Monoclonal Antibodies as Probes for Functional Domains in cAMP-dependent Protein Kinase II*

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The antigenic regions of the type II regulatory subunit of cAMP-dependent kinase from bovine heart have been correlated with the previously established domain structure of the molecule. Immunoblotting with both serum and monoclonal antibodies of fragments generated by limited proteolysis or chemical cleavage of the R-subunit established that the major antigenic sites were confined to the amino-terminal portion of the polypeptide chain (residues 1–145). Radioimmunoassays using two different antisera suggested that one or more of the high affinity serum antibody recognition sites were further restricted to residues 91–145.

This amino-terminal portion of the R-subunit includes the hinge region which is particularly sensitive to proteolysis, allowing the R-subunit to be cleaved readily into a COOH-terminal domain which retains the CAMP-binding sites and an NH2-terminal fragment which appears to be the major site for interaction of the R-subunits in the native dimer. Monoclonal antibodies that recognized determinants on both sides of this hinge region were characterized and their specific recognition sites localized.

Accessibility of antigenic sites in the holoenzyme in contrast to free R2 was compared. Although cAMP did tend to slightly increase the affinity of the holoenzyme for one of the monoclonal antibodies, all of the antigenic sites clearly were exposed and accessible in the holoenzyme. Furthermore, despite the presumed close proximity of antigenic sites to interaction sites between the R- and C-subunits, in no case did binding of antibody to the holoenzyme promote dissociation of the complex.

The fact that the monoclonal antibodies would precipitate holoenzyme as well as free R2 was used to ascertain the importance of specific amino acid residues in the interaction of the R- and C-subunits. cAMP-binding domains were isolated following limited proteolysis with chymotrypsin and thermolysin. These fragments differed by only three amino acid residues at the NH2-terminal end. Use of these fragments in conjunction with immunoadsorption established that the chymotryptic fragment, which contained the Asp-Arg-Arg preceding the site of autoprophosphorylation, was capable of forming a stable complex with the C-subunit. In contrast, the thermolysin fragment which differed by only those three residues no longer com-plexed with the C-subunit, indicating that the arginine residues not only contribute to the specificity of the phosphorylation site but also are an essential component for energetically stabilizing the holoenzyme complex.

The regulatory subunit of cAMP-dependent protein kinase has a well-defined domain structure that has been characterized by a variety of approaches. The domain structure was first apparent from distinct proteolytic fragments that were generated during purification (1). Limited proteolysis was utilized subsequently to more precisely define that domain structure. To summarize, these studies demonstrated that the regulatory subunit, normally present as a dimer, has a so-called "hinge" region which is susceptible to proteolytic cleavage by a variety of proteases (2). Following such limited proteolytic cleavage, each regulatory subunit is cleaved into two fragments, a COOH-terminal domain (31,000–37,000) which retains both of the high affinity cAMP-binding sites of the native protein but which no longer dimerizes and an NH2-terminal domain (10,500–19,000) which appears to retain the sites that are important for interaction between the two promoters of the native dimeric protein. More extended proteolysis suggested that the domain structure in the COOH-terminal cAMP-binding region of the molecule could be defined even further, since proteolytic fragments of molecular weights of 12,000–14,000 could be generated which retained the ability to bind cAMP (3).

The domain structure in the COOH-terminal region of the molecule was reinforced when the homology between the R-subunit and the CAMP-binding protein from Escherichia coli became apparent. This protein has two domains which correspond to a COOH-terminal DNA-binding domain and an NH2-terminal cAMP-binding domain (4). Sequence homology strongly suggested that the in-tandem gene duplicated regions in the COOH-terminal portion of bovine heart R (5) corresponded to two cAMP-binding domains which are both homologous to the cAMP-binding domain of the cAMP-binding protein of E. coli (6).

The antigenic properties of the R-subunit also have been characterized using a variety of methods. Although all R-subunits have the general domain structure described above, the major forms of cAMP-dependent protein kinase in cells are antigenically distinctive, and these differences are associated exclusively with the R-subunits (7, 8). In the present studies we have correlated the antigenic structure of the type

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1 The abbreviations used are: R, the regulatory subunit of cAMP-dependent protein kinase; C, the catalytic subunit of cAMP-dependent protein kinase; R2, type II R-subunit; MES, 2-(N-morpho-

ino)ethanesulfonic acid; PIA, radioimmunoassay; dansyl, 5-dimethyl-

amino naphtalene-1-sulfonyl; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.
II R-subunit with the overall domain structure of the molecule using serum antibodies as a more general probe and monoclonal antibodies (9) as probes for very specific regions of the molecule. Finally, the monoclonal antibodies have been used to define specific functional properties that are associated with isolated domains of the R-subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were purchased as follows: cytochrome c, Sigma Chemical Co., St. Louis, Mo.; [γ-32P]ATP (3000 Ci/mmol, in stabilized water), Amersham; chymotrypsin, L-1-tryosylamido-2-phenethyl chloromethyl ketone and L-1-tryosylamido-2-phenethyl chloromethyl ketone, Worthington Biochemicals, New Jersey; Staphylococcus aureus V8 protease, Miles Laboratories; nitrocellulose membranes (0.1 μm), Schleicher & Schuell; goat biotinyl anti-rabbit IgG (H + L), horse biotinyl anti-mouse IgG (H + L), avidin biotinylated horseradish peroxidase, Vector Laboratories, Inc. (Burlingame, CA); polyamide sheets and trifluoracetic acid (Sequenom grade), Pierce Chemical Co.; Pansorbin, Calbiochem-Behring; Nonidet P-40, Shell; iodoacetic acid and histone H11A, Sigma; acetonitrile (HPLC grade), Fisher; protein A-Sepharose, Pharmacia; [2,8-3H]cAMP (15 Ci/mmol), ICN; Cytocint, West Chem (San Diego, CA).

**Protein Purifications**—The regulatory subunit of type II cAMP-dependent protein kinase was purified from porcine cardiac tissue as described previously (11) except that it was eluted from CAMP-Sepharose with buffer containing 25 mM cGMP. Bovine R1 was prepared in a similar fashion. The C-subunit of type I cAMP-dependent protein kinase, purified from bovine lung (13), was provided by Gill (Department of Medicine; University of California, San Diego).

**Radioimmunoassay of R1**—All radioimmunoassays were performed as described previously (17) except that bovine heart R1 was used as the competing label.

**Isolation of Peptides for Localization of Antigenic Sites**—[^32P]-labeled R1 (3 mg) was dialyzed in 50 mM NH4HCO3 (pH 8.3), 0.5 mM EDTA, and carbamylmethylated as described by Kerlavage and Taylor (15). The protein was extensively dialyzed versus 50 mM NH4HCO3 (pH 8.3), 2 mM EDTA, and digested with 50:1 S. aureus V8 protease for 18 h at 37 °C in the presence of 20 μg of 1-chloro-3-tryosylamido-7-azino-2-heptanone. Subsequent cleavage of the immunoreactive S. aureus V8 peptide was performed with 50:1 l-1-tryosylamido-2-phenethyl chloromethyl ketone-trypsin for 18 h at 37 °C. Peptides were separated by HPLC as outlined below.

**Preparation of Type II Holoenzyme**—Type II regulatory (or cAMP-binding fragments) and catalytic subunits, purified to homogeneity from porcine heart, were combined in equal weights and dialyzed for 24 h at 4 °C versus 10 mM potassium phosphate (pH 6.5), 1 mM EDTA, 10 mM 2-mercaptoethanol. To remove excess C and verify holoenzyme formation, the complex was bound to a column of DEAE-Sephacel (2 ml) equilibrated with 40 mM potassium phosphate (pH 6.5), washed with the same buffer, and eluted with a 200-mM linear gradient of 40-250 mM potassium phosphate (pH 6.5), containing 1 mM EDTA and 10 mM 2-mercaptoethanol. Fractions exhibiting both phosphotransferase activity and CAMP-binding activity were pooled and concentrated.

**RESULTS**

In order to correlate the antigenic properties of R1 with its domain structure, fragments were prepared following proteolysis with a variety of proteases. The proteases used were chymotrypsin, thermolysin, and S. aureus V8 protease, and the specific sites of cleavage for these proteases are indicated in the figure legends.
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Fig. 1. Cleavage scheme for production of domains of the type II regulatory subunit of cAMP-dependent protein kinase. The fragments derived from limited proteolysis and cyanogen bromide digestion are shown in the sequence of RII. Sites for limited proteolysis by chymotrypsin (Ch), trypsin (T), thermolysin (Th), and S. aureus V8 protease (St) are indicated by arrows in the hinge region. P, auto-
phosphorylation site; cAMP, residue modified by 8-Ns-cAMP; N, NH2-terminal and C, COOH-terminal ends of the polypeptide chain.

Fig. 2. Formation of functional domains in RII by limited proteolysis. Coomassie staining of protein in 15% polyacrylamide gels is as follows: lane 1, 32P-phosphorylated bovine heart RII; lane 2, limited proteolysis with chymotrypsin; lane 3, limited proteolysis with S. aureus V8 protease. Lanes 4–6 show autoradiographs of lanes 1–3, respectively.

in Fig. 1. These sites of cleavage were verified by sequencing the NH2-terminal residues of the larger isolated domains. Visualization of these proteolytic fragments and correlation with the site of autophosphorylation are shown in Fig. 2. In contrast to digestion with chymotrypsin, thermolysin, and trypsin, proteolysis with the S. aureus V8 protease results in the autophosphorylation site being retained with the smaller NH2-terminal fragment. Fragments also were generated by chemical cleavage with cyanogen bromide, and the results likewise are summarized in Fig. 1.

Immunoblotting following gel electrophoresis was used as a tool for qualitatively identifying immunoreactive proteins and peptide fragments whereas RIAs were utilized for a more quantitative measure of cross-reactivity. The serum antibodies were generated against porcine heart RII and were specific for RII versus other functionally related proteins. Immunoblotting with the serum antibodies showed no cross-reactivity with porcine RII or C or with bovine cGMP-dependent protein kinase (data not shown). These polyclonal antibodies were used initially to ascertain whether the R-subunit contains select regions that are preferentially antigenic. Antigenicity was monitored by immunoblotting following polyacrylamide gel electrophoresis in the presence of SDS as described under "Experimental Procedures." When a total cyanogen bromide digest was characterized, the results (Fig. 3, lane 1) indicated that all of the cross-reactivity was associated with one major CNBr peptide having a molecular weight of 26,000 on SDS gels, which corresponded to CNBr I. The only other cross-reactive band that was visualized was a minor band at 37,000 which corresponded to an incomplete digestion product con-
taining CNBr I since the autophosphorylation site was retained in this fragment. Immunoblotting of RII that had first been subjected to limited proteolysis with either chymotrypsin or S. aureus V8 protease showed that both the NH2-terminal fragment and the COOH-terminal domain were immunoreactive, indicating that antigenic determinants were localized on both sides of the hinge region (Fig. 3, lanes 3 and 4). A more quantitative assessment of the relative antigenicity of the COOH-terminal domain is seen with the competitive displacement RIA shown in Fig. 4. Under these conditions the major- ity of the immunoreactivity was associated with the domain generated by chymotrypsin. The fact that a large portion of the immunoreactivity was lost with the thermolytic fragment suggests that the charged residues associated with the hinge region may be dominant determinants. Similar results were observed with antisera from two different rabbits.

Antigenic sites on RII were more carefully defined using monoclonal antibodies that were originally generated to bovine heart RII. Immunoblotting of the CNBr peptides and the fragments generated by limited proteolysis gave the patterns shown in Fig. 3 for the two monoclonal antibodies, B10 and H5. Both antibodies cross reacted only with CNBr I. However, immunoblotting of the proteolytic fragments showed that the recognition sites for these antibodies were located on opposite sides of the hinge region. Unlike H5 which consistently cross-reacted with the COOH-terminal domain generated by both proteases, B10 was found to cross-react with the smaller NH2-terminal fragment. A third antibody, B6, was more difficult to evaluate in that although cross-reactivity was also associated with CNBrI, immunoreactivity was lost following digestion with trypsin, chymotrypsin, or thermolysin. Antigenicity was retained only when RII was proteolysed with S. aureus V8 protease, and this cross-reactivity was associated with the NH2-terminal fragment (data not shown).

The antigenic site recognized by H5 was previously characterized (17) and localized to a 20-residue peptide (residues 102-121) located just beyond the site of autophosphorylation, serine 95. This is consistent with the immunoblotting pattern seen in Fig. 3. Like H5, the second monoclonal antibody, B10, also recognized fragments of RII in immunoblotting experi-

ment. Based on these results, further localization of the B10 antigenic site was pursued. Peptides generated from an extended V8 protease digestion of RII autophosphorylated with [γ-32P]ATP were separated by HPLC. Immunoreactivity of the resultant fractions coincided with the 32P-labeled autophosphorylation site as seen in Fig. 5. The major antigenic peptide (A) was cleaved with trypsin which removed the autophosphorylation site (A2). The largest tryptic peptide (A1) retained the immunoreactivity and its amino acid composition (Table I) indicated that the peptide was located in region 46-89 of the polypeptide chain in the native protein.

Although no catalytic activity is associated with the regulatory subunit, it has a number of functional properties. Specifically, the R-subunit serves as a receptor for cAMP, is a substrate for autophosphorylation, and functions as a reversible inhibitor of the catalytic subunit. Previous studies (17) have suggested that interaction of R with monoclonal antibodies H5 and B10 was not altered by autophosphorylation and also did not significantly affect the affinity or maximal levels of cAMP binding. The other important role of the R-subunit is its function as an inhibitor of the C-subunit. Since a number of indications implicate the hinge region in R-C interaction and since the antigenic sites described above lie close to this hinge region, the interaction of the monoclonal antibodies with holoenzyme was characterized to see if aggregation with the C-subunit masked any antigenic sites.

Holoenzyme competed as effectively as R2(cAMP)4 in competitive displacement RIAs with 32P-R11 for most of the monoclonal antibodies studied. However, intact holoenzyme was not as efficient as the free subunit or holoenzyme incubated with cAMP for monoclonal antibody H5. In this case, the addition of cAMP to the reconstituted holoenzyme increased the relative affinity of RII in the complex to that of R2(cAMP)4 at concentrations of holoenzyme above 5 nM (Fig. 6). To determine whether binding of the antibody promoted disso- ciation of the holoenzyme, the H5 monoclonal antibody was immobilized on protein A-Sepharose and subsequently mixed with holoenzyme that had been reconstituted as described under “Experimental Procedures.” The gel was collected on a small fritted glass filter, washed with buffer, and eluted with buffer containing 0.3 mM cAMP. Fractions were monitored for absorbance and for phosphotransferase activity. As seen in Fig. 7, when a control gel containing immunoglobulins from a nonimmunized rabbit bound to protein A-Sepharose was used, no protein was bound to the gel; both R- and C-subunits were found in the initial eluant. In contrast, when the holoenzyme was bound to H5-protein A-Sepharose no protein or phosphotransferase activity appeared in the eluant until the column was washed with cAMP. Only at that time did the C-subunit appear in the eluate. If the holoenzyme was incubated with cAMP prior to mixing with H5-protein A-Sepharose, the phosphotransferase activity did not bind to the column. Sim-ilar results were obtained with B10 and B6 and serum antibodies. Immunoprecipitation from crude extracts confirmed that both R- and C-subunits were precipitated by the antibodies when cAMP was absent (data not shown).

Since the above experiment indicated that binding of antibody to the holoenzyme did not promote dissociation, H5-protein A-Sepharose was utilized to determine whether or not any of the fragments of RII generated by limited proteolysis retained the ability to form a stable complex with the C-subunit. Two proteinic fragments were used, the COOH-terminal domains generated by chymotrypsin and thermolysin. The individual fragments were initially recombined with C-subunit under conditions normally sufficient to regenerate holoenzyme. The mixture was then combined with H5-protein
FIG. 5. HPLC separation of 5′-au- reus V8 peptides from phosphorylated porcine and bovine Rβ. The upper panel shows the peptides obtained from 32P-phosphorylated bovine heart Rβ (3 mg) separated in a 140-min gradient from 0.1% trifluoroacetic acid to 40% acetonitrile on reverse-phase HPLC. The 32P cpm associated with the phosphorylated peptides from bovine heart (A— -A) in the upper panel is compared to the profile of B10 immunoactive peptides in the lower panel.

| Amino Acid | Theoretical | Experimental |
|------------|-------------|--------------|
| Asp        | 8 6.6       | 8 6.5        |
| Thr        | 1 1.0       | 0.7 0        |
| Ser        | 6 4.2       | 5 3.1        |
| Glu        | 6 6.0       | 5 5.0        |
| Pro        | 7 5.8       | 7 5.8        |
| Gly        | 4 4.5       | 4 4.1        |
| Ala        | 8 6.9       | 7 5.8        |
| Cys        | 1 1.0       | 0 0          |
| Val        | 5 4.5       | 3 3.2        |
| Met        | 0 0         | 0 0          |
| Ile        | 1 1.1       | 0.9 0        |
| Leu        | 2 2.5       | 2 2.0        |
| Tyr        | 0 0         | 0 0          |
| Phe        | 2 2.2       | 2 1.8        |
| His        | 0 0.3       | 0 0          |
| Lys        | 0 0         | 0 0          |
| Arg        | 3 3.8       | 2 1.2        |

+9P cpm remained associated with this peptide.

Peptide contains phosphorylated serine.

A-Sepharose, collected on a fritted glass filter, and eluted first with buffer and then with buffer containing cAMP. The results shown in Fig. 8 indicated that phosphotransferase activity associated with the C-subunit was retained on the protein A-Sepharose column only with the chymotryptic fragment, in which case the C-subunit could be selectively eluted with cAMP. When the thermolysin fragment was utilized, no phosphotransferase activity was retained, indicating that no stable complex was formed between this fragment and the C-subunit.

DISCUSSION

The domain structure of the R-subunit of CAMP-dependent protein kinase can be defined experimentally by limited pro-
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Fragment that includes residues 1–145. Radioimmunoassays using antisera from two rabbits suggested that most of the high affinity sites are further localized between residues 91 and 145. The COOH-terminal two-thirds of the molecule which contains the two in-tandem cAMP-binding domains is remarkably nonantigenic.

Monoclonal antibodies confirm the results obtained with serum antibodies in that those sites which have been localized to specific regions are NH2-terminal to the two cAMP-binding sites. The hinge region which joins NH2-terminal domain with the COOH-terminal cAMP-binding domain is included within this antigenic segment. Most sites of proteolytic cleavage in this hinge region fall on either side of the site of autophosphorylation at serine 95. Since the antigenic site for one of the monoclonal antibodies, B10, lies NH2-terminal to the hinge region and another, H5, is COOH-terminal to the hinge region, these two antibodies in particular promise to be useful tools for further investigating the domain structure of CAMP-dependent protein kinase II.

Fig. 7. Immunoadsorption of holoenzyme. The upper panel shows the effect of adding holoenzyme to the control column containing immunoglobulins from normal rabbit serum (NRS) bound to protein A-Sepharose. The presence of the C-subunit was determined by phosphotransferase activity (O—O). cAMP (0.3 mM) was added at fraction 42. Adsorption of holoenzyme to H5-protein A-Sepharose in the presence and absence of 0.3 mM CAMP is indicated in the middle and lower panels, respectively.

Fig. 8. Immunoadsorption of cAMP-binding domains and C. The upper panel indicates the adsorption of holoenzyme to H5-protein A-Sepharose where the elution of C-subunit is monitored by phosphotransferase activity (O—O). ▼ indicates the addition of CAMP. The parallel experiments with the CAMP-binding domains generated by thermolysin and chymotrypsin are indicated in the middle and lower panels, respectively.

Fig. 9. Localization of the immunoreactive region and the H5 and R10 antigenic sites near the hinge region of the type II regulatory subunit of CAMP-dependent protein kinase.
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these regulatory subunits, especially with regard to potential interaction sites with membranes and other proteins.

Although the antigenic sites recognized by these monoclonal antibodies are in close proximity in terms of the sequence to several functional sites, binding of antibody does not appear to markedly affect a number of functional properties. For example, antibody binding does not significantly alter CAMP binding, and antibody interaction with R' is not affected by antibodies in close proximity in terms of the sequence (2). The autophosphorylation site is clearly a recognition site for the C-subunit, and since autophosphorylation can occur in the absence of dissociation of the holoenzyme, it has been assumed that this site masks a portion of the catalytic site of the C-subunit in the inactive holoenzyme complex. The importance of this autophosphorylation site for R-C recognition was indicated by Weber and Hilz (19) who demonstrated that R' was still capable of being autophosphorylated following limited proteolysis with chymotrypsin. They showed, furthermore, that the chymotryptic digest, but not the tryptic digest, was still capable of inhibiting the catalytic subunit. Nelson and Taylor (21) showed that Cys 97 in R' is also protected against alkylation in the holoenzyme but not in R' further supporting the importance of this region for interaction between the R- and C-subunits. Finally, Takio et al. (5) have predicted that the two anionic sites flanking the autophosphorylation site at serine 95 are important components of R-C interaction since basic proteins such as histones will promote dissociation of the holoenzyme.

In light of the apparent importance of the hinge region for providing an interaction site for the C-subunit, it was somewhat surprising to find that all of the sites recognized by the monoclonal antibodies were just as accessible in the holoenzyme as in the dissociated regulatory subunit. The affinity of H5 for R did appear to be reduced in the holoenzyme in the absence of cAMP; however, the extent of this difference is difficult to evaluate experimentally since the $K_{d}$ for antigen interaction with the antibody is very close to the $K_{d}$ (10$^{-4}$ M) for R-C interaction. These results suggest that perhaps only a small region of R is masked by the C-subunit. In any case, it is apparent that even though some of the antigenic sites may be somewhat constrained in the holoenzyme, the major conformational integrity of those sites is retained and accessible. The results also demonstrate that binding by antibody did not dissociate the R- and C-subunits.

It was possible to evaluate the role of specific residues because of the known amino acid sequence in the hinge region and because functional proteolytic fragments differing by only a few amino acid residues can be purified. Immunoabsorption was used for these studies by immobilizing H5 to protein A-Sepharose. Limited proteolysis with chymotrypsin and thermolysin generated two large fragments which were compared for their respective ability to bind the C-subunit. These COOH-terminal fragments both retain the cAMP-binding site and serine 95. Both are also monomeric, in contrast to the native R-subunit which is dimeric. The fragments differ by only three residues, the Asp-Arg-Arg that precedes the site of autophosphorylation being present in the chymotryptic fragment but missing in the thermolysin fragment. The results show that the chymotryptic fragment retains the ability to form a stable complex with the catalytic subunit and that dissociation of the complex is promoted by cAMP. The inability of the thermolysin fragment to form a stable complex with C indicates that the two arginine residues that precede the site of autophosphorylation not only provide a site that is recognized by the active site of the C-subunit, but that these residues are essential for interaction between the R- and C-subunits and make a major contribution to the energy of binding between the subunits. Since the chymotryptic cAMP-binding domain is missing the first acidic region and H5 binds to at least part of the second, the formation of a stable complex between the chymotryptic cAMP-binding domain and the C-subunit indicates that the presence of the two arginine residues is sufficient for R-C interaction.

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