modulation of the Ca2+ homeostasis in skeletal muscle cells. J Clin Invest 100:1440–1447.

Bengur AR, Robinson EA, Appella E, Sellers JR. 1987. Sequence of the sites phosphorylated by protein kinase C in the smooth muscle myosin light chain. J Biol Chem 262:7613–7617.

Breitenlechner CB, Friebe WG, Brunet E, Werner G, Graul K, Thomas U, Kunkele KP, Schafer W, Gassel M, Bossemeyer D, Huber R, Engh RA, Masjost B. 2005. Design and crystal structures of protein kinase B-selective inhibitors in complex with protein kinase A and mutants. J Med Chem 48:163–170.

Broers JL, Machiels BM, van Eys GJ, Kuijpers HJ, Manders EM, van Driel R, Ramaekers FC. 1999. Dynamics of the nuclear lamina as monitored by GFP-tagged A-type lamins. J Cell Sci 112(Pt 20):3463–3475.

Bush EW, Helmke SM, Birnbaum RA, Perryman MB. 2000. Myotonic dystrophy protein kinase domains mediate localization, oligomerization, novel catalytic activity, and autoinhibition. Biochemistry 39:8480–8490.

Chan KF, Hurst MO, Graves DJ. 1982. Phosphorylase kinase specificity. A comparative study with cAMP-dependent protein kinase on synthetic peptides and peptide analogs of glycogen synthase and phosphorylase. J Biol Chem 257:3655–3659.

Cho W, Shin J, Kim J, Lee M, Hong K, Lee J-H, Koo K, Park J, Kim K-S. 2009. miR-372 regulates cell cycle and apoptosis of ags human gastric cancer cell line through direct regulation of LATS2. Mol Cells 28:521–527.

Cuppen E, Gerrits H, Pepers B, Wieringa B, Hendriks W. 1998. PDZ motifs in PTP-BL and RIL bind to internal protein segments in the LIM domain protein RIL. Mol Biol Cell 9:671–683.

Cuppen E, van Ham M, Wansink DG, de Leeuw A, Wieringa B, Hendriks W. 2000. The zyxin-related protein TRIP6 interacts with PDZ motifs in the adaptor protein RIL and the protein tyrosine phosphatase PTP-BL. Eur J Cell Biol 79:283–293.

Elkins JM, Amos A, Niesen FH, Pike AC, Fedorov O, Knapp S. 2009. Structure of dystrophia myotonica protein kinase. Protein Sci 18:782–791.

Geering K. 2006. FXYD proteins: New regulators of Na-K-ATPase. Am J Physiol Renal Physiol 290:F241–F250.

Gerrits L, Overheul GJ, Derks RC, Wieringa B, Hendriks WJAJ, Wansink DG. 2012. Gene duplication and conversion events shaped three homologous, differentially expressed myosin regulatory light chain (MLC2) genes. Eur J Cell Biol in press.

Groenen PJ, Wansink DG, Coerwinkel M, van den Broek W, Jansen G, Wieringa B. 2000. Constitutive and regulated modes of splicing produce six major myotonic dystrophy protein kinase (DMPK) isoforms with distinct properties. Hum Mol Genet 9:605–616.

Halle M, Liu YC, Hardy S, Theberge JF, Blanchetot C, Bourdeau A, Meng TC, Tremblay ML. 2007. Caspase-3 regulates catalytic activity and scaffolding functions of the protein tyrosine phosphatase PEST, a novel modulator of the apoptotic response. Mol Cell Biol 27:1172–1190.

Hao Y, Chun A, Cheung K, Rashidi B, Yang X. 2008. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. J Biol Chem 283:5496–5509.

Harmon EB, Harmon ML, Larsen TD, Yang J, Glasford JW, Perryman MB. 2011. Myotonic dystrophy protein kinase is critical for nuclear envelope integrity. J Biol Chem 286:40296–40306.

Hergovich A, Hemmings BA. 2009. Mammalian NDR/LATS protein kinases in hippo tumor suppressor signaling. Biofactors 35:338–345.

Hunter T. 2000. Signaling–2000 and beyond. Cell 100:113–127.

Iyer D, Belaguli N, Fluck M, Rowan BG, Wei L, Weigel NL, Booth FW, Epstein HF, Schwartz RJ, Balasubramanyam A. 2003. Novel phosphorylation target in the serum response factor MADS box regulates alpha-actin transcription. Biochemistry 42:7477–7486.

Jin S, Shimizu M, Balasubramanyam A, Epstein HF. 2000. Myotonic dystrophy protein kinase (DMPK) induces actin cytoskeletal reorganization and apoptotic-like blebbing in lens cells. Cell Motil Cytoskeleton 45:133–148.

Kaliman P, Catalucci D, Lam JT, Kondo R, Gutierrez JC, Reddy S, Palacin M, Zorzano A, Chien KR, Ruiz-Lozano P. 2005. Myotonic dystrophy protein kinase phosphorylates phospholamban and regulates calcium uptake in cardiomyocyte sarcoplasmic reticulum. J Biol Chem 280:8016–8021.

Kamikubo Y, Takaori-Kondo A, Uchiyama T, Hori T. 2003. Inhibition of cell growth by conditional expression of kpm, a human homologue of Drosophila warts/lats tumor suppressor. J Biol Chem 278:17609–17614.

Ke H, Pei J, Ni Z, Xia H, Qi H, Woods T, Kelekar A, Tao W. 2004. Putative tumor suppressor Lats2 induces apoptosis through downregulation of Bcl-2 and Bcl-x(L). Exp Cell Res 298:329–338.

Krieger E, Koraimann G, Vriend G. 2002. Increasing the precision of comparative models with YASARA NOVA–a self-parameterizing force field. Proteins 47:393–402.

Lee KH, Goan YG, Hsiao M, Lee CH, Jian SH, Lin JT, Chen YL, Lu PJ. 2009. MicroRNA-373 (miR-373) post-transcriptionally regulates large tumor suppressor, homolog 2 (LATS2) and stimulates proliferation in human esophageal cancer. Exp Cell Res 315:2529–2538.

Lei QY, Zhang H, Zhao B, Zha ZY, Bai F, Pei XH, Zhao S, Xiong Y, Guan KL. 2008. TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. Mol Cell Biol 28:2426–2436.

Li Y, Pei J, Xia H, Ke H, Wang H, Tao W. 2003. Lats2, a putative tumor suppressor, inhibits G1/S transition. Oncogene 22:4398–4405.

Mah AS, Elia AE, Devgan G, Ptacek J, Schutkowski M, Snyder M, Yaffe MB, Deshaies RJ. 2005. Substrate specificity analysis of protein kinase complex Dbf2-Mob1 by peptide library and proteome array screening. BMC Biochem 6:22.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. 2002. The protein kinase complement of the human genome. Science 298:1912–1934.

McPherson JP, Tamblyn L, Elia A, Migon E, Shehabeldin A, Matysiak-Zablocki E, Lemmers B, Salmena L, Hakem A, Fish J, Kassam F, Squire J, Bruneau BG, Hande MP, Hakem R. 2004. Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. EMBO J 23:3677–3688.

Millward TA, Heizmann CW, Schafer BW, Hemmings BA. 1998. Calcium regulation of Ndr protein kinase mediated by S100 calcium-binding proteins. EMBO J 17:5913–5922.

Millward TA, Hess D, Hemmings BA. 1999. Ndr protein kinase is regulated by phosphorylation on two conserved sequence motifs. J Biol Chem 274:33847–33850.

Mounsey JP, John JE 3rd, Helmke SM, Bush EW, Gilbert J, Roses AD, Perryman MB, Jones LR, Moorman JR. 2000a. Phospholemman is a substrate for myotonic dystrophy protein kinase. J Biol Chem 275:23362–23367.

Mounsey JP, Mistry DJ, Ai CW, Reddy S, Moorman JR. 2000b. Skeletal muscle sodium channel gating in mice deficient in myotonic dystrophy protein kinase. Hum Mol Genet 9:2313–2320.

Mulders SA, van Horssen R, Gerrits L, Bennink MB, Pluk H, de Boer-van Huizen RT, Croes HJ, Wijers M, van de Loo FA, Fransen J, Wieringa B, Wansink DG. 2011. Abnormal actomyosin assembly in proliferating and differentiating myoblasts upon expression of a cytosolic DMPK isoform. Biochim Biophys Acta 1813:867–877.

Muranyi A, Zhang R, Liu F, Hirano K, Ito M, Epstein HF, Hartshorne DJ. 2001. Myotonic dystrophy protein kinase phosphorylates the myosin phosphatase targeting subunit and inhibits myosin phosphatase activity. FEBS Lett 493:80–84.

Pearce LR, Komander D, Alessi DR. 2010. The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol 11:9–22.

Roberts R, Timchenko NA, Miller JW, Reddy S, Caskey CT, Swanson MS, Timchenko LT. 1997. Altered phosphorylation and intracellular distribution of a (CUG)n triplet repeat RNA-binding protein in patients with myotonic dystrophy and in myotonin protein kinase knockout mice. Proc Natl Acad Sci USA 94:13221–13226.

Sicot G, Gourdon G, Gomes-Pereira M. 2011. Myotonic dystrophy, when simple repeats reveal complex pathogenic entities: New findings and future challenges. Hum Mol Genet 15:R116–R123.

Tanaka M, Gupta R, Mayer BJ. 1995. Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. Mol Cell Biol 15:6829–6837.

Torshin I. 2000. Direct and reversed amino acid sequence pattern analysis: Structural reasons for activity of reversed sequence sites and results of kinase site mutagenesis. Biochem J 345(Pt 3):733–740.

Turner MS, Fen Fen L, Trauger JW, Stephens J, LoGrasso P. 2002. Characterization and purification of truncated human Rho-kinase II expressed in Sf-21 cells. Arch Biochem Biophys 405:13–20.

Ubersax JA, Ferrell JE Jr. 2007. Mechanisms of specificity in protein phosphorylation. Nat Rev Mol Cell Biol 8:530–541.

van Ham M, Croes H, Schepens J, Fransen J, Wieringa B, Hendriks W. 2003. Cloning and characterization of mCRIP2, a mouse LIM-only protein that interacts with PDZ domain IV of PTP-BL. Genes Cells 8:631–644.

Vicente-Manzanares M, Ma X, Adelstein RS, Horwitz AR. 2009. Non-muscle myosin II takes centre stage in cell adhesion and migration. Nat Rev Mol Cell Biol 10:778–790.

Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH, Agami R. 2006. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 124:1169–1181.

Vriend G. 1990. WHAT IF: A molecular modeling and drug design program. J Mol Graph 8:52–56 29.

Wansink DG, van Herpen RE, Coerwinkel-Driessen MM, Groenen PJ, Hemmings BA, Wieringa B. 2003. Alternative splicing controls myotonic dystrophy protein kinase structure, enzymatic activity, and subcellular localization. Mol Cell Biol 23:5489–5501.

Yabuta N, Okada N, Ito A, Hosomi T, Nishihara S, Sasayama Y, Fujimori A, Okuzaki D, Zhao H, Ikawa M, Okabe M, Nojima H. 2007. Lats2 is an essential mitotic regulator required for the coordination of cell division. J Biol Chem 282:19259–19271.

Yang J, Cron P, Thompson V, Good VM, Hess D, Hemmings BA, Barford D. 2002. Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. Mol Cell 9:1227–1240.

Zaliani A, Pinori M, Ball HL, DiGregorio G, Cremonesi P, Mascagni P. 1998. The interaction of myristylated peptides with the catalytic domain of protein kinase C revealed by their sequence palindromy and the identification of a myristyl binding site. Protein Eng 11:803–810.