Localisation of an ATP-binding site on adenylyl cyclase type I after chemical and enzymatic fragmentation

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Abstract Photolabeling of partially purified bovine brain adenylyl cyclase (AC I) with [γ^{32}P]8-N_3-ATP led to incorporation of ^{32}P into the 115 kDa catalyst. Further treatment with *N*-chlorosuccinimide, which cleaves proteins at tryptophan residues, yielded a 14 kDa ^{32}P-labeled fragment. The latter was immunoprecipitated by antibody BBC1, recognizing the extreme C-terminus of AC I, but not by antibody BBC2, recognizing a more remote epitope. Further fragmentation of photolabeled AC I by the proteases Glu-C and Asp-N yielded ^{32}P-labeled peptides corresponding to 2.9 kDa and 5.6 kDa fragments, which were not recognized by any of these antibodies. This narrows the ATP binding site down to a 25 amino acid sequence containing a general motif G(X_{0-7})KG(X_{0-4})L/M(X_{5-7})S/T present in all eukaryotic adenylyl cyclases so far cloned, but also in a variety of bacterial adenylyl cyclases (Peterkofsky et al. (1993) Progr. Nucleic Acids Res. Mol. Biol. 44, 31–65).

Key words: Adenylyl cyclase type I; Affinity labeling; ATP site; Monoclonal antibodies

1. Introduction

To date at least eight eukaryotic adenylyl cyclases from mammals, insects and slime molds have been cloned and sequenced. For type V adenylyl cyclase several subforms exist [1,2]. The presumptive catalytic domains of these adenylyl cyclases but also of various guanylyl cyclases exhibit a high degree of homology. For adenylyl cyclases the greatest homology resides in the C1a and C2a segments of the cytosolic loops [3]. However, the catalytic ATP binding site has not been identified so far, because consensus sequences characteristic for classical nucleotide binding sites GXXXXGKS as present in ATPases or GTPases are missing [4,5].

Therefore we started out to localize the ATP binding site(s) on type I adenylyl cyclase by reaction with the affinity label [γ^{32}P]8-N_3-ATP and consecutive chemical or enzymatic fragmentation of labeled adenylyl cyclase. This ATP analog has been previously used by Castets et al. [6] for labeling of a partially purified adenylyl cyclase from rat brain synaptosomes. Since photoaffinity labeling usually proceeds with low efficiency and ^{32}P-labeled peptides, due to addition of several negative charges, may migrate differently from unlabeled peptides, precipitation by specific antibodies against adenylyl cyclase was used to check coincidence of labeling and immunoreactivity. Among the antibodies used, antibody BBC1 was shown to be specific for the calmodulin-dependent adenylyl cyclase (M_r 115 kDa) from bovine brain while antibody BBC2 (and BBC4) was recognizing a broad variety of adenylyl cyclases from different tissues and species [7].

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Abbreviations: 8-N_3-ATP, 8-azido-adenosine triphosphate; DTT, dithiothreitol; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; NCS, *N*-chlorosuccinimide; BPB, bromophenol blue

2. Materials and methods

2.1. Materials

N-Chlorosuccinimide, *N*-acetylmethionine, 2'-deoxy-3'-AMP, 8-N_3-ATP were from Sigma; creatine phosphate, creatine kinase, alkaline phosphatase (molecular biology grade) and endoproteinases Asp-N and Glu-C (sequencing grade) were from Boehringer Mannheim; Pansorbin cells were from Calbiochem and CSPD was purchased from Serva. Immobilon transfer membranes were obtained from Millipore. [^{125}I]NaI (carrier free) and [γ^{32}P]8-N_3-ATP (5–15 Ci/mmol) were from ICN, [γ^{32}P]ATP (760 Ci/mmol) and [^{3}H]cAMP (15–30 Ci/mmol) were purchased from Amersham. Peptides derived from the sequence of AC I (P^{1120}-A^{1134} and G^{985}-T^{1005}) were kindly synthesized by Drs. J. Wallach and R. Frank, ZMBH, Heidelberg.

2.2. Methods

2.2.1. Miscellaneous. Polyacrylamide gel electrophoresis and immunoblotting were conducted as described previously [7]. Antibodies were either radioiodinated [7,8] or labeled with alkaline phosphatase for detection by chemiluminescence with CSPD as substrate. Phosphatase was coupled to antibody according to the specifications of the distributor. Radioiodinated (Fig. 1) or rainbow protein markers (Amersham) were used as M_r standards. Bovine brain cortex adenylyl cyclase was prepared according to Pfeuffer et al. [9,10]. The preparation after the first forskolin affinity chromatography was used. It had a specific activity of 1.58 μmol mg^{-1} min^{-1}. Adenylyl cyclase assays were performed in the presence of 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 100 μM forskolin as described earlier [10]. Radioiodination was performed according to Greenwood and Hunter [11].

2.2.2. Cleavage with N-chlorosuccinimide (NCS). This was performed essentially according to Lischwe and Ochs [12]. Purified bovine brain adenylyl cyclase [9] in buffer A (10 mM MOPS, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM Tween 60 pH 7.4) was treated with 2.5 volumes of cleavage solution (1 g urea and 2 ml of 50% acetic acid) including the indicated concentrations of NCS for 30 min at 22°C. The reaction was stopped by addition of a 5-fold molar excess of *N*-acetylmethionine and left for a further 30 min at 22°C. The reaction mixture was lyophilized, dissolved in one volume H_2O and neutralized with Tris base. Protein samples were used either directly for SDS gel electrophoresis after application of sample buffer (Fig. 1) or for immunoprecipitation with the monoclonal antibody BBC1 after diluting the solution to 1 M urea and adjusting to RIPA buffer conditions (0.5% SDS, 1% NP40 and 1% deoxycholate, 150 mM NaCl, 10 mM sodium phosphate, pH 7.4) (Fig. 3).

2.2.3. Photolabeling of adenylyl cyclase. Unless specified otherwise, adenylyl cyclase in buffer A was concentrated 5–10-fold by centrifugation through a Microcon 30 filter device (Amicon). Solutions were adjusted to 5 mM $MnCl_2$ following the protocol of Castets et al. [6]. Photolabeling was performed on ice in microtiter plates (30–60 μl per well). Reaction was started by addition of 50 μM [γ^{32}P]8-N_3-ATP (5–15 Ci/mmol) and irradiation at 254 nm with a low intensity UV lamp (Camag) for 10 min directly on top of the wells. Photolysis was followed either by TCA precipitation or by immunoprecipitation in the dark. For precipitation by TCA, the reaction was stopped by addition of 10 mM DTT, 1 mg/ml soybean trypsin inhibitor (as carrier protein) and 9 volumes of ice-cold 10% TCA and left on ice for 5

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min. Protein was collected by centrifugation and the pellet washed with ice-cold acetone. The dried protein was dissolved in sample buffer and applied to 5–15% polyacrylamide SDS gel electrophoresis according to Laemmli [13].

2.2.4. Immunoprecipitation with monoclonal antibody BBC1. For immunoprecipitation, monoclonal antibody BBC1 was coupled to Pansorbin as described earlier [8]. Prior to precipitation, solutions were adjusted to RIPA buffer conditions and mixed with the BBC1/Pansorbin complex (200 µl of a 20% slurry per ml adenylyl cyclase). The slurry was agitated for 2 h at room temperature. Following centrifugation, the pellet was washed twice with RIPA buffer, once with buffer A, suspended in buffer A (50% slurry) and finally stored at −20°C. For fragmentation experiments, adenylyl cyclase was eluted from the BBC1/Pansorbin complex with 0.5% SDS at 50°C for 10 min and separated from the pansorbin by centrifugation.

2.2.5. Fragmentation of labeled adenylyl cyclase by endoproteases. BBC1-purified radiolabeled adenylyl cyclase in buffer A was diluted with 4 volumes of 50 mM sodium phosphate pH 8.0 (Asp-N) or 25 mM $(NH_4)_2CO_3$ buffer pH 7.8 (Glu-C) to a final SDS concentration of 0.1%. For optimal fragmentation the mixtures were supplemented with 0.2% Triton X-100. Reactions were started by adding 0.2 µg Asp-N or 5 µg Glu-C per 100 µl, maintained at 37°C (Asp-N) or 25°C (Glu-C) for 6 h and stopped either with Laemmli sample buffer or with SDS (final concentration 0.5%) at 60°C for 10 min. For immunoprecipitation solutions were adjusted to RIPA buffer conditions.

3. Results and discussion

Fig. 1 shows the fragmentation pattern of bovine brain adenylyl cyclase obtained by controlled NCS cleavage at tryptophan residues visualized by two different monoclonal antibodies. Antibody BBC1 detected four fragments with M_rs 14, 35, 46 and 79 kDa, expected from the amino acid sequence of

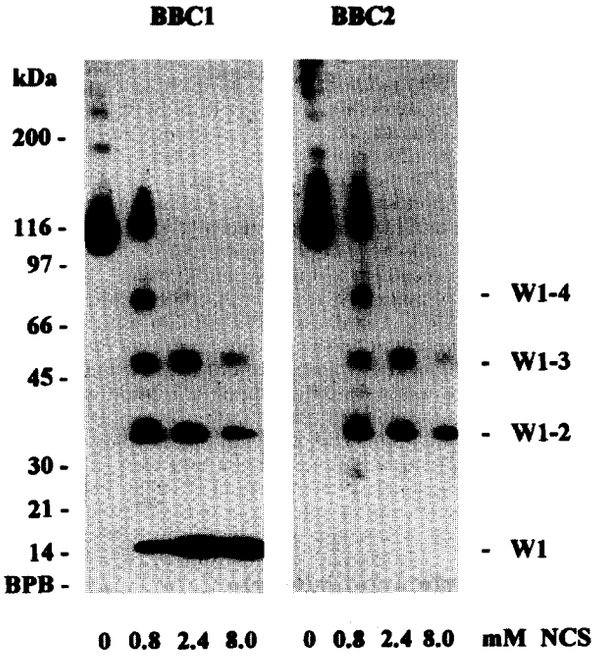

Fig. 1. Analysis of NCS-treated bovine brain adenylyl cyclase with monoclonal antibodies BBC1 and BBC2. 365 ng each of purified adenylyl cyclase were cleaved at tryptophan residues, using the indicated NCS concentration as described in Section 2. Samples were separated on a 5–15% SDS polyacrylamide gel, blotted onto nitrocellulose and probed with radioiodinated antibodies BBC1 or BBC2. Molecular masses of radioiodinated standards (BioRad) and NCS fragments are marked on the left hand and right hand side, respectively.

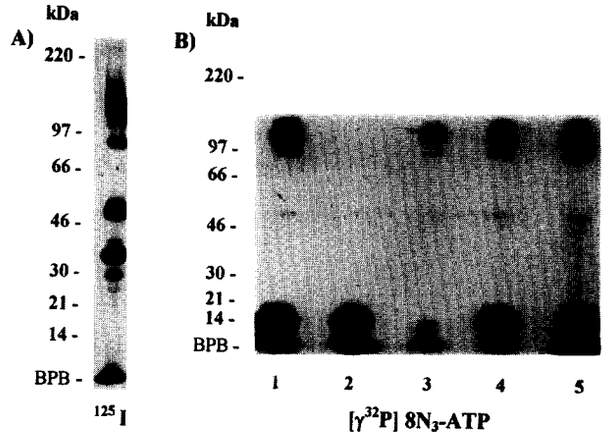

Fig. 2. Photoaffinity labeling of purified bovine brain adenylyl cyclase with [γ^{32}P]8-N_3-ATP. A: Purity of adenylyl cyclase preparation. 15 ng of purified adenylyl cyclase was radioiodinated as described in Section 2 and electrophoresed on a 5–15% SDS polyacrylamide gel according to Laemmli [13]. The gel was dried and exposed to a film for 15 h. B: Specificity of radiolabeling. 290 ng each of purified adenylyl cyclase were labeled with [γ^{32}P]8-N_3-ATP without prior concentration, precipitated with TCA as described in Section 2 and electrophoresed on a 5–15% SDS polyacrylamide gel. The dried gel was autoradiographed for 2 days. The labeled 115 kDa bands were excised from the gel and measured by Cerenkov radiation. The percentage of radiolabeling relative to control is given in brackets. lane 1: control [100%]; lane 2: no UV irradiation [0%]; lane 3: addition of 500 µM ATP [42%]; lane 4: addition of 500 µM 2′-deoxy,3′-AMP [80%]; lane 5: addition of 500 µM adenosine [85%].

type I adenylyl cyclase [3]. No fragments due to the closely spaced tryptophans W^{166} and W^{213} could be observed. From a series of synthesized oligopeptides covering the 14 kDa fragment (W^1), one 15 amino acid peptide representing the extreme C-terminus, completely blocked immunostaining by BBC1 (not shown). Antibody BBC2, in contrast, was recognizing only the three higher M_r fragments. At the same time its total immunoreactivity gradually disappeared when the concentration of NCS increased. This suggested that the site of recognition must be located around the C-terminal tryptophan (W^{1002}). Consequently the synthetic 21 amino acid peptide G^{985}–T^{1005}, incidentally a highly conserved sequence, including this tryptophan was able to compete effectively with the cyclase for antibody BBC2 in Western blots and immunoprecipitation (not shown). The characteristic fragmentation pattern and the fact that BBC2 recognizes a well conserved epitope argue that purified brain adenylyl cyclase used in this study consists of only one adenylyl cyclase isoform, namely AC I. This was remarkable, since the enzyme was purified by forskolin-affinity chromatography from bovine brain cortex known to contain more than one adenylyl cyclase isoform [7]. Since purification was conducted with 'basal' enzyme (i.e. not activated with AlF_4^- or GTP analogs), other isoforms apparently have been lost due to limiting affinity for forskolin. Contamination by other impurities was further precluded since fragmentation was performed after the labeled enzyme had been isolated with the type I specific antibody BBC1.

8-N_3-ATP used as affinity label had an affinity comparable to that of natural substrate ATP. Lineweaver-Burk analysis revealed a K_m of 49 µM for ATP and a K_i of 49 µM for 8-N_3-ATP, in the presence of 5 mM $MnCl_2$ and 100 µM forskolin, comparable to previously obtained K_m values of 30–60 µM

for AC I [14], AC II and AC VI [15]. Inhibition by 8-N_3-ATP proved to be of purely competitive type (not shown).

Fig. 2A shows an autoradiograph of the radioiodinated, partially purified type I adenylyl cyclase used in this study, after SDS electrophoresis. Aside from the M_r 115 kDa band corresponding to AC I several impurities of M_rs 90, 53, 35 and 29 kDa are obvious.

Photoinduced incorporation of [γ^{32}P]8-N_3-ATP into this adenylyl cyclase sample is demonstrated in Fig. 2B which shows an autoradiograph after SDS gel electrophoresis. Specificity of labeling of the 115 kDa protein was evident from inclusion of excessive unlabeled ATP (10-fold) reducing incorporated radioactivity by some 60%, while the P-site inhibitors adenosine and 2′-deoxy-3′-AMP at the same concentration were less (15–20%) efficient. Practically no radiolabeling was measurable when the UV irradiation was omitted, demonstrating that incorporation was not due to phosphate transfer by a kinase contamination.

In the following the location of the ATP binding site was approached by fragmentation of the labeled enzyme by chemical and enzymatic means. Fig. 3A shows that [γ^{32}P]8-N_3-ATP incorporated into the M_r 115 kDa protein was completely precipitated by the AC I specific antibody BBC1 coupled to Pansorbin cells (lane P). Following cleavage of the labeled catalyst at tryptophan (W) residues, the ^{32}P was mainly found in a fragment of M_r 14 kDa (Fig. 3B, lane T). As expected from the results in Fig. 1, the labeled 14 kDa fragment was almost quantitatively precipitated by the antibody BBC1 (lane P). These findings together were indicative for the ATP label being attached to the C-terminal 14 kDa fragment of AC I.

To further narrow down the site of ATP binding, fragmentation of labeled adenylyl cyclase by the proteases Asp-N and

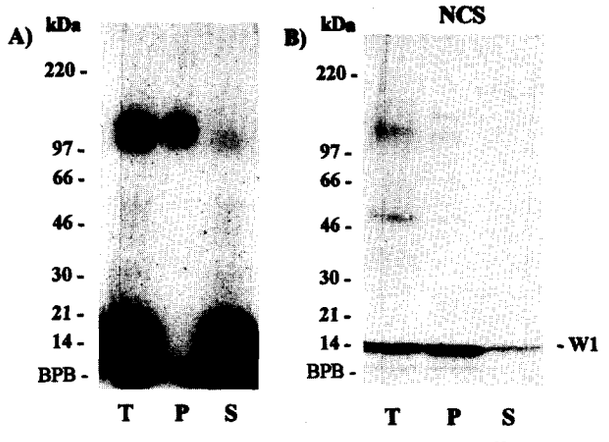

Fig. 3. Immunoprecipitation by antibody BBC1 of [γ^{32}P]8-N_3-ATP labeled adenylyl cyclase before (A) and following (B) NCS treatment. A: 180 ng of purified brain adenylyl cyclase was labeled and immunoprecipitated with antibody BBC1 as described in Section 2. Protein samples were supplied with Laemmli sample buffer and electrophoresed on a 5–15% SDS-polyacrylamide gel. The dried gel was exposed to the film for 3 days. B: 630 ng of purified bovine brain adenylyl cyclase was radiolabeled, immunoprecipitated with the monoclonal antibody BBC1 and specifically cleaved at tryptophan residues with 5 mM NCS as described in Section 2. The lyophilized protein pellet was dissolved in RIPA buffer and immunoprecipitated with BBC1. Protein samples were supplied with Laemmli sample buffer, electrophoresed on a 5–15% SDS-polyacrylamide gel and blotted onto Immobilon membrane. The membrane was autoradiographed for 3 days. T = total sample; P = immunoprecipitate; S = supernatant from immunoprecipitation.

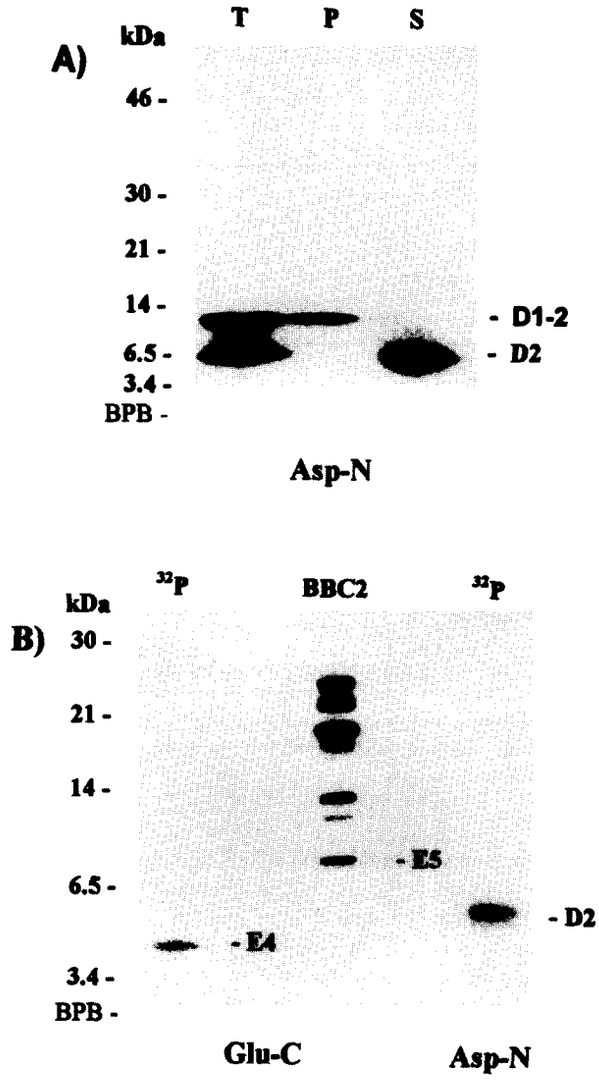

Fig. 4. Fragmentation of [γ^{32}P]8-N_3-ATP labeled AC I by endoproteases Asp-N and Glu-C. A: Analysis of Asp-N fragments by immunoprecipitation with BBC1. 420 ng radiolabeled and immunoprecipitated AC I was treated with Asp-N as described in Section 2. Peptides were immunoprecipitated with BBC1 and separated on a 7–25% SDS-polyacrylamide gel. Autoradiography of the dried gel was performed for 5 days. T = total sample; P = immunoprecipitate; S = supernatant from immunoprecipitation. B: Analysis of Glu-C and Asp-N fragments on a low M_r range SDS gel. [γ^{32}P]8-N_3-ATP labeled adenylyl cyclase, 230 ng each, was treated with Glu-C (lanes 1 and 2) or Asp-N (lane 3) as described in Section 2. Samples were separated by SDS-gel electrophoresis (16.5% acrylamide, 0.5% bisacrylamide [16]) and transferred onto Immobilon membrane. Blotted peptides were either autoradiographed for 5 days (lanes 1 and 3) or immunostained with antibody BBC2 (lane 2).

Glu-C was performed (Fig. 4). Treatment of labeled adenylyl cyclase with Asp-N yielded two fragments of M_rs 12.7 (D1–D2) and 6.5 kDa (D2, theoretical M_r 5.6 kDa) (Fig. 4A). Only the larger one was precipitated by the BBC1 antibody (lane P), indicating that the label was not attached to the extreme C-terminus. Regarding the sequence of AC I, the smaller peptide should result from cleavage at D^{1013} and D^{1062} (D2) while the 12.7 kDa peptide results from cleavage at D^{1013} (D1–D2) (see scheme in Fig. 5).

Further information was obtained from the application of the endoprotease Glu-C. The results shown in Fig. 4B are

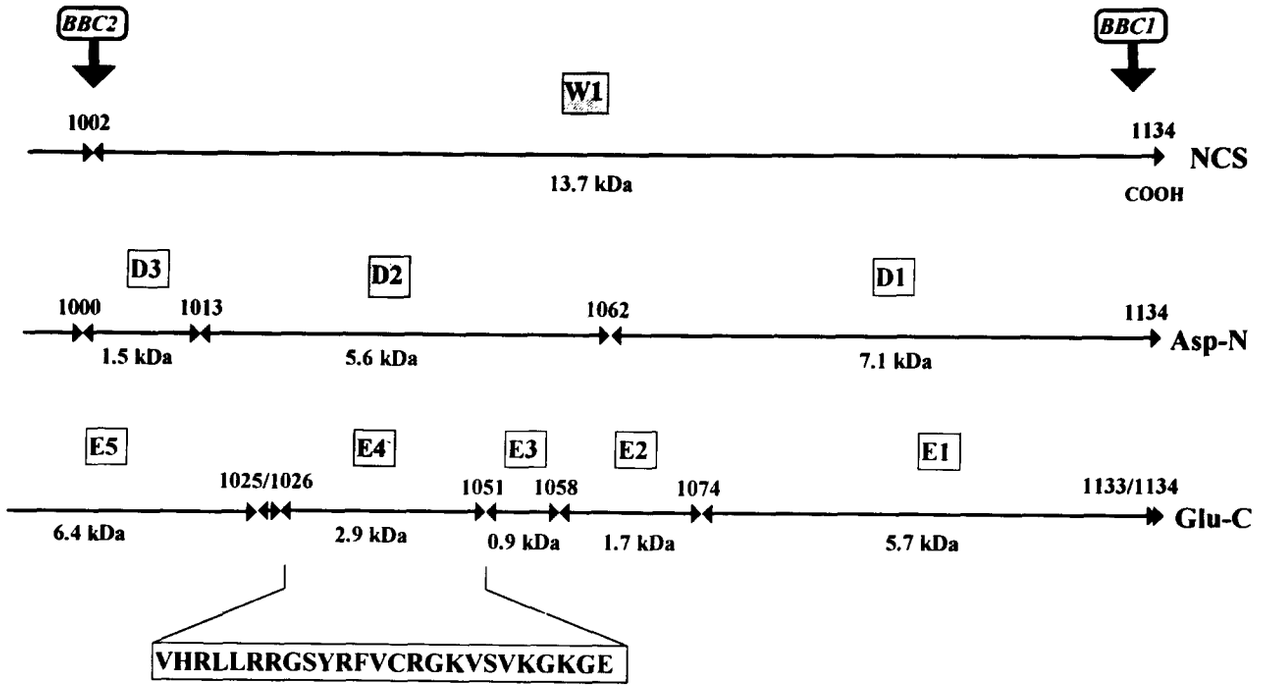

Fig. 5. Carboxy-terminal cleavage sites of AC I. Numbers indicate theoretical cleavage sites by NCS (W), Asp-N (D), Glu-C (E) and the C terminus (1134). Putative binding sites of monoclonal antibodies BBC1 and BBC2 are also indicated. Radiolabeled fragments are marked by shaded boxes.

representing an autoradiograph of an immobilon blot of an SDS gel prepared according to Schägger and von Jagow [16] which is useful for the separation of small peptides. From lane 1 it is obvious that the smallest radiolabeled peptide obtained had an apparent M_r of 4 kDa (E4, theoretical M_r 2.9 kDa). As a further criterion however, antibody BBC2 failed to detect a peptide of this size in Western blots (lane 2) as it failed to precipitate protein-bound radioactivity (not shown). It did however detect a somewhat larger peptide (E5) running at approx. M_r of 7.3 kDa (theoretical M_r 6.4 kDa, lane 2). For a proper comparison, lane 3 shows the radiolabeled D2 fragment from another (more complete) Asp-N cleavage experiment, running in the Schägger gel system at an M_r of 5.0 kDa.

Knowledge of the topology of adenylyl cyclases is extremely scarce and information on contact sites with G-protein α and βγ subunits is missing. The sole exception is the calmodulin binding site on subform AC I, which has been localized by site-directed peptides [17] and site-directed mutagenesis [18]. There is also no information on where divalent cations like Mg^{2+} or Ca^{2+} bind. The latter has been shown to inhibit several subforms of adenylyl cyclase at submicromolar concentration most likely via direct interaction with the catalytic moiety of the enzyme [1,19]. Likewise missing is information on binding sites of the common activator forskolin and on nucleotide binding sites for substrate ATP or the so-called P-site inhibitors.

The primary structure of adenylyl cyclases does not exhibit typical nucleotide binding sequences as present in ATPases and GTPases; hence the common motif GXXXXGKS is missing [4]. Therefore Peterkofsky et al. suggested an alternative sequence $G(X_{0-7})KG(X_{0-4})L(X_{0-5})S/T$ found in 12 adenylyl cyclases of mammalian, bacterial and yeast origin [5]. In this modified motif the basic amino acid, mostly lysine, precedes rather than follows the second glycine residue. The authors have argued that this may be due to the specific mechanistic difference, namely release of P-P_i as opposed to that of P_i.

The purified type I adenylyl cyclase used in this study had similar affinities for ATP and 8-N_3-ATP which was in contrast to the findings of Castets et al. [6] who found a comparable affinity for 8-N_3-ATP (22 μM) but a 5-fold higher affinity for the substrate ATP. However, this difference may be due to a different isoform of adenylyl cyclase for which the authors claimed a >100-fold higher specific activity than that reported here. Using bovine brain adenylyl cyclase type I, [γ^{32}P]8-N_3-ATP labeled a single peptide. Incorporation of label was efficiently competed by the substrate ATP, but adenosine or the more potent P-site inhibitor 2′-deoxy-3′-AMP were also competitors, although to a lesser degree (Fig. 2B).

Although the existence of two adenine nucleotide/nucleoside binding sites in mammalian adenylyl cyclases is widely accepted, the specificity towards different ligands remains to be established. A recent study on AC I, expressed in Sf9 cells, has indicated that substrate ATP also interfered with P-site ligands [20]. On the other hand, typical P-site ligands, as 2′,5′-dideoxyadenosine derivatives, affected the catalytic site as well. In a recent paper, Tang et al. [14] set up a series of point mutations of conserved charged amino acids in AC I. They observed in several cases linkage of reduction of affinities for substrate ATP and P-site inhibitors and therefore suggested that both ligands may bind to different conformational states of the same site.

A straightforward approach for identification of a distinct binding site on a polypeptide chain consists in the use of affinity labeling in combination with chemical and enzymatic

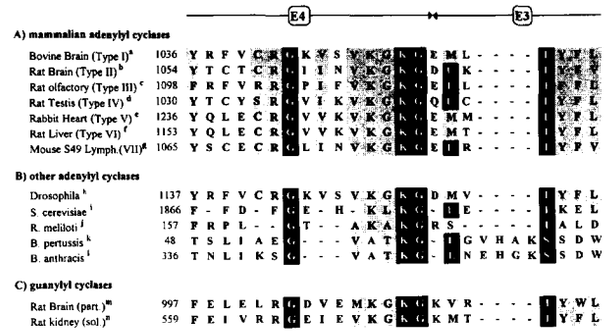

Fig. 6. Sequence alignment of different adenylyl cyclases and guanylyl cyclases. Shown are regions homologous to the proposed ATP binding site of AC I. Alignment follows the consensus sequence of the ATP binding motif $G(X_{0-7})KG(X_{0-5})L/M(X_{0-7})S/T$ suggested by Peterkofsky et al. [5], with a slight modification (L/M instead of L). Amino acid symbols of this motif are highlighted by dark boxes, symbols of other identical or conserved amino acids are marked by gray boxes. Numbers refer to homologous sequences from adenylyl or guanylyl cyclases taken from the following references: (a) Krupinski et al. [3]; (b) Feinstein et al. [25]; (c) Bakalyar and Reed [26]; (d) Gao and Gilman [27]; (e) Wallach et al. [1]; (f) Premont et al. [28]; (g) Watson et al. [29]; (h) Levin et al. [30]; (i) Young et al. [31]; (j) Beuve et al. [32]; (k) Glaser et al. [33]; (l) Robertson et al. [34]; (m) Chinkers et al. [35]; (n) Yuen et al. [36]. The localization of the E3 and E4 peptides obtained from Glu-C cleavage (this study) of AC I is also indicated.

fragmentation. In the present study, this has been achieved with NCS (cleaving at tryptophan residues) and with the endoproteinases Asp-N and Glu-C.

Fig. 5 is a schematic presentation of the C-terminal part of AC I displaying the numbers of amino acids at which chemical and enzymatic fragmentation are likely to occur. The presumptive epitopes of the monoclonal antibodies are shown in context. The present study infers that the smallest labeled peptide that could be detected was the 25 amino acid sequence V^{1027}–E^{1051}. The identity of this 2.9 kDa peptide, termed E4, was further corroborated by its failure to be precipitable by antibody BBC2 (not shown). E4 comprises a GXI/VXVKGKG sequence present in eight of the nine mammalian adenylyl cyclases so far cloned. Only type VIII adenylyl cyclase has a somewhat modified motif [21].

Fig. 6 shows the alignment of relevant sequences of several adenylyl cyclases and guanylyl cyclases. It is evident that the consensus sequence $G(X_{0-7})KG(X_{0-4})L(X_{0-5})S/T$ [5] is essentially verified for most eukaryotic and prokaryotic adenylyl cyclases. Furthermore a membrane bound and a soluble guanylyl cyclase were included, also fitting this motif. The lysine residue in this motif has been shown to be essential for catalytic activity in the case of the enzymes from *B. pertussis* (K^{58}) and *B. anthracis* (K^{346}) by mutagenesis [22,23]. In contrast, Tang et al. [14] have mutated K^{1049} in AC I and found no change in K_m and V_{max} but enhancement of K_i for 2′-deoxy,3′-AMP by a factor of 8. However, since bacterial adenylyl cyclases have a KG instead of a KGKG motif, the equivalence of the lysines is questionable.

Recently it has been found that an active adenylyl cyclase may be constructed solely from cytosolic segments C1a and C2a, segments C1b and C2b therefore being dispensable for catalytic activity [24]. The putative ATP binding site suggested in the present paper would be located at the C-terminal end of domain C2a.

Although the structure of eukaryotic adenylyl cyclases is symmetric, no labeling of a corresponding site in subdomain C1a could be detected here. It is of interest that the labeled E4 peptide also has the protein kinase A consensus sequence RRGSYR. Nevertheless, labeling by [γ^{32}P]8-N_3-ATP was clearly due to photolysis rather than phosphoryl transfer.

The precise identification of the labeled amino acid remains to be determined, e.g. by advanced mass spectrometric techniques. Because of the uncertainty in discrimination between substrate and inhibitor sites, similar studies with radiolabeled adenosine derivatives, as developed by Shoshani et al. [20], seem to be necessary.

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