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Identification of a novel phosphorylation site in c-jun directly targeted *in vitro* by protein kinase D

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Abstract

Protein kinase D (PKD) phosphorylates the c-jun amino-terminal *in vitro* at site(s) distinct from JNK [C. Hurd, R.T. Waldron, E. Rozengurt, Protein kinase D complexes with c-jun N-terminal kinase via activation loop phosphorylation and phosphorylates the c-jun N-terminus, *Oncogene* 21 (2002) 2154–2160], but the sites have not been identified. Here, metabolic ³²P-labeling of c-jun protein in COS-7 cells indicated that PKD phosphorylates c-jun *in vivo* at a site(s) between aa 43–93, a region containing important functional elements. On this basis, the PKD-mediated phosphorylation site(s) was further characterized *in vitro* using GST-c-jun fusion proteins. PKD did not incorporate phosphate into Ser63 and Ser73, the JNK sites in GST-c-jun(1–89). Rather, PKD and JNK could sequentially phosphorylate distinct site(s) simultaneously. By mass spectrometry of tryptic phosphopeptides, Ser58 interposed between the JNK-binding portion of the delta domain and the adjacent TAD1 was identified as a prominent site phosphorylated *in vitro* by PKD. These data were further supported by kinase reactions using truncations or point-mutations of GST-c-jun. Together, these data suggest that PKD-mediated phosphorylation modulates c-jun at the level of its N-terminal functional domains.

Keywords

Protein kinase D; Protein kinase C; PKCmu; c-jun; c-jun N-terminal kinase; AP-1; Phosphorylation site; Activation loop; Phosphopeptide; Mass spectrometry

The basic domain-leucine zipper (bZIP) protein c-jun is a central component of AP-1 transcription factor complexes required for gene expression responses to extracellular stimuli and stresses [1]. Amino-terminal domains contribute to c-jun regulation, thereby linking cellular stimuli to the functions of more distal domains in c-jun [1–3]. The so-called delta domain (aa 34–60) plays a central role, together with the adjacent MAP kinase (MAPK) phosphorylation sites (Ser63, Ser73, Thr91, and Thr93), through their involvement in conformational changes, ubiquitination, and binding to ancillary factors/coactivators and/or inhibitory complexes [3–5]. The differences in transcriptional activity between c-jun and an oncogenic viral counterpart, v-jun, which lacks the delta region [2], further support this view.

Taken together, these observations suggest that identification of distinct regulatory inputs to the delta region, such as novel phosphorylation sites, may help to explain the cooperation between c-jun functional domains.

Protein kinase D (PKD) represents a family (PKC μ /PKD1, PKD2, and PKC ν /PKD3) of CAMK-related protein kinases with distinct structural, enzymological and dynamic properties [6]. PKDs, as well as several protein kinase C (PKC) isoforms, are directly stimulated by the second messenger diacylglycerol. Further, PKDs have emerged as effectors of novel PKC isoforms including PKC ϵ and PKC η , which directly phosphorylate and activate PKDs in novel protein kinase cascades [6,7]. PKD, activated via PKC-mediated activation loop phosphorylation, exhibits dynamic spatiotemporal behavior including nucleocytoplasmic shuttling [6]. This ability to access multiple cellular compartments implies that PKD signaling responses include targeting of nuclear proteins.

PKD was proposed to exert a negative effect on epidermal growth factor (EGF) signaling to c-jun N-terminal kinase (JNK) [8]. In agreement with this, inducible overexpression of kinase-active PKD-S744E/S748E reduced c-jun Ser63 phosphorylation in response to EGF stimulation in HEK 293 cells [9]. More recent reports advance the concept that PKD activity modulates JNK signaling to c-jun at multiple levels. Thus, PKD activates distinct MAP3Ks operating in JNK activation pathways, including hematopoietic progenitor kinase, HPK1 [10] and, when activated in response to oxidative stress [11], the apoptosis signaling kinase ASK1 [12]. In our own previous studies we found that purified PKD directly phosphorylated a GST-c-jun(1-89) (N-terminal) fusion protein *in vitro*, but the sites were not identified [13].

Using mass spectrometry of tryptic phosphopeptides, we report here the identification of Ser58 as a prominent PKD phosphorylation site, and Ser37 as an additional, minor site in the c-jun N-terminus. The location of these novel sites in close proximity to important c-jun functional domains suggests a previously unrecognized possibility of AP-1 modulation by PKD.

Materials and methods

Expression constructs

Wild-type pcDNA3-PKD [14] and kinase-deficient pcDNA3-PKDK618N [15] were described previously. 3XHA-JNK1 α 1 and pSR α 3-3XHA-JNK2 α 2 were gifts from R. Chiu, UCLA and M. Karin, UCSD, respectively. pGEX2T-GST-c-jun-1-135 bacterial vector, described by Kyriakis and colleagues [16], was a gift from J. Kabarowski, UAB.

Full-length human c-jun [17] (Open Biosystems) was inserted into pcDNA3.1-myc-His(B) (Invitrogen) to generate pcDNA3.1-c-jun-myc-HIS (jun-myc) by standard cloning procedures. The deletion mutant, c-jun- Δ 43-93-myc, was generated by in-frame excision of an internal *Bmr*I cassette. All mutations were confirmed by sequence analysis using the UCLA genotyping and sequencing CORE services.

Transfection and immunoprecipitation of protein kinases

COS-7 cells, routinely cultured in DMEM/10% FBS in a humidified incubator with 90% air/10% CO₂, were transfected with PKD, K618N, HA-JNK1 or HA-JNK2 using Lipofectin and OPTI-MEM (Invitrogen). After 72 h, cells expressing PKD forms were left untreated or treated with PDB to induce PKD activation [15], and lysed in buffer (1% Triton X-100, 2 mM EGTA, 2 mM EDTA in 50 mM Tris-HCl, pH 7.4, with 2 mM dithiothreitol, 1 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1 mM AEBSF). To induce JNK activation [1], cells were stimulated with UV light (5000 μ J/m²) for 3.5 min and incubated at 37 °C for 15 min, washed with ice-cold PBS, and extracted in buffer comprising 0.1% Triton X-100 and (in mM) 25 Hepes, 300 NaCl, 1.5 MgCl₂, 0.2 EDTA, 0.5 DTT, 20 β -glycerophosphate, 0.1 Na₂VO₄. Kinases were

immunoprecipitated from clarified lysates, and washed with lysis/extraction buffer and twice with buffer (10 mM MgCl₂ in 30 mM Tris-HCl, pH 7.4) before being used in assays.

Metabolic labeling of c-jun

COS-7 cells transfected with either c-jun-myc or c-jun-Δ43-myc, with or without PKD, were labeled with carrier-free ³²P (0.25 mCi/ml) in fresh DMEM lacking phosphate with L-glutamine (200 μM) for 5 h, then either left unstimulated or stimulated with 10% FBS for 30 min and lysed, and c-jun-myc proteins immunoprecipitated using the anti-c-myc mAb, washed (4× with lysis buffer) and separated by SDS-PAGE. Phosphorylation was quantitated using a Molecular Dynamics STORM 820 phosphorimager at the Bio-imaging facility, Department of Biological Chemistry, UCLA School of Medicine.

GST fusion proteins

pGEX2T-GST-c-jun(1-72) was derived from pGEX2T-GST-c-jun-1-135 by standard cloning procedures. Additional truncations (GST-c-jun(1-34), and GST-c-jun(1-46)) or Ala for Ser substitutions at positions 37 and/or 58, or 63 (GST-c-jun(1-72/S37A), GST-c-jun(1-72/S58A), GST-c-jun(1-72/S37A/S58A), and GST-c-jun(1-72/S63A)) were generated using the Quikchange kit and pGEX2T-GST-c-Jun(1-72) as template. GST fusion proteins were purified from BL21 bacterial cultures using the B-Per Kit (Pierce), then washed with kinase buffer using Amicon Ultra-4 devices with a 10,000 MW cutoff (Millipore). Protein concentrations were assessed using QuickStart Bradford reagent (Bio-Rad).

GST-c-jun fusion protein and c-jun peptide phosphorylation reactions

GST-c-jun(1-89) (2 μg) was incubated with activated PKD and JNK in 30 μl containing ATP (100 μM) in a 30 °C water bath, and reactions terminated by addition of SDS-PAGE sample buffer (0.2 M Tris-HCl, pH 6.8, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, and 10% glycerol). GST or GST-c-jun fusion proteins (4 μg/time point) were phosphorylated by GST-PKD (90 ng/time point), in the presence of PS/PDB vesicles [7] and ATP (100 μM, with 2 μCi/time point [γ -³²P]ATP). SDS-PAGE gels were stained with Coomassie blue and dried, and c-jun fusion protein phosphorylation quantitated with a scintillation counter. KPHLRAKNSDLL (2 mg/ml) was phosphorylated by incubation with PKD and 100 μM ATP containing 2.5 μCi/reaction of [γ -³²P]ATP for 10 min at 30 °C. Reactions were terminated with 100 μl of 75 mM phosphoric acid, spotted to P81 paper, washed in phosphoric acid, and counted in a scintillation counter [7].

In-gel trypsinization, phosphopeptide extraction, and HPLC of phosphopeptides

³²P-labeled GST-c-jun(1-89) was in-gel digested with trypsin by established methods [18]. Samples (200 μl) in 0.1% TFA/water were separated by HPLC on a Polaris C18-A5u PN2000 (150 × 20 mm) column, into 1-ml fractions at 0.5 ml/min on a HP1090 liquid chromatograph using a 40 min continuous (0-70%) acetonitrile gradient for elution. ³²P radioactivity in fractions was quantitated by scintillation counting.

Mass spectrometric identification of phosphopeptides in HPLC fractions

Radiolabeled HPLC fractions were subjected to liquid chromatography-nanoelectrospray tandem mass spectrometry (nLC-MSMS; QSTAR XL, Applied Biosystems) using Analyst QS software with the input GST-c-jun(1-89) sequence as a database. The Mascot search engine (Matrix Science) identified phosphopeptides with the scores (for JNK-mediated Ser⁶³ phosphorylation) 56 for NSDLLTpSPDVGLLK, (for JNK-mediated Ser⁷³ phosphorylation) 50 for LApSPELER, and (for PKD-mediated Ser⁵⁸ phosphorylation) 84 for AKNPDDLTPSPDVGLLK.

Western blot analysis

Proteins, resolved by SDS-PAGE, were transferred to Immobilon-P (Millipore) PVDF membranes [19]. Blocking was with either 5% non-fat dried milk in PBS, pH 7.2 (for anti-HA MAb or C-20) or 5% BSA/0.1% Tween 20 in PBS (for pS744) for 2-3 h at RT. PKD (C-20) was used at 1 µg/ml, pS744 at 1:1000 dilution. Immunoreactive bands were detected using HRP-conjugated secondary IgG and enhanced chemiluminescence (ECL, GE Healthcare).

Materials

GST-PKD was from Biosource. Anti-PKD (C-20) was from Santa Cruz. HRP-anti-GST was from Amersham. Anti-HA and anti-myc mAbs, phospho-c-Jun Ser63 and phospho-c-jun Ser73, PKD activation loop phospho-Ser744/Ser748 and GST-c-jun(1-89) were from Cell Signaling. Protein A agarose was from Roche. KPHLRAKNSDLL peptide (>90% pure) was from Bio-Synthesis, Inc.

Results and discussion

PKD promotes c-jun phosphorylation in vivo

To determine whether PKD increases c-jun phosphorylation *in vivo*, we metabolically labeled COS-7 cells transfected with either c-jun-myc or c-jun Δ 43-myc, with or without PKD, with 32 P (Fig. 1). The cells were then either stimulated with FBS or left unstimulated, and the extent of phosphorylation of the myc-tagged c-jun proteins determined. As shown in Fig. 1, basal levels of phosphorylation present in c-jun were increased by serum stimulation of cells, by approximately 25%. Interestingly, in cells cotransfected with c-jun and PKD, the basal level was increased by approximately 50%, and the serum-induced increase was greater, approximately two-fold. These data suggested that PKD could phosphorylate the c-jun protein *in vivo*. Consistent with the phosphorylation being within the transcriptional activation domain, no increases were detected in c-jun Δ 43. Western blot analysis confirmed that the phosphorylation increases in the cells cotransfected with PKD were not due to increased levels of c-jun protein (Fig. 1D).

PKD does not phosphorylate c-jun at JNK target sites

Whereas JNK is known to phosphorylate Ser⁶³ and Ser⁷³, the site(s) targeted by PKD in GST-c-jun(1-89) remain undefined. PKD preferentially targets residues within sequences containing a basic residue at -3 as well as a leucine at the -5 position in model peptide substrates [14,20]. In the region of c-jun phosphorylated by PKD, only Ser73 preceded by LLKLA fits this description. However, our previous data indicating that PKD and JNK did not utilize phosphorylation sites in common suggested that Ser73 was not phosphorylated by PKD. To investigate this more conclusively, we analyzed c-jun(1-89) phosphorylation by Western blot analysis using phosphospecific antibodies.

Activated PKD, PKD-K618N, or JNK were incubated with GST-c-jun(1-89) in the presence of ATP, and the proteins resolved by SDS-PAGE and transferred to membranes. PKD and K618N used for the assays were strongly phosphorylated at the activation loop sites Ser744/Ser748 that trigger persistent PKD activation in response to cell stimulation with PDB [6], as shown by Western blot analysis using a phosphospecific antibody [19] (Fig. 2A). Western blot analysis also confirmed the presence of HA-JNK1 in the anti-HA immunoprecipitates (Fig. 2A).

Analysis of *in vitro* phosphorylation reactions using phosphospecific antibodies recognizing pS63 or pS73 (Fig. 2A) indicated that JNK1 robustly phosphorylated both Ser63 and Ser73 in GST-c-jun(1-89), both in its basal activation state from unstimulated cells, and especially, after UV stimulation. In contrast, the fusion protein incubated with activation loop-phosphorylated

PKD or K618N exhibited no detectable phosphorylation at either Ser63 or Ser73. These results substantiate that neither activated PKD nor any coimmunoprecipitating proteins phosphorylate the proline-directed JNK sites Ser63 and Ser73.

The extent of GST-c-jun(1-89) phosphorylation by unactivated or activated, immunoprecipitated PKD or JNK or both kinases sequentially was next assayed. Results shown in Fig. 2B indicate that activated PKD, or activated JNK, incorporated significant ^{32}P into GST-c-jun(1-89) within 5 min. The phosphorylation by JNK was similar when the fusion protein was incubated first with unactivated PKD. In contrast, JNK phosphorylation was additive with that catalyzed by activated PKD, irrespective of the order in which these reactions were performed. Taken together, these experiments indicate that distinct PKD and JNK sites in the c-jun N-terminus can be phosphorylated simultaneously.

Ser58 in the c-jun N-terminus is targeted by activated PKD

We next sought to identify the site(s) in GST-c-jun(1-89) phosphorylated *in vitro* by activated PKD or JNK using mass spectrometric analysis. As shown in Fig. 3A, activated PKD, but not PKD from unstimulated cells or K618N from PDB-stimulated cells, markedly phosphorylated GST-c-jun(1-89) (Fig. 3A). These data provide strong evidence that c-jun N-terminal phosphorylation is catalyzed by PKD itself, rather than a co-immunoprecipitated protein kinase. This assay also readily detected GST-c-jun(1-89) phosphorylation by HA-JNK1 isolated from UV-stimulated cells (Fig. 3A).

As shown by the data in Fig. 3B, digestion of GST-c-jun(1-89) phosphorylated by PKD into tryptic phosphopeptides yielded a single HPLC fraction containing the majority of the associated ^{32}P (typically, approximately 50% of the total recovered radioactivity). In contrast, digestion of the JNK-phosphorylated c-jun N-terminal produced two well-separated, prominent peaks (comprising approximately 40% and 30% of recovered counts, respectively), consistent with previous studies [1] (Fig. 3B).

Radiolabeled HPLC fractions were analyzed by nLC-MSMS to yield sequence data from the detected mass peaks. The data shown in Fig. 3C identify a single peptide phosphorylated by PKD as AKNpSDLLTSPDVGLLK, revealing a novel, PKD-mediated site in c-jun, at Ser58. Interestingly, phosphorylation of Ser58 apparently prevents efficient cleavage by trypsin at the lysine residue at position 2 (Lys56), extending this peptide by two amino acids. Our analysis also detected JNK-phosphorylated phosphopeptides, corresponding to Ser63 (NSDLLTSPDVGLLK) and Ser73 (LApSPELER) (data not shown).

PKD phosphorylation of GST-c-jun(1-72) fusion proteins is selectively diminished by point mutations including Ser58

Identification of a peptide containing phosphorylated Ser58 prompted a further evaluation of the specificity of this site using phosphorylation of truncated or mutated c-jun fusion proteins (Fig. 4). Only two of the 13 Ser/Thr residues in GST-c-jun(1-72) have a basic residue at the -2, or preferred -3 position, together with an aliphatic residue (I, L, or V) at the -4 or -5 position, relative to the phosphorylation site, i.e., Ser37 and Ser58. A series of GST-c-jun fusion proteins were generated, as described in Materials and methods, and characterized by Coomassie blue staining and Western blot analysis (Fig. 4A).

We first investigated phosphorylation of GST or GST-c-jun fusion proteins of different lengths by purified GST-PKD. As shown by the data in Fig. 4B, GST-PKD did not phosphorylate any residues in either GST or GST-c-jun(1-34) containing the initial seven amino-terminal Ser/Thr residues in c-jun. Intriguingly, GST-c-jun(1-46) containing Ser37 and Thr 39 was phosphorylated to a low, but significant extent. Maximal phosphorylation by GST-PKD was

obtained when GST-c-jun(1-72) was used for the assay (Fig. 4B). Consistent with all the above data, this phosphorylation was preserved when GST-c-jun(1-72/S63A) was substituted for GST-c-jun(1-72) (Fig. 4B).

Next, phosphorylation of fusion proteins with mutations at Ser58 was evaluated using activated, PKD, JNK1, and JNK2. GST-c-jun(1-72/S58A) was poorly phosphorylated when incubated with activated PKD (Fig. 4C). Whereas the double mutant GST-c-jun(1-72/S37A/S58A) reduced the phosphorylation only slightly further, PKD phosphorylated GST-c-jun(1-72/S37A) well, suggesting that Ser37 is a relatively minor site. In contrast, these proteins were robustly phosphorylated by either JNK1 (Fig. 4C) or JNK2 (data not shown). However, wild-type GST-c-Jun(1-72) was phosphorylated strongly by either PKD or JNK1 (Fig. 4C) or JNK2 (data not shown). Further, GST-c-jun(1-72/S63A) was well phosphorylated by PKD, but not by JNK (Fig. 4C). Taken together, these data support the conclusion that the majority of phosphorylation catalyzed by PKD *in vitro* takes place on Ser58.

To independently confirm that PKD could phosphorylate Ser58 in the context of its surrounding sequence, we generated a peptide, KPHLRAKNSDLL for *in vitro* phosphorylation assays. This peptide was robustly phosphorylated by immunoprecipitated, active PKD, but not by PKD-K618N (Fig. 4D). These data provide additional evidence that PKD, and not a co-immunoprecipitating protein kinase, directly phosphorylates c-Jun Ser58.

Concluding remarks

Previous studies determined MAPK sites Ser63, Ser73, Thr91, and Thr93 in the c-jun N-terminus [1,3]. Although it is well accepted that phosphorylation of these sites in response to stimuli regulates the activity and protein stability of c-jun, these events do not exclude the possibility of additional modulatory inputs. In two prior reports, Ser58 phosphorylation *in vivo* was implied by data obtained in response to phosphorylation cascades induced by either adenovirus E1A expression [21] or UV stimulation [22]. However, as no putative kinase was identified in these studies, and the evidence of this site remained ambiguous, its phosphorylation has not subsequently been pursued. Mass spectrometric and other data described here for the first time identify c-jun Ser58 as a PKD-mediated phosphorylation site *in vitro*. These data strongly substantiate our previous evidence of PKD-mediated, direct phosphorylation of the c-jun amino terminal at site(s) distinct from those targeted by JNK.

The sequences surrounding residues Ser37 and Ser58, identified here as minor and prominent PKD phosphorylation sites, respectively, do not conform precisely to optimal PKD recognition sequences. This indicates that PKD recognizes phosphorylation sites with certain flexibility, rather than strictly adhering to the previously defined motif (e.g., a Scansite [23] search at low stringency did not identify c-jun Ser58 as a phosphorylation motif). Thus, future target predictions might consider sites with features similar to those presently identified in c-jun.

The identification of a new phosphorylation site in c-jun with potential to be targeted by PKD or other kinase(s) *in vivo* raises important issues related to regulation of AP-1 transcriptional activity. Further studies will be required to establish the kinetics, cell cycle timing and range of stimuli leading to Ser58 phosphorylation *in vivo*. The prospect that phosphorylation of this novel site by PKD constitutes a distinct modulatory component of the interplay between functional domains of c-jun warrants further investigation.

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Abbreviations

PKC, protein kinase C
 PKD, protein kinase D
 CAMK, Ca²⁺/calmodulin-dependent protein kinase
 JNK, c-jun N-terminal kinase
 MAPK, mitogen-activated protein kinase
 PS, phosphatidylserine
 PDB, phorbol 12,13 dibutyrate
 PBS, phosphate-buffered saline
 PAGE, polyacrylamide gel electrophoresis
 AP-1, activator protein 1 transcription factor
 AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride.

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Fig. 1.

Metabolic ^{32}P labeling of c-jun-myc proteins. (A) Scheme of c-jun-myc and c-jun-Δ43-myc. (B) COS-7 cells were transfected with c-jun-myc or c-jun-Δ43-myc, with or without PKD, labeled with ^{32}P , and stimulated with 10% FBS, then jun-myc proteins were separated by SDS-PAGE. A representative autoradiogram of a dried gel is shown. (C) Quantitation of cumulative data from experiments shown in (B). Results shown are the average plus SEM of four experiments. (D) Western blot analysis of c-jun-myc protein expression levels in cell lysates normalized to equivalent cell numbers, with actin as a loading control. Results shown are typical of four experiments.

Fig. 2.

JNK selectively phosphorylates Ser63 and Ser73. PKD, PKD-K618N or HA-JNK1 were incubated with ATP and 2 μ g of GST-c-jun(1-89), and the proteins analyzed by Western blot using the indicated antibodies. (A) pS744/748, α -HA, and pS63, anti-c-jun phospho-Ser63, pS73, anti-c-jun phospho-Ser73. (B) GST-c-jun(1-89) (2 μ g) was incubated with activated or unactivated PKD, or activated HA-JNK, with PKD and then JNK, or JNK and then activated PKD for five min each, as indicated, in buffer containing 100 μ M ATP plus 2 μ Ci [γ -³²P]ATP. Autoradiograms were generated from dried SDS-PAGE gels. Similar results were obtained in three separate experiments.

Fig. 3.

Identification of a PKD-mediated c-jun peptide containing phospho-Ser58. (A) Activated PKD, PKD-K618N or HA-JNK1 were incubated in phosphorylation reactions with [γ - ^{32}P] ATP and with or without GST-c-jun(1-89) (2 μg). (A) Representative autoradiogram developed from a dried SDS-PAGE gel is shown. (B) HPLC of radiolabeled tryptic peptides from GST-c-jun(1-89). Radioactivity present in HPLC fractions is depicted by striped bars. (C) Data from an MS/MS experiment depicting peptide fragmentation obtained from the radiolabeled fraction 10 peak shown in the PKD panel in (B) Inset, sequence of the phosphopeptide identified by the data. Similar results were obtained in three separate experiments.

Fig. 4.

Phosphorylation of GST-c-jun by PKD versus JNK. (A) Characterization of fusion proteins by Western blot analysis (60 ng protein/lane, above), or Coomassie blue (5 μ g protein/lane, below). (B) GST or GST-c-jun fusion proteins were phosphorylation by GST-PKD as described in Materials and methods. Coomassie blue stained fusion proteins are depicted in the inset. Results are representative of three independent experiments. (C) Phosphorylation of GST-c-jun(1-72) fusion proteins (4 μ g/lane) by activated PKD and HA-JNK1. A representative gel stained with Coomassie blue and corresponding autoradiogram are shown. Similar results were obtained in at least three experiments. (D) Phosphorylation of Ser58-containing c-jun peptide KPHLRKNSDLL by PKD immunoprecipitates from unstimulated or PDB-stimulated cells, either mock-transfected (-) or transfected with PKD or PKD-K618N. The data shown represent means $\pm$ SEM of three separate determinations.