Substrate specificities for yeast and mammalian cAMP-dependent protein kinases are similar but not identical

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The substrate specificity of the cAMP-dependent protein kinase (cAPK) from *Saccharomyces cerevisiae* has been investigated using synthetic peptides corresponding to the local phosphorylation site sequence around Ser-230 in the yeast transcriptional activator ADR1. ADR1 is required for the expression of the glucose-repressible alcohol dehydrogenase. Yeast cAPK (encoded by the *TPK1* gene) phosphorylated Ser-230 in the synthetic peptide ADR1-217–234, VRKKYKL窖LRRAFSQ-NH₂, with a *K₅₅* of 5.3 μM compared with 46 μM for *LRRAFSQ* (Kemptide). Porcine heart cAPK phosphorylated the ADR1 peptide and Kemptide with the considerably lower *K₅₅* values of 0.23 and 1.6 μM, respectively. These results indicate that the ADR1 peptide is an excellent substrate for cAPK. Both the yeast and mammalian protein kinases qualitatively shared a number of substrate specificity determinants in common involving residues on the proximal NH₂-terminal side and up to the +4 position of the COOH-terminal side of the phosphoacceptor. The mammalian enzyme, however, had a much higher affinity for its substrates than did the yeast enzyme. In addition, the yeast and mammalian enzymes displayed several quantitative differences in their preferences for particular peptide substrates. In particular, the mammalian enzyme strongly preferred substrates with NH₂-terminal extensions beyond the –4 position relative to the phosphoacceptor. These results suggest that all eukaryotic cAPKs recognize similar but not identical substrate specificity determinants. They also suggest that the different affinities for substrates that inhere to the individual enzymes could influence their physiological roles.

Yeast and mammalian cAMP-dependent protein kinases (cAPKs) exhibit 50% sequence similarity in their catalytic domains (1). Whereas the substrate specificity determinants for mammalian cAPK have been analyzed extensively the specificity determinants for the yeast enzyme are poorly understood. The yeast enzyme can phosphorylate *LRRAFSQ* (Kemptide) as can the mammalian enzyme (2), and several of the RRXS sequences which have been shown to be very important to mammalian cAPK substrate recognition (3–5). Since the mammalian cAPK is physiologically interchangeable with the yeast enzyme, both enzymes must recognize an overlapping set of determinants (6). The mammalian and yeast enzymes phosphorylate Kemptide with similar *K₅₅* values, but the mammalian cAPK phosphorylates it with a *K₅₅* that is 25-fold lower than that found using the yeast enzyme (2). This potential difference in substrate affinity between the two enzymes suggests that other differences in substrate specificity might be present.

We have used peptide analogs modeled on the cAPK phosphorylation site of a natural substrate, yeast transcriptional activator ADR1, for analyzing yeast cAPK substrate specificity determinants. ADR1 has been shown to be phosphorylated in vitro at serine 230 by yeast and mammalian cAPK, and genetic evidence suggests that this site is phosphorylated in vivo by yeast cAPK (3). Our findings indicate that the ADR1 peptides serve as excellent substrates for yeast and mammalian cAPK. In addition, they indicate that yeast and mammalian cAPKs recognize similar but not identical substrate determinants.

**MATERIALS AND METHODS**

**Synthesis of Peptides**—Peptides were synthesized as the free COOH-terminal amides by the Merrifield method (7) on an Applied Biosystems 430A peptide synthesizer. Peptides were purified by both ion-exchange and reversed-phase chromatography (8). All peptides eluted as a single peak following reversed phase HPLC using a acetonitrile gradient in the presence of 0.1% (v/v) trifluoroacetic acid. All peptides contained the expected ratios of amino acids as determined by amino acid analysis (9).

**Peptide Phosphorylation**—Phosphorylation of synthetic peptides was carried out as described previously (10) in a 40-μl reaction mix containing 50 mM MES, pH 6.8, 1 mg/ml bovine serum albumin, 0.25% Tween 20, 1 mM EGTA, 12.5 mM magnesium acetate, 14.7 μM 2-mercaptoethanol, 200 μM [*γ-32P*]ATP (1,000–3,000 cpm/pmol), and synthetic peptide. The *K₅₅* for ATP for the mammalian enzyme is 7.6 μM and for the yeast enzyme is 33 μM (2). Control experiments indicated that 200 μM ATP was saturating for the yeast enzyme. Incubations were at 30°C for 4 min for mammalian cAPK and 5 min for yeast cAPK at which time 20-μl aliquots were removed from the reaction mix and applied to P-81 paper (10). Mammalian and yeast cAPK concentrations were chosen such that all reactions were linear to at least 10 min, and not more than 10% of the substrate peptide was consumed. Phosphorylation of all peptides was stoichiometric as assessed by HPLC and kinetic analysis. Yeast cAPK (TPK1 subunit) was purified as described previously (2). Porcine heart cAPK was provided by Dr. S. Taylor (University of California, San Diego).

**Detection of Phosphorylated Residues**—The identity of the phos-
phorylated residues was determined to be serine for all peptides after partial acid hydrolysis and high voltage electrophoresis at pH 3.5 as described previously (9). For those peptides containing more than 1 serine residue, the phosphorylated peptides (about 40 nmol) were incubated overnight at 30 °C in 200 μl of 0.1 M NH₄HCO₃ containing 14 μg of chymotrypsin. Chymotryptic cleavage separated serine 230 from the other serine residues. The reaction was terminated with the addition of 0.1% trifluoroacetic acid, and the chymotryptic peptides were isolated on a Shimadzu HPLC system using gradient elution (0–60% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) over 30 min with a flow rate of 1 ml/min on a reversed-phase RP300 column (4.6 × 30 mm, 7-mm particle size, Brownlee). Amino acid analysis as described above was used to determine the composition of the chymotryptic peptides. 99% of the radioactive phosphate transferred was accounted for in the chymotryptic fragments containing serine 230.

RESULTS AND DISCUSSION

The ADR1 synthetic peptide ADR1-217–234 was phosphorylated at Ser-230 by yeast cAPK with a Kₘ that was 9-fold lower than that found for Kemptide (Table I). This result indicated that a synthetic ADR1 peptide could serve as a very good substrate for yeast cAPK. Several other ADR1 peptides were synthesized subsequently to investigate the features of yeast cAPK substrate specificity. The importance of residues to the NH₂-terminal side of residue 225 was studied using peptides ADR1-212–234 and ADR1-225–234. Basic residues at the −6 position relative to the phosphorylation site (compare ADR1–222–234 with ADR1–225–234) have been shown to be important for mammalian cAPK phosphorylation (11). As expected from the mammalian studies, peptide ADR1–222–234 was phosphorylated with a Kₘ that was 2-fold lower than that found for peptide ADR1–225–234. However, adding more residues to the NH₂-terminal side as in peptide ADR1–217–234 had little or no effect on the Kₘ of phosphorylation by yeast cAPK (Table I). The Vₘₐₓ values for these three peptides were comparable (Table I).

The importance of sequences to the COOH-terminal side of the phosphoacceptor was also analyzed. Peptide ADR1–217–231, which is 3 residues shorter at the COOH-terminal end than peptide ADR1–217–234, was phosphorylated with a Kₘ that was 2-fold higher than peptide ADR1–217–234. This result indicates that at least 2 residues on the COOH-terminal side of the phosphoacceptor are preferred. Adding 7 residues to the COOH-terminal end as in peptide ADR1–225–241 decreased the Kₘ of phosphorylation 2-fold relative to peptide ADR1–225–234 (Table I), indicating that yeast cAPK phosphorylation is affected by distal carboxyl-terminal residues. However, the Vₘₐₓ value for peptide ADR1–225–241 was found to be slightly lower than for ADR1–225–234.

To compare directly mammalian and yeast substrate specificities, the kinetic properties of the ADR1 peptide were analyzed using porcine heart cAPK. As observed previously (2) Kemptide had a Kₘ 20–30-fold lower with mammalian cAPK than with yeast cAPK (Table II). ADR1 peptides ADR1–217–234 and ADR1–222–234, which are much better substrates than Kemptide for yeast cAPK, also were phosphorylated with Kₘ values 20–30-fold lower with mammalian cAPK than with yeast cAPK (Table II). Indeed, ADR1 peptides are exemplary substrates for mammalian cAPK (with Kₘ values in the 200–300 nM region). Since the Vₘₐₓ values of mammalian and yeast cAPK for these peptides are similar (Tables I and II; Ref. 2), these results also suggest that the mammalian cAPK associates much better with its substrates than yeast cAPK.

Mammalian cAPK, like yeast cAPK, phosphorylated peptide ADR1–222–234 with a lower Kₘ than did peptide ADR1–225–234, confirming the importance of additional basic residues in the −6 and −7 positions (11). However, residues 222–225 were much more important to the mammalian enzyme than the yeast cAPK. Peptides extending to at least residue 222 were phosphorylated by the porcine heart cAPK with Kₘ values that were 3–5-fold lower and Vₘₐₓ values 2–4-fold higher than for peptides with shorter amino-terminal extensions (Tables I and II; also see Table III for Vₘₐₓ/Kₘ ratios). Sequences beyond −8 were not important for substrate recognition by mammalian cAPK (compare peptides ADR1–222–234 and ADR1–217–234) (Tables II and III). Mammalian cAPK also displayed a slight preference for at least 2 residues to the COOH-terminal side of the phosphoacceptor (see peptides ADR1–217–231 and ADR1–217–234) (Tables II and III). Sequences beyond +4, however, played no role in mammalian cAPK substrate recognition (compare peptides ADR1–225–234 and ADR1–225–241) although they were important for the yeast enzyme.

These analyses using peptides with varying lengths surrounding Ser-230 of ADR1 yield several conclusions. First, peptides modeled on ADR1 serve as much better substrates than Kemptide regardless of which protein kinase was studied. Second, mammalian cAPK differs significantly from yeast cAPK in its strength of binding to substrates. The mammalian enzyme generally displayed Kₘ values of phosphorylation 20–30-fold lower than that of the yeast cAPK (Table II). Third, although additional residues at the −8 through −5 position are important for substrate recognition for both the yeast and mammalian enzymes they play a much more important role for the mammalian enzyme than for the yeast cAPK (Tables I and III). Sequences further NH₂-terminal, however, seem to have little effect on substrate recognition for either enzyme. Also, yeast and mammalian cAPK prefer substrates with more than 1 residue on the COOH-terminal side of the phosphoacceptor site. Finally, the kinetic properties of yeast cAPK, in marked contrast to mammalian cAPK, were affected by substrates with COOH-terminal extensions beyond the +4 position.

ADRI peptide ADR1–222–234 was chosen for further study to analyze the importance of specific residues in yeast cAPK.

| Peptide    | Sequence            | Kₘ  | Vₘₐₓ        |
|------------|---------------------|-----|-------------|
| Kemptide   | LRRASLG             | 46 ± 5.5 | 14 ± 2.1 |
| ADR1–217–234 | VRKVLKLTRASFSAQ     | 5.3 ± 1.0 | 26 ± 1.7 |
| ADR1–222–234 | LKKLTRASFSAQ       | 6.8 ± 0.85 | 29 ± 1.8 |
| ADR1–225–234 | LTRASFSAQ          | 13 ± 2.4 | 35 ± 7.4 |
| ADR1–217–231 | VRKVLKLTRASF       | 11 ± 1.6 | 27 ± 0.0 |
| ADR1–225–241 | LTRASFSQSASSYAL    | 7.0 ± 0.35 | 20 ± 3.7 |
enzymes (Table IV). Interestingly, the leucine substitution at position 227, although having little effect on the peptide ADR1-222-234, dramatically affected peptide ADR1-222-234(P229) with a leucine substitution in the -1 position (P229) has little or no effect on mammalian cAPK phosphorylation of its peptide substrate. Similarly, yeast cAPK phosphorylation increased dramatically for both the yeast and mammalian enzymes (Table IV). In each case the $K_a$ for phosphorylation was particularly sensitive to the lysine substitution at position -2 (ADR1-222-234(K228)) (Table IV), suggesting that recognition of the -2 arginine plays a more important role in yeast cAPK recognition than it does for the mammalian enzyme. In contrast, the lysine substitution at position -3 dramatically affected the $V_{max}$ value for mammalian phosphorylation but had little effect on the yeast enzyme (Table IV). These differences suggest that the proteins do not share identical binding and active sites.

We tested an ADR1 peptide analog for its ability to inhibit cAPK activity. ADR1-222-234(A230) serves as a poor inhibitor of both mammalian and yeast cAPK (Table IV). The $K_a$ was 860 $\mu$M for the yeast enzyme and 150 $\mu$M for the mammalian cAPK. In contrast, the $K_a$ for the Kemptide inhibitor, which is replaced by alanine, is about 350 $\mu$M for mammalian cAPK (14). This value is only slightly greater than that for the ADR1 peptide inhibitor although the ADR1 peptide serves as a much better substrate than Kemptide. It also remains unclear why ADR1-222-234(A230) gives a $K_a$ for the yeast enzyme which is only 6-fold greater than that for the mammalian enzyme when the $K_a$ values for ADR1-222-234 differ by 26-fold.

Our finding that the yeast and mammalian cAPK share a number of recognition features suggest that all eukaryotic cAPKs recognize similar substrate determinants. Yet because of the several quantitative differences we detected, specific differences in peptide affinities and catalytic efficiencies do inhere to the individual enzymes. Because of the number of isoforms of cAPK found in individual cell types (for example, three in yeast), the physiological roles of these enzymes may depend not only on differences in abundance, location, regulation, and timing of expression but in subtle kinetic differ-

### Table II

**Comparison of mammalian and yeast cAPKs substrate specificities**

| Peptide | Yeast cAPK $K_a$ | Mammalian cAPK $K_a$ | Ratio of yeast to mammalian $K_a$ | V$_{max}$ | Mammalian $V_{max}$ | Ratio of yeast to mammalian $V_{max}$ |
|---------|-----------------|---------------------|---------------------------------|---------|----------------------|---------------------------------|
|         | $\mu$M          | $\mu$M              |                                  | $\mu$mol/min/mg | $\mu$mol/min/mg      |                                 |
| Kemptide| 46              | 1.6 ± 0.37          | 15 ± 0.98                        | 29      |                      |                                 |
| ADR1-217-234 | 5.3          | 0.23 ± 0.026        | 24 ± 1.0                         | 23      |                      |                                 |
| ADR1-222-234 | 6.8          | 0.26 ± 0.011        | 19 ± 1.8                         | 26      |                      |                                 |
| ADR1-225-234 | 13            | 1.3 ± 0.12          | 0.1 ± 1.1                        | 10      |                      |                                 |
| ADR1-217-231 | 11            | 0.39 ± 0.051        | 26 ± 1.1                         | 28      |                      |                                 |
| ADR1-225-241 | 7.0            | 1.4 ± 0.19          | 8.6 ± 0.80                       | 5.0     |                      |                                 |

### Table III

**V$_{max}$/K$_{max}$ ratios for yeast and mammalian cAPK**

| Peptide     | Yeast V$_{max}/K_{max}$ | Mammalian V$_{max}/K_{max}$ | Ratio of yeast to mammalian V$_{max}/K_{max}$ |
|-------------|-------------------------|----------------------------|-----------------------------------------------|
| Kemptide    | 0.30                     | 9.4                       | 31                                            |
| ADR1-217-234| 4.9                      | 104                       | 21                                            |
| ADR1-222-234| 4.3                      | 73                        | 17                                            |
| ADR1-225-234| 2.7                      | 4.7                       | 1.7                                           |
| ADR1-217-231| 2.5                      | 67                        | 27                                            |
| ADR1-225-241| 2.9                      | 6.1                       | 2.1                                           |
| ADR1-222-234(L227)| 0.0068               | 0.0064                   | 0.94                                          |
| ADR1-222-234(K228)| 0.10                  | 3.4                       | 34                                            |
| ADR1-222-234(R232)| 0.20                   | 2.4                       | 12                                            |
| ADR1-222-234(P229)| 5.1                    | 62                        | 12                                            |

### Table IV

**Influence of specific residues on yeast and mammalian cAPK peptide phosphorylation**

| Peptide     | Sequence     | Yeast cAPK $K_a$ | Mammalian cAPK $K_a$ | Yeast cAPK $V_{max}$ | Mammalian cAPK $V_{max}$ | Ratio of yeast to mammalian $K_a$ | Ratio of yeast to mammalian $V_{max}$ |
|-------------|--------------|-----------------|---------------------|----------------------|--------------------------|---------------------------------|---------------------------------|
| ADR1-222-234| LKLTRASFSQAQ | 6.8 ± 0.85      | 29                  | 0.26 ± 0.011         | 19                       | 26                              |                                 |
| ADR1-222-234(L227)| 2,500 ± 140 | 17 ± 3.5        | 170 ± 23            | 1.1 ± 0.12           | 15                       |                                 |
| ADR1-222-234(K228)| 3 ± 18      | 23 ± 4.0        | 3.5 ± 0.70          | 12 ± 1.4             | 66                       |                                 |
| ADR1-222-234(R232)| 140 ± 43    | 28 ± 2.0        | 6.6 ± 1.7           | 16 ± 1.2             | 21                       |                                 |
| ADR1-222-234(P229)| 4.9 ± 0.86  | 25 ± 2.6        | 0.34 ± 0.009        | 21 ± 2.9             | 14                       |                                 |
| ADR1-222-234(A230)$^*$| 860 ± 50    | 159 ± 33        | 5.7                 |                       |                          |                                 |

*The values represent the $K_a$ of inhibition.
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ences. Peptide analogs can be used to probe these differences although in general we do not expect large differences to be identified readily by this method. Since the three-dimensional structures of the mammalian and yeast cAPKs are presently being determined, both the similarities and differences in kinetic properties we have observed may assist in understanding the fine structure of the active site in its role in catalysis. Our results predict that the mammalian and yeast cAPKs will display significant differences in their peptide binding regions.

Our results also suggest that the construction of hybrid yeast and mammalian cAPKs may be used for identifying features of the proteins involved in recognition of substrates. They may, for example, aid in localizing regions of the cAPK involved in recognition of sequences to the NH₂- or COOH-terminal side of the phosphoacceptor and in characterizing the domains that endow the mammalian enzyme with a greater overall affinity for its substrates than the yeast enzyme.

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Note Added in Proof—The recently determined crystal structure of mouse cAPK (15) clarifies why the mammalian cAPK, in contrast to the yeast enzyme, strongly preferred peptide substrates containing a basic residue at the -6 position. The mammalian enzyme uses residue Glu-203 to contact the basic residue at -6 whereas the yeast enzyme has Asp-247 at the homologous position. It would be expected, therefore, that the mammalian enzyme would make stronger contact to the basic residue at -6 than the yeast enzyme. Explanations for the several other differences in phosphorylation between the two enzymes was not obvious from the crystal structure of the mouse enzyme.

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