Sites of Phosphorylation and Mutation in Regulatory Subunit of Cyclic AMP-dependent Protein Kinase from S49 Mouse Lymphoma Cells: Mapping to Structural Domains

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ABSTRACT A novel peptide mapping approach has been used to map sites of charge modification to major structural domains of regulatory subunit (R) of type I cAMP-dependent protein kinase from S49 mouse lymphoma cells. Proteolytic fragments of crude, radiolabeled R were purified by cAMP affinity chromatography and displayed by two-dimensional polyacrylamide gel electrophoresis. [35S]methionine-labeled peptides containing sites of mutation or phosphorylation exhibited charge heterogeneity attributable to the modification. Phosphate-containing fragments were also labeled with [32P]orthophosphate to confirm their phosphorylation. Major fragments from [35S]methionine-labeled S49 cell R corresponded in size to carboxyterminal cAMP-binding fragments reported from proteolysis of purified type I Rs from various mammalian species; additional fragments were also visualized. End-specific markers in Rs from some mutant S49 sublines confirmed that cAMP-binding fragments extended to the carboxyterminus of R. Aminoterminal endpoints of fragments could be deduced, therefore, from peptide molecular weights. Clustering of proteolytic cleavage sites within the "hinge-region" separating aminoterminal and carboxyterminal domains of R permitted high resolution mapping in this region: the endogenous phosphate and a "phenotypically-silent" electrophoretic marker mutation fell within a 2.5-kdalton interval at its aminoterminal end. On the other hand, $K_a$ mutations that increase the apparent constant for activation of kinase by cAMP mapped within the large cAMP-binding region of R. A map of charge density distribution within the hinge-region of R was constructed to facilitate structural comparisons between Rs from S49 cells and from other mammalian sources.

As the intracellular transducers for hormones acting through elevation of cAMP, cAMP-dependent protein kinases play central roles in the regulation of animal cell metabolism. Accordingly, these enzymes have been subjected to intensive structural, functional, and metabolic study. cAMP-dependent protein kinases have tetrameric structures consisting of two catalytic subunits (Cs) and a regulatory subunit (R) dimer (1, 2). Two major classes of mammalian kinases, designated as types I and II, appear to differ only in Rs (2-4). Kinase holoenzyme is inactive; activation proceeds by a concerted reaction in which cAMP binds to holoenzyme releasing active Cs and an R dimer complexed with cAMP (2, 5, 6). R has two sites for binding cAMP (7-9) as well as sites for R dimer formation and for interaction with C. Furthermore, conformational interaction between cAMP-binding and C-binding regions of R is suggested by the concerted mode of kinase activation and by studies showing structural alterations in R that accompany binding of cAMP (5, 6, 10, 11).

Native Rs purified from a variety of mammalian tissues exhibit similar structures with proteolytically-sensitive "hinge-regions" extending from ~25 to 30% of the way from aminoterminal to carboxyterminal ends of the molecules (7, 8, 12). The hinge-region divides R into an aminoterminal domain involved in dimer association and a carboxyterminal domain containing cAMP-binding sites (7, 8, 12); the hinge-
region has been implicated in binding and inactivation of C
(7, 8, 13, 14). An autophosphorylation site in R maps to the
carboxyterminal end of the hinge-region (13, 15), and phos-
phorylation at this site reduces both the proteolytic suscepti-
bility of R (13) and the affinity of R for C (16-18).
A homologous region in R can be phosphorylated with purified
cAMP-dependent protein kinase causing reductions in binding
of both cAMP and C (19-21), but this phosphorylation
does not occur under physiological conditions (21). Addi-
tional phosphorylation sites have been found in the amino-
terminal end of bovine R: serines 74 and/or 76 are phos-
phorylated in vitro to a considerable extent and can be phosho-
rilylated in vitro by casein kinase II (glycogen synthase kinase
5; 22, 23); serines 44 and 47 can be phosphorylated in vitro
by glycogen synthase kinase 3 and are phosphorylated in vivo
to a small extent (23). R is not phosphorylated in vitro by
glycogen synthase kinases 3, 4, or 5 or by phosphorylase
kinase (23), but it is phosphorylated to a significant extent in
vivo at a site that has not yet been reported (21, 24, 25).
Endogenous phosphorylation does not affect the ability of R
to bind either cAMP or C subunit (21).

Mutants with lesions affecting cAMP-dependent protein
kinase have been isolated from cultures of S49 mouse lympho-
phoma cells by clonal selection in the presence of dibutyryl
AMP (26-28); wild-type cells are killed by this analog (29).
In some such mutants the apparent constant for activation
of kinase by cAMP is increased to 5 to 20 times that for wild-
type kinase (27, 28). cAMP-dependent protein kinase from
S49 cells is mostly of the type I isozyme, and these “Kc”
mutants carry structural mutations in R (24, 30, 31). Many
Kc mutations cause changes in R charge that provide electro-
phoretic markers for the mutational change and reveal S49
cells to be diploid for Rm genes (24). A cAMP-sensitive S49
subline that carries an electrophoretic marker mutation in
one R allele has also been described (32). In S49 cells, R
phosphorylation is at a single serine residue and is modulated
in vivo by both cAMP and mutations affecting kinase activity
(24, 32, 33).

In the present report, sites of phosphorylation and mutation
in S49 cell R are mapped to structural domains of the protein
by studying cAMP-binding fragments from wild-type and
mutant cells. Procedures used for analysis of purified Rs (7,
8, 12-14) have been modified for use with crude preparations
from radiolabeled cells. cAMP-affinity columns (34) were
used to purify cAMP-binding fragments, and two-dimen-
sional polyacrylamide gel electrophoresis (35) was used both
to resolve R fragments from contaminating peptides and to
reveal charge heterogeneity attributable to either phosphory-
lation or mutations. Sites of endogenous phosphorylation
and of a phenotypically-silent marker allele mutation (above)
were mapped to a small region at the aminoterminal end of the
hinge-region of R. Several Kc mutations fell within the cAMP-
bounding region of R. Subsequent reports will present methods
for mapping mutations within the carboxyterminal domain
of R with greater precision (R. A. Steinberg, submitted man-
uscripts).

MATERIALS AND METHODS

Chemicals and Radiochemicals: Ovalbumin (albumin, egg, 5 x
crystalline) and thermolysin were obtained from Calbiochem-Behring Corp.
(San Diego, CA), and a-chymotrypsin was obtained from Miles Laboratories,
Inc. (Elkhart, IN). N*-(2-aminomethyl)cAMP Sepharose (34) and control Sepha-
roase were prepared using CNBr-activated Sepharose 4B from Pharmacia Fine
Chemicals (Piscataway, NJ), as described previously (33). [35S]methionine
(>900 Ci/mmol) and ACS scintillation mixture were obtained from Amersham
Corp. (Arlington Heights, IL), and [32P]orthophosphate (carrier-free in water)
was from New England Nuclear (Boston, MA). X-ray film and photographic
chemicals were from Eastman Kodak (Rochester, NY), and intensifying screens
were from E. I. Du Pont de Nemours & Co. (Wilmington, DE). Chemicals for
solutions used in extraction and purification of R and for two-dimensional gel
electrophoresis were obtained as reported previously (33).

Cell Culture: Clonal sublines of S49.1 mouse lymphoma cells (36)
were grown in suspension culture as described previously (37). The wild-type
subline was from 24.3.2 was obtained by serial subcloning of S49.1 under nonselective
conditions (26); the allelically-marked cAMP-sensitive subline U36 was derived from
a thymidine kinase-negative subline of S49.1 that was mutagenized with
ethyl methanesulfonate and selected for resistance to 10 mM ouabain (32). Kc
mutant subline U200.65 was selected from a population of 24.3.2 mutagenized
with N-methyl-N-nitro-N-nitrosoguanidine (24, 28); mutants U36.B1.R5 and
U36.B2.R1 were selected from freshly subcloned populations of U36
were used in mutagenesis.

Radiolabeling and Preparation of Cell Extracts: Concentrated
suspensions of cells in low methionine or low phosphate media were labeled
with [35S]methionine or [32P]orthophosphate as described elsewhere (38, 39)
except that, for the experiments of Figs. 5 and 6, cells were concentrated to
5 x 10^6/100 ml instead of 2.5 x 10^6/100 ml, and preincubation times were
reduced to 2 to 1 h. [35S]Methionine labeling was for 3 h at 100 #Ci per ml
(Figs. 2 and 3), 5 h at 60 #Ci per ml (Fig. 4), and 18 h at 20 #Ci per ml
(Fig. 6, 7); [32P] labeling was for 6 h at 100 #Ci per ml. After labeling, cells
were harvested by centrifugation and washed once with ice-cold PBS
(100 #Ci 32P-labeled cells; binding to cAMP-Sepharose or control Sepharose
at room temperature as described
elsewhere (33, 39). After labeling, loaded samples were washed successively with
portions of “starting buffer” containing 10 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 2 mM EDTA
(ethylenediamine tetraacetate), 2 mM L-methionine, and 0.5% wt/vol Nonidet
P-40 to give protein concentrations of ~ 5 mg per ml. Extracts were clarified
by centrifugation for 5 min at 8 x 10^4 g in a Fisher microcentrifuge (Fisher
Scientific Co., Pittsburgh, PA). (For Figs. 5 and 6 centrifugation was for 10 min
at about 160,000 g in a Beckman airfuge centrifuge [Beckman Instruments,
Inc., Fullerton, CA], but this modified procedure did not appreciably affect R
phosphorylation.

Affinity and Control Sepharose Purifications: Undigested or
proteolyzed cell extracts were loaded onto 20-μl columns of N*-(2-aminoethyl)-
cAMP Sepharose or control Sepharose at room temperature as described
elsewhere (33, 39). After loading, columns were washed successively with
glycerol. The “starting buffer” containing 10 mM 4-morpholineethanesulfonate,
ph 6.6, 1 mM dithiothreitol, 2 mM EDTA
ternal to the kinase domain (24, 39). Bound radioactivity was eluted with gel sample
buffer: 2-5 μl portions were subjected to scintillation counting in 6 ml ACS
scintillation mixture and samples were stored frozen at ~70°C as described
elsewhere (24, 39).

Thermolysin and Chymotrypsin Proteolysis: 1 mg/ml-stock
solutions of thermolysin in water and chymotrypsin in 1 mM hydrochloric acid
were stored in small portions at ~70°C. For proteolysis in cell extracts, proteases
were diluted with a 5 mg/ml-solution of ovalbumin in water, then 10-μl portions of
the diluted proteases were added to mixtures of 10-μl cell extracts and 5 μl
0.2 M sodium bicarbonate. Mixtures were incubated for 1 h at 30°C, then
subjected to affinity or control Sepharose purification as above. Concentrations
of proteases in incubation mixtures were 8 μg per ml (Figs. 3 and 4) or 20 μg
per ml (Fig. 2a and b) to give ratios of protease to protein of ~1:500 or 1:200.
For column proteolysis, extracts were loaded onto affinity or control Sepharose
columns, and columns washed as above through the second set of starting
buffer washes. Columns were then washed twice with 0.2 ml of “digestion
solution” containing 20 mM sodium bicarbonate, 1 mM dithiothreitol, 1 mg
per ml ovalbumin, and 0.5% wt/vol Nonidet P-40; washed twice with 0.2 ml
of digestion solution containing, in addition, thermolysin at 2 μg per ml (Fig.
2a, c and d) or chymotrypsin at 2.5 μg per ml (Fig. 6); and incubated with a third
portion of protease-containing solutions for 1 h at room temperature. Columns
were then washed twice with 0.2 ml starting buffer and twice with
0.2 ml starting buffer lacking methionine and ovalbumin before eluting as
above. Eluted samples were counted and stored as above.

Two-dimensional Polyacrylamide Gel Electrophoresis: The
O’Farrell two-dimensional gel procedure (35) was used with modifications
2 Details of the isolation and characterization of these mutants will be presented in a subsequent report.

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described previously (38). Second dimension SDS gels were 12.5% in polyacrylamide. To calibrate peptide molecular weights in the two-dimensional gel patterns, samples of C′-labeled proteins from bacteriophage T4-infected Escherichia coli (40) were loaded on selected second dimension gels in wells adjacent to first dimension gels; calibration curves from the bacteriophage T4 proteins were adjusted for cellular actin (Mr 43,000) to give an apparent subunit molecular weight of 49,000 for R. All gel patterns are shown with the acidic end of the isoelectric focusing dimension at the right and the low molecular weight region of the SDS gel dimension at the bottom.

**Autoradiography and Fluorography:** For Figs. 1, 3, and 4, 35S-labeled peptides were detected by direct autoradiography of dried gels; autoradiography of 32P-labeled peptides (Fig. 4, b and c) was enhanced by using Lightning-Plus intensifying screens as described previously (33). For the gels of Figs. 2, 5, and 6, 35S-labeled species were detected by the fluorographic procedure of Bonner and Laskey (41).

**RESULTS**

Fig. 1 shows portions of two-dimensional gel patterns of affinity-purified R from wild-type and mutant S49 cells to illustrate the charge heterogeneity in R caused by phosphorylation and mutation. The positions of nonphosphorylated (left) and phosphorylated (right) forms of wild-type R are indicated by downward-pointing arrowheads; corresponding forms of mutant Rs are indicated by upward-pointing arrowheads. Under the labeling conditions used for this experiment and for the other experiments described in this report, ~90% of labeled R in wild-type cells (Fig. 1 a) was in the phosphorylated form (33). In subline U36 (Fig. 1 b), which has a wild-type phenotype (32), recoveries and phosphorylations of wild-type and mutant Rs were approximately equal. In subline **FIGURE 1**

Two-dimensional gel electrophoresis patterns of affinity-purified R from wild-type and mutant S49 cells. Cells were labeled with [35S]methionine, extracts prepared, and portions purified on N6-(2-aminoethyl)-cAMP Sepharose as described in Materials and Methods. 2 x 10⁴ cpm of the purified samples were subjected to two-dimensional gel electrophoresis, and autoradiograms of dried gels were exposed for 29 d. (a) R from wild-type subline 24.3.2; (b) R from the allelically-marked subline U36; (c) R from K₆ mutant subline U200.65. Arrowheads indicate positions of the various forms of R as described in the text.

**FIGURE 2**

Two-dimensional gel patterns of affinity or control Sepharose-bound thermolytic peptides from [35S]methionine-labeled wild-type cells. Wild-type S49 cells were labeled with [35S]methionine, extracts prepared, and portions digested with thermolysin directly or after binding to columns as described in Materials and Methods. 5 x 10⁴ cpm of column-purified samples for a and b and 2 x 10⁴ cpm for c and d were subjected to two-dimensional electrophoresis; fluorographic exposures were for 6 d. (a) Affinity Sepharose-bound, and (b) control Sepharose-bound, peptides from a cell extract proteolyzed with thermolysin; (c) thermolytic peptides from affinity Sepharose-bound proteins; and (d) thermolytic peptides from control Sepharose-bound proteins. Arrowheads indicate positions of cAMP-binding fragments. A contaminating protein common to all patterns is enriched for reference. Values (K) indicate approximate molecular weights of fragments.
U200.65 (Fig. 1 c), which has a $K_+$ phenotype (24, 28), recovery and phosphorylation of wild-type R were less than for mutant R.

Since two-dimensional gel electrophoresis resolved forms of R modified in charge by phosphorylation or mutation, it was adopted as a method for identifying fragments of R bearing modified residues. For the studies presented in this report, fragments were generated by proteolysis of native R with thermolysin or chymotrypsin and purified by cAMP-affinity chromatography. Two experimental protocols were used: in the first, R was digested in crude extracts of radiolabeled cells; and, in the second, radiolabeled R was digested after binding to an affinity column. The experiment of Fig. 2 shows that specific R-derived fragments were generated by both of these protocols. Fig. 2, a and c shows two-dimensional gel patterns of thermolytic peptides bound by N$^6$-(2-amino-ethyl)-cAMP Sepharose from extracts of $^{35}$S methionine-labeled wild-type S49 cells; Fig. 2, b and d shows corresponding patterns of peptides bound by control Sepharose. For Fig. 2, a and b, thermolysin was incubated with crude cell extracts, and then fragments were purified by chromatography on affinity or control Sepharose columns. For Fig. 2, c and d, column-binding species from a crude cell extract were immobilized on affinity or control Sepharose, washed free of most contaminating cell proteins, and proteolized in place with thermolysin; columns were eluted after washing to remove protease and unbound fragments. Thermolysin yielded a major cAMP-binding fragment of $\sim$37 kdaltons and minor fragments of $\sim$40.5, 33, 32.5, 30.2, and 16–18 kdaltons; when digestion was on column-bound R, almost all label was in the major 37-kdalton peptide. Patterns of cAMP-binding chymotryptic peptides from wild-type cell preparations are shown in Figs. 3a, 4a, and 6a; specifically-bound fragments (indicated by arrowheads) were identified with reference to control Sepharose-bound material as for Fig. 2 (data not shown). The major chymotryptic cAMP-binding fragment was a 33-kdalton species, and minor species of $\sim$41.5, 32, and 28.5 kdaltons were also observed. When digestion was on column-bound R, chymotrypsin yielded, in addition, a prominent fragment of $\sim$38 kdaltons and several minor fragments between 28.5 and 38 kdaltons (Fig. 6a). Many of the larger fragments generated in crude cell extracts with either thermolysin or chymotrypsin formed doublets in the SDS gel dimension; the corresponding fragments from column-bound R were single species that migrated with the larger forms generated in extracts. This difference in fragment patterns from proteolyzed extracts and from column-bound R suggested that many of the large fragments shared an end sensitive to a protease endogenous to S49 cell extracts. This suggestion was confirmed (below, experiment of Fig. 6) by using structural mutations that sensitized this end to proteolysis in the absence of exogenous proteases.

Fig. 3 shows patterns of cAMP-binding fragments generated from the three sublines of S49 cells whose R patterns are shown in Fig. 1; digestions were performed in cell extracts with either chymotrypsin (Fig. 3, a–c) or thermolysin (Fig. 3, d–f). Mutant forms of R or its peptides are indicated with upward-pointing arrowheads. Patterns from the allelically-marked “wild-type” subline U36 (Fig. 3, b and e) were virtually identical to those from the wild-type subline 24.3.2 (Fig. 3, a and d) except for several minor high molecular weight peptides that had counterparts more basic by $\sim$2 charge units (U) in the patterns from U36. The U36 electrophoretic marker appeared to be within the 40.5- and 41.5-kdalton fragments generated, respectively, by thermolysin and chymotrypsin but outside the major 37-kdalton thermolytic fragment. In contrast to results with U36, all of the cAMP-binding fragments in wild-type cells had more basic counterparts in peptide patterns from the $K_+$ mutant subline U200.65 (Fig. 3, c and f).

Since the wild-type R used for the experiments of Figs. 2 and 3 was phosphorylated to an extent of $\sim$90%, R fragments containing the phosphorylation site should have appeared in two forms differing in charge by 1 to 2 U with the more basic forms labeled to $\sim$10% the intensities of their more acidic counterparts. The major cAMP-binding fragments generated with thermolysin or chymotrypsin did not exhibit such charge heterogeneity (Figs. 2, a and c, 3, a and d), but the minor 41.5-kdalton chymotryptic fragment did (Fig. 3a). In several experiments putative nonphosphorylated and phosphorylated forms of the 40.5-kdalton thermolytic fragment also could be distinguished (e.g., Fig. 2a).3 Peptide patterns from U200.65 (Fig. 3, c and f) provided further support for the presence of the phosphorylation site in the 41.5- and 40.5-kdalton fragments; reduced labeling of the phosphorylated form of wild-type R in this $K_+$ mutant strain resulted in reduced labeling of the more acidic forms of wild-type 41.5- and 40.5-kdalton fragments (compare with patterns from U36 in Fig. 3, b and c).

Fig. 4 compares patterns of cAMP-binding chymotryptic fragments from extracts of $^{32}$P-labeled cells with those from extracts of $^{34}$S methionine-labeled cells to determine directly which peptides contain the phosphorylation site. Fig. 4a shows $^{34}$S methionine-labeled peptides, Fig. 4b shows $^{32}$P-labeled peptides, and Fig. 4c shows a mixture of the two preparations. The ratio of $^{32}$P to $^{35}$S cpm loaded onto gels was such that from undigested material $^{32}$P-labeled R gave a stronger autoradiographic response than did $^{34}$S methionine-labeled R (33). In the chymotryptic peptide patterns $^{32}$P-labeling was restricted to undigested R and minor 48.5- and 41.5-kdalton species that also labeled with $^{34}$S methionine; the major 33-kdalton fragment from $^{34}$S methionine-labeled R was not detected among peptides from $^{32}$P-labeled R. A parallel experiment confirmed the absence of phosphate from the major 37-kdalton cAMP-binding fragment generated from R by proteolysis with thermolysin (not shown).

To map sites of mutation and phosphorylation using the data of Figs. 3 and 4, it is necessary to know the positions of fragment endpoints in the R polypeptide chain. Published reports have localized cAMP-binding regions of R to the carboxyterminal portion of the molecule (12, 42). If cAMP-binding fragments actually contained the carboxyterminus of R, their molecular weights would estimate the positions of their aminoterminal ends. Fig. 5 shows gel patterns of Rs from U36 (Fig. 5a) and from two $K_+$ mutant sublines of U36 that carry charge-shift mutations in the wild-type allele of U36. In U36.B1.R5 (Fig. 5b) the mutation caused an acidic shift of 1 U in R, and in U36.B2.R1 (Fig. 5c) the mutation caused an acidic shift of about 2 U in R. In addition to

3 Relative labeling of the putative nonphosphorylated form of the 40.5-kdalton thermolytic peptide was less than expected from the R subunit pattern; this disproportion appears to reflect preferential production of the 40.5-kdalton peptide from kinase holozyme and partitioning of most nonphosphorylated R in a “free” compartment (unpublished observations).
altering the electrostatic charge of R, the mutations of U36.B1.R5 and U36.B2.R1 caused heterogeneity in the size of the \( K_a \) mutant Rs. The proportion of \( K_a \) mutant R in smaller forms varied between R preparations from the same strain, suggesting that the heterogeneity resulted from limited proteolysis during extraction and purification. This size heterogeneity marks an end (or ends) of the mutant Rs and, as shown below, can be used to demonstrate that large cAMP-binding fragments of R contain its carboxyterminus.

Fig. 6 shows two-dimensional gel patterns of chymotryptic fragments from column-bound R from sublines 24.3.2 (Fig. 6a), U36 (Fig. 6b), U36.B1.R5 (Fig. 6c), or U36.B2.R1 (Fig. 6d). Filled arrowheads indicate positions of wild-type peptides, upward-pointing open arrowheads indicate positions of peptides specific to the U36 marker allele product, and downward-pointing arrowheads indicate positions of peptides specific to the \( K_a \) mutant Rs. Consistent with the results of the experiment of Fig. 3, charge heterogeneity attributable to the U36 marker allele mutation was observed in 41.5-kdalton fragments, but not in 38-kdalton or smaller fragments. Acidic charge shifts attributable to the \( K_a \) mutations in U36.B1.R5 and U36.B2.R1 were found in the major 33-kdalton fragment and all larger fragments. \( K_a \) mutant forms of the 38- and 41.5-
kdalton fragments exhibited size heterogeneity similar to that seen in the positions of their "undigested" R counterparts, confirming that wild-type forms of these two fragments contained the same (carboxyterminal) end of R. (Fluorographic exposures longer than those shown in Fig. 6 clearly revealed mutant forms of the 41.5-kdalton fragments.) The 33-kdalton fragments from the K, mutant Rs were also heterogeneous in size, although relative labeling among the different forms was somewhat different from that of undigested mutant R. Since the U36 marker allele mutation fell within the 40.5-kdalton thermolytic fragment (Fig. 3) but outside the 38-kdalton chymotryptic fragment (Fig. 6), carboxyterminal localization of the 38-kdalton fragment placed the mutation within an interval from ~8.5 to 11 kdaltons from the aminoterminus of R (assuming that undigested R is 49 kdaltons). Patterns from the two K, derivatives of U36, by resolving 38-kdalton fragments from the two R allele products, also proved that the 38-kdalton fragment from the U36 marker allele product had wild-type charge. Without this control, it remained possible that the absence of charge heterogeneity in the 38-kdalton fragment from U36 reflected aberrant proteolysis of the mutant R. From the experiments of Figs. 2, 3, and 4, the endogenous phosphorylation site fell in the same fragments as did the U36 mutation. This result was also confirmed in the experiment of Figs. 5 and 6 by noting the effects of the K, mutations in U36.B1.R5 and U36.B2.R1 on labeling patterns of the U36 marker allele product and its 38- and 41.5-kdalton peptides.

Fig. 7 summarizes in diagrammatic form the results of the experiments presented in this report. Major cleavage sites for thermolysin (Th) and chymotrypsin (Ch) are shown in heavy arrows, and minor cleavage sites are shown with lighter arrows. The region susceptible to endogenous proteolysis is also indicated (E). Sites of phosphorylation (ser-P) and mutation were localized to intervals delimited by the proteolytic cleavage sites. For the marker allele mutation in U36 and the endogenous phosphorylation site, localization was to an interval comprising ~5% of the R polypeptide. For the K, mutations in U200.65, U36.B1.R5, and U36.B2.R1, however, localization was only to the carboxyterminal 67% of the molecule. Since two-dimensional gel electrophoresis provided information on both charge and size of R fragments, it was possible to estimate net charge differences between fragments and, thereby, construct a map of charge density distribution for the protein. For the charge density histogram shown in Fig. 7, distances between fragments were calibrated for charge shifts using patterns from a variety of mutant sublines including, but not limited to, those shown in Figs. 3 and 6. Locations shown in Fig. 7 for structural domains implicated in R dimer formation (R--R), interaction between R and C (R--C), and cAMP-binding are based on published information (7, 8, 12–21). The allele marker mutation in U36 and the endogenous phosphorylation site fell at the aminoterminus of the hinge-region between regions implicated in dimer formation...
and binding of C. Three $K_a$ mutations fell within the cAMP-binding domain of R, but they were not localized with great precision.

**DISCUSSION**

Type I R from S49 mouse lymphoma cells, like the homologous proteins from other mammalian sources, has a proteolytically-stable cAMP-binding domain adjacent to a proteolytically-sensitive hinge-region. The major cAMP-binding thermolytic and chymotryptic fragments from S49 R were similar in molecular weights to the corresponding fragments from purified porcine R; (12, 14) except that the apparent values for S49 R and its fragments were ~6% greater than those reported for the porcine species; this small discrepancy is probably attributable to differences in gel calibrations. Partial amino acid sequences are available for the regions surrounding the major thermolysin cleavage sites in porcine and bovine R's (12, 14); data is more extensive for the bovine protein (20), but, where they overlap, the sequences are in complete agreement. Among the eight residues aminoterminal to the thermolysin cleavage site in bovine R, there are four arginines and a lysine; the next 10 aminoterminal residues are neutral amino acids. A similar very basic region to the aminoterminal side of the thermolysin cleavage site in S49 R can be deduced by the 5-charge unit-difference between the 38-kdalton chymotryptic and 37-kdalton thermolytic cAMP-binding peptides (Fig. 7). Also in common with bovine and porcine R's is that the region between endpoints of the 37- and 38-kdalton fragments from S49 cell R is susceptible to tryptic cleavage at several sites (R. A. Steinberg and M. Stroczykowski, unpublished results). Three lines of evidence implicate this basic region in interactions of R with C: the C-inhibitory activity of R is sensitive to the arginine-specific reagent 2,3-butanedione (15); phosphorylation at a serine four residues carboxyterminal to the four arginines abolishes the C-inhibitory activity of R (20, 21); and C protects this basic region from tryptic digestion in partially-purified kinase holoenzyme (14). C also appears to protect this region from proteolysis in S49 cell R. Chymotryptic digestion of affinity column-bound R yielded a prominent 38-kdalton cAMP-binding fragment (Fig. 6) that was absent from digests of cell extracts (Figs. 3 and 4). Thermolysin digestion of partially-purified kinase holoenzyme from S49 cells yielded the 37-kdalton fragment seen in the experiments of Figs. 2 and 3 only when cAMP was present during digestion; in the absence of cAMP, the major product was the 40.5-kdalton fragment produced as a minor species in digests of crude extracts (Figs. 2 and 3; R. A. Steinberg, unpublished results). It is not clear why purification was needed to reveal protection of the thermolysin site but not that of the chymotrypsin site.

Immediately carboxyterminal to the basic region discussed above is a region delimited by the major cleavage sites for thermolysin and chymotrypsin that carries a net negative charge of 4 U (Fig. 7). In the hinge-region sequence from bovine R (20), there are 14 residues between the site cleaved by thermolysin and a tyr-val bond that appears to be the major cleavage site for chymotrypsin (14); this interval contains four acidic residues and no basic residues. This 14 amino acid interval is grossly overestimated in size by the differences in SDS mobilities of carboxyterminal thermolytic and chymotryptic fragments from porcine R, and, presumably, from S49 R as well. The acidic region extending from ~12 to 16 kdaaltons from the aminoterminus of R in the map of Fig. 7, therefore, actually comprises only ~1,500 daltons of protein. When sequence data is available for the entire protein, similar corrections may have to be applied to additional regions of

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4 The aminoterminus of the carboxyterminal chymotryptic fragment from porcine R differed in two residues from the sequence carboxyterminal to the proposed chymotrypsin cleavage site in the bovine sequence; both changes could have resulted from single base substitutions in the gene sequence.
the S49 R_I map. An acidic region occupies a position in the bovine R_II sequence equivalent to that of the acidic region in bovine and S49 R_S; it has been argued that, along with the arginine-rich region discussed above, this acidic region may be important for the interaction of R with C (40).

In contrast to the autophosphorylation site in R_I (13, 15, 42) and the site in R_I phosphorylated by cGMP-dependent protein kinase (20), the endogenous phosphoserine in S49 R is aminoterminal to the major cleavage sites for both chymotrypsin and thermolysin. The endogenous phosphorylation site in S49 cell R and the allele marker mutation in subline U36 map to the aminoterminal end of the hinge-region at site in S49 cell R and the allele marker mutation in subline U36 has little or no effect on R phosphorylation observed in S49 cell mutants deficient in C activity (32, 33). Nevertheless, it has not been possible to demonstrate phosphorylation of R by C in purified preparations of type I protein kinase (3, 19, 43). Despite its close proximity to the site of endogenous phosphorylation, the marker allele mutation in subline U36 has little or no effect on R phosphorylation (Figs. 1 and 5).

Although the charge changes caused by the U36 mutation or endogenous phosphorylation might be expected to alter the local configuration of R, neither modification has a discernible effect on the activation parameters of kinase (21, 32). This suggests that the aminoterminal portion of the hinge-region is not involved in binding or inhibition of C. On the other hand, three different K_0 mutations that increase the apparent constant for activation of S49 kinase by cAMP map within the carboxyterminal cAMP-binding region of R; more refined mapping of these and 22 additional charge-shift K_0 mutations has localized them all to a few sites in a region from ~7 to 25 kdaltons from the carboxyterminus of R (A. Steinberg, submitted manuscripts; and R. A. Steinberg and E. F. McHugh, unpublished results). Thus, despite the suggestion that some K_0 mutations primarily affect interactions between R and C (31), none of those we have mapped falls near the region thought to be important in these interactions.

K_0 mutants are heterozygous for expression of mutant and wild-type R alleles (24, 44, and this report), yet <25% of kinase from K_0 mutant cells has wild-type activation parameters (44, 45). This dominance of the mutant allele product appears to be explained in part by its modification of wild-type R properties through heterodimer formation (45). If R dimer interaction is limited to the aminoterminal domain implicated by proteolysis studies (14), the ability of K_0 mutations in the carboxyterminal half of R to affect properties of wild-type R through heterodimer formation suggests that there is significant conformational interaction between aminoterminal and carboxyterminal domains and between monomers linked through dimer association. Alternatively, there may be additional dimer interaction regions in the carboxyterminus of R. In this regard, it is interesting to note that considerable homology has been found between cAMP-binding site sequences in bovine R_II and in the catabolite gene activator protein of _Escherichia coli_ (46); in the _E. coli_ protein, cAMP-binding sites are formed by interactions of sequences from both monomers of the dimeric protein (47).

Two-dimensional partial proteolysis peptide maps have the potential for providing a rapid and inexpensive method for mapping mutations in R_I and for comparing R_I structures from various animal species. In the studies presented in this report, cAMP-binding fragments from native R have been used to localize mutations to major structural domains of the protein. For high resolution mapping using partial proteolytic digests, it is important to introduce a large number of cleavage sites within relatively small regions of the protein. The fragments used in the present studies allow mapping of features within the hinge-region of R to intervals comprising 5% or less of the R polypeptide chain. To achieve comparable mapping of mutations within the cAMP-binding carboxyterminus of the protein, it has been necessary to generate proteolytic fragments from denatured R. In subsequent reports I will present experiments that map endpoints of fragments generated from denatured R and map mutations to intervals delimited by these fragment endpoints (R. A. Steinberg, submitted manuscripts).

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