Tyrosine Versus Serine/Threonine Phosphorylation by Protein Kinase Casein Kinase-2

A STUDY WITH PEPTIDE SUBSTRATES DERIVED FROM IMMUNOPHILIN Fpr3*

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Protein kinase casein kinase-2 (CK2) is a spontaneously active, ubiquitous, and pleiotropic enzyme that phosphorylates seryl/threonyl residues specified by multiple negatively charged side chains, the one at position \( n + 3 \) being of crucial importance (minimum consensus S/T-x-x-E/D/S(P)/T(P)). Recently CK2 has been reported to catalyze phosphorylation of the yeast nuclear immunophilin Fpr3 at a tyrosine residue (Tyr184) fulfilling the consensus sequence of Ser/Thr substrates (Wilson, L.K., Dhillon, N., Thorner, J., and Martin, G.S. (1997) *J. Biol. Chem.* 272, 12961–12967). Here we show that, by contrast to other tyrosyl peptides fulfilling the consensus sequence for CK2, a peptide reproducing the sequence around Fpr3 Tyr184 (DEDADITY184DEEDYDL) is phosphorylated by CK2, albeit with much higher \( K_m \) (384 versus 4.3 \( \mu \)M) and lower \( V_{\text{max}} \) (8.4 versus 1,132 nmol/min\(^{-1}\), mg\(^{-1}\)) than its derivative with Tyr184 replaced by serine.

The replacement of Asp at position \( n + 1 \) with alanine and, to a lesser extent, of Ile at \( n - 1 \) with Asp are especially detrimental to tyrosine phosphorylation as compared with serine phosphorylation, which is actually stimulated by the Ile to Asp modification. In contrast the replacement of Glu at \( n + 3 \) with alanine almost suppresses serine phosphorylation but not tyrosine phosphorylation. It can be concluded that CK2 is capable to phosphorylate, under special circumstances, tyrosyl residues, which are specified by structural features partially different from those that optimize Ser/Thr phosphorylation.

Protein kinase CK2† (also termed "casein kinase-2 or II") is a ubiquitous Ser/Thr-specific protein kinase, essential for cell viability, independent of second messengers or phosphorylation for activation, generally composed of two catalytic (\( \alpha \) and/or \( \alpha' \)) and two noncatalytic \( \beta \) subunits and implicated in cell proliferation and transformation (for review, see Refs. 1–5).

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‡ The abbreviations used are: CK2, casein kinase 2; Fmo, N-(9-fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; PKA, protein kinase A.

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addition to the crucial acidic determinant at \( n + 3 \) several other acidic residues nearby. Consequently a peptide substrate reproducing this sequence was also phosphorylated by CK2 at Tyr\(^{184} \). Such a phosphorylation was improved by previous phosphorylation of the seryl residue at position \( n + 2 \) or by its replacement with a glutamic acid, suggesting that in this special case the determinant at position \( n + 2 \) might play a relevant role. The relevance of the other residues nearby, however, was not assessed nor was the phosphorylation of the tyrosyl peptide compared with that of canonical seryl peptide substrates of CK2. Here we show that the phosphorylation efficiency of the Fpr3 tyrosyl peptide is very low compared with that of its seryl counterpart, both in terms of \( V_{\text{max}} \) and \( K_m \) and depending on structural features that are partially distinct from those determining the phosphorylation of canonical substrates.

**Experimental Procedures**

**Materials**—DEAR-cellulose filters (NA45) were from Schleicher & Schuell. P51 phosophocellulose papers were from Whatman. \( [\gamma-^3\text{P}]\text{ATP} \) (3,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. All other reagents were from Sigma. Native CK2 was purified from rat liver cytosol as described previously (24). Recombinant forms of CK2 either wild type or its mutants defective in substrate recognition were obtained as described elsewhere (16).

**Synthetic Peptides**—The tyrosyl peptides DEDADYDEDEDDYDL, DEDADDYID(p)EDYDL, DEDADDYAEDEYDL, DEDADDYDAEDYDL, DEDADDYDEDEYDL, DEDADDYDDDD, and RRRADDDDDDD as well as their seryl analogues DEDADDYDDDL, DEDADDYAEEDYDL, DEDADDYDEEDYDL, and DEDADDYDDDD were synthesized by an automated peptide synthesizer ABI 431-A (Applied Biosystems) on 4-hydroxymethyl-phenoxymethylpolyostirene-1% divinylbenzene resin (1.04 mmol/g). The synthesis of the peptides EEEEEEEEEE and PEGDYEEEEE was described previously (19, 25). The synthesizer was equipped for using Fmoc chemistry (26) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole protocol (27) in a 0.05 mmol scale.

The incorporation of phosphate into the Ser residue has been performed according to a building block approach using Fmoc-Ser(P(O(benzyl))OH (from Novabiochem) during the synthesis.

Cleavage of peptides from the resin and side chain deprotection was carried out by treatment of the peptide resin with trifluoroacetic acid/anisole/ethanedithiol/ethylmethylsulfide (95:3:1:1). In the presence of the benzyl protecting group on phosphoserine a mixture of trifluoroacetic acid/trimethylpropylsilane/water (95:2.5:2.5) was preferred.

The crude peptides were purified by high-performance liquid chromatography (HPLC) on a preparative reverse phase column Prep NovaPak HR C18, 6 μm, 25 × 10 mm (Waters). The analytical HPLC and matrix-assisted laser desorption ionization/time of flight mass spectrometry analysis of the purified peptides showed a correct sequence and a >95% purity.

**Phosphorylation Assay**—Synthetic peptide substrates (0.5 mm) were phosphorylated by incubation in a medium (25 μl final volume) containing 50 mM Tris-HCl buffer, pH 7.5, 10 mM MgCl\(_2\), 50 mM KCl, and 100 μM [\( \gamma-^3\text{P} \)]ATP (specific radioactivity 500–1,000 cpm/pmol) unless otherwise indicated. The reaction was started with the addition of the enzyme (0.23 μg of either native or recombinant CK2) and stopped by the addition of 10 μl of 0.25 M EDTA and cooling in ice. Phosphorylation of Fpr3-derived synthetic peptides was evaluated according to the procedure described by Wilson et al. (23) by using DEAE-cellulose filters. Phosphorylation of RRRADDDDDDDD and of its tyrosyl derivative was quantitated by the phosphocellulose paper procedure (28). Control experiments were also performed with both types of peptides by determining phosphate incorporation after conversion of \( [\gamma-^3\text{P}]\text{ATP} \) into P, and phosphomolybdc complex extraction as described previously (29).

**HPLC Separation of Tyrosine-phosphorylated Fpr3 Peptide**—The peptide DEDADYDEDEDDYDL, previously phosphorylated by CK2 as described above, was loaded onto a SuperPac Pep-5 (Amersham Pharmacia Biotech) C2/18 (5 μm) column (4.6 × 250 mm). Elution was carried out at a flow rate of 1 ml/min with a gradient of acetonitrile in 0.08% trifluoroacetic acid from 0 to 40% for 30 min and then from 40 to 100% for 10 min. The effluent was monitored by absorbance measurements at 220 nm. The radioactivity of the fractions collected (1 ml) was determined by counting in a liquid scintillation apparatus (Canberra-Packard).

**Results**

The time courses for CK2-catalyzed phosphorylation of the Fpr3 peptide with Glu at position \( n + 2 \) relative to Tyr\(^{184} \) instead of serine and of its derivative where Tyr\(^{184} \) was replaced by serine are compared in Fig. 1A. It can be seen that the phosphorylation of the tyrosyl peptide is negligible as compared with that of its seryl counterpart, especially after short incubation times. By prolonging incubation the phosphorylation of the seryl peptide reaches a plateau when ATP is nearly exhausted, whereas the phosphorylation of the tyrosyl peptide slowly increases up to 2 h of incubation. By increasing ATP concentration (1 mM) over that of the peptides (0.5 mM) a stoichiometric phosphorylation of the seryl peptide was attained after 30 min of incubation, whereas the phosphorylation of the tyrosyl peptide was still far from stoichiometric (around 0.03 mol/mol) after 2 h (not shown). Albeit slow, the phosphorylation of the Fpr3 tyrosyl peptide was quite significant especially if compared with that of another tyrosyl peptide featured after the optimal peptide substrate routinely used for assaying CK2 (Fig. 1B). In this case, no detectable phosphorylation of the tyrosyl peptide could be observed, although that of its seryl counterpart was even faster than that of the seryl derivative of the Fpr3 peptide. The stoichiometry of the Fpr3 tyrosyl peptide phosphorylation was confirmed by HPLC where the phosphodervative can be resolved from the nonphosphorylated peptide (Fig. 2), thus allowing their relative quantitation (~3% versus 97%).

The suspicion that tyrosine phosphorylation in the Fpr3 peptide might rely on structural features different from those described above was corroborated by the finding that two other tyrosyl peptides, in addition to RRRADDDDDDDD, namely EEEEEEEEEE and PEGDYEEEEE, are totally unaffected by CK2 despite the fact that they fulfill optimal conditions for Ser/Thr phosphorylation (see Table 1).

To shed light on the uncommon phosphorylation of tyrosine

**Fig. 1.** Time course phosphorylation of seryl peptides and their tyrosyl counterparts by CK2. Phosphorylation was performed for the times indicated and evaluated as described under “Experimental Procedures.” The concentrations of ATP and of peptide substrates were 0.2 mM and 0.5 mM, respectively.

**Molecular Modeling**—Calculations were made on an Indy R4000 (Silicon Graphics) workstation. For human CK2α subunit modeling, the crystal structure of Zea mays CK2α was used (Protein Data Bank number 1A60). The two sequences were aligned using the Insight II package (Molecular Simulation Inc.) with the protein homology tools. The elaboration of the model of human CK2α, the prelu modeler (30) was used. Five structures were obtained from Modeler, and the one with the highest structural homology with respect to maize CK2α was used further. The backbone of the central part of the Fpr3 peptide (DIYDEE) has been shaped using as a template the equivalent sequence of protein kinase inhibitor bound to the catalytic subunit of PKA (Protein Data Bank number 1atp). The peptide was manually placed in CK2α catalytic pocket avoiding physical interference with residues of the kinase. The obtained structure was minimized using the Discover module of Insight II package until the energy reached a minimum.

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To shed light on the uncommon phosphorylation of tyrosine
The peptide was phosphorylated by CK2 for 90 min as described under “Experimental Procedures” and directly subjected to HPLC analysis. The absorbance at 220 nm (A) and the radioactivity incorporated (B) are shown. The arrows denote the position of the phosphorylated (left) and unphosphorylated (right) peptide, respectively.

**TABLE I**

Kinetic constants for the phosphorylation of tyrosyl and seryl peptides by CK2

Phosphorylation conditions and evaluation of phosphate incorporated are described under “Experimental Procedures.” The data are the mean of at least three independent experiments with standard errors not exceeding 10%. Substituted residues relative to the reference peptide are in bold type. The residue phosphorylated is underlined.

| Peptide          | \( V_{\text{max}} \) | \( K_{M} \) | \( V_{\text{max}}/K_{M} \) |
|------------------|----------------------|-------------|-----------------|
| DEDADYDEEDYDL    | 8.4                  | 384         | 0.0216          |
| DEDADYDEcEDYDL   | 20.7                 | 370         | 0.0559          |
| DEDADYDEEYDL     | 8.4                  | 729         | 0.0115          |
| DEDADYDAEEDYDL   | 8.4                  | 740         | 0.0113          |
| DEDADYAEEDYDL    | 0.5                  | 787         | 0.0006          |
| DEDADDYDEEDYDL   | 2.1                  | 166         | 0.0128          |
| DEDADDYDEEDYDL   | ND                   | ND          | ND              |
| EEEEYEEEEEEEE    | ND                   | ND          | ND              |
| PEGDYEELE        | ND                   | ND          | ND              |
| DEDADISDEEDYDL   | 1132                 | 4.3         | 263.2558        |
| DEDADDISDEEDYDL  | 1311                 | 4.0         | 327.7500        |
| DEDADDISAEEDYDL  | 586                  | 66.0        | 8.5787          |
| DEDADISAEEDYDL   | 394                  | 125.0       | 3.1520          |

*ND = not determined due to undetectable phosphorylation.

in the Fpr3 peptide a number of derivatives with individual substitutions were synthesized and compared with the parent peptide. This latter includes a glutamic acid at position \( n+3 \), which is the main determinant for CK2 recognition (13) and an aspartic acid at position \( n+1 \) where also an acidic residue is very important (17). Position \( n+2 \) is occupied in the wild type peptide by a serine, whose primary phosphorylation by CK2 is believed to improve subsequent phosphorylation of tyrosine, because its replacement by alanine, but not by glutamic acid, was detrimental (23); thus it appeared that a negatively charged side chain at \( n+2 \), a position rather unimportant for serine phosphorylation (13), might be crucial for tyrosine phosphorylation. In contrast, position \( n-1 \) in the Fpr3 site is occupied by isoleucine, a rare feature in CK2 sites, where an acidic residue was shown to be preferred and is actually found in about 50% of the natural substrates (10). We therefore decided to check how much the substitution of this Ile by Asp, of Asp (+1) by Ala, of Glu (+2) by Ala or phosphoserine, and of Glu (+3) by Ala might affect phosphorylation by CK2. As shown by the time courses in Fig. 3, the most detrimental substitution (a part of Tyr<sup>184</sup>, confirming that this one and not the other tyrosine, Tyr<sup>198</sup> undergoes phosphorylation) is that of alanine instead of glutamic acid at \( n+1 \), followed by aspartic acid instead of isoleucine at \( n-1 \), whereas the replacement of the most important acidic determinant in canonical CK2 substrates, i.e. Glu at \( n+3 \), by alanine is in this case much better tolerated. In contrast to a previous report (23), the replacement of Glu at \( n+2 \) by alanine has no effect, whereas a phosphoserine in this position significantly improves phosphorylation (Fig. 3). As shown in Table I, the differences in phosphorylation outlined by the time courses of Fig. 3 are variably accounted for by changes in \( V_{\text{max}} \) and/or \( K_{M} \). Especially remarkable is the drop in phosphorylation efficiency due to both a dramatic decrease of \( V_{\text{max}} \) and an increase of \( K_{M} \) promoted by the Ala for Asp substitution at \( n+1 \). In comparison, the effect of a similar substitution at the "crucial" \( n+3 \) position, due to just a 2-fold increase in \( K_{M} \), is negligible. The detrimental effect of replacing Ile at \( n-1 \) with Asp (see Fig. 3), appears to be exclusively due to a drop in \( V_{\text{max}} \) (Table I).

Table I also highlights the striking superiority of the seryl derivative of the Fpr3 peptide over the tyrosyl peptide both in terms of \( V_{\text{max}} \) (>100-fold higher) and of \( K_{M} \) (almost 90-fold lower), and it corroborates the view that the specificity determinants are different with the two peptides. In fact the replacement of Ile at \( n-1 \) by Asp, far from being detrimental, is actually improving the phosphorylation efficiency of the seryl derivative and the replacement at position \( n+3 \) is more detrimental than that at \( n+1 \).

The particularly crucial role of the acidic determinant at position \( n+1 \) as opposed to that at position \( n+3 \) in determining tyrosine phosphorylation was also confirmed by experiments with CK2 mutants, which are defective in the recognition of individual determinants. As shown in Fig. 4, mutant K198A with reduced ability to bind the determinant at position +1 (17) is much more defective toward the tyrosyl peptide than...
compared with the dramatic drop of efficiency promoted by Asp of Ile (substrate recognition.

peptides by wild type CK2 and CK2 mutants defective in sub-

effectors and/or experimental conditions, which are known to

specificity of CK2 appears not to be affected by a number of

n cluster, which is responsible for tight interaction with the

side chain of isoleucine, is not considered.

1 aspartate at position

substrate side chains is the one between the carboxylate of

respectively) (32). The only evident interaction with peptide

between PKA and a seryl peptide substrate (2.7 and 3.0 Å,

PKA (31) has been placed into the active site of CK2
detergent to the peptide during the catalytic event (32). The distances between the phosphoac-
tceptor hydroxyl group of tyrosine and either Asp156 (2.67 Å) or

Lys168 believed to bridge the phosphate to the peptide during

addition of 336 nM polylysine to the holoenzyme (about 2-fold)

DISCUSSION

Nearly all members of the huge eukaryotic protein kinase

family fall into two categories, Ser/Thr- and Tyr-specific en-
zymes, respectively (33). In most instances the borderline be-
tween these two groups, empirically drawn using substrates of
the two sorts, is quite sharp and clear-cut, being also based on
differences in primary structure motifs (34). In a few instances,
however, protein kinases may display a dual specificity in that
they are able to phosphorylate both Ser/Thr and Tyr residues
(35). The only known sensu stricto dual specificity protein ki-
nases are probably those of the mitogen-activated protein ki-
nase kinase family, which are highly dedicated kinases that
catalyze the simultaneous phosphorylation of both Thr and Tyr
in a conserved sequence, T-x-Y present in the activation loop
of mitogen-activated protein kinases. In several instances, how-
ever, bona fide Ser/Thr protein kinases have been reported to
display tyrosine kinase activity as well under certain circum-
cstances, detectable either as autophosphorylation or toward
trans substrates. In general the latter is negligible compared
with that observed toward canonical Ser/Thr sites. The list of
these Ser/Thr kinases exhibiting low Tyr kinase activity as well
includes among others phosphorylase kinase (36), calcium-cal-
modulin-dependent protein kinase II and Spk1 (37), CK1 (38,
39), CK2 (40), and even PKA (37). Conversely it should be noted
that also a classical tyrosine kinase like Src can phosphorylate
aliphatic as well as aromatic alcohols in a stretch of peptide
(41). It is likely therefore that in several instances this hardly
detectable “dual” specificity of bona fide Ser/Thr protein ki-
nases is a mechanistic curiosity devoid of practical conse-
quences. That this may not always be the case, however, is
supported by the observation that in lower eukaryotes, like
yeast, where bona fide tyrosine kinases are absent, tyrosine-
specific protein phosphatases are nevertheless present (22),
tyrosine phosphorylation of proteins other than kinases of
the mitogen-activated protein kinase family has been reported
to take place. It is tempting therefore to assume that tyrosine
phosphorylation by bona fide Ser/Thr kinases is a physiologi-
cally relevant event in these cases.

A notable example of this situation is provided by immu-
nophilin Fpr3, an abundant nuclear protein, a member of the
FK506-binding subfamily of immunophilins (42), which is
a substrate of S. cerevisiae protein-tyrosine phosphatase Ptp1.
Phosphotyrosine dephosphorylation by Ptp1 prevents nucleo-
ar accumulation of Fpr3 in vivo (21). The tyrosyl residue
phosphorylated in Fpr3 was identified as Tyr184, residing in a
highly acidic sequence fulfilling optimal conditions for CK2
catalyzed phosphorylation, and indeed CK2 was shown to be
responsible for Tyr phosphorylation of Fpr3 and to be able to
phosphorylate in vitro a tyrosyl peptide reproducing the se-
quence around Tyr184 (33). This observation would include CK2
in the growing list of sensu lato dual specificity protein kinases,
despite the previous failure to demonstrate any activity of CK2
toward tyrosyl peptides fulfilling its consensus sequence. This
prompted us to undertake a study of the structural features
underlying tyrosine phosphorylation by CK2 and to compare
this activity with that toward canonical Ser/Thr substrates.
The main outcomes of this investigation are the following.

1) The phosphorylation of Fpr3-derived tyrosyl peptide, al-

![Figure 4. Phosphorylation of tyrosyl versus seryl Fpr3-derived peptides by wild type CK2 and CK2 mutants defective in substrate recognition.](Image)
beit quite significant, is negligible compared with that of an identical peptide where tyrosine is replaced by serine. This shows that even in this case serine is by far preferred over tyrosine by CK2. The superiority of the seryl peptide is due to both much lower $K_m$ and much higher $V_{\text{max}}$. The low $V_{\text{max}}$ of the tyrosyl peptide suggests that its phosphorylated product leaves the active site of the kinase very slowly, a situation that could give rise to remarkable product inhibition. Also, consistent with this interpretation would be the low phosphorylation stoichiometry of the tyrosyl peptide ($\leq 5\%$). It is possible that in vivo the kinetics of Tyr$^{184}$ phosphorylation are improved either by features intrinsic to the full-length Fpr3 protein conformation or by the fast translocation of phospho-Fpr3 to separate compartment(s). In this respect a comparison between the kinetic constants of Tyr$^{184}$ peptides and those of full-length Fpr3 would be misleading since Tyr$^{184}$ is just a minor Fpr3 phosphoacceptor site (23); consequently the parameters calculated with Fpr3 would mainly reflect the phosphorylation of residues other than Tyr$^{184}$. It is worth noting, however, that a significant phosphorylation of Tyr$^{184}$ by CK2 is observable under conditions where GST-Fpr3 is about 1 $\mu M$ (23), although at this concentration the phosphorylation of the best peptide substrate, whose $K_m$ is 370 $\mu M$, would be flatly undetectable. It is likely therefore that indeed the phosphorylation efficiency of Tyr$^{184}$ is improved by features that are lacking in the peptides used in our study. On the other hand the reason for the discrepancy between our data and that reported by Wilson et al. (23) where a 1 to 1 phosphorylation stoichiometry of the same peptide is reported to occur after less than 2 h of incubation with recombinant human CK2, is not clear. It should be noted, however, that the stoichiometry reported in that paper is not consistent with the experimental conditions used, where ATP and the peptide are 100 and 580 $\mu M$, respectively, allowing at best a 20% phosphorylation stoichiometry.

2) CK2 activity ratio toward the tyrosyl versus the seryl derivative of the Fpr3 peptide is not significantly affected by either the subunit composition of CK2 (catalytic subunit versus holoenzyme), or the addition of polycationic stimulators, or the nature of the phosphate donor (GTP versus ATP) and of the activatory cation (Mn$^{2+}$ versus Mg$^{2+}$). This last outcome is worth noting, because in the case of phosphorylase kinase, Mn$^{2+}$ instead of Mg$^{2+}$ is required to see tyrosine-phosphorylating activity (36).

3) Ability to phosphorylate tyrosine is not a general feature of CK2 toward any kind of substrate fulfilling its consensus sequence, because the replacement of serine (or threonine) by tyrosine in several outstanding peptide substrates of CK2 different from the Fpr3-derived peptide rendered their phosphorylation undetectable.

4) Among the local structural determinants favoring tyrosine versus serine phosphorylation, the isoleucine at position $n-1$ and the acidic residue at position $n+1$ appear to play a prominent role. Interestingly, hydrophobic residues are only sporadically found in CK2 substrates at position $n-1$, where
instead of acidic residues are quite frequent (10). Consequently, the replacement of Ile by Asp at this position in the seryl derivative of the Fpr3 peptide increases its phosphorylation efficiency, but it is detrimental in the tyrosyl peptide. The molecular basis for such an opposite behavior is presently unclear. The favorable effect of an acidic residue at position \( n + 1 \), on the other hand, is a well known feature of all CK2 phosphoacceptor sites, including Ser/Thr (10, 12). In the latter case, however, the \( n + 1 \) acidic determinant is generally less important than the one at \( n + 3 \) (10, 12), whereas the opposite applies to the Fpr3 tyrosyl peptide where the replacement of the \( n + 3 \) Glu by alanine is exceptionally well tolerated. Computer-aided modeling (Fig. 5) corroborates the concept that in the case of Fpr3-derived tyrosyl peptide the acidic determinant at \( n + 1 \) is much more important than the one at \( n + 3 \) in making contacts with the enzyme. The replacement of glutamate at \( n + 2 \) with phosphoserine improves 2–3-fold the \( V_{\text{max}} \) and phosphorylation efficiency of the peptide, consistent with the knowledge that phosphoserine is a better determinant than Glu especially at positions \( n + 1 \) and \( n + 2 \) (14); it is quite possible therefore that, as suggested by others (23), the phosphorylation of Fpr3 \textit{in vivo} is enhanced by a hierarchical effect where previous phosphorylation of Ser\(^{186} \) will improve subsequent phosphorylation of Tyr\(^{184} \). The replacement of Glu at \( n + 2 \) with Ala, however, has only a modest effect, similar to that caused by the same substitution in canonical seryl peptide substrates of CK2 (12). Therefore, it seems unlikely that the phosphorylation of Tyr\(^{184} \) in the Fpr3 peptide is entirely dependent on the presence of a negatively charged side chain at \( n + 2 \), as suggested by others (23). The reasons why the same peptide, DEDADID\( \Delta \)EDYDL, which in our hands is nearly as good as the parent peptide with Glu instead of the underlined Ala (see Fig. 3 and Table I), was not phosphorylated at all (23) is unclear to us.

5) The different mode of binding of tyrosyl \textit{versus} seryl peptide substrates was also corroborated by experiments with CK2 mutants with a reduced ability to recognize individual determinants. Especially remarkable is the behavior of the mutant K198A, which is much more defective toward the Fpr3 tyrosyl peptide as compared with its seryl derivative.

In conclusion, CK2 can be legitimately included in the list of \textit{sensu lato} dual specificity protein kinases. Clearly, however, its ability to phosphorylate tyrosine in peptide substrates is very modest as compared with its canonical Ser/Thr kinase activity and, more important, it depends on structural features that are partially different from those determining Ser phosphorylation. Among these the most striking are the absolute requirement for the acidic determinant at position \( n + 1 \) as opposed to the tolerance toward the replacement of the one at position \( n + 3 \), and the preference for a hydrophobic over an acidic residue at position \( n - 1 \).