Structural Features Underlying the Multisite Phosphorylation of the A Domain of the NF-AT4 Transcription Factor by Protein Kinase CK1[†]

Oriano Marin,[‡,||] Veronica Burzio,[§,||] Marco Boschetti,[‡] Flavio Meggio,[‡] Catherine C. Allende,[§] Jorge E. Allende,[§] and Lorenzo A. Pinna*[,‡]

Dipartimento di Chimica Biologica and Centro di Studio delle Biomembrane del CNR, Università di Padova, viale G. Colombo 3, 35121 Padova, Italy, and Programa de Biología Celular y Molecular, Instituto de Ciencias Biomedicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile

Received June 14, 2001; Revised Manuscript Received September 27, 2001

ABSTRACT: The phosphorylation and dephosphorylation of the NF-AT family of transcription factors play a key role in the activation of T lymphocytes and in the control of the immune response. The mechanistic aspects of NF-AT4 phosphorylation by protein kinase CK1 have been studied in this work with the aid of a series of 27 peptides, reproducing with suitable modifications the regions of NF-AT4 that have been reported to be phosphorylated by this protein kinase. The largest parent peptide, representing the three regions A, Z, and L spanning amino acids 173−218, is readily phosphorylated by CK1 at seryl residues belonging to the A2 segment, none of which fulfill the canonical consensus sequence for CK1. An acidic cluster of amino acids in the linker region between domains A and Z is essential for high-efficiency phosphorylation of the A2 domain, as shown by the increase in K_m caused by a deletion of the linker region or a substitution of the acidic residues with glycines. Individual substitutions with alanine of each of the five serines in the A2 domain (S-177, S-180, S-181, S-184, and S-186) reduce the phosphorylation rate, the most detrimental effect being caused by Ser177 substitution which results in a 10-fold drop in V_{max}. On the contrary, the replacement of Ser177 with phosphoserine triggers a hierarchical effect with a dramatic improvement in phosphorylation efficiency, which no longer depends on the linker region for optimal efficiency. These data are consistent with a two-phase phosphorylation mechanism of NF-AT4 by CK1, initiated by the linker region which provides a functional docking site for CK1 and allows the unorthodox phosphorylation of Ser177; once achieved, this phosphoserine residue primes the phosphorylation of other downstream seryl residues, according to a hierarchical mechanism typically exploited by CK1.

The large number of protein kinases in eukaryotes, with over 800 genes found in the human genome (*1*), raises multiple questions as to the function and specificity of these important enzymes.

In recent years, several laboratories, including ours, have approached the study of the substrate specificity of protein kinases. These studies have concentrated on the analysis of the amino acid sequences surrounding the immediate vicinity of the sites that are phosphorylated in vivo and in vitro by specific kinases and on the preparation of synthetic peptides that contain these sequences and serve as substrates for these particular enzymes (*2*−*5*). These studies have been very useful in determining the consensus sequence recognized preferentially by the active center of these kinases and in predicting the domains of new proteins that are probably phosphorylated by these enzymes. In addition, this approach has allowed us to design several peptides that are highly specific for kinases and that can be employed in assaying for the activity of these kinases in crude extracts of cells and tissues (e.g., refs *5*−*7*).

The studies with short peptides, however, demonstrated that these model molecules are sometimes less efficient than the true physiological substrates. In addition, several sequences that contain the defined consensus for phosphorylation by these kinases are not phosphorylated in the native proteins. Conversely, atypical sites that are not acted upon in model peptides serve as good substrates within the context of whole proteins (*5*). These results clearly indicate that the phosphorylation of proteins by protein kinases involves recognition and interactions that go beyond the immediate vicinity of the acceptor serines or threonines in the substrates. The recent discovery that several protein kinases recognize "docking sites" which are distant from the phosphorylatable residues in their protein substrates constitutes an important step toward the understanding of some of the complexities that provide specificity in kinase−protein substrate interactions (*8*).

[†] This work was supported by the Italian Ministero per l'Università e per la Ricerca Scientifica e Tecnologica (Grant PRIN 2000), the Armenise-Harvard Foundation, the Associazione Italiana per la Ricerca sul Cancro, the Italian Ministry of Health (Project AIDS), and the Consiglio Nazionale delle Ricerche (Grant 00.00369.ST74 and T.P. on Biotechnology), and by FONDECYT-Chile and the ICGEB-Trieste, Italy, to J.A.

* To whom correspondence should be addressed. Phone: 39-049-8276108. Fax: 39-049-8073310. E-mail: pinna@civ.bio.unipd.it.

[‡] Università di Padova.

[§] Universidad de Chile.

[||] These authors contributed equally to the work.

10.1021/bi0112309 CCC: $22.00
Published on Web 12/13/2001

Protein kinase CK1[1] (originally known as casein kinase I) is a family of related monomeric enzymes which, in mammalian cells, contains seven members: α, β, γ1, γ2, γ3, δ, and ε (*9*). The α and β isoforms are the shortest (37−39 kDa), while the γ, δ, and ε isoforms have carboxyl terminal extensions and vary from 45 to 49 kDa. CK1 isoforms are ubiquitous in all eukaryotes, from yeast to man. In addition, CK1α has four splice variants that contain insertions of two short exons (*10*). There are a large number and variety of CK1 protein substrates including, among others, the large T antigen of SV-40 (*11*, *12*), the insulin receptor (*13*), p53 (*14*), DARPP-32 (*15*), and the transcription factor NF-AT of T cells (*16*). There are also a number of cellular processes in which CK1 has been involved. These include radiation-induced DNA repair (*17, 18*), circadian rhythm in Drosophila (*19*) and humans (*20*), the Wnt signaling cascade that regulates morphogenesis in *Xenopus laevis* (*21*−*23*), the centrosome function in cell division (*24*), and neurotransmitter regulation (*25*, *26*).

The site specificity of CK1 enzymes has been studied previously. Initial studies indicated that CK1 could only phosphorylate serines or threonines that were three amino acids downstream from previously modified phosphoserines or phosphothreonines (Sp/Tp-X-X-S*, where S* is the target serine for CK1) (*27*−*29*). Such a stringent requirement for a previously existing phosphoamino acid restricted CK1 to a function in hierarchical phosphorylation. Subsequent studies demonstrated that this stringent requirement did not hold for many substrates and that an acidic amino acid in position n-3 could replace the phosphoamino acid, especially when there are a number of additional acidic residues at the amino side of the target serine (*30*, *31*). A recent detailed study with several isoforms of CK1 purified from rat liver and recombinant CK1α from *X. laevis* has been carried out to re-evaluate the specificity requirements (*31*).

The publication of Zhu et al. (*16*) which reported the phosphorylation of the NF-AT4 transcription factor by protein kinase CK1 was of great interest to us because it seemed relevant to the problem of CK1 recognition of substrates. These researchers set out to define the factors that regulate the nuclear import of NF-AT4, a transcriptional regulator that plays a key role in T cell activation. They found that CK1α antagonized the Ca^{2+}-mediated activity of the calcineurin phosphatase, which was responsible for the translocation of NF-AT4 to the nucleus. The CK1α effect was clearly related to its capacity to phosphorylate NF-AT4 because an inactive dominant-negative mutant of CK1 would counteract the effect of the catalytically active protein kinase. Analyzing the regions of the NF-AT4 molecule responsible for the CK1 effect on its nuclear import, these authors concluded that CK1 is bound to the A domain (aa 165−184) of NF-AT4, specifically to the sequence S_{177}DASSCES$_{184}$ which they then designated A-2, and phosphorylated several serine residues of that domain and nearly in the Z domain (aa 204−236). They also reported that MEKK1 strengthened the binding of CK1 to the A domain.

An interesting aspect of the report of Zhu et al. (*16*) was the proposal that subdomain A-2 of NF-AT was a binding region necessary for the phosphorylation of sites in the Z domain. This fits the definition of a docking site, although it was complicated by the fact that A-2 was also a substrate site. A more detailed study of the phosphorylation of this region of NF-AT by CK1α was therefore undertaken to confirm and amplify some of these observations.

In the present study, we have used a whole family of synthetic peptides variably overlapping the A and Z subdomains of NF-AT4, including the linker region between the two. These peptides have been used in vitro to test their phosphorylation with recombinant CK1α and with CK1 purified from rat liver, measuring the kinetic constants and their efficiency as substrates. The results presented in this report differ significantly from those of Zhu et al. (*16*), especially in the fact that the Z region of our peptides is not detectably phosphorylated by CK1 in the presence or absence of A2. Nevertheless, this study does indicate that a non-phosphorylated linker region between domains A and Z (aa 190−198), which is rich in acidic residues, greatly enhances the phosphorylation of the serines in the A-2 region which per se would lack the consensus for primary (nonphosphate-directed) phosphorylation by CK1. This linker region would conform to the functional definition of a docking site. In addition, it is observed that hierarchical phosphorylation also plays a major role in the phosphorylation of subdomains A1 and A2 by CK1, once two serines located near their N termini have been phosphorylated.

EXPERIMENTAL PROCEDURES

Materials. Native protein kinase CK1 was isolated and partially purified from rat liver cytosol, as previously described (*32*). Its specific activity was about 11 000 units/mg, one unit being defined as the amount of enzyme that transfers 1 pmol of phosphate per min into the specific peptide substrate RRKHAAIGDDDDDAYSITA. The preparation was free from other protein kinases, as judged from its failure to phosphorylate histones, poly Glu/Tyr (4:1), and specific peptide substrates of CK2, GSK3, and proline-directed protein kinases. P81 phosphocellulose paper was from Whatman (Kent, U.K.). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Cleveland, OH), and nitrilotriacetic acid (NTA) agarose was from Qiagen (Valencia, CA). All other reagents were from Sigma (St. Louis, MO).

Recombinant CK1α *Isoforms from Zebrafish (Danio rerio)*. cDNA clones coding for the splice variants of CK1α (CK1α, CK1αS, CK1αL, and CK1αLS) were obtained from a *D. rerio* embryo cDNA library in λ2AP and introduced into pT7−7H6 expression vectors for *Escherichia coli* (V.B. et al., in preparation). For each CK1 isoform, 1.5 L of LB containing 100 μg/mL ampicillin was inoculated with 1.5 mL of an overnight culture of *E. coli* BLZ1 cells transformed with the CK1 vector constructs and grown at 37 °C with shaking until an OD_{600nm} of 1.0. The cultures were cooled to 15 °C and 0.4 mM isopropyl-β-D-thiogalactopyranoside was added. The cultures were then incubated overnight at 15 °C with shaking. The cell pellet was obtained by centrifuging at 5000*g* for 20 min at 4 °C and later resuspended in $^1/_{20}$ of a volume of a lysis buffer (20 mM Tris (pH 7.9), 500 mM NaCl, and 0.5% Triton X-100), after which 1 mg/mL lysozyme was added, and incubation

[1] Abbreviations: CK1, protein kinase CK1; NF-AT, nuclear factor of activated T cells; GSK-3, glycogen synthase kinase-3; NLS, nuclear localization signal.

continued on ice for 30 min. The lysates were sonicated for 1 min at 4 °C and centrifuged at 30 000*g* for 30 min at 4 °C. The supernatant was incubated with rocking for 1 h at 4 °C with $^1/_{1000}$ of the original culture volume of resuspended NTA-Ni^{2+} agarose. The lysate with the resin was then mounted on a 5 mL syringe column and washed with 50 column volumes of a lysis buffer containing 10 mM imidazole. The enzyme was eluted with 5 volumes of a lysis buffer containing 250 mM imidazole, and fractions were assayed for activity using 5 mg/mL β-casein as a substrate. In the work presented here, assays were carried out using CK1α isoform, unless specifically indicated otherwise.

Synthetic Peptides. The original peptides derived from the NF-AT4 sequence as well as their substituted derivatives were synthesized by automatic solid-phase methods on 4-hydroxymethyl-*co*-polystyrene-1% divinylbenzene resin (0.95 mmol/g, 0.05 meq) (Applied Biosystems, Foster City, CA) and fast 9-fluorenilmethoxycarbonyl (Fmoc) chemistry utilizing 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzo-triazole (HOBt) coupling (*33*) with a 431A peptide synthesizer (Applied Biosystems), as improved in our laboratories (*34*). The incorporation of phosphoserine was performed by a building-block strategy, using the phosphoamino acid derivative Fmoc-Ser(PO(benzyl))OH as previously described (*35*). Peptidyl resins were cleaved and deprotected following the procedure of King et al. (*36*). The crude peptides (50−100 mg in 10 mL of water) were pumped onto a preparative reversed-phase (RP) column (prepNova-Pak HR C18, 6 μm, 25 × 10 mm; Waters Corp., Milford, MA) and eluted with a linear gradient of 10−45% acetonitrile at 12 mL/min. The purity of peptides was >90% by analytical RP-HPLC on a 5 μm C18 Symmetry300 column (4.6 × 250 mm; Waters) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at 1 mL/min. The molecular weights of the peptides were confirmed by mass spectroscopy using a matrix-assisted laser desorption ionization time-of-flight (MALDI-Tof) spectrometer (Maldi-1; Kratos-Schimadzu, Manchester, U.K.).

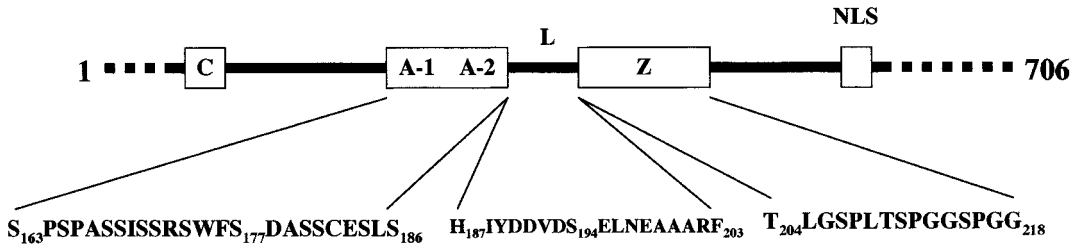
Peptide Phosphorylation Assay. Synthetic peptide substrates (0.2 mM) were phosphorylated by incubation in a medium (25 μL final volume) containing 50 mM Tris HCl buffer (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, and 40 μM [γ-^{32}P]ATP (specific radioactivity 500−1000 cpm/pmol). The reaction was started with the addition of the protein kinase and stopped by cooling in ice and absorption onto phosphocellulose paper. Experiments were done typically with 5−20 units per assay. Phosphorylation of all peptides was evaluated by a phosphocellulose paper procedure (Pinna and Ruzzene, 1996) except when peptides were lacking the two additional N-terminal arginines and containing only two basic residues in which case phosphate incorporation was determined in parallel by measuring [γ-^{32}P]ATP converted into P_i and extracting a phosphomolybdic complex as described previously (*37*). Control experiments were also performed by subjecting phosphorylated samples to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and subsequent scanning on an instant imager apparatus (Canberra-Packard, Meriden, CT) or autoradiography. Kinetic constants were determined by regression analysis of double-reciprocal plots constructed from initial-rate measurements. Phosphoamino acid analysis of radiolabeled peptides was performed as previously described (*38*).

Tryptic Digestion. Phosphorylated peptide reaction products were separated by 18% SDS−PAGE, and the area corresponding to ^{32}P-labeled peptides was cut out and eluted several times with freshly prepared 50 mM ammonium hydrogencarbonate. Eluted radiolabeled peptides were lyophylized and dissolved in Tris HCl 100 mM (pH 7.5). Digestion was started by the addition of trypsin (roughly 1:50 w/w). After 2 h, an equal amount of freshly prepared trypsin was added. The digestion was stopped after 4 h by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.1%. The reaction mixture was processed by RP-HPLC using a Source 15 RPC ST (46 × 100 mm) column (Amersham Pharmacia Biotech) eluting at 3 mL/min with a linear gradient of acetonitrile containing 0.08% TFA from 0% to 50% for 15 min. The identification of main fragments was obtained by MALDI-Tof mass spectroscopy using α-cyano-4-hydroxycinnamic acid as a matrix, while the radioactivity of the collected fractions (1 mL) was determined by counting in a liquid scintillation apparatus (Canberra-Packard).

Resolution of Variably Phosphorylated Peptide AL-13 by HPLC. Peptide AL-13 (200 μM) was extensively phosphorylated by a 60 min incubation at 37 °C in the phosphorylation medium (see the previous paragraph) in the presence of 1 mM ATP. The reaction started with the addition of 20 units of rat liver CK1 (repeated after 30 min). The phosphorylated products were resolved on an RP-HPLC C18 column (0.46 × 25 cm) and eluted at 1 mL/min with a linear gradient of acetonitrile containing 0.08% TFA from 0% to 50% for 60 min. The identification of the collected peaks was performed by MALDI-Tof mass spectrometry as described previously.

RESULTS

Synthetic Peptides Encompassing Both the A and Z Domains of NF-AT4 Are Readily Phosphorylated by CK1. Figure 1 schematically shows the region of NF-AT4 that includes the regulatory domains controlling its nuclear translocation. Upon calcium influx, activated calcineurin binds to the C domain and catalyzes the dephosphorylation of residues which are believed to be located in both the A and Z domains. Dephosphorylation by calcineurin is necessary for nuclear localization (*39*). Kinases opposing the nuclear translocation of NF-AT4 are proposed to be CK1, MEKK1 (*16*), and GSK-3 (*40*). While the molecular implication of MEKK1 is unclear, the role of CK1 in establishing an intramolecular masking of the nuclear localization signal on NF-AT4 is substantiated by in vivo experiments showing that CK1α associates with NF-AT4 and that a dominant-negative mutant of CK1α induces the nuclear import of NF-AT4 (*16*). The same authors provide evidence suggesting that CK1α directly phosphorylates and binds to the carboxyl terminal moiety of the A domain (A2 subdomain) and subsequently phosphorylates the Z domain (*16*). Examination of the primary structure (Figure 1) shows that neither the A nor the Z domains includes a canonical consensus sequence for primary (i.e., nonphosphate-directed) phosphorylation by CK1, which is specified by multiple acidic residues upstream from Ser/Thr at positions n-3 and further upstream (*30*, *31*). However, one serine (Ser194) fulfilling this feature is present in the linker region between domains A and Z. To check if this serine could be a major



NF-AT4 amino acids	Peptides
173-218 (ALZ1)	**RR**RSWFSDASSAESLSHIYDDVDSELNEAAARFTLGSPLTSPGGSPGG
173-202 (AL1)	**RR**RSWFSDASSAESLSHIYDDVDAELNEAAAR
189-218 (LZ1)	YDDVDSELNEAAARFTLGSPLTSPGGSPGG
173-188 (A1)	**RR**RSWFSDASSAESLSHI
163-188 (A5)	**RR**SPSPASSISSRSWFSDASSAESLSHI

FIGURE 1: Schematic representation of NF-AT4 region encompassing domains C, A, Z, and NLS and the linker region designated L. (Top) Shown is the sequence between the amino terminal of domain A and carboxyl terminal of domain Z. (Bottom) Amino acid sequences are presented for parent peptides, which are those peptides with minimal conserved substitutions prepared for comparative studies with a series of peptides containing further substitutions (see Table 1). In all peptides, Cys182 was replaced by alanine for technical reasons. The numbering of peptides refers to the natural sequence without taking into account the two N-terminal arginines (boldface) added for technical reasons. In one peptide, Ser194 has been replaced by alanine (underlined).

target for CK1, we have synthesized two peptides encompassing the 173−218 sequence including subdomain A2, the linker region, and the Z domain with either natural Ser194 (peptide ALZ-1) or with alanine (peptide ALZ-2) at the 194 position (Table 1). Both peptides were readily phosphorylated by either rat liver CK1 (a mixture of various isoforms (*31*)) or by *D. rerio* recombinant CK1α, with similar time courses (not shown) and kinetic constants (Tables 1 and 2). This finding rules out the exclusive implication of Ser194 in the phosphorylation of the NF-AT4 peptide by CK1, strongly suggesting, at the same time, that phosphorylation at residues other than Ser194 must rely on structural features other than those dictated by a canonical consensus sequence. This prompted us to study these structural features by synthesizing a number of substituted or deleted peptides. To eliminate the incidence of Ser194, this residue was replaced by alanine in all the derived peptides, unless explicitly mentioned (see Table 1).

Z Domain Is Not Phosphorylated by CK1. To evaluate the contribution of domains A2 and Z to the phosphorylation of the parent peptide 173−218, which includes both domains as well as the linker region, advantage was taken of an arginyl residue (Arg202) at the end of the linker region. By tryptic digestion of the phospho-radiolabeled peptide ALZ-2, the two fragments 173−202 and 203−218 were isolated by HPLC. As shown in Figure 2, the former accounts for the total radioactivity incorporated, while the latter was not radiolabeled. The idea that the Z domain is not a substrate for CK1 was further validated by synthesizing two shorter peptides including either subdomain A2 (173−202) or the Z domain (189−218) (peptides AL-1 and LZ-1, respectively) and assaying them for CK1-mediated phosphorylation. As shown in Table 1, AL-1 was phosphorylated nearly as readily as the parent 173−218 peptide (ALZ-2 peptide), while LZ-1 was phosphorylated much more slowly, a circumstance that hindered its kinetic analysis. The slow phosphorylation of the Z-domain peptide LZ-1 (189−218) disappeared if the seryl residue at position 194 in the linker region was replaced by alanine (peptide LZ-2). Thus, the weak phosphorylation of the 189−218 LZ-1 peptide must be attributed to this serine and not to any of the five phosphorylatable residues (three serines and two threonines) in the Z domain.

A derivative of the AL-1 peptide was also synthesized in which the two arginines, added for technical reasons to the N terminus, were replaced by the two serines found in the native sequence of NF-AT4. This peptide (AL-12), the phosphorylation of which had to be assayed by a different procedure (see Experimental Procedures), displayed a time course phosphorylation curve (not shown) comparable to AL-1, supporting the view that the two arginines, added to the N terminus of most peptides for methodological reasons, do not influence significantly the phosphorylation response.

Altogether, these data show that the Z domain, either alone or linked to the A2 domain, is not targeted to any appreciable extent by CK1 nor is it relevant to CK1-mediated phosphorylation of the peptide encompassing both A2 and Z domains. Similar data were obtained using either rat liver CK1 or recombinant *D. rerio* CK1α.

Acidic Residues in the Linker and in the A-2 Regions Are Required for High-Efficiency Phosphorylation of the NF-AT4 Peptides by CK1. The role of other serines present in the NF-AT4 parent peptide was explored in relation to CK1 phosphorylation. Ser181 and Ser186 both have acidic residues (Asp and Glu, respectively) at position n-3, a feature which has been claimed to be sufficient to trigger phosphorylation by CK1 (*4*). The replacement of these serines by alanine was checked to determine whether these substitutions were detrimental to the phosphorylation of the 173−202 (AL-1) peptide. As shown in Tables 1 and 2, both substitutions (peptides AL-4 and AL-6) were still compatible with phosphorylation efficiencies not much lower than those of the parent peptide AL-1. In an attempt to evaluate the contribution of the remaining three serines as well, these were also individually mutated to alanine. Each of these substitu-

Table 1: NF-AT4-Derived Synthetic Peptides Used in This Study and Kinetic Constants for Their Phosphorylation by Rat Liver CK1[a]

Peptide	Sequence	K_m (mM)	V_{max} (pmol/min)	V_{max}/K_m
ALZ-1 (173-218)	RRRSWFSDASSAESLSHIYDDVDSELNEAAARFTLGSPLTSPGGSPGG	0.553	31.6	57
ALZ-2	RRRSWFSDASSAESLSHIYDDVD**A**ELNEAAARFTLGSPLTSPGGSPGG	0.320	27.4	85
AL-1 (173-202)	RRRSWFSDASSAESLSHIYDDVDAELNEAAAR	0.606	33.3	54
AL-2	RRRSWF**A**DASSAESLSHIYDDVDAELNEAAAR	0.285	3.3	11
AL-3	RRRSWFSDA**A**SAESLSHIYDDVDAELNEAAAR	0.680	13.6	20
AL-4	RRRSWFSDAS**A**AESLSHIYDDVDAELNEAAAR	0.722	17.1	23
AL-5	RRRSWFSDASSAE**A**LSHIYDDVDAELNEAAAR	1.052	16.0	15
AL-6	RRRSWFSDASSAESL**A**HIYDDVDAELNEAAAR	0.419	9.5	22
AL-7	RRRSWFSDAS**G**AESL**G**HIYDDVDAELNEAAAR	0.312	10.0	32
AL-8	RRR**G**WF**G**DA**G**SAE**G**LSHIYDDVDAELNEAAAR	0.215	6.1	28
AL-9	RRRSWFS**G**ASSA**G**SLSHIYDDVDAELNEAAAR	n.d.	n.d.	-
AL-10	RRRSWFSDASSAESLSHIY**GG**VG**A**GLNGAAAR	1.428	6.3	4
AL-11	RRRSWFS**A**ASS**A**ASLSHIYDDVDAELNEAAAR	n.d.	n.d.	-
AL-12	**SS**RSWFSDASSAESLSHIYDDVDAELNEAAAR	0.156	34.1	218
AL-13	RRRSWF**pS**DASSAESLSHIYDDVDAELNEAAAR	0.033	201.5	6106
AL-14	RRRSWF**pSG**ASSA**G**SLSHIYDDVDAELNEAAAR	0.027	210.2	7785
A-1 (173-188)	RRRSWFSDASSAESLSHI	3.225	35.0	10
A-2	**SS**RSWFSDASSAESLSHI	1.170	27.6	23
A-3	RRRSWF**pS**DASSAESLSHI	0.106	258.7	2440
A-4	RRRSWF**pSG**ASSA**G**SLSHI	0.105	223.4	2127
A-5 (163-188)	RRSPSPASSISSRSWFSDASSAESLSHI	1.923	43.0	22
A-6	RRSP**pS**PASSISSRSWFSDASSAESLSHI	0.058	25.7	443
A-7	RRSP**pS**PA**GG**ISSRSWFSDASSAESLSHI	1.538	16.5	10
A-8	RRSP**pS**PASSISSRSWF**A**DASSAESLSHI	0.047	21.5	457
A-9	RRSPSPASSISSRSWF**pS**DASSAESLSHI	0.090	213.8	2375
LZ-1 (189-218)	YDDVDSELNEAAARFTLGSPLTSPGGSPGG	n.d.	n.d.	-
LZ-2	YDDVD**A**ELNEAAARFTLGSPLTSPGGSPGG	n.d.	n.d.	-

[a] Capital letters A, L, and Z denote the presence in the peptides of sequences encompassing the A domain, the linker region (L), and the Z domain, respectively. Peptide ALZ-1 is also referred to in the text as the parent peptide. Residues also substituted in the parent peptide or added for methodological reasons are underlined. Individual and multiple substitutions are in boldface. Amino acids are indicated by their one letter codes. pS is phosphoserine. The kinetic constants are the mean of values obtained in triplicate with SD not exceeding 15%.

tions (peptides AL-2, AL-3, and AL-5, respectively) significantly decreased the phosphorylation efficiency, with special reference to the Ser177 substitution (peptide AL-2) which caused a 10-fold drop in V_{max} with rat liver CK1. Nevertheless, the quadruple substitution of serines 175, 177, 180, and 184 (peptide AL-8) did not fully abrogate phosphorylation, its effect on V_{max} being paradoxically less detrimental than that of an individual substitution of Ser177 (Table 1). Interestingly, this substitution (peptide AL-2), which is very detrimental in terms of the phosphorylation rate (V_{max}), affects a residue (Ser177) which is devoid of any feature of putative CK1 sites. Also remarkable is the fact that the substitution of serine residues 177, 180, 181, and 186 affects V_{max} without causing any dramatic increase in the K_m value, which reflects the affinity of the peptides for rat liver CK1 (Table 1). A

Table 2: Kinetic constants for the phosphorylation of representative NF-AT4-derived synthetic peptides by *D. rerio* CK1αS[a]

	CK1αS		
peptide	K_m (mM)	V_{max} (pmol/min)	V_{max}/K_m
ALZ-1	0.259	2.740	10.58
ALZ-2	0.279	6.013	21.55
AL-1	0.284	3.311	11.66
AL-2	0.271	1.551	5.72
AL-3	1.254	7.350	5.86
AL-4	0.349	2.911	8.34
AL-5	1.423	7.977	5.61
AL-6	0.304	1.603	5.27
AL-10	2.370	3.591	1.52
A-1	2.732	5.900	2.16

[a] The nomenclature of the peptides is the same as that in Table 1.

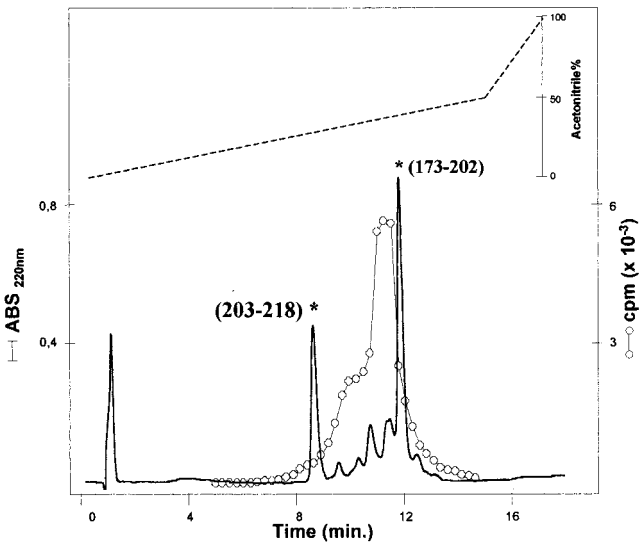


FIGURE 2: HPLC separation of CK1-phosphorylated ALZ-2 peptide after trypsin digestion. The peptide was phosphorylated for 60 min under the conditions described in the Experimental Section and then subjected to 18% SDS−PAGE. The corresponding band was localized on the gel after scanning on an instant imager (Packard), was cut out and eluted with 50 mM ammonium hydrogencarbonate, and was lyophylized. After tryptic digestion (see Experimental Section), the radiolabeled hydrolysate was analytically resolved on RP-HPLC. The absorbance and the radioactivity (o- - -o) of the collected fractions are shown. The identification of the main fragments as those encompassing residues 173−202 and 203−218, respectively (denoted by an asterisk), was performed by MALDI-TOF mass spectrometry.

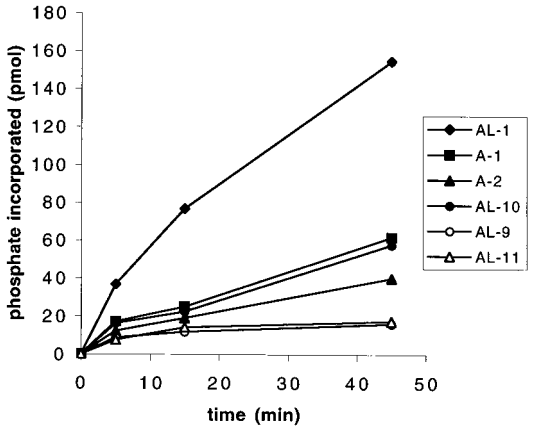


FIGURE 3: Relevance of the linker region to the CK1-catalyzed phosphorylation of the NF-AT4 A2 domain. The peptides were phosphorylated at a 200 μM concentration for variable periods of time (as indicated), and the phosphate incorporated was quantified as detailed in the Experimental Section. The data represent the mean of at least three independent experiments, with SD never exceeding 12%.

significant yet not dramatic increase in K_m is observed only upon the substitution of Ser184 (peptide AL-5) and, only in the case of zebrafish recombinant CK1α, of Ser180 (peptide AL-3, Table 2). Similar results were found with recombinant CK1αS, CK1αL, and CK1αLs, the splice variants of CK!α of the zebrafish enzyme (not shown). These data suggested that the binding of the 173−202 peptide does not rely specifically on a single one of the six phosphorylatable residues but that these various serines are rather interchangeable as targets for CK1.

To check the possibility that the entire linker region (in which Ser194 is consistently changed to alanine and is, thus, devoid of phosphorylatable residues) might cooperate in the relatively high binding affinity of the "AL" peptides, a deleted peptide, A-1, was generated, which comprised residues 173−188 (see Table 1). Although peptide A-1 includes the six serines also present in peptide 173−202 (AL-1), its phosphorylation, tested at 200 μM, is much lower than that of AL-1 (not shown). This drop in phosphorylation efficiency is entirely due to a 5- to 10-fold increase in K_m (Tables 1 and 2) observed with CK1 from both species. It may be concluded, therefore, that the linker segment between domains A and Z, in contrast to the phosphorylatable residues in the A2 subdomain (see the previous discussion), is essential for high-affinity binding to CK1. An obvious feature of this region is a cluster of acidic residues with three aspartic and two glutamic acids located in its central part. The possible relevance of these acidic residues was assessed by generating a derivative of the 173−202 peptide (AL-10) in which all of the acidic residues in the linker region were replaced by glycine. As shown by the kinetic constants (Tables 1 and 2), the peptide AL-10 lacking this acidic cluster is much less efficient as a substrate than the parent one. As seen in Table 1, with rat liver CK1, such a drop in phosphorylation efficiency is accounted for by both a dramatic decrease in V_{max} and an increase in K_m. With recombinant CK1α, the increase in K_m is even more pronounced, reaching the same level of the peptide A-1 which is entirely devoid of the linker segment. This leaves little doubt that the acidic residues downstream from domain A2, either directly or through conformational effects, play an important role in determining the high-efficiency phosphorylation of residues located in the A2 domain itself.

In addition to the effect of the acidic cluster of the linker region, some acidic residues of the A2 domain also have a striking effect, as seen by replacing two amino acids of domain A2, Asp178 and Glu183, with either glycine or alanine (peptides AL-9 and AL-11, respectively). Both of these substitutions almost abolish phosphorylation by CK1 (Figure 3 and Table 1) and render the peptide a powerful noncompetitive inhibitor of CK1 with respect to the phosphorylation of a canonical peptide substrate (Figure 4A). Such an inhibition is not observed with the AL-10 peptide in which the five acidic residues in the linker region had been substituted.

Also noteworthy is the fact that peptide AL-9 is also an inhibitor of the phosphorylation of peptide ALZ-1, which lacks a canonical consensus sequence for CK1 but contains the linker region. Interestingly, however, in this case, inhibition is of the competitive type (Figure 4B).

Phosphorylation at Ser177 and Ser165 Primes Hierarchical Phosphorylation of NF-AT4-Derived Peptides by CK1. The detrimental effect on V_{max} observed by the substitution of Ser177 for alanine would be consistent with the idea that while the affinity is mostly dictated by the linker region downstream from subdomain A2, Ser177 plays a prominent role among the phosphoacceptor residues of this region. This observation and the location of Ser177 upstream from a row of serines, each separated by 2−3 residues, suggested the possibility that once Ser177 becomes phosphorylated, it might initiate a cascade of hierarchical phosphorylation

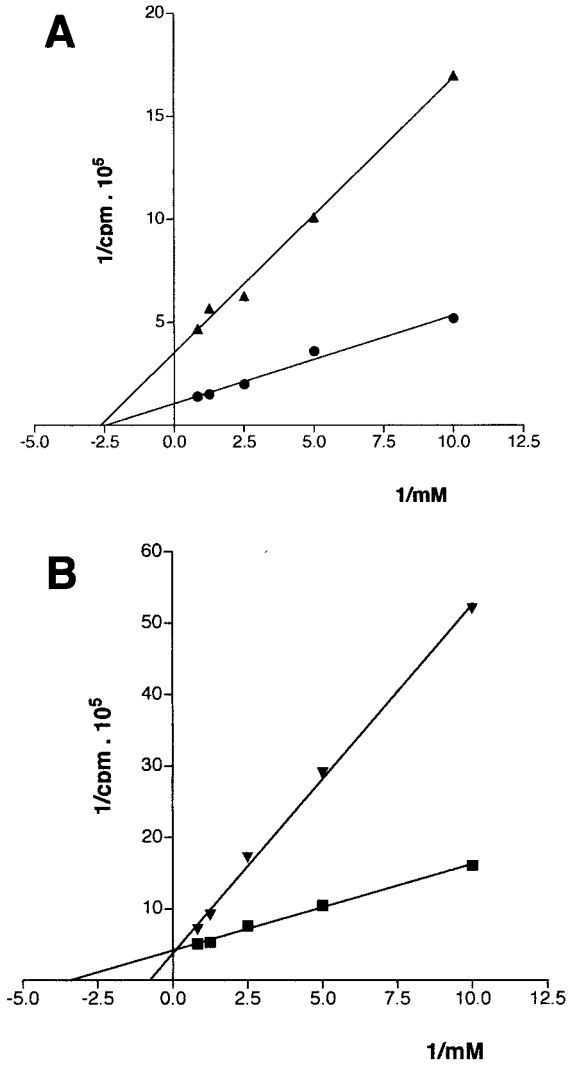


FIGURE 4: Variable inhibition of CK1 activity by peptide AL-9 derived from the NF-AT4 A2 domain. Increasing amounts of (A) inhibitor2-derived RRKHAAIGDDDDAYSITA peptide and of (B) peptide ALZ-1 were phosphorylated as described in the Experimental Section in the absence and in the presence of 40 and 200 μM AL-9 peptide, respectively. Shown are the double-reciprocal plots of the data calculated according to Lineweaver−Burk.

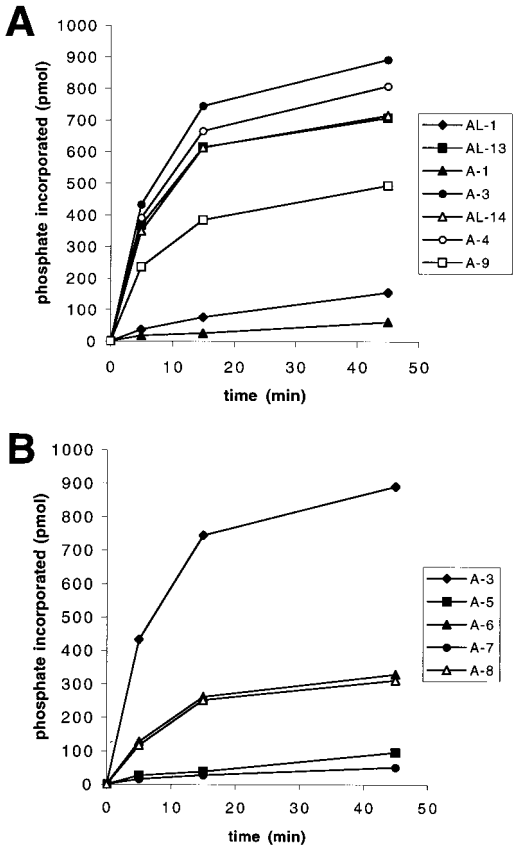


FIGURE 5: Phosphorylation of Ser177 and Ser165 primes hierarchical phosphorylation of NF-AT4 peptides by CK1. (A) The time courses of phosphorylation by CK1 of the control (A-1 and AL-1) and the chemically phosphorylated at Ser177 (A-3, A-4, A-9, AL-13, and AL-14) peptides reproducing the A domain and linker region of NF-AT4 are shown. Phosphorylation conditions are described in the Experimental Section. (B) The time courses with peptides A-5, A-6, A-7, and A-8 reproducing the N terminally extended A domain as such or including the chemically phosphorylated Ser165 are shown.

through the whole A2 subdomain, ending with Ser186, at the very beginning of the linker region (see Figure 1). The implication of CK1 in this kind of sequential multiphosphorylation is very well documented (e.g., refs *27* and *41*). To test this possibility, a derivative of the 173−202 parent peptide, in which Ser177 had been replaced by a phosphoserine, was synthesized (peptide AL-13) (see Experimental Procedures) and assayed as a substrate for CK1. Replacement of Ser177 by a phosphoserine was expected to have opposite effects, depending on the role of this serine. If Ser177 were only an important phosphoacceptor residue without consequences on the phosphorylation of other residues, then its previous chemical phosphorylation would deprive CK1 of a crucial target, causing a drop in phosphorylation efficiency. However, if the phosphorylation of Ser177 were priming the subsequent phosphorylation of several serines, one would expect the phosphopeptide to be an improved target for CK1. As shown in Figure 5A and Table 1, the latter hypothesis proved correct, the Ser177 phosphorylated derivative (AL-13) of the 173−202 peptide being an outstanding substrate for CK1 with a 20-fold lower K_m and a 6-fold higher V_{max} than those of the parent peptide AL-1. An even more striking favorable effect is observed if Ser177 is replaced by phosphoserine in the shorter peptide 173−188. In this case, the parent peptide A-1 displays a huge K_m value, in the millimolar range, which is decreased almost 30-fold by the phosphorylation of Ser177 (peptide A-3), while the V_{max} increases 7-fold. The same favorable effect of PSer177 is observed if the A-1 peptide is extended on its N-terminal side (compare peptides A-5 and its phosphoderivative A-9 in Table 1). Thus, the complete phosphorylation of Ser177 makes the linker region less necessary in the efficiency of the phosphorylation of substrate peptides (Table 1, compare peptides A-3, A-9, and AL-13). The concept that the presence of phosphoserine in position 177 overrides the other effects seen with the unphosphorylated peptide is corroborated by the observation that replacement of D-178 and E-183 by glycines (which completely blocks the capacity of the phosphorylation of peptide AL-9) has no effect at all in peptides AL-14 and A-4 in which serine 177 is totally phosphorylated. AL-14 and A-4 are, in fact, as good substrates as AL-13 and A-3, respectively, which have the wild-type acidic amino acids in these positions (Table 1). In

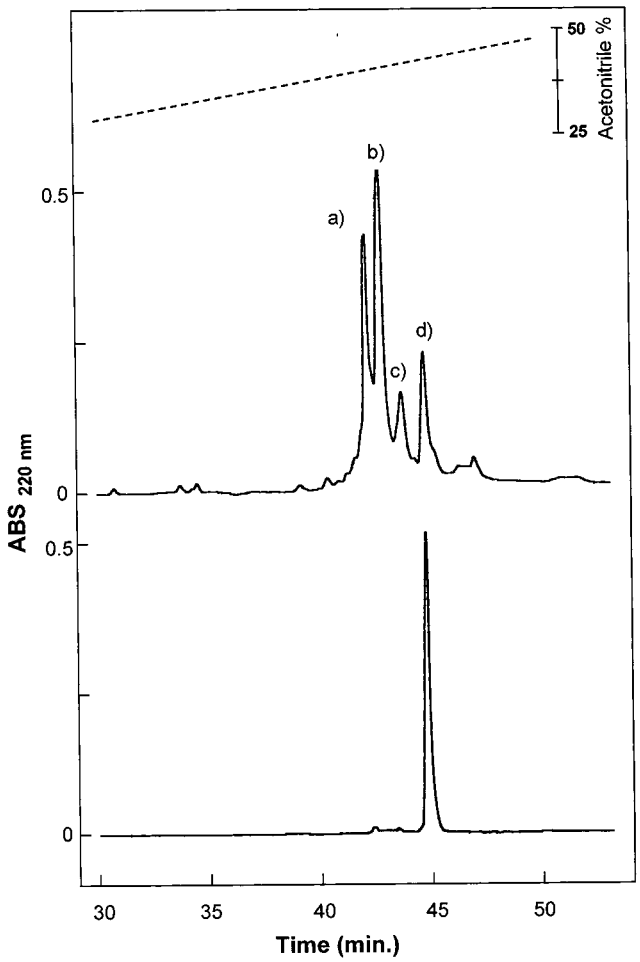


FIGURE 6: HPLC and MALDI-Tof analysis of the phosphorylation products of peptide AL13. Peptide AL-13 was extensively phosphorylated for 60 min by rat liver CK1 as described in the Experimental Section in the presence (upper panel) and in the absence (lower panel) of a 5-fold molar excess of ATP and directly subjected to RP-HPLC analysis. The absorbance at 220 nm is shown. The four main peaks obtained (indicated as a−d) were individually analyzed by mass spectrometry (see text).

the attempt to provide additional evidence about the occurrence of hierarchical phosphorylation, peptide AL-13 was incubated with CK1 in the presence or absence of a 5-fold molar excess of ATP, and the phosphorylation products were analyzed to see whether multiple species are, in fact, generated during incubation with ATP. As shown in Figure 6 the phosphorylated sample subjected to HPLC gives rise to a series of four sharp peaks (a−d), while only the most retarded peak (d) is present in the sample incubated without ATP. The identification of this peak with unmodified AL-13 was confirmed by MALDI-Tof spectrometric analysis providing a molecular mass almost identical to the one expected (3709 vs 3706). Peak b could also be characterized spectrometrically, revealing a molecular mass (3871) almost perfectly coincident to that of the triply phosphorylated peptide (3866). Thus, peaks b and d represent the triply and singly phosphorylated peptides, respectively. By analogy, we conclude that peaks a and c represent the quadruply and doubly phosphorylated peptide, although attempts to determine their molecular mass were not successful for technical reasons.

A second hierarchical phosphorylation cascade was explored by examining the possibility that upstream serines in the A1 region of NF-AT4 could induce phosphorylation of the A2 region. For that purpose, a series of peptides encompassing residues 163−188 (the complete A domain) were synthesized (peptides A-5 → A-8 in Table 1). In Figure 5B and Table 1, it can be seen that peptide A-6, which has a phosphoserine in position 165, is a much better substrate than A-5, which has the unphosphorylated residue in that position. This figure shows two additional results. Interruption of the hierarchical cascade by replacement of serines 168 and 169 (peptide A-7) eliminates the positive hierarchical effect of phosphoserine in position 165. Additionally, the replacement of Ser177 with alanine (peptide A-8) does not significantly diminish the effectiveness of the phosphopeptide as a CK1 substrate. This latter result would suggest that upstream hierarchical phosphorylation probably does not greatly affect Ser177, which we have seen previously to be a key residue in the phosphorylation of the A2 region by CK1.

DISCUSSION

The phosphorylation of the A and Z region of NF-AT proteins is of great importance because it can determine the cellular localization of these transcription factors and define the activation or quiescence of T cells and the triggering of the immune response (*40*). There is a general agreement that Ca^{2+}-regulated calcineurin is responsible for the dephosphorylation of this key region of the NF-AT family of proteins (*42*). However, there are discrepancies regarding the opposing kinases that phosphorylate the serine and threonine residues which abound in their A and Z domains and which block the translocation of NF-AT proteins into the nucleus. Glycogen synthase kinase-3 has been reported to be responsible for the phosphorylation of these regions (*39*). Evidence has also been provided to implicate protein kinase CK1 and, in a secondary role, MEKK (*16*). Another report (*43*) has claimed that other kinases including JNK, ERK, p38, and CK2 may be involved in the phosphorylation of this region. In the work presented here, we have mapped and studied the regulatory region through the synthesis of a large number of peptides in an attempt to define the structural features that allow CK1 phosphorylation.

The results obtained tell us that the Z domain of our peptides was not significantly phosphorylated by CK1. This result is at variance with the conclusions of the publication of Zhu et al. (*16*), who claimed that CK1 strongly phosphorylated this domain. We cannot readily explain this discrepancy except with the obvious statement that our methods and preparations were different. The most important difference was that they used the A2 domain expressed as a GST fusion protein and a 165-amino acid recombinant fragment of NF-AT4 (aa 187−351) starting downstream from the linker region and extended on its C-terminal side to also include NLS (see Figure 1). The analysis of a family of peptides encompassing the A region together with the linker (residues 186−203) that joins the A and Z domains rendered some new insights about the complexity of factors that determine the specificity of CK1 phosphorylation. The linker region has a single serine residue (194) that has a consensus sequence for CK1. This residue has only a moderate influence in the capacity of the parent peptide (ALZ-1) to

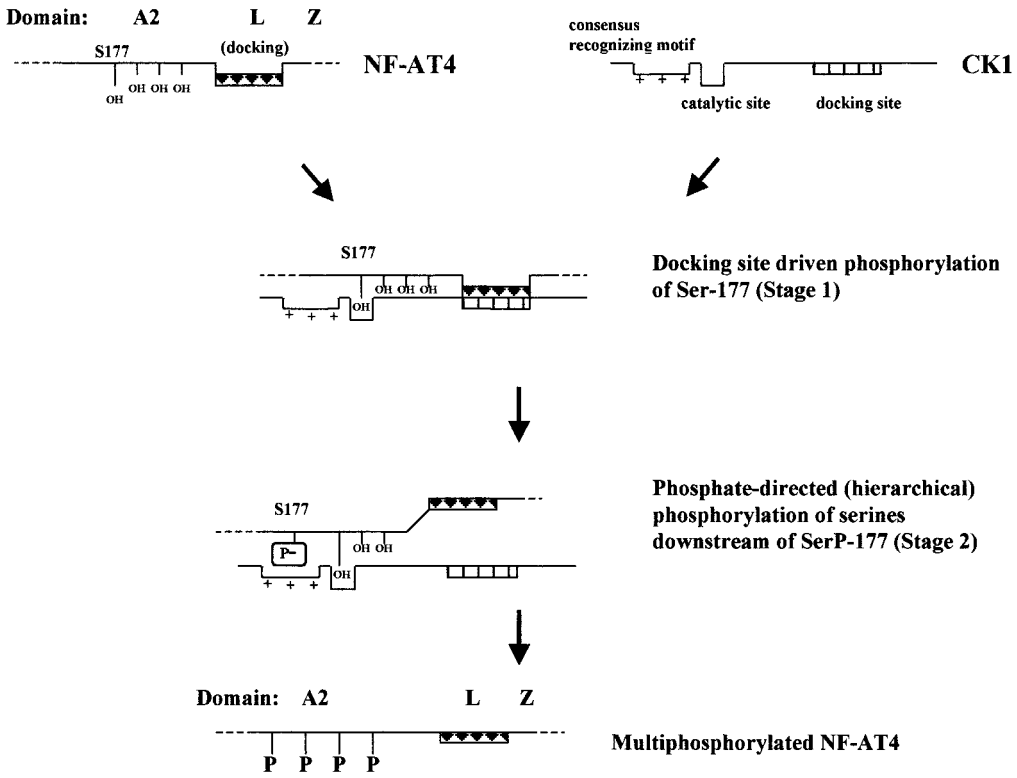


FIGURE 7: Schematic representation of a two-stage mechanism of phosphorylation of NF-AT4 peptides by protein kinase CK1. As a first step, NF-AT4 and CK1 interact through docking elements outside their phosphoacceptor and catalytic sites, respectively. In NF-AT4, this element is included in the linker region between domains A and Z. Association makes possible the positioning of NF-AT4 Ser177 into the catalytic site of CK1, and its subsequent phosphorylation despite Ser177 does not fulfill the consensus sequence for CK1. Once Ser177 is phosphorylated, a canonical pS-X-X-S consensus is created which primes the hierarchical phosphorylation of downstream serines. This takes place independently of the association between CK1 and NF-AT4 through their docking sites.

serve as a substrate for CK1, and because of this fact, the majority of the studies were carried out with peptides that contained a substitution of Ser194 by alanine. Although the linker that contained this substitution was not phosphorylated, it did have a major positive influence on the efficiency of the peptides containing the A2 region to serve as substrates for CK1, provided its central acidic cluster is not substituted by neutral residues. Because the linker region is rather distant from the phosphorylation site and downstream from it, whereas the specificity determinants for CK1 are located upstream, it may be considered as a functional docking site for CK1 rather than as part of the phosphoacceptor site itself.

The presence of such a downstream docking site seems to be especially required for the phosphorylation of the serines in the A2 region, which are targets for CK1 despite the fact that their amino acid context does not conform to the consensus for the enzyme.

Interestingly, in fact, once Ser177 is phosphorylated, the presence of the linker region or its acidic cluster becomes irrelevant, and the hierarchical phosphorylation of downstream residues, which is a typical property of CK1, proceeds equally well also in the absence of the linker region.

In summary, these data allow us to dissect the phosphorylation of the peptides that contain NF-AT4 regulatory sequences into two stages (see the scheme in Figure 7). In the first stage, the CK1 binds to a docking site located in the linker region. The binding to this site is dependent on a cluster of acidic residues in it and somehow positions the enzyme for efficient phosphorylation of the noncanonical site Ser177. Although the docking site intriguingly resembles a canonical phosphoacceptor site of CK1 by having a cluster of acidic residues upstream from the Ser194, several arguments suggest that it is not recognized as a canonical consensus by the catalytic site of CK1. First, the phosphorylation of Ser194 is quite modest. Second, phosphate incorporation mainly occurs at residues (notably Ser177) which are located far away upstream (and not downstream) from the acidic cluster that spans residues 191−199. Third, and most important, the association between the peptides that contain the linker region and CK1 cannot be overcome by canonical peptide substrates, whose phosphorylation is inhibited by peptides AL-9 and AL-11 in a noncompetitive way. Therefore, while the structural element of CK1 responsible for the binding of the linker region is presently unknown, it is likely to be outside the catalytic site; the possibility that it might coincide with the basic loop shown to interact with members of the centaurin-α family (*44*) will be taken into account.

In the second stage, Ser177, once phosphorylated, greatly enhances the efficiency of the enzyme to move downstream, phosphorylating other serines of the A2 region according to a hierarchical mode of phosphorylation typical of CK1. It is interesting to note that another kinase that has been known to participate in hierarchical phosphorylation, protein kinase CK2, has also been implicated in the phosphorylation of an equivalent region of NF-ATc (*43*).

It is also possible that, in an in vivo situation, the presence of the phosphates introduced by CK1 in region A2 may induce a hierarchical phosphorylation by GSK-3 of the A-1 region serines in a third hypothetical stage. Indeed, GSK-3

has also been identified as a phosphate-directed kinase, whose action is specified by phosphorylated residues located on the C-terminal side of its target serines and threonines (*16*, *45*, *46*). It is, therefore, possible that the existing discrepancy between those who claim that either CK1 or GSK3 are responsible for phosphorylating this regulatory region of NF-AT proteins may be resolved by future data showing that both of these kinases participate in a complementary of synergistic fashion.

ACKNOWLEDGMENT

The skillful technical assistance of Ms. Maria Patrizia Schiappelli is gratefully acknowledged.

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BI0112309