A High Throughput Proteomics Screen Identifies Novel Substrates of Death-associated Protein Kinase*

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Death-associated protein kinase (DAPk) is a Ser/Thr kinase whose activity is necessary for different cell death phenotypes. Although its contribution to cell death is well established, only a handful of direct substrates have been identified; these do not fully account for the multiple cellular effects of DAPk. To identify such substrates on a large scale, we developed an in vitro, unbiased, proteomics-based assay to search for novel DAPk substrates. Biochemical fractionation and mass spectrometric analysis were used to purify and identify several potential substrates from HeLa cell lysate. Here we report the identification of two such candidate substrates, the ribosomal protein L5 and MCM3, a replication licensing factor. Although L5 proved to be a weak substrate, MCM3 was efficiently and specifically phosphorylated by DAPk on a unique site, Ser160. Significantly, DAPk phosphorylated this site in vivo upon overexpression in 293T cells. Activation of endogenous DAPk by increasing intracellular Ca2+ also led to increased phosphorylation of MCM3. Importantly short hairpin RNA-mediated knockdown of endogenous DAPk blocked both basal phosphorylation and Ca2+-induced phosphorylation, indicating that DAPk is both necessary and sufficient for MCM3 Ser160 phosphorylation in vivo. Identification of MCM3 as an in vivo DAPk substrate indicates the usefulness of this approach for identification of physiologically relevant substrates that may shed light on novel functions of the kinase. Molecular & Cellular Proteomics 7:1089–1098, 2008.

Death-associated protein kinase (DAPk), 1 a Ca2+/calmodulin (CaM)-activated Ser/Thr kinase that localizes to the cytoskeleton, has been linked to cell death and is a potent tumor suppressor (for a review, see Ref. 1). Originally identified in a screen for genes whose functions were necessary for interferon-γ-induced death (2), it has since been shown to be necessary for the regulation or execution of cell death in response to numerous stimuli, including death receptor activation (3), transforming growth factor-β (4), oncogene expression (5), UNC5H2 signaling (6), ceramide (7, 8), and matrix detachment (9). The specific cellular phenotype induced by DAPk activity can vary from one cell setting to another. It has been linked to both type I apoptotic and type II autophagic cell death in both caspase-dependent and caspase-independent manners (1). Specifically DAPk expression can lead to various actin-dependent death-associated morphologic changes, which include membrane blebbing and cell rounding (e.g. Refs. 10 and 11). Detachment from the extracellular matrix often accompanies these phenotypes due to inhibition of integrin function (12). Even in the absence of cell death, the effects of DAPk on the cytoskeleton can lead to the induction of stress fiber formation (13) and interference with cell polarity and directed cell motility (14). DAPk has also been linked to p53-dependent apoptosis through the induction of p53 in a p19ARF-dependent manner (5) and can lead to the up-regulation of autophagy (11). In addition to the chromatin fragmentation that accompanies caspase-dependent apoptosis (e.g. Refs. 4–6), DAPk expression can also lead to caspase-independent nuclear changes that include chromatin condensation (11). Each of these phenotypes can be considered an independent functional arm of DAPk.

Catalytic activity is required for all DAPk-associated phenotypes, implying that phosphorylation of specific substrates mediates the various functional arms. To date, only a limited number of substrates have been identified. These include myosin II regulatory light chain (MLC) whose phosphorylation and subsequent activation of myosin-based contractility leads to membrane blebbing (10, 13, 15, 16). A second substrate identified is syntaxin-1A, a component of the v-SNARE (vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, which mediates docking and fusion of synaptic vesicles with the membrane (17). Although kinetically syntaxin-1A is an efficient in vitro DAPk substrate, its physiologic relevance is not yet known. DAPk was also shown to phosphorylate ribosomal protein S6, thereby reducing translation rates in reticulocyte lysates (18). DAPk may also participate in kinase signaling cascades as it has been shown to phosphorylate and regulate other kinases. For example, phosphorylation of the highly related zipper-interacting protein kinase (ZIPk) influences its intracellular localization.
and enhances its death-promoting activity (19). DAPk can also efficiently phosphorylate Ca\(^{2+}\)/CaM-dependent protein kinase kinase (CaMKK) in vitro and in vivo; this inhibits the ability of CaMKK to undergo CaM-activated autophosphorylation in vitro (20). During oxidative stress, DAPk phosphorlates protein kinase D, leading to activation of c-Jun N-terminal kinase (JNK) and subsequently caspase-independent cell death (21). Although identification of these substrates has shed light on some of the mechanisms of action of DAPk, there are still many gaps that remain in our understanding of how DAPk activity leads to the multiple functional outcomes discussed above. To fully understand these gaps, a more thorough understanding of the complete substrate profile of DAPk needs to be attained.

Here we undertook a large-scale, unbiased proteomics-based screen whose aim was to identify DAPk substrates in vitro to be followed by in vivo confirmation. This was based on a recently described method for searching for kinase substrates, called KESTREL, which has been successfully used to identify substrates for several closely related kinases (22, 23). In this manner, we identified two novel DAPk substrates, ribosomal protein L5 and the MCM3 replication initiation factor.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—Plasmids encoding N-terminal FLAG- and hemagglutinin-tagged human full-length DAPk (pcDNA3-DAPk), the activated kinase deleted of its calmodulin regulatory domain (pcDNA3-DAPkCaM), or the catalytically inactive mutant (pcDNA3-DAPkK42A) have been described previously (10). A pASK-IBA3 vector encoding the catalytic domain of DAPk (amino acids 1–285) tagged at its C terminus with streptavidin was obtained from M. Watterson (Northwestern University, Chicago, IL) and used to produce recombinant DK1. pcDNA3 encoding the non-relevant protein luciferase was used as a control. FLAG-tagged L5 was generated by PCR cloning of the rat L5 cDNA (a kind gift from O. Meyuhas, Hebrew University, Jerusalem, Israel) into pcDNA3. FLAG-tagged human MCM3 in pcDNA3 was a kind gift from Dr. M. Gossen (Max Delbruck Center for Molecular Medicine, Berlin, Germany) (25). Ser\(^{160}\) was mutated to either Ala or Asp by PCR-based site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) according to the recommended protocol. pSuper-based vectors containing shRNA targeting DAPk (nucleotides 5776–5784, GenBank\(^{TM}\) accession number NM_004938.2) or Hc-Red (nucleotides 99–117, GenBank accession number AF363776) were used for knockdown experiments. Chemicals and inhibitors were purchased from Sigma-Aldrich unless otherwise indicated.

**Cell Culture**—293T human embryonic kidney cells and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 50 units/ml penicillin, and 50 \(\mu\)g/ml streptomycin (Invitrogen). Cells were transiently transfected by the calcium sonicated. Following dialysis against 25 mM Tris, pH 7.4, and further dialyzed against 25 mM Tris, pH 7.4. 1% of the dialyzed protein was subjected to an in vitro kinase assay with recombinant catalytic domain of DAPk (DK1) to monitor the presence of particular substrates. The remaining portion of each salt precipitant was applied to a HiTrap phenyl-HP hydrophobic column (GE Healthcare) after addition of NH\(_4\)SO\(_4\) to a final concentration of 1 M. The column was washed with buffer, and then proteins were eluted over a NH\(_4\)SO\(_4\) concentration gradient ranging from 1 to 0 M. Fractions collected were dialyzed against 25 mM Tris, pH 7.4, and concentrated on a YM-10 CentriPlus Spin column (Millipore) to \(\sim\)1/10 volume. A small portion (1–5%) of the total protein from selected fractions was assayed with DK1 for the presence of substrate. Fractions selected for the highest substrate content were resolved by SDS-PAGE in parallel with the products of the in vitro kinase assay. The half of the gel containing the kinase assay was silver-stained to detect the lower levels of protein present, whereas the remaining half of the gel was stained with GelCode (Pierce). The location of each substrate on the gel was denoted by comparison of the autoradiogram and silver-stained gels, and the equivalent portion of the gel was then excised from the GelCode-stained gel. The protein sample was digested with trypsin, fractionated into individual peptides by liquid chromatography, and then analyzed by mass spectrometry (MS/MS) at the Biological Mass Spectrometry unit of the Weizmann Institute of Science using an API Q-Star Pulsar\(^\text{a}\) electrospray-quadruple TOF tandem mass spectrometer with a collision cell (Applied Biosystems/MDS Sciex) equipped with a nanoelectrospray source (MDS Proteomics, Odense, Denmark). Peak lists were generated from the raw data using Analyst QS1.1 and BioAnalyst 1.1.5 (MDS Sciex) and Mascot 2.1 (Matrix Science) software. Centroid and deisotoping parameters were used, and peaks less than 10% of the maximum were removed from analysis. A Mascot MS/MS ion search of the Swiss-Prot 51.0 human database (27,0778 entries, 99,412,397 residues) was performed with a peptide tolerance of \(\pm 0.8\) Da and a MS/MS tolerance of \(\pm 0.8\) Da. Up to two missed cleavages, four positive charges, and modifications by oxidation, deamidation, and carbamidomethylation were allowed.

**Protein Purification and Immunoprecipitation**—DK1 was expressed in TOP10 bacteria (Invitrogen) upon induction with tetracycline and affinity-purified from bacterial lysates using the StrepTactin column (Genosys Biotechnologies) according to the manufacturer’s instructions. Full-length FLAG-DAPk or FLAG-tagged substrates were expressed in 293T cells, which were lysed in B buffer (20 mM HEPES, pH 7.6, 100 mM KCl, 0.5 mM EDTA, 0.4% Nonidet P-40, 20% glycerol) supplemented with protease inhibitors. For experiments using DAPk K42A, which expresses much lower levels than the WT kinase, cells expressing either K42A or WT DAPk constructs were first treated with the actin-depolymerizing agent latrunculin B (20 \(\mu\)M) for 30 min to release all of the kinase from the actin cytoskeleton and then immediately lysed in B buffer. Extracts were immunoprecipitated with an anti-FLAG M2 monoclonal antibody conjugated to protein G beads, and proteins were eluted with excess FLAG peptide. MCM3-bound beads were first washed stringently in 0.5 M KCl and 0.5 M LiCl\(_2\) to remove co-precipitating kinases that resulted in high basal levels of phosphorylation. For immunoprecipitation of phosphorylated MCM3, cells were lysed in PLB (10 mM NaPO\(_4\), pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (100 \(\mu\)M okadaic acid, 20 \(\mu\)M \(\beta\)-glycerophosphate, 1 mM NaF) and immunoprecipitated with monoclonal anti-MCM3 antibodies (Stressgen) prebound to protein G-agarose.
Kinase Assays—Kinase assays of total cell lysate or fractionated lysates followed a modified protocol based on the KESTREL method described previously (22). In brief, samples were incubated with DK1 (0.5 mg) in DK kinase buffer (50 mM β-glycerophosphate, 20 mM MnCl₂) in the presence of 57 nM (5 μCi) [γ-33P]ATP (Amersham Biosciences) and the following inhibitors for 2 min at 30 °C: 1 mM PMSF, 1% protease inhibitor mixture, 1 mM NaF, 5 mM EGTA, and 10 μM protease inhibitor cocktail (PI) (Sigma-Aldrich). Reactions were terminated by the addition of sample buffer and boiled prior to electrophoresis on SDS-polyacrylamide gels. Depending on the amount of protein present, gels were either silver-stained or stained with GelCode, dried, and exposed to MR x-ray film (Eastman Kodak Co.). For kinase assays using purified FLAG-tagged substrate, reactions were incubated for 10 min with DK1 as described above. For assays using full-length DAPk as the kinase, immunopurified FLAG-DAPk and FLAG-tagged substrate at kinase:substrate molar ratios of ~1:5 or 1:10 were incubated for 10 min at 30 °C in kinase buffer (50 mM Hepes, pH 7.5, 20 mM MgCl₂) supplemented with 0.16 μCi/μL [γ-32P]ATP, 50 μM ATP, 1 μM bovine calmodulin, and 0.5 mM CaCl₂. For stoichiometric analysis, kinase assays were performed over a time course ranging from 1 min to 2 h. Reactions were terminated by boiling in SDS loading buffer and resolved on 7.5% acrylamide gels, which were stained with GelCode and dried. Levels of ATP incorporated were measured by phosphorimaging and comparison with a standard curve of known ATP concentrations. For determination of Kₘ and Vₘₐₓ values, a peptide derived from the MCM3 phosphorylation site was synthesized and used in P-81 Whatman filter assays. 20 ng of DK1 was incubated for 15 min at 30 °C with increasing concentrations of peptide (0.25–1000 μM) in reaction buffer (50 mM Hepes, pH 7.5, 20 mM MgCl₂, 100 μM ATP, 4 μCi [γ-32P]ATP). Reactions were applied to P-81 filters and washed extensively in 1% phosphoric acid. Total counts were measured by scintillation counting. Total levels of ATP incorporated were calculated and plotted against substrate concentration. Double reciprocal plots were generated and analyzed by linear regression to obtain kinetic parameters.

Western Blot Analysis—Total cell lysates, protein immunoprecipitates, or kinase assays were resolved on 7.5 or 10% polyacrylamide gels, transferred to nitrocellulose membranes blots, and incubated with monoclonal antibodies to MCM3 (Stressgen), DAPk (clone 55, Sigma), actin (Sigma), or ZIPk (BD Transduction Laboratories) or with affinity-purified rabbit polyclonal antibodies to the MCM3 phosphopeptide KKTIERRYpS 160DLT (where pS is phosphoserine) (generated by PhosphoSolutions, Aurora, CO). Secondary antibodies consisted of horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratories), which were detected by SuperSignal enhanced chemiluminescence (Pierce).

RESULTS

In Vitro Screen for DAPk Substrates—To identify novel substrates of DAPk whose phosphorylation may mediate the various functional cellular effects of DAPk, an unbiased, high throughput screen was undertaken. An in vitro kinase assay was performed on HeLa cell lysate in the presence of a recombinant protein that consisted of the catalytic domain of DAPk (DK1). DK1 lacks the regulatory domains of DAPk and is constitutively active even in the absence of Ca²⁺/CaM. Furthermore it lacks the major autophosphorylation sites of the full-length kinase (24). To suppress the activity of endogenous kinases present in the lysates, specific kinase inhibitors were added to the reaction buffer (i.e. PKI to inhibit cAMP-dependent protein kinase and EGTA to inhibit Ca²⁺-dependent kinases). Furthermore the reactions were run under stringent conditions including short incubations times and the use of limiting quantities of Mn²⁺-ATP (γ-32P-labeled). Preliminary experiments confirmed that the replacement of the usual Mg²⁺ with Mn²⁺ in the reaction buffer had no effect on DK1 activity (data not shown). The addition of DK1 to the reaction mixture led to the phosphorylation of up to nine prominent substrates, which are referred to as S1–S9 (Fig. 1). The strongest phosphorylation was observed at a band of 20 kDa (S2), which corresponds to the molecular mass of MLC, a known in vitro and in vivo substrate of DAPk (13, 15, 16). In fact, a protein at the same position as S2 was recognized by antibodies to MLC, suggesting that S2 is MLC (data not shown). The identification in this manner of a known DAPk substrate validates this strategy as an effective tool for identifying relevant substrates.

To semipurify substrates to enable identification, HeLa cell lysate was subjected to consecutive fractionation steps. The first round consisted of stepwise precipitation with NH₄SO₄ (i.e. 25, 40, 50, 60, and 80% final concentration). The majority of proteins precipitated at the 50 and 60% NH₄SO₄ concentrations. Each salt fraction was then applied to a phenyl-HP hydrophobic column, and proteins were eluted with decreasing concentrations of NH₄SO₄. DK1 kinase assays were performed on 1–5% of the total protein in selected fractions. Resolution of the kinase reactions by electrophoresis followed by silver staining of the gel and autoradiography revealed the elution profiles of individual substrates. For example, the strongest S5 signal was observed to elute in fractions 19–22 upon fractionation of the 60% salt cut (Fig. 2A). Interestingly S5 was a weak and barely detectable substrate in the original
total cell lysate yet gave rise to a strongly phosphorylated band after the final fractionation. This enhanced reactivity may be due to enrichment of the total amount of protein present or its isolation from additional cellular factors that inhibit its phosphorylation. The S9 substrate, which was most prominent in the 50% NH₄SO₄ fraction, eluted in fractions 32–46 upon application to the column with particular enrichment in fractions 36–42, corresponding to 0 mM salt (Fig. 2B).

To identify the proteins corresponding to individual substrates, specific fractions were subjected to more precise resolution by SDS-PAGE. For example, for S5 identification, fraction 20 from the corresponding column was resolved on a 12% gel. For S9 identification, fractions 36 and 41, from either end of the range of elution, were each resolved on 6% gels. In each case, the band that ran at the position of the substrate was excised from each lane and analyzed by mass spectrometry. The complete results for S5 and S9 are presented in Tables I and II. In each case, several candidate proteins were present in the band of interest, migrating at the position of the DAPk substrate. The abundance of a particular protein in the sample (i.e. a high score in the MS results) does not necessarily correlate with its likelihood to be the true substrate; a strong phosphorylation signal may indicate a highly efficient substrate rather than high levels of protein present in the fraction. Thus, among the candidate proteins identified by MS, each was tested by in vitro kinase assays until the true substrate was identified by the process of elimination. To this end, each candidate was expressed as a FLAG-tagged protein in 293T cells, immunopurified with anti-FLAG antibodies, and incubated with DK1 in in vitro kinase assays. Among the candidates for S5, annexin II failed to undergo phosphorylation (data not shown). In contrast, ribosomal protein L5 was phosphorylated in a specific manner by DK1 (Fig. 3A). Furthermore full-length DAPk phosphorylated the L5 protein in the presence of Ca²⁺/CaM (Fig. 3B). However, compared with the known DAPk substrate MLC, L5 was a relatively weak substrate in these assays.

The S9 substrate, a 110-kDa protein, was of particular interest because, like DAPk, it was insoluble in mild detergent buffers (data not shown). One of the highest scoring candidates for S9 identified by mass spectrometry was MCM3, a component of the replication licensing complex that undergoes caspase-mediated cleavage during apoptosis (25, 26). Addition of DK1 to FLAG-tagged MCM3 led to significant

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**Table I**

Results of MS/MS analysis of S5

The band corresponding to substrate S5 in phenyl-HP hydrophobic column fraction 20 (fractionation of the 60% NH₄SO₄ precipitant) was excised from an SDS-acrylamide gel and subjected to MS/MS analysis. Annexin II was eliminated as a candidate after it failed to undergo phosphorylation in in vitro kinase assays with either DK1 or full-length DAPk (data not shown).

**Fig. 2. Purification of DAPk substrates.** A, the 60% salt precipitate (Input) was fractionated by hydrophobic column chromatography with decreasing concentrations of NH₄SO₄, and selected fractions were analyzed for the presence of S5 substrate in kinase assays. Reactions were resolved on a 10% gel that was stained with silver (left), dried, and exposed to film (right). FT, flow-through and washes. As a control for specificity, fraction 29 was incubated in the absence of DK1 (−). B, the 50% salt precipitate was fractionated, and alternate fractions were analyzed for the presence of S9 substrate in kinase assays as described above. Fractions below 16 represent flow-through and washes. As a control for specificity, fraction 30 was incubated in the absence of DK1 (−).
phosphorylation, comparable to the phosphorylation levels attained with equimolar concentrations of recombinant MLC (Fig. 4A). MCM3 was also phosphorylated by full-length DAPk in a Ca\(^{2+}\)/CaM-dependent manner in contrast to α-actinin-4, a second high scoring candidate for S9, that failed to undergo phosphorylation and is used here as a negative control (Fig. 4B). To exclude the possibility that the phosphorylation observed was due to a contaminating kinase that co-precipitated with DAPk, kinase assays were also performed with immunopurified DAPk K42A, a mutant that has greatly reduced catalytic activity. In contrast to the WT DAPk, K42A failed to induce phosphorylation of MCM3 in the absence or presence of Ca\(^{2+}\)/CaM (Fig. 4C). Thus, the identity of the S9 substrate was confirmed as MCM3.

Kinetic Analysis of MCM3 Phosphorylation and Mapping of the Phosphorylation Site—Analysis of the kinetics of phosphorylation of MCM3 by full-length DAPk indicated a time-dependent increase in phosphorylation, achieving a maximum of 0.7 mol of ATP incorporated/mol of MCM3 after 1.5 h (Fig. 4D). Longer incubations did not result in increased phosphorylation as the overall activity of the kinase (including autophosphorylation) tended to decline beyond 2 h. This suggests that there is one unique DAPk phosphorylation site within MCM3.

A scan of the MCM3 sequence indicated a potential DAPk phosphorylation site at Ser 160, which matched a proposed consensus sequence for efficient phosphorylation (27) (Fig. 5A). A mutant MCM3 in which Ser160 was changed to Ala was generated and subjected to in vitro kinase assays with full-length DAPk. Significantly mutation of Ser 160 almost completely abolished the DAPk-dependent phosphorylation without affecting the basal phosphorylation observed in the absence of DAPk (Fig. 5B). To further confirm Ser\(^{160}\) as the site of modification by DAPk, an antibody was generated that specifically recognizes the phosphorylated Ser160 site. Increasing concentrations of both WT and S160A MCM3 were subjected to in vitro kinase assays phosphorylation and is used here as a negative control (Fig. 5C). To exclude the possibility that the phosphorylation observed was due to a contaminating kinase that co-precipitated with DAPk, kinase assays were also performed with immunopurified DAPk K42A, a mutant that has greatly reduced catalytic activity. In contrast to the WT DAPk, K42A failed to induce phosphorylation of MCM3 in the absence or presence of Ca\(^{2+}\)/CaM (Fig. 4C). Thus, the identity of the S9 substrate was confirmed as MCM3.

**TABLE II**

**Results of MS/MS analysis of S9**

The bands corresponding to substrate S9 in phenyl-HP hydrophobic column fractions 36 and 41 (fractionation of the 50% NH\(_4\)SO\(_4\) precipitant) were excised from SDS-acrylamide gels and subjected to MS/MS analysis. The use of two fractions from either end of the elution range enabled elimination of contaminating proteins that were present in one, but not both, fractions, and only those common to both are listed. α-Actinin-4 was eliminated as a candidate after it failed to undergo phosphorylation in in vitro kinase assays with either DK1 or full-length DAPk (data not shown).

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**Fig. 3. Identification of S5 as ribosomal protein L5.** A, FLAG-tagged L5 was expressed in and immunopurified from 293T cells, and 400 ng of the purified protein was subjected to kinase assays in the presence or absence of DK1. Equimolar concentrations of recombinant His-tagged MLC or FLAG-tagged green fluorescent protein (GFP) were used as positive and negative controls, respectively. Reactions were resolved on 10% gels that were stained with GelCode (bottom), dried, and exposed to film to visualize substrates (top). B, FLAG-tagged L5 (400 ng) was incubated with full-length FLAG-tagged DAPk (at a kinase:substrate ratio of 1:10) in the presence of Ca\(^{2+}\)/CaM or EGTA with limiting concentrations of hot ATP and assayed as above. *, unidentified phosphorylated bands that co-immunoprecipitate with L5; IgG HC and LC, contaminating heavy and light chains of antibody used to purify FLAG-DAPk.
with DAPk that were then Western blotted with the phosphospecific antibody. The antibody recognized the wild type, but not mutant MCM3, in a concentration-dependent manner (Fig. 5D). Furthermore a peptide derived from the region encompassing the phosphorylation site (bold), corresponding to the sequence KKTIERRY\(^{S308}\)DLT, was phosphorylated in vitro by DK1. To measure the efficiency of MCM3 as a DAPk substrate, the rate of ATP incorporation was measured over increasing concentrations of peptide (Fig. 6A). Double reciprocal plot analysis of the resulting curve predicted a \(K_m\) of 16 \(\mu\)M and a \(V_{\text{max}}\) of 9.7 pmol of ATP/min/pmol of DK1 (Fig. 6B). These values are comparable to those reported for additional DAPk substrates, syntaxin-1A, ribosomal protein S6, and CaMKK (18, 20). Thus DAPk phosphorylates MCM3 on Ser\(^{160}\) in an efficient manner.

**DAPk Phosphorylates MCM3 in Vivo**—The kinetic properties of MCM3 are well in the range of physiologic substrate/kinase relationships. To verify that MCM3 is in fact phosphorylated in vivo by DAPk, an activated form of DAPk lacking the Ca\(^{2+}\)/CaM binding domain (\(\Delta\text{CaM}\)), was introduced into 293T cells. As controls, the catalytically inactive K42A mutant and an irrelevant protein (luciferase) were used. Total MCM3 protein was immunoprecipitated from lysates from these transfected cells and then blotted with the phosphospecific antibody. Interestingly MCM3 exhibited a low degree of phosphorylation in control cells that was significantly enhanced in cells expressing active DAPk (Fig. 7A). This phosphorylation required DAPk catalytic activity as the levels were not enhanced by the presence of DAPk K42A. To determine whether DAPk phosphorylates endogenous MCM3 under conditions when it is not grossly overexpressed, endogenous DAPk was activated by inducing a rise in cellular Ca\(^{2+}\) by treating 293T cells with ionomycin for 30 min. Significantly phosphospecific Western blotting of MCM3 immunoprecipitates from ionomycin-treated cells indicated an enhanced phosphorylation of MCM3 at the DAPk recognition site compared with control, non-treated cells (Fig. 7B). To prove that the enhanced phosphorylation of MCM3 on Ser\(^{160}\) was dependent on DAPk and not due to other signaling effects of increasing cellular Ca\(^{2+}\), shRNA was used to knockdown DAPk expression in cells treated with either DMSO or ionomycin. DAPk expression was successfully reduced following transfection with shRNA vectors targeted to DAPk but not the Hc-Red control protein (Fig. 7C). Significantly the ionomycin-induced phosphorylation of MCM3 was partially abrogated by knockdown of DAPk. Thus DAPk is necessary for phosphorylation of MCM3 at Ser\(^{160}\) in response to increases in cellular Ca\(^{2+}\). The residual phosphorylation observed may be attributed to the low levels of DAPk still present or to the activation of other Ca\(^{2+}\)-dependent kinases.
such as the closely related DAPk homologue DRP-1. In addition, phosphorylation of MCM3 in the basal state was also partially dependent on DAPk. Again the residual phosphorylation observed may be due to redundancy or to incomplete knockdown of DAPk. Nevertheless these experiments proved that endogenous DAPk is responsible for phosphorylation of MCM3 on Ser160 both in the basal state and upon increases in cellular Ca$^{2+}$.

**DISCUSSION**

Protein kinases are the most common mediators of signaling transduction, yet for many, the full repertoire of substrates that mediate their cellular effects is not known. It is no trivial task to identify physiologically relevant kinase substrates. For some kinases for which a consensus phosphorylation site has been determined, substrates can be predicted based on the presence of such sites within their published sequences. Phosphorylation must then be confirmed by *in vitro* and *in vivo* kinase assays. Unfortunately for DAPk, there are not yet enough known substrates with similar phosphorylation sequences to reliably predict a phosphorylation consensus. To solve this problem, Watterson and co-workers (27) used a positional scanning peptide library to generate an optimal peptide substrate sequence starting from the MLC phosphorylation sequence. The sequence obtained can theoretically be used to scan protein databases for substrates and in fact was used to correctly predict the phosphorylation site within CaMKK (20) and more recently ribosomal protein S6 (18). Database searches predict nearly 200 human proteins containing the proposed consensus. Several of these, however, have been subjected to experimental validation assays and proven not to be phosphorylated by DAPk (data not shown). Considering the large number of potential substrates without additional data indicating the relevance of a particular candidate, it is technically impractical to experimentally assess each one. Moreover the phosphorylation sites in other known DAPk substrates, such as syntaxin-1A (17) and the extracatalytic domain of ZIPk (19), lack the critical features of the proposed consensus. Therefore, this phosphorylation sequence alone is not a definitive tool for predicting DAPk substrates. Other methods for identifying kinase substrates include screening peptide expression libraries, which are limited because the targets are not in their native form and are not full-length proteins. Several years ago, an alternative ap-
A proteomic approach called KESTREL was described and applied to the identification of mitogen-activated protein kinase kinase (MAPKK) and stress-activated protein kinase substrates (22). Since then it has been extended and successfully used to identify substrates of several kinases (23). Here we adapted this strategy to successfully screen for DAPk substrates. We report the identification of two substrates, ribosomal protein L5 and MCM3.

L5 proved to be a weak *in vitro* substrate, and its identification in this screen may be due to its high abundance. This contrasts with the S3 substrate, which gave a very strong phosphorylation signal yet proved refractory to MS analysis due to the low levels of the corresponding protein present (data not shown). Ribosomal proteins are often present as contaminants in MS analysis. However, the successful and specific phosphorylation of L5 by DAPk suggests that it is the true identity of the S5 substrate. Furthermore DAPk has been shown previously to phosphorylate a ribosomal protein and influence protein translation rates at least *in vitro* (18). The ability of DAPk to modify ribosomal proteins may be part of a larger signaling checkpoint that monitors ribosomal integrity and translation rates. However, the weak phosphorylation observed precluded further analysis to determine whether L5 is in fact a true *in vivo* substrate of DAPk.

The second substrate discussed here, MCM3, proved, however, to be a physiologically relevant substrate for DAPk. We showed that DAPk phosphorylates MCM3 on Ser^{160}, both *in vitro* and *in vivo*, upon overexpression of the active kinase. Moreover activation of endogenous DAPk led to increased Ser^{160} phosphorylation, and the endogenous kinase was necessary for phosphorylation of MCM3. MCM3 is one of six proteins, MCM2–7, that form a multiprotein complex that...
binds and licenses chromatin for initiation of replication (28, 29). Like many of the MCM proteins, MCM3 is a known phosphoprotein that is phosphorylated in a cell cycle-dependent manner. These phosphorylation events regulate the cell cycle-dependent recruitment of the MCM complex to the origin of replication (28, 29). An additional phosphorylation occurs at Ser160 by ataxia telangiectasia-mutated in response to ionizing radiation (30). The phosphorylation of MCM3 on Ser160 is a novel modification with no a priori connection to replication. Ser160 lies within the zinc finger motif of MCM3; this motif is found in all MCM proteins, although that of MCM3 differs from the canonical sequence (28). This motif has been shown to be critical for complex assembly (31). However, mutation of this site to Ala, to mimic dephosphorylation, or to Asp, to mimic constitutive phosphorylation, had no effect on its interaction with other MCM subunits or on overall chromatin binding, which requires prior complex assembly (data not shown). Notably other non-replicative functions of MCM proteins have been described recently, such as interactions with transcription factors (e.g. Refs. 32–34) and gene silencing through the induction of histone deacetylation and heterochromatization (35). The functional effects of DAPk-mediated phosphorylation and any connection to these functions remain to be determined.

In conclusion, the proteomics-based in vitro substrate screen used here has proven to be an effective strategy for identifying relevant DAPk substrates. It can be broadened to identify the additional substrates that were not addressed in this study. Furthermore it will serve as the basis for further cellular and molecular studies to elucidate the functional significance of the phosphorylation events.

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