The use of phosphorylation state-specific antibodies has revolutionized the field of cellular signaling by Ser/Thr protein kinases. A more recent application of this technology is the development of phospho-specific antibodies that specifically recognize the consensus substrate phosphorylated motif of a given protein kinase. Here, we describe the development and use of such an antibody which is directed against the optimal phosphorylation motif of protein kinase D (PKD). A degenerate phosphopeptide library with fixed residues corresponding to the consensus LXR(Q/K/E/M)(M/L/K/E/Q/A)8-XXXX was used as an antigen to generate an antibody that recognizes this motif. We characterized the antibody by enzyme-linked immunosorbent assay and with immobilized peptide arrays and also detected immunoreactive phosphoproteins in HeLa cells stimulated with agonists known to activate PKD. Silencing PKD expression using RNA interference validated the specificity of this antibody immunoreactive against putative substrates. The antibody also detected the PKD substrates RIN1 and HDAC5. Knowledge of the PKD consensus motif also enabled us to identify Ser82 in the human heat shock protein Hsp27 as a novel substrate for PKD. We term this antibody anti-PKD pMOTIF and predict that it will enable the discovery of novel PKD substrate proteins in cells.

Approximately one-third of all proteins in eukaryotic cells are phosphorylated on either serine, threonine, or tyrosine, a reaction that is catalyzed by members of the protein kinase superfamily (1). Over 500 protein kinases comprise the human Kinome, and they fall into seven distinct families (2). The identification of protein substrates of distinct protein kinases lags significantly behind knowledge of their regulatory mechanisms. However, recent technological advances in the identification of phosphorylation sites, such chemical genetics as well as mass spectrometry, have enabled progress in this area (3). In addition, the advent of phosphorylation state-specific antibodies which recognize phosphorylated Ser/Thr residues in a sequence-specific context has provided much needed insight into the specific function of many protein kinases (4). A more recent application of this technique is the development of substrate-directed phospho-specific antibodies that recognize the optimal consensus motif of a specific protein kinase or family of kinases (5). This has been possible through the identification of consensus phosphorylation motifs by degenerate peptide library approaches, such that the knowledge that many kinases prefer to phosphorylate, for example, basophilic, acidophilic, or Ser-Pro-directed motifs has been further refined to obtain a specific consensus for amino acids surrounding the phosphorylatable Ser or Thr (6). As an example, identification of the consensus motif phosphorylated by Akt/PKB, an AGC kinase, enabled the development of an antibody which recognizes this motif and which was used to identify novel Akt/PKB substrates, such as the TSC2 gene product, tuberin (5, 7, 8).

PKD, originally cloned and termed PKCμ, and identified as a PKC (protein kinase C) family member, comprises a family of three closely related isoforms, PKD1, PKD2, and PKD3/PKCν. Based on sequence similarities, PKDs are now grouped into the CAMK (calcium and calmodulin-dependent kinases) family of kinases. PKDs regulate a plethora of cellular responses, ranging from cell growth, cell survival, Golgi organization, and trafficking and immune cell responses in B cells (reviewed in Ref. 9). Similarly, the regulation of PKD catalytic activity by cellular location, binding to adapter proteins, and by phosphorylation is also well documented. What has remained elusive, however, is the identification of specific protein substrates of PKD, which relay the signal to downstream responses. Notable exceptions are Kidins220 (kinase D-interacting substrate of 220 kDa), a neuronal PKD substrate protein (10), RIN1, a PKD substrate involved in the modulation of Ras signaling (11), and HDAC5 (histone deacetylase 5), a class II deacetylase implicated in suppression of cardiac hypertrophy (12). Clearly, numerous other PKD substrates must exist given the large number of cellular responses attributed to this protein kinase.

We took advantage of the known optimal consensus phosphorylation motif preferred by PKD to develop a substrate-directed, phospho-specific antibody that is immunoreactive against proteins phosphorylated by PKD in cells. Using this antibody, we have detected multiple phosphoproteins in stimulated cells and have used it to identify a previously unidentified PKD substrate, the heat shock protein Hsp27.

**EXPERIMENTAL PROCEDURES**

Cell Culture, Antibodies, and cDNA Expression Plasmids—The HeLa and HEK293E cell lines were purchased from ATCC and maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The anti-PKD (C-20) and anti-Hsp27 antibo...
ies were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-FLAG (M2), and anti-actin from Sigma. Anti-HDAC5, anti-phospho-(Ser/Thr) Akt/PKB substrate antibody (Akt/PKB pMOTIF), and anti-phospho-Hsp27 (pSer28) were purchased from Santa Cruz Biotechnology. The anti-PKD3 antibody was a generous gift of Dr. Zhifeng Liu (University of Florida). The anti-PKB substrate antibody was a generous gift of Dr. Jeffrey M. Blenis (Brandeis University). The anti-PKB substrate antibody was a generous gift of Dr. David M. Sablina (University of Zurich). The anti-PKB substrate antibody was a generous gift of Dr. Christof Frohman (University of Oregon). The anti-PKB substrate antibody was a generous gift of Dr. Christian Benesch (University of Nebraska). 

**RESULTS AND DISCUSSION**

To develop an antibody-based method for PKD substrate identification, we first considered the optimal phospho-phosphorylation motif of PKD. An oriented depleting phosphopeptide library approach originally devised by Cantley and colleagues (1) revealed that PKD strongly selects for aliphatic residues (leucine, valine, and isoleucine) at the −5 position relative to the phospho-acceptor. In this original study, arginine was locked at the −3 position to orient the library, but a more recent application of this technique made use of a positional scanning peptide library to reveal that, in fact, PKD does strongly select arginine at −3 (18). We have confirmed these findings using an immobilized degenerate peptide library array, which was phosphorylated by purified, recombinant PKD.

The library was arrayed such that each row represents a fixed position in the indicated library, and this position was systematically fixed with a specific amino acid as indicated in single amino acid letter code above each column. The first column from the left contains the nonglycosylated peptide library (Fig. 1C). The purified antibodies and fractions eluting from the phosphopeptide chromatography were tested against this array by incubating the antibody at a dilution of 1:1000 with membranes for 4 h at room temperature in 1% bovine serum albumin in PBST (PBS + 0.2% Tween 20), followed by three washes with PBST. Secondary horseradish peroxidase-conjugated antibody was incubated at a dilution of 1:2000 in PBST for 1 h at room temperature. After three washes with PBST the signal was revealed by Lumiglo (Cell Signaling Technology).

**Immunoblotting and Immunoprecipitation**—Cells were stimulated or harvested 24 h after transfection and lysed in lysis buffer (50 mM Tris/HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, pH 7.4) plus protease inhibitor mixture (Sigma-Aldrich). The lysates were used either for immunoblot analysis or were immunoprecipitated by a 1-h incubation with the respective antibody (2 μg), followed by a 30-min incubation with protein G-agarose (Amersham Biosciences). Immune complexes were washed three times with TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl) and resolved by SDS-PAGE or subjected to kinase assays.

**In Vitro Kinase Assay**—For in vitro kinase assays with GST fusion proteins or immobilized substrates, the reaction was carried out by adding 0.5 μg of purified PKD to 2 μg of purified protein in a volume of 20 μl of kinase buffer. The kinase reaction was started by adding 10 μl of kinase substrate mix (100 μM ATP (cold assay) or 100 μM ATP and 10 μCi (γ-32P)ATP in kinase buffer) and was carried out for 30 min at room temperature. To terminate the kinase reaction, SDS sample buffer was added, and the samples were resolved by SDS-PAGE.

**RNA Interference**—RNAs plasmids for PKD1 and PKD2 silencing have been described (14, 15). To silence human Hsp27 the following oligonucleotide sequences were cloned in pSUPER: 5’-GATCCCCGAGTGGCAGGATCTTCAAGAGAGATCTCCACCAGCATTCTTTTGAAAA-3’ and 5’-GATCCTTCCTCAAAAGAATGGGCTGTGGAGATCTCTTGGATAGACATTCCCTGACCTCACCACCATGGGG-3’. HEK293E and HeLa cells were transfected with pSUPER or pSUPER-RNAs using the TransIT HEK293 or HeLa-Monster reagents, respectively (Mirus). In all experiments the cells were transfected at 30% confluence. Transfection efficiencies (80–90%) were controlled using a green fluorescent protein expression vector. Reduced expression of target proteins was measured by immunoblotting.

**ELISA**—ELISA was performed according to established protocols (16). Briefly, 50 μl of 1 μM synthetic phospho- and non-phosphopeptides were used to coat each well in 96-well plates. Coating was carried out overnight at 4 °C. Phospho-PKD substrate antibody was used at a 1:1000 dilution. The plates were incubated at 37 °C for 2 h after addition of primary antibody. An alkaline phosphatase-conjugated goat anti-rabbit antibody (Cell Signaling Technology) was used as a secondary antibody, and p-nitrophenyl phosphate (Sigma) was used for color development. Absorbance at 405 nm was read on an ELISA plate reader.

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fixed positions. The results reveal that anti-PKD pMOTIF has modest selectivity for amino acids at the −7 and −6 positions, although methionine and tryptophan showed some selectivity at −7, whereas histidine, asparagine, and proline were preferred at −6. As expected, only leucine was selected at −5. Although in the immunizing peptide the −4 position was left degenerate, the antibody preferentially bound basic amino acids such as arginine and lysine, as well as isoleucine, leucine, and methionine. This conforms well to aliphatic and basic amino acids preferred by PKD (Fig. 1C). As expected, peptides with arginine revealed the strongest binding at −3. At −2, glutamate, lysine, methionine, and, to a lesser extent, glutamine were preferred. Anti-PKD pMOTIF selected phosphoserine at the phospho-acceptor position, and, to a lesser extent, phosphothreonine. There was no detectable binding of phosphotyrosine or any other amino acid at this position. Carboxyl-terminal to the phosho-acceptor, there was modest selectivity for hydrophobic amino acids at +1 and +2. Taken together, these results show that anti-PKD pMOTIF binds to phosphopeptides, which accurately reflect the optimal PKD consensus motif.

We next evaluated the ability of this antibody to detect putative PKD substrates in cells. PKD is activated in response to a wide variety of agonists that stimulate activation of PKC, which in turn phosphorylates and activates PKD (19). NIH-3T3 fibroblasts were stimulated with either bombesin, bradykinin, or PDGF. We also used PMA and pervanadate, which are well known activators of PKD. In response to all of these agonists, increased phosphorylation of a number of putative PKD substrates was detected (Fig. 2A). Specifically, three proteins of 85, 100, and 150 kDa were detected in fibroblasts stimulated with PDGF, PMA, and pervanadate. A strong immunoreactive
**Specificity of the PKD pMOTIF antibody using various phospho- and non-phosphopeptides containing different versions of the consensus PKD substrate pMOTIF as determined by ELISA**

Reactivity is expressed as a percentage of ELISA reading of each peptide relative to that of the optimal PKD consensus motif (Fig. 1C). Uppercase S or T represents non-phospho-Ser/Thr, and lowercase s or t represents phospho-Ser/Thr.

| Peptide no. | Peptide sequence | % pMOTIF |
|-------------|------------------|----------|
| 64          | CTRDRVPQYQYNM    | 16.3     |
| 65          | CTRDRVPTQYQYN    | 3.0      |
| 66          | CIRDNRGHDLAG     | 3.4      |
| 67          | CIRDNRGHTDLAG    | 3.1      |
| 68          | CRMRLEGGGGFFNVN  | 3.5      |
| 69          | CRMRLEGGGGFNV    | 3.8      |
| 70          | VLSLPRsQAMDLLC   | 3.1      |
| 71          | CELQTDGQASRRS    | 3.1      |
| 72          | CLEQGHSsQPSNYPS  | 3.1      |
| 73          | CVKYSsQPEFRTGT   | 3.1      |
| 74          | CPEVPsARsRRsIS   | 3.1      |
| 75          | CEEVAAKsPVKATAP  | 3.1      |
| 76          | VIPHPHLVTRVMNTC  | 3.1      |
| 77          | GLGPSLsEDQPFYPLAGGILGSNIHQGQR | 3.4 |
| 78          | CFGLSVQMEdVY     | 4.4      |
| 79          | CGLMTDssEEDLDP   | 3.3      |
| 80          | IGLDCAsEFKK      | 3.1      |
| 81          | CSEKFLQGQsFVAPs  | 4.2      |
| 82          | CLLTTGGLssKSDl   | 3.2      |
| 83          | CLEPQsLssDEG     | 2.9      |
| 84          | CILSsELssRRRls   | 2.9      |
| 85          | CXXs    |          |
| 86          | CXXs    |          |
| 87          | CXXs    |          |
| 88          | CXXs    |          |
| 89          | CXXs    |          |
| 90          | CXXs    |          |
| 91          | CXXs    |          |
| 92          | CXXs    |          |
| 93          | CXXs    |          |
| 94          | CXXs    |          |
| 95          | CXXs    |          |
| 96          | CXXs    |          |
| 97          | CXXs    |          |
| 98          | CXXs    |          |
| 99          | CXXs    |          |
| 100         | CXXs    |          |
| 101         | CXXs    |          |
| 102         | CXXs    |          |
| 103         | CXXs    |          |
| 104         | CXXs    |          |
| 105         | CXXs    |          |
| 106         | CXXs    |          |
| 107         | CXXs    |          |
| 108         | CXXs    |          |
| 109         | CXXs    |          |
| 110         | CXXs    |          |
| 111         | CXXs    |          |
| 112         | CXXs    |          |
| 113         | CXXs    |          |
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| 116         | CXXs    |          |
| 117         | CXXs    |          |
| 118         | CXXs    |          |
| 119         | CXXs    |          |
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| 140         | CXXs    |          |
| 141         | CXXs    |          |
| 142         | CXXs    |          |
| 143         | CXXs    |          |
| 144         | CXXs    |          |
| 145         | CXXs    |          |
| 146         | CXXs    |          |
| 147         | CXXs    |          |

**Verification of the PKD Substrate-directed Phospho-antibody**

Specificity of the PKD pMOTIF antibody was determined using various phospho- and non-phosphopeptides containing different versions of the consensus PKD substrate pMOTIF, as determined by ELISA. Reactivity is expressed as a percentage of ELISA reading of each peptide relative to that of the optimal PKD consensus motif (Fig. 1C). Uppercase S or T represents non-phospho-Ser/Thr, and lowercase s or t represents phospho-Ser/Thr. The table presents the reactivity of various peptides tested with the PKD pMOTIF antibody, showing the percentage of reactivity compared to the optimal motif. The specificity of the antibody was confirmed by testing against known PKD substrates and finding that the antibody was specific to the phospho motif, while not reacting to non-phospho peptides.
Fig. 2. PKD pMOTIF antibody recognizes putative substrates. A, NIH-3T3 cells were stimulated with Bombesin (50 ng/ml, 10 min), PDGF (50 ng/ml, 10 min), PMA (100 nM, 10 min), or pervanadate (75 μM, 15 min). Cells were lysed, and samples were immunoblotted against the PKD pMOTIF antibody. The open arrow indicates a 27-kDa protein, and filled arrows indicate other putative PKD substrates. B, HEK293E cells were transfected with vector control (pSUPER) or PKD RNAi (pSUPER PKD1/2) for 48 h. Cells were then stimulated with H2O2 (10 μM, 10 min), pervanadate (75 μM, 15 min), or PMA (100 nM, 10 min). Lysates were immunoblotted with the PKD pMOTIF antibody. The open arrow indicates a putative PKD substrate of 27 kDa. PKD silencing by PKD-specific RNAi and equal loading were determined in control blots using α-PKD or α-actin. C, HeLa cells were serum-starved for 24 h and then stimulated with IGF-1 (50 ng/ml) or PMA (100 nM) for 10 min. Lysates were immunoblotted with anti-Akt/PKB pMOTIF (left panel) and anti-PKD pMOTIF (right panel). Open arrows indicate putative Akt/PKB substrates, and solid arrows indicate putative PKD substrates. D, top panel, HeLa cells were transfected with RIN1 or vector control (Ctrl) and then stimulated with PMA (100 nM, 10 min) and lyasates immunoblotted with anti-PKD pMOTIF or anti-RIN1. Bottom panel, HeLa cells were stimulated with PMA (100 nM, 10 min), and HDAC5 was immunoprecipitated. Immunoprecipitates were immunoblotted with anti-PKD pMOTIF, stripped, and reprobed with anti-HDAC5.

stimulation of HeLa cells. Therefore, PKD pMOTIF detects both putative and known PKD substrate proteins.

To further demonstrate the feasibility of this antibody to discover novel PKD substrates, we performed a protein BLAST search on the Swiss-Prot data base using the peptide sequences from the ELISA screen. The search consistently returned the heat shock protein Hsp27 with the highest score for a protein containing the PKD consensus phosphorylation motif. We noted that one of the most prominent immunoreactive bands in Fig. 2B migrated at 27 kDa. To validate that this indeed represents Hsp27, we first reduced expression of endogenous Hsp27 using RNAi, followed by immunoblotting with anti-PKD pMOTIF. As predicted, immunoreactivity of the 27-kDa band was significantly reduced following H2O2 stimulation of HeLa cells (Fig. 3A). Analysis of the Hsp27 amino acid sequence reveals two optimal putative PKD phosphorylation sites at Ser15 and Ser82 (Fig. 3B). Shown for comparison is the minimal PKD consensus phosphorylation sequence and the PKD phosphorylation sites in RIN1 and HDAC5. Next, we evaluated immunoreactivity of Hsp27 in cells transfected with PKD RNAi. There was a marked reduction in the phosphorylation of the 27kDa band as detected by immunoblotting total cell lysates with anti-PKD pMOTIF, whereas total Hsp27 levels were unaffected (Fig. 3C, left panel). That this band represents Hsp27 was confirmed by immunoprecipitation of Hsp27, followed by immunoblotting with PKD pMOTIF, and again there was a reduction in immunoreactivity in cells transfected with PKD RNAi (Fig. 3C, right panel). Because this antibody is phospho-specific, we conclude that PKD activation leads to the phosphorylation of Hsp27, which is detected by PKD pMOTIF.

Next, we investigated on which residue Hsp27 is phosphorylated by PKD. GST-Hsp27 fusion proteins, either wild-type, Ser15 → Ala, or Ser82 → Ala mutations, were incubated with purified, recombinant PKD in in vitro kinase assays. Both wild-type and Ser15 → Ala GST-Hsp27 were efficiently phosphorylated by PKD, whereas the Ser82 → Ala mutant showed no detectable phosphorylation (Fig. 3D, left panel). This was confirmed by immunoblotting separate in vitro kinase assays either with anti-PKD pMOTIF or with a phospho-antibody specific to Ser82 in Hsp27. Both antibodies recognized wild-type Hsp27, whereas Ser82 → Ala Hsp27 immunoreactivity was reduced (Fig. 3D, right panel). These results demonstrate that: (i) PKD directly phosphorylates Hsp27 and that (ii) Ser82, and not Ser15, is the relevant site, at least in vitro. This is also true in cells, because both wild-type and Ser15 → Ala Hsp27 are efficiently detected by anti-PKD pMOTIF upon stimulation with H2O2, whereas no appreciable immunoreactivity was evident with the Ser82 → Ala mutant (Fig. 3E, top panel). Again, the same result was obtained by immunoblotting with anti-pSer82 (Fig. 3E, bottom panel). We therefore conclude that Hsp27 is phosphorylated at Ser82 by PKD in stimulated cells.

Although much is known about the mechanisms of regula-
tion of PKD and its importance in cell biology, the identification of specific protein substrates that relay the PKD signal has remained elusive. Here we have used an antibody-based method, which we speculate will aid in the identification of such substrates. We have shown that the anti-PKD pMOTIF antibody reacts with peptides that conform to the preferred phosphorylation motif of this kinase and furthermore validate its use and show that the Hsp27 protein is a previously unidentified in vivo PKD substrate. Phosphorylation of Hsp27 at Ser^{15} and Ser^{82} has previously been demonstrated in response to treatment of cells with a variety of stresses such as oxidative stress and heat shock (21, 22), and the MAPKAP kinases 2/3 have been shown to phosphorylate Hsp27 in vitro (21). However, the identity of the physiological kinase(s) for Hsp27 phosphorylation at these residues has not been determined. Using a combination of the anti-PKD pMOTIF antibody, PKD-specific RNAi, and in vitro kinase assays, we show that PKD is the relevant kinase for Hsp27 phosphorylation at Ser^{82}. Hsp27...
phosphorylation at Ser\textsuperscript{15} and Ser\textsuperscript{82} modulates oligomerization and chaperone function, leading to protection of cells from injury due stress (23). Because PKD plays a major role in protecting cells from oxidative stress (14), we further speculate that Hsp27 phosphorylation by PKD may play a key role in this response. The use of the PKD pMOTIF antibody in combination with proteome-wide screens should yield much needed information concerning the identify of additional PKD substrates. Because other kinases in the human Kinome may reveal optimal phosphorylation motifs similar to that of PKD, it will be important to perform combinatorial screens with PKD-specific RNAi, as shown here. Additional in vitro validation by direct phosphorylation of identified putative PKD substrates will also be important to confirm the newly identified substrate. Given the present lack of any other rapid, substrate-directed methods to discover substrates of PKD in cells, this method should be well suited for analysis of PKD signaling in cells.

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