Proteomic Analysis of Calcium/Calmodulin-dependent Protein Kinase I and IV in Vitro Substrates Reveals Distinct Catalytic Preferences

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The multifunctional calcium/calmodulin-dependent protein kinases I and IV (CaMKI and CaMKIV) are closely related by primary sequence and predicted to have similar substrate specificities based on peptide studies. We identified a fragment of p300-(1–117) that is a substrate of both kinases, and through both mutagenesis and Edman phosphate (32P) release sequencing, established that CaMKI and CaMKIV phosphorylate completely different sites. The CaMKI site, Ser19 (84LLRSGSSPNL93), fits the expected consensus whereas the CaMKIV site, Ser24 (19SSPALSASAS28), is novel. To compare kinase substrate preferences more generally, we employed a proteomic display technique that allowed comparison of complex cell extracts phosphorylated by each kinase in a rapid in vitro assay, thereby demonstrating substrate preferences that overlapped but were clearly distinct. To validate this approach, one of the proteins labeled in this assay was identified by microsequencing as HSP25, purified as a recombinant protein, and examined as a substrate for both CaMKI and CaMKIV. Again, CaMKI and CaMKIV were different, this time in kinetics and stoichiometry of the phosphorylation sites, with CaMKI preferring Ser15 (10LLRTPSWGPF19) to Ser85 (80LNRQLSSGVS89) 3:1, but CaMKIV phosphorylating the two sites equally. These differences in substrate specificities emphasize the need to consider these protein kinases independently despite their close homology.

The calcium (Ca2+) receptor calmodulin (CaM) translates changes in intracellular calcium concentration to changes in biochemical functions of a variety of proteins that participate in a wide range of cellular processes, including transcription, cell cycle regulation, and differentiation (1). Ca2+/CaM binds target enzymes to elicit alterations in their confirmations and activities (2). Among the Ca2+/CaM-regulated enzymes, the multifunctional Ca2+/calmodulin-dependent protein kinases (CaMKs) occupy positions of influence because they communicate the Ca2+ signal via phosphorylation to a broad range of substrates. Within this kinase family, CaMKII clearly has distinct regulation and function, whereas CaMKI and CaMKIV are more difficult to discern as they share substantial sequence homology and behave similarly in many biochemical analyses. Nevertheless, there are many indications that at a physiological level their functions are nonredundant.

Differences in CaMKI and CaMKIV localization probably play important roles in the regulation of these kinases. Although CaMKI is a ubiquitously expressed, primarily cytoplasmic enzyme, CaMKIV is found in the nuclei of only a select group of cells including cells in the thymus, brain, testes, and ovaries. However, these expression and subcellular localization differences are not sufficient to account either for the distinct behaviors of CaMKI and IV in vitro or for their lack of redundancy in vivo. All cells that express CaMKIV also contain CaMKI, and CaMKI is capable of nuclear entry in some contexts (3). The only identified Caenorhabditis elegans CaMKI/IV homologue, cCaMKI, further blurs this distinction. Although this enzyme is more highly homologous to CaMKI by primary sequence, like CaMKIV it is nuclear and only expressed in a limited set of cells (4, 5). As we sought to clarify the ambiguity of the homology of cCaMKI, we observed biochemical differences between CaMKI and CaMKIV that led us to evaluate more thoroughly the substrate preferences of the two mammalian enzymes.

The similarities between CaMKI and CaMKIV relative to CaMKII extend to substrate preferences to the degrees that they have been characterized. All known CaMKI substrates (but not all CaMKII substrates) can be phosphorylated by CaMKIV. For example, CREB Ser133 can be phosphorylated by CaMKs I, IV, and II, while only the later kinase also phosphorylates Ser142 (6); C/EBPβ Ser276 is a CaMKII substrate but not a CaMKIV substrate (7, 8); CaMKIV phosphorylates sites 1 of synapsin I, whereas CaMKII efficiently phosphorylates sites 2 and 3 (9), and CaMKIV can phosphorylate all three (10). As-protein kinase IV; CaMKKS, calcium/calmodulin-dependent protein kinase β; cCaMKK, C. elegans calcium/calmodulin-dependent protein kinase; CREB, cAMP response element-binding protein; HSP25, mouse 25-kDa heat shock protein; MEF, mouse embryonic fibroblast; CBP, CREB-binding protein; hsCaMKI, human α isoform; rmCaMKIV, rat α isoform.
essment of CaMK activities toward a panel of site 1 peptide variants identified various primary sequence determinants in the vicinity of the phosphoacceptor site (P = 0). CaMKII phosphorylates efficiently only those peptides that incorporate a nonbasic residue in the P–2 position and a hydrophobic residue in the P+1 position (11), whereas CaMKIV prefers hydrophobic residues at the P+4 position (12). All three kinases favor a hydrophobic residue at P–5 and a basic residue, preferably Arg, at P–3. Thus, although kinetic differences between the kinases have been documented in these peptide studies, this definition of substrate discrimination indicates similar and highly degenerate consensus sequences, and implies that CaMKIV will phosphorylate a superset of CaMKI targets.

Few studies compare the catalytic activities of CaMKI and CaMK IV toward substrates other than short synthetic peptides. Whereas the peptide-derived consensus sequences appear to reflect the behavior of the kinases toward the few known protein substrates, their limited predictive value is demonstrated in our analysis of the phosphorylation by CaMKIV of an N-terminal fragment (1–117) of the transcriptional coactivator, p300. N-terminal fragments of p300 have previously been shown to be in vitro substrates for PKC, AMP kinase, and CaMKIV (13–15), and here we show that it is also a CaMKIV substrate. Among the 20 serine and threonine residues in the first 117 amino acids, a single serine, Ser49, emerges as a likely candidate phosphorycceptor site based on CaMKIV consensus comparisons. Indeed, mutation of Ser49 to Ala abolished the ability of CaMKIV to phosphorylate the protein. However, despite the overlapping consensus determinants of CaMKI and CaMKIV, the mutant remained a robust substrate of CaMKIV. This observation, and the repeated failure of efforts to identify new substrates through degenerate consensus-directed searching, indicate that determinants must exist beyond the known consensus, either hidden within the primary sequence or in the higher level folded structure. We undertook further investigations of CaMKI and CaMKIV protein substrate preferences to try to define their degree of overlap and to develop methods for identifying new substrates.

Here we compare the preferences of the mammalian kinases CaMKI and CaMKIV to those in lower organisms. CaMK II from C elegans (CaMKI) and CaMKIV (rat a isoform, mmCaMKIV) and the CaMK homologue from C elegans (CeCaMKI). Our first experiments identify a novel CaMKIV phosphorylation site in the N-terminal fragment p300 (1–117). Recognizing that additional determinants of phosphorylation could potentially include protein binding, binding partners, or posttranslational modifications of substrates, we examine the spectrum of proteins phosphorylated by the kinases in complex cell extracts using methodology similar to that described for mitogen-activated protein kinases by Nef et al. (16). The application of this proteomic approach reveals dramatic differences between the sets of substrates phosphorylated by CaMKs I and IV, and supports the classification of CeCaMKI as a CaMKI-like enzyme. Furthermore, applying microsequencing techniques in conjunction with proteomic display, we identify a novel and differentially phosphorylated CaMKI/IV substrate, HSP25, in mouse embryo fibroblast (MEF) extracts. Using purified recombinant HSP25, we characterize it as a CaMK IV substrate both to identify sites and to validate our approach for evaluating CaMK substrates in complex mixtures.

EXPERIMENTAL PROCEDURES

Recombinant DNA and Protein Expression—Vectors for production of human glutathione S-transferase-CaMKI and rat glutathione S-transferase-CaMKIV and myelin basic protein-CaMKK8 were provided by S. Hook (17) and K. Anderson (18). Wild type, S89A, S19A/S20A/S89A, and S19A/S20A/S82A/S89A variations of pET30a-p300-(1–117) were provided by C. Kane (15). Additional mutants were generated by PCR mutagenesis using pET30a-p300-(1–117) as template. The vector for His6–CaMKII was the gift of M. Montminy (19). Recombinant proteins were prepared using these vectors as previously described.

CaMKIV cDNA was independently isolated from mixed stage C. elegans RNA by reverse transcriptase-PCR, sequenced on both strands, and subcloned into pGEX-2T,4 and recombinant protein was prepared according to the vector protocols from the manufacturers. The vector pQE9-HSP25 was the gift of L. Cooper (20). His6–HSP25 was prepared under denaturing conditions as previously described, and renatured by dialysis (3500 Da MWCO) at 4 °C against gradually decreasing concentrations of urea in 50 mM Tris, 125 mM NaCl, 0.5 mM dithiothreitol, pH 7.5. The dialyzed protein was concentrated ~5-fold on a centrifugal filter unit (10 kDa MWCO, Millipore) and quantified by standard methods.

Standard Kinase Assays—Kinase reactions were carried out at 30 °C in 50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 0.1% Tween 20, and 10 mM MgCl2. Unless otherwise specified, 50 nM kinase, 200 μM ATP, 1 mM CaCl2, and 1 μM CaM were incubated for 5 min with 2 μM recombinant substrate. For calcium-free reactions, 1 mM EDTA was included in place of CaCl2. In activation assays, the kinase and 50 mM kinase (or mock controls) were preincubated in the reaction buffer 20 min before the addition of [γ-32P]ATP and substrate. Specific radioactivity was determined for each set of reactions, generally falling between 500 and 2500 cpm/mole of ATP. Assays were stopped by either addition of Laemmli buffer and boiling (for reactions to be run on SDS-PAGE, dried, and autoradiographed) or spotting on Whatman 3MM paper and washing (for liquid scintillation quantification of counts incorporated) (21). To determine phosphorylation stoichiometry, 2 μM recombinant substrate was incubated in standard reaction buffer with 100 nM kinase and 500 μM ATP (1000 cpm/mole) for 60 min, and specific incorporation observed around the quantified using the 3MM filter assay described above. Recombinant CEBP, which has a stoichiometry of phosphorylation by CaMKIV (1.0 (22)), was used to normalize stoichiometry results.

Determination of Phosphorylation Sites—Recombinant, His6-tagged proteins were labeled for analysis of phosphorylation sites using 50 μM kinase and 20 μM substrate incubated for 30 min at 30 °C, with 400 μM ATP and specific activities of 5000–10000 cpm/mole. To purify the substrates away from the kinases, the reaction was diluted 1:1 with 20 mM Tris, pH 7.5, containing 20 μl of packed Ni2+–chelating resin, and allowed to bind for 60 min at 4 °C. The resin was washed in batch with 2 x 200 μl of 20 mM Tris, 0.5 mM NaCl, 5 mM imidazole and then with 2 x 200 μl of 20 mM Tris, 10 mM imidazole. Protein was eluted in 2 x 50 μl of 100 mM EDTA, which was diluted 1:1 with 20 mM Tris, pH 7.5, and run over a BioSpin-6 column (Bio-Rad) to exchange buffers and remove any remaining unincorporated ATP. Labeled proteins were digested with endo-Lys-C, cyanogen bromide, or trypsin (as indicated), and acidified by the addition of trifluoroacetic acid. In some cases, the peptide mixture was bound directly to Immobilon membrane, and the site of phosphorylation determined by Edman degradation and analysis of pels of liquid chromatography to determine 32P release (23). In other cases (as indicated), the resulting peptide mixture was resolved on a reverse phase column (Zorbax SB-C18, 4.6 x 250 mm) by a linear acetonitrile gradient in 0.1% trifluoroacetic acid. Peak fractions of radiolabeled peptides were analyzed on Immobilon membrane by Edman cycle 32P release. In some cases, a portion of the phosphopeptide sample was analyzed by TOF/NEGS mass spectroscopy using a precursor ion scan in negative mode to identify parent ions that liberated the characteristic 78.997 m/z phosphate ion. TOF/POS was then used to measure the masses of peptide fragments produced by collision-induced dissociation (MS/MS), and predict the primary sequence of the unknown phosphopeptide.

MEF Extract Assays—The mouse embryonic fibroblasts were the generous gift of Christina Kahl, who had derived them from wild type C57/B6 mice as previously reported (25). The cells were stored at passage 2 under liquid nitrogen until thawing and expansion by standard methods (DMEM, 10% with fetal bovine serum). All cells were collected by trypsinization to passage 2 under liquid nitrogen until thawing and expansion by standard methods (DMEM, 10% with fetal bovine serum). All cells were collected by trypsinization and centrifugation at or before passage 6. After two washes with Hanks’ saline solution, cell pellets were flash frozen and stored at −80 °C until use.

MEF extract was prepared by resuspending ~2 x 106 previously frozen MEF cells in extract lysate buffer (20 mM Tris, 0.1 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 8.0, with protease inhibitors: 200 μg/ml Pefabloc, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 2 μg/ml aprotinin), pH 8.0, with protease inhibitors: 200 μg/ml Pefabloc, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 2 μg/ml aprotinin),
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...and addition of Triton X-100 to 0.5%. After rocking at 4 °C for 2 h, extracts were diluted 5-fold with lysis buffer bringing the Triton X-100 concentration to 0.1%. Following clarification by centrifugation at 15,000 × g, the extract was filtered (0.45 μm), aliquoted, and flash frozen for storage at −80 °C.

For phosphorylation of cell extracts, reactions were carried out at 30 °C in reaction buffer (50 mM Tris, pH 7.5 with 1 mM diithiothreitol and 0.1% Triton X-100). The indicated kinases, with or without kinase each, at 2 μM (for 0.5 μM final), were preincubated 20 min in 50 μl of reaction buffer supplemented with 20 mM MgCl₂, 5 mM CaCl₂, 100 μM ATP, and 8 μM purified bovine CaM. This mixture was diluted to 200 μl by the addition of 50 μl of [γ³²P]ATP (~35,000 cpm/pmol ATP) and 200 μg of cell extract, starting the reaction. The reaction was stopped by precipitation with 50 μl of 100% trichloroacetic acid (for two-dimensional electrophoresis), incubated on ice for 20 min, centrifuged 20 min at 14,000 × g, washed 3 times with ether, and air dried. The protein pellet was resuspended directly in IEF sample buffer and frozen at −20 °C. Two-dimensional gels were run using a Bio-Rad Mini-IEF apparatus, with pH 3 to 10 isoelectric focusing as the first dimension using a standard mixture of ampholytes (0.8% 5/7 ampholyte, 0.8% 6/8 ampholyte, and 0.4% 3/10 ampholyte) between 100 mM NaOH and 10 mM H₂PO₄ overnight at 300 volts. After an additional hour at 600 volts, gels were extruded from their tubes, equilibrated in 4× Laemmli buffer, and run on 10% SDS-PAGE for the second dimension, fixed, silver-stained, and exposed to film.

Substrate Identification—Samples for mass spectrometry could be recovered directly from wet silver-stained gels using well-stained, radioactive extract protein landmarks for alignment. Samples from up to eight duplicate gels or membranes were pooled to increase sample quantity. Gel fragments were treated in series with 50 mM ammonium bicarbonate, 100 mM sodium hyposulfate, and 30 mM potassium ferrocyanide for 15 min per solution. The gel slice was then soaked in 50 mM ammonium bicarbonate for 5 min washes until transparent, then incubated in trypsin overnight at 37 °C. Resulting peptides were extracted with several washes of 5% formic acid, 60% acetonitrile, and subjected to electrospray mass spectrometry on a QSTAR-Pulsar mass spectrometer (Applied Biosystems). TOFPOS was used to measure the peptide fingerprint, and then to determine primary sequence of specific peptides by collision-induced dissociation (MS/MS). Matches were made using the FASTS program to search public data bases.

RESULTS

p300-(1–117) Is Differentially Phosphorylated by the Multifunctional CaMKs—The ability of hsCaMKI to phosphorylate the N-terminal 1–117-amino acid fragment of p300 was previously reported (15), but the phosphorylation site was not identified. We characterized this substrate in more detail, to compare the multifunctional kinases and further define their substrate requirements. First, purified bacterial expressed, HIs₆-tagged p300-(1–117) (referred to as 117(wt), Fig. 1A) was used as a substrate for recombinant ceCaMKI, hsCaMKI, and rnCaMKIV (Fig. 1B). 117(wt) was phosphorylated in vitro by all three of these CaMKs, demonstrated by [³²P] incorporation. Because [³²P] incorporation was dramatically enhanced by preincubation of the kinases with recombinant rnCaMKKβ although incubation with rnCaMKKβ alone failed to incorporate [³²P] into 117(wt), 117(wt) is clearly an activation-dependent substrate of the CaMKs. However, only rnCaMKIV phosphorylation retarded the electrophoretic mobility of the protein (Fig. 1B). Phosphorylation by any of the kinases did not seem to require any specific secondary structure, because boiling the substrate for 5 min prior to the kinase reaction did not affect the results of the assay (data not shown). Thus, assuming that the amino acid sequence was the primary site determinant, Ser⁸⁹ was identified the likely phosphoacceptor by analogy to CaMK peptide substrate requirements (26); the site includes hydrophobic residues at P−5 and P+4, and Arg at P−3. Mutation to Ala⁸⁹ eliminated detectable phosphorylation by hsCaMKI and ceCaMKI but not by rnCaMKIV.

Molar phosphate incorporation into 117(wt) was determined relative to recombinant CREB in our assay conditions, to estimate stoichiometry of 117 phosphorylation by the kinases based on the well characterized CaMK phosphorylation profile...
phorylation by CaMKIV, confirming the biochemical results (Fig. 1B). This site had not been among the candidate sites identified; it contained none of the amino acid determinants normally associated with CaMK phosphorylation. This fragment of p300, therefore, contains two atypical CaMK sites; Ser89 is the only known site phosphorylated by CaMKI but not by CaMKIV, whereas Ser24 is a nonconsensus CaMKIV site.

CaMKI and CaMKIV Phosphorylate Distinct Subsets of Proteins in Complex Mixtures—To evaluate more thoroughly the degree to which CaMKI and CaMKIV protein substrate sets are coincident, an assay was developed to visualize in vitro substrate proteomes within complex cell extracts generated from MEF or mixed stage N2 C. elegans. In MEF extract, the patterns observed with activated ceCaMKI and hsCaMKI were remarkably similar, although the ceCaMKI pattern was fainter, and they were quite different from that of rnCaMKIV (Fig. 2). In particular, a group of high molecular weight acidic proteins was phosphorylated exclusively by ceCaMKI and hsCaMKI. In contrast, activated rnCaMKIV phosphorylated several low molecular weight basic proteins that were only weak CaMKI targets. Less dramatic results were obtained when performing the assay with nematode extract (Fig. 2). A few proteins were phosphorylated by mammalian CaMKI and not by CaMKIV (most easily seen in the acidic, low Mr region), and vice versa (particularly in the middle of the gels). Interestingly, several proteins were phosphorylated by both mammalian kinases but not by ceCaMKI. In both extract types, the pattern of proteins phosphorylated by the downstream kinases alone, hsCaMKI, ceCaMKI, and rnCaMKIV, without the addition of rnCaMKKβ, were nearly identical to the extract alone. Thus, phosphorylation of the recombinant kinases by endogenous activators did not contribute substantially to the observed patterns. Likewise, rnCaMKKβ alone did not alter significantly the extract pattern. Because the kinases were preincubated in unlabeled ATP for 20 min, it was not surprising to find that the control gels had minimal signal, although the protein was readily detected by silver staining.

HSP25 Is a CaMK Substrate in MEF Extracts—The identity of one low molecular weight protein phosphorylated by the CaMKs, and favored by CaMKIV, was assigned by mass spectrometry. The two-dimensional gel spot (Fig. 3A) was excised, digested with trypsin, and the primary sequence was obtained by analysis of the collision-induced dissociation spectra. The peptide, AVTQSAEITIPVTFEAR (Fig. 3B), was determined through FASTS data base alignment comparison (27) to be an exact match for the mouse 25-kDa heat shock protein (mmHSP25). The molecular mass (25 kDa) and pI values (5.7, 5.9) previously observed for the related rat HSP27 (28) agree with the position of the unknown substrate on the two-dimensional gel.

To confirm that the assays we developed in fact reflected patterns of substrate preference, phosphorylation of recombinant His$_6$-mmHSP25 by hsCaMKI and rnCaMKIV was analyzed. Both CaMKs could phosphorylate the protein efficiently. The apparent $K_m$ for phosphorylation by CaMKI was 36 ± 4 μM and by CaMKIV was 45 ± 5 μM (Fig. 4B). The observed $k_{cat}$ for CaMKI was twice that for CaMKIV, and the stoichiometry was
corresponding to the underlined peptides in major peaks of radiation (labeled I and II) are identifiable in all traces indicated kinases and purified by affinity resin before digestion. Two tography purification of trypsin-digested HSP25 phosphorylated by the CaMKI and CaMKIV, respectively.

Reactions; curves are direct Michaelis-Menten fits to the data. Apparent incorporation as Ser 15 and Ser 85 (data not shown). Correlat-

ysis of these peptides identified the specific sites of phosphate from HSP25 revealed two peaks (Fig. 4C)

Ser15 over Ser 85 in a 3:1 ratio (Fig. 4C).

In the extract analyses, unlike combinatorial peptide library screens that restrict one to iterating from defined sequence parameters, the kinases are presented with native cellular proteins. Reaction conditions were designed to provide the best possible definition of the substrate preference within the given extract mixture. They allowed reactions to be performed in solution using high concentrations of exogenous kinase and short reaction times to approximate initial rate conditions and thereby minimize bias toward detection of abundant sub-

strates. Importantly, these assays were not designed to identify solely physiologic substrates. Whereas the proteins phosphorylated should represent the best rates of phosphorylation among the proteins available to the kinase, the use of whole extract and exogenous kinase makes it impossible to attribute physiologic relevance to any particular phosphorylation in this assay. Because local concentrations of kinase and substrate in a cell are likely to be regulated, and vary widely from the total cellular concentrations, the relevance of a phosphorylation cannot be determined by in vitro kinetics alone. Nonetheless, the comparison between different kinases allows some prediction as to how the kinases might behave given the same opportunity, and may narrow the field of candidate kinases responsible for a given physiologic phosphorylation event. Furthermore, by comparing the pattern similarities and differences between substrate sets in whole proteomes arrayed in two dimensions, we assess the degree to which the catalytic functions of these enzymes are interchangeable. For example, if CaMKI and CaMKIV exhibited nearly identical substrate preferences, differences in function could be wholly attributed to differences in localization and regulation by other pathways.

Using autoradiograms aligned with the Melanie software package based on landmark phosphoproteins present in the extract control, the patterns for CaMKKβ-activated CaMKI and CaMKIV were overlaid in the indicated color channels onto extract alone to visualize similarities and differences (Supple-

mental Materials). Clear global differences between the sets of proteins phosphorylated by CaMKs I and IV are apparent, which are particularly useful for examining the related kinases from other organisms. Looking more closely at ceCaMKI, it phosphorylated a similar set of proteins to hsCaMKI, with high specific incorporation into a cluster of acidic, high molecular weight proteins. This is in contrast to high incorporation into more basic, lower molecular weight proteins of rnCaMKIV. Overlapping substrate sets were also observed, including a prominent cluster in the range of 50 kDa, near the middle of the IEF range. Thus, CaMKI and CaMKIV could be easily distinguished by this approach, and the biochemical similarity between ceCaMKI and mammalian CaMKI over CaMKIV was reinforced.

Given the highly degenerate definition of substrate consen-

sus sequence currently available, it is not surprising that some proteins with the “required” minimal characteristics are not CaMK substrates as predicted, and the patterns of substrate choice are not simply subsets of each other. p300-(1–117) is one

Fig. 4. Kinetics of HSP25 phosphorylation by CaMKs. A, deduced amino acid sequence of recombinant His 10-tagged mouse HSP25 used to study the initial rate kinetics. Underlined sequences correspond to the phosphopeptides identified by MS/MS following high performance liquid chromatography purification. Major consensus determi-
nants are indicated in bold, and phosphorylated Ser residues are indicated by asterisks. B, phosphorylation by 0.5 μM CaMKs in 2-min reactions; curves are direct Michaelis-Menten fits to the data. Apparent K m values for HSP25 phosphorylation were 36 ± 4 and 45 ± 5 μM for CaMKI and CaMKIV, respectively. C, high performance liquid chromatography purification of trypsin-digested HSP25 phosphorylated by the indicated kinases and purified by affinity resin before digestion. Two major peaks of radiation (labeled I and II) are identifiable in all traces corresponding to the underlined peptides in A that include Ser 15 and Ser 85, respectively.

confirm that HSP25 is an in vitro CaMK substrate, and identify two new sites that are differentially phosphorylated by CaMKI and CaMKIV.

DISCUSSION

We have compared kinase specificity profiles of mammalian CaMKI and CaMKIV both by identification of specific sites in individual proteins and by evaluating the set of proteins in a homogenized cell extract that can be in vitro substrates for a particular kinase. These approaches are complimentary, and they lead to the same overall conclusion, the substrates preferred by CaMKI and CaMKIV are different, and do not always follow the peptide-derived consensus sequences.

low even after 2 h; incorporation reached 44% with CaMKIV and 27% with CaMKI (data not shown). Radiation release by Edman degradation of trypsin-digested, labeled HSP25 indicated that the major site(s) of phosphate incorporation for both kinases was three residues C-terminal to a basic residue, whereas radiation released from CNBr-digested peptides indicated that Ser 15 was at least one site (data not shown). Reverse phase high performance liquid chromatography pu-

rification of CaMKI or CaMKIV 32P-labeled tryptic peptides from HSP25 revealed two peaks (Fig. 4C), and MS/MS analysis of these peptides identified the specific sites of phosphate incorporation as Ser 15 and Ser 85 (data not shown). Correlating the peptide identification with radioactive incorporation by quantitative high performance liquid chromatography, it can be seen that two sites are phosphorylated equally well by CaMKIV, but that CaMKI preferentially phosphorylated Ser 15 over Ser 85 in a 3:1 ratio (Fig. 4C). These experiments
of those proteins. Among its many serines and threonines, Ser163 meets the consensus requirements for phosphorylation by any of the CaMKs, but while clearly a CaMK site for both C. elegans and human CaMK homologues, it is not the predominant CaMKIV site. In agreement with these results, another p300 fragment, p300(74–163), was not phosphorylated by CaMKIV in vitro, although it was a PRC substrate (19). The CaMKIV site we have identified in p300, Ser291, lacks any of the determinants found in previous studies. It is sufficiently different from peptides used as starting points that it could represent a completely different type of substrate, with different primary sequence determinants, not likely to be identified through systematic variation of a known sequence. We also considered but ruled out tertiary structure as a determinant to explain this difference from previous peptide data as denaturing the 117(wt) by boiling had no apparent effects on phospho-

Ser301 is not conserved in p300, but both Ser89 and Ser24 appear to be conserved in CBP, albeit with substantial sequence differences C-terminal to the sites, yet neither was identified as a target in the CBP experiments. Such differences in phosphorylation sites may help explain differences between p300 and CBP, as well as between CaMKI and CaMKIV effects, but much work remains to sort out potential biological relevance.

HSP25, identified directly from our proteomic approach as a CaMK substrate, follows the peptide consensus sequences more closely. Of the two phosphorylation sites identified, Ser15 most closely resembles the CaMKI-phosphorylated synapsin site-1 peptide sequence with a hydrophobic residue at P+4, and it is, in fact, preferred by CaMKI. CaMKIV is able to phosphorylate both sites, which would be predicted based on reported preferences. Interestingly, the primary sequence context surrounding Thr163 would indicate that it is equally suited to be a CaMKIV phosphorylation site, but is not phosphorylated to a significant degree by either of these CaMKs.

Because both tested CaMKs were capable of phosphorylating recombinant His-HSP25 in vitro, we analyzed phosphorylation kinetics in more detail to determine the source of differences observed in the initial extract phosphorylation. The \( K_{\text{cat}} \) for initial rates with CaMKI and CaMKIV were essentially identical in vitro, but \( k_{\text{cat}} \) of CaMKI was almost twice that of CaMKIV for this substrate. Based on these kinetics alone, CaMKI would be predicted to outpace CaMKIV in our short term extract phosphorylation assay. However, CaMKI preferred Ser15 whereas CaMKIV phosphorylated both sites equally, and in a 30-min assay phosphorylation by CaMKIV outpaced CaMKI. This could result from incorporation into a slow phosphoacceptor site or from changes in oligomerization of HSP25 known to follow phosphorylation. In this light, differences between tissue-purified and recombinant HSP25, such as post-translational modification and pre-formed oligomerization, may be important to selection as a CaMK substrate and could result in differential phosphorylation.

Although our methods do not necessarily indicate a physiological substrate relationship, they do not exclude this possibility. The small HSPs form complex quaternary structures that are regulated at least in part by phosphorylation, and can act as chaperones, regulate actin dynamics, and block apopto-

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