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FOR THE RECORD

Characterization and crystallization of a minimal catalytic core domain from mammalian type II adenylyl cyclase

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Abstract: Adenylyl cyclases play a pivotal role in signal transduction by carrying out the regulated synthesis of cyclic AMP. The nine cloned mammalian adenylyl cyclases all share two conserved regions of sequence, C_1 and C_2, which are homologous to each other and are together responsible for catalytic activity. Recombinant C_1 and C_2 domains catalyze the synthesis of cyclic AMP when they are mixed and activated by forskolin, and C_2 domains alone also manifest reduced levels of forskolin-stimulated enzyme activity. Using limited proteolysis and mass spectrometry, we have mapped the boundaries of a minimal stable and active C_2 catalytic domain to residues 871–1090 of type II adenylyl cyclase. We report the properties and crystallization of this trimmed domain, termed IIC_2-Δ4. Crystals belong to space group $P4_n2_12$, where $n = 1$ or 3; $a = b = 81.3$, and $c = 180.5$ Å; and there are two molecules per asymmetric unit related by an approximate body centering operation. Flash-frozen crystals diffract anisotropically to 2.2 Å along the c^* direction and to 2.8 Å along the a^* and b^* directions using synchrotron radiation.

Keywords: cyclic AMP; forskolin; G-protein; mass spectrometry, protein engineering, transmembrane signaling, X-ray crystallography

Adenylyl cyclases (E.C. 4.6.1.1) play a pivotal role in signal transduction by catalyzing the regulated conversion of ATP to 3′, 5′ cyclic AMP (cAMP). cAMP is a second messenger of ubiquitous importance in biology by virtue of its activation of the cAMP-dependent protein kinase, cyclic nucleotide-gated ion channels, the bacterial catabolite gene activator protein, and the cAMP receptor of *Dictyostelium* (Robison et al., 1968; Rodbell, 1980; Taussig & Gilman, 1995; Sunahara et al., 1996; Tang et al., 1997). Nine isoforms of adenylyl cyclase have been cloned from mammalian tissues (Taussig and Gilman, 1995; Sunahara, et al., 1996; Tang et al., 1997). All of the cloned mammalian isoforms have similar activation by $G_{s\alpha}$, and all but IX are activated by forskolin. In contrast, they are quite variable in their responses to the α subunits of G_i, G_o, and G_z; and $G_{\beta\gamma}$, Ca^{2+}, Ca^{2+}/calmodulin, protein kinase C, cAMP-dependent protein kinase, and other regulators (Taussig & Gilman 1995; Sunahara et al., 1996; Tang et al., 1997).

The nine cloned mammalian adenylyl cyclases have a similar structure, with two hydrophobic domains comprising six predicted transmembrane helices each, and two ~40 kDa cytoplasmic domains (C_1 and C_2) (Taussig & Gilman 1995; Sunahara et al., 1996; Tang et al., 1997). The C_1 and C_2 domains are homologous to each other (roughly 30% identity) and confer all of the known catalytic and regulatory properties of mammalian adenylyl cyclases (Taussig & Gilman 1995; Sunahara et al., 1996; Tang et al., 1997). The C_1 and C_2 domains are homologous to a large family of adenylyl cyclases that have the common catalytic domains but otherwise diverse primary structures. These include adenylyl cyclases from bacteria, parasites, slime mold, and yeast (Tang et al., 1997). In addition, the C_1 and C_2 domains are homologous to membrane-bound and soluble guanylyl cyclases which catalyze the conversion of GTP to 3′, 5′ cyclic GMP (Tang et al., 1997). Thus, the three-dimensional structure of the conserved cyclase core will provide crucial information on the related catalytic mechanisms of all of these enzymes.

In a discovery with major implications for the structure and mechanism of adenylyl cyclases, Gilman and co-workers (Tang & Gilman, 1995; Dessauer & Gilman 1996) found that a recombinant protein consisting of the C_1 and C_2 regions connected by a linker was catalytically active and responsive to forskolin and $G_{s\alpha}$ stimulation. Furthermore, the type I C_1 (IC_1) and type II C_2 (IIC_2) regions expressed as separate recombinant proteins and then mixed at 10 μM concentrations have the same properties as the linked

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construct (Yan et al. 1996; Whisnant et al. 1996). Finally, the C_2 region alone is catalytically active as detected by genetic complementation (Tang & Gilman, 1995).

A working model has been proposed for cyclase activation on the basis of this dimerization and on the homology between the C_1 and C_2 regions (Tang et al., 1995, Whisnant et al. 1996, Yan et al., 1996). In this model, the juxtaposition of the two domains in the appropriate arrangement is required for catalytic activity. The appropriate juxtaposition is formed readily by the C_1 and C_2 heterodimer. The activity of C_2 alone strongly suggests that a C_2 homodimer can also be formed, although with a somewhat lower affinity, and that its stereochemistry is close enough to that of the C_1–C_2 heterodimer to enable catalysis. This suggests that three-dimensional crystal structure of either a C_1–C_2 heterodimer or a C_2 homodimer would lead to definitive insights into the mechanism and regulation of adenylyl cyclase. Here we describe the mapping of a protease-resistant core of the C_2 region of type II adenylyl cyclase, the properties of this C_2 domain variant, and its crystallization in the presence of forskolin.

Results and discussion: Our attention focused initially on recombinant protein containing residues 855–1090 of rat type II adenylyl cyclase preceded by a non-cleavable 21-residue tag MHHHHH HAAAYPYDVPDYAGI, for which Tang and co-workers have reported higher levels of recombinant protein expression than for other constructs (Yan et al. 1996). This construct, designated IIC_2-Δ3 (Yan et al., 1996) crystallized from ammonium sulfate in the first attempt in space group $I4_122$. These crystals had cell parameters of $a = b = 118$ Å, $c = 170$ Å and diffracted to 3.8 Å in the lab. Self-rotation and self-Patterson analysis suggested a single molecule was present in the asymmetric unit, yielding an unusually large packing parameter $V_m = 5.15$ Å^3/Da (Matthews, 1968). Crystals grew as large as 1.0 mM along each direction (Fig. 1), but crystals of a wide range of sizes diffracted to roughly the same limit. These crystals exhibited strong non-Bragg diffuse scattering. The crystals were also highly sensitive to any changes in their storage liquor, which precluded freezing and impeded searches for heavy atom derivatives. Finally, the diffraction limit measured on a charge-coupled device (CCD) detector at the A1 beamline at the Cornell High Energy Synchrotron Source (CHESS) was no better than that measured in the lab. In combination, this demonstrated that the diffraction limit of these IIC_2-Δ3 crystals was limited by a lack of internal order which could not be overcome by increasing scattering power.

In an effort to remove flexible regions of IIC_2-Δ3 and improve the order in the crystals, we proceeded to map the boundaries of potential rigid core structures by limited proteolysis and mass spectrometry (Cohen et al., 1995). Digestion of the 29 kDa IIC_2-Δ3 protein with subtilisin, chymotrypsin, and endoproteinases LysC and GluC revealed a relatively stable GluC product of approximately 25 kDa. The mass spectrum of the digest showed this product has a molecular weight of 25,058 Da (Fig. 2). Because only one cleavage product appeared strongly, a singly-cleaved product was judged more plausible than a multiply cut product. Of all potential GluC cleavage products, the most plausible singly-cut product was at Glu 865, with a predicted MW of 25,046.

In addition to the major 25,058 Da product, a number of lower MW products of 14.7 kDa and below were also present. Therefore, we chose not to attempt to purify and crystallize the cleavage product itself, but rather to engineer a new IIC_2 variant based on the calculated GluC cleavage site. The calculated Glu-C cleavage site was converted into a thrombin site by site-directed mutagenesis. The mutant protein, designated pro-IIC_2-Δ4, yields only one thrombin product visible on a 16% SDS-PAGE gel at the expected MW. Mature IIC_2-Δ4 has a mass of 24,490 Da by mass spectrometry, less than the mass of 25,391 Da which was calculated for the C-terminal portion of the engineered thrombin site (GS) fused to residues 864–1090. The N-terminal amino-acid sequence of mature IIC_2-Δ4 is SLKNEELYHQSYD as obtained by automated Edman degradation. The mature protein therefore begins at residue 871. Thrombin cleavage occurred at the site FLAR^SL, which is atypical but has some resemblance to a typical thrombin site. We subsequently found that cleavage at this site occurs with equal efficiency in both IIC_2-Δ3 and IIC_2-Δ4. The IC_1 and IIC_2-Δ4 mixture shows approximately 20% of the catalytic activity of the IC_1 and IIC_2-Δ3 mixture at the same amount of forskolin (Fig. 3A). The IIC_2-Δ4 alone also showed low but detectable forskolin-stimulated adenylyl cyclase activity (Fig. 3B).

Tetragonal crystals (form II) of IIC_2-Δ4 were readily obtained at room temperature from ammonium sulfate solutions. The observed morphology is entirely different from that of form I crystals of IIC_2-Δ3. Form II grows as rods rather than bi-pyramids. These crystals grow as clusters and were micro-surgically de-twinned for data collection. Despite a less attractive visible morphology than form I, form II crystals diffract far better, up to 2.8 Å in the laboratory and 2.2 Å at the A1 beamline at CHESS. The best diffraction is only observed in the c^* direction, with the diffraction limit in the a^* and b^* directions lower by about 0.6 Å. Prior to scaling, integrated intensities were subjected to an anisotropic resolution cut-off defined by an ellipsoid of rotation with axes of 1/2.8 Å along a^* and b^*, and 1/2.2 Å along c^*. The ellipsoid contains a volume of reciprocal space equivalent to that of a sphere with radius 1/2.58 Å, hence we believe the data will yield a 2.6 Å-equivalent refined structure. The R_{merge} ($\Sigma|I_i - \langle I\rangle|/\Sigma\langle I\rangle$) for the 17,326 unique reflections measured with non-negative intensity within the ellipsoid is 0.067. The ratio of merged intensity to its standard error $\langle I\rangle/\sigma\langle I\rangle$ is 14.4 overall and 2.9 for the 2.28 to 2.20 Å bin. The space group is $P4_n2_12$ ($n = 1,3$) with $a = b = 81.3$ Å and $c = 180.5$ Å. A reasonable V_m value of 3.04 Da/Å^3 is obtained if two molecules are assumed per asymmetric unit. The self-rotation function does not reveal any significant peaks corresponding to non-crystallographic rotations. The self-Patterson, however, has a large peak at 0.5,0.5,0.47 indicating approximate body centering. Indeed, manual inspection of intensities revealed a characteristic pattern of alternating strong and weak intensities as a function of index. We believe the approximate extinctions are partially responsible for the high proportion of weak data for form II.

Proteolytic "trimming" has proved to be a powerful approach to the crystallization of otherwise intransigent proteins (Noel et al., 1993). Here, we have found that trimming the N-terminus of a recombinant domain has yielded crystals which diffract as far as 2.2 Å resolution in one direction, whereas the untrimmed domain never yielded crystals which diffracted better than 3.8 Å. This demonstrates the power of the approach not only in *de novo* crystallization of a previously uncrystallizable protein, but also in greatly improving the quality of crystals of a protein already known to crystallize. IIC_2-Δ3 is relatively unique among reported adenylyl cyclase fragments in that it can be readily expressed at high levels in *Escherichia coli* without proteolytic degradation (Yan et al., 1996). To avoid the risk of altering these desirable expression properties, we opted not to express a further shortened form of

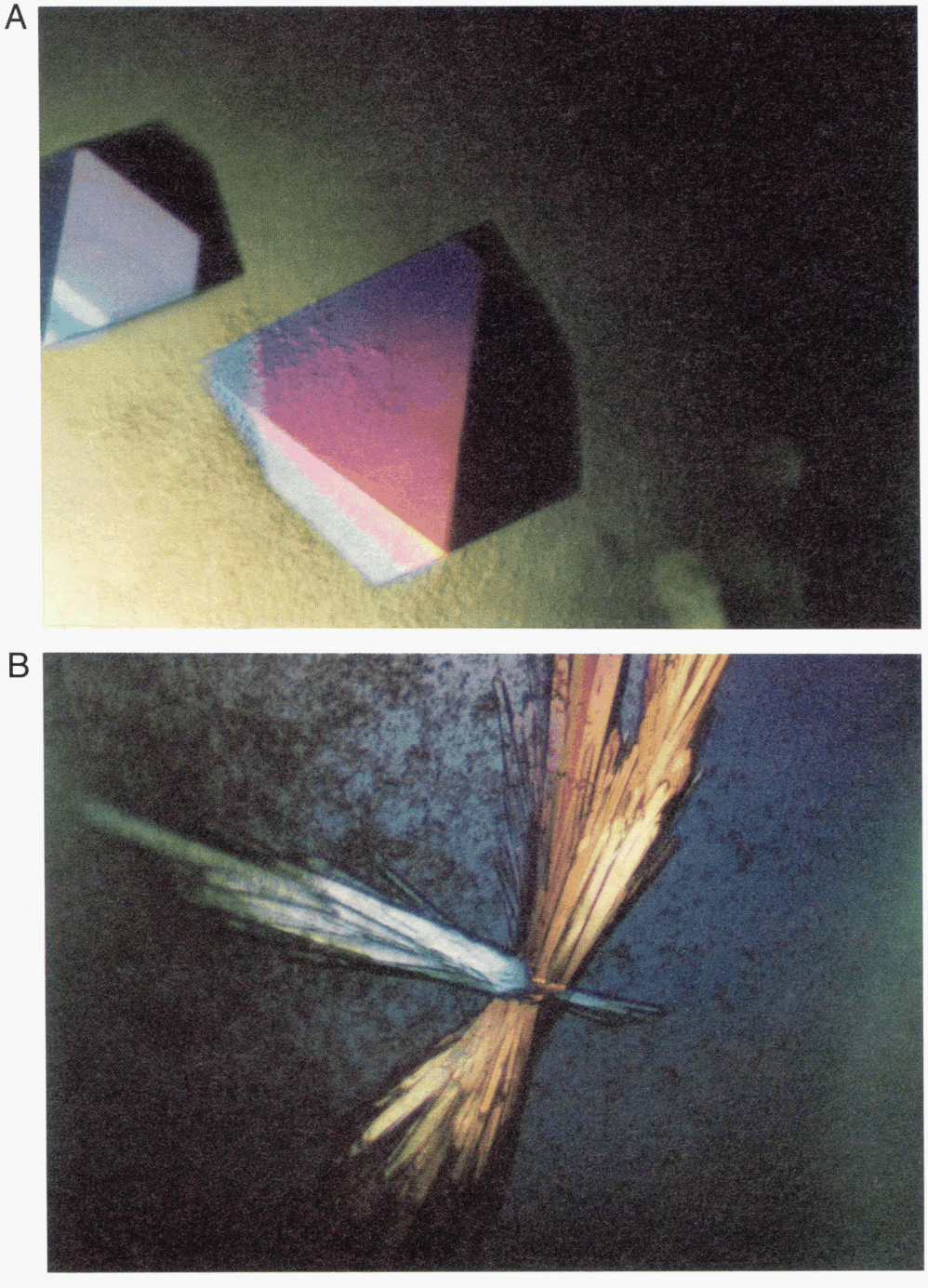

Fig. 1. Crystals of **(A)** IIC_2-Δ3 (form I) and **(B)** IIC_2-Δ4 (form II). The horizontal edge of the photographs is approximately 2.0 mM long.

IIC_2-Δ3, but to engineer a more specific protease site instead. The serendipitous finding of a naturally occurring thrombin site in IIC_2-Δ3 showed that the second tactic was unnecessary in this case. Because it is uncommon for highly specific natural protease sites to be found precisely where needed to yield crystallizable protein fragments, we feel that the strategy outlined here should be useful in other systems.

Excluding the polyhistidine tag, 17 residues of type II adenylyl cyclase are removed from IIC_2-Δ4 as compared to IIC_2-Δ3. The remaining 220 residues include all of the catalytically important residues identified to date (Tang et al., 1995). Therefore it is not surprising that the catalytic properties of IIC_2-Δ4 are similar to those of IIC_2-Δ3.

Growth of form I and II crystals requires the presence of forskolin. Previous studies of by gel filtration chromatography by some workers have shown that forskolin promotes dimerization of adenylyl cyclase catalytic domains (Yan et al. 1996), while other workers have found similar domains are constitutive dimers (Whis-

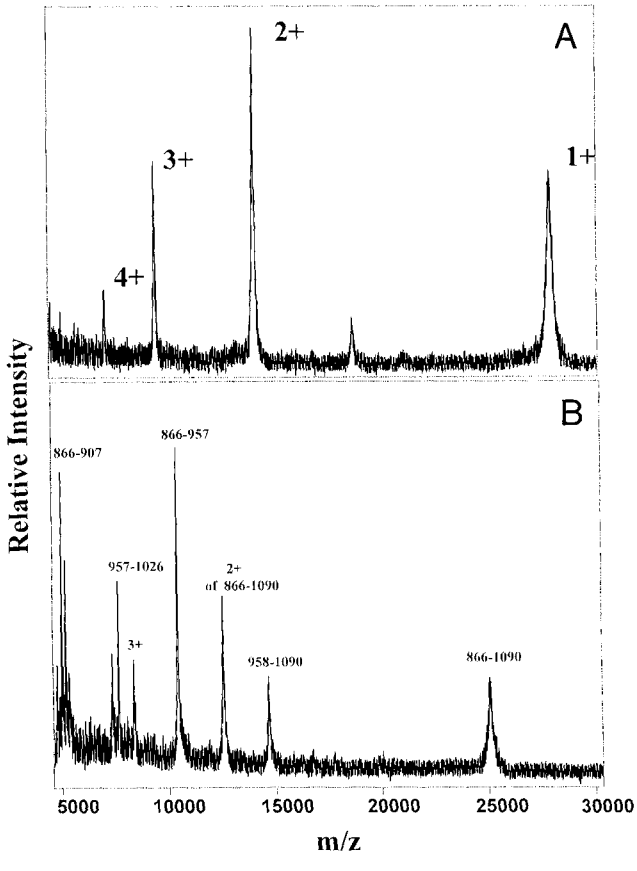

Fig. 2. Mass spectra of **(A)** IIC_2-Δ3, with the presumed ionization state of each peak indicated, and **(B)** the GluC digest of IIC_2-Δ3, showing peaks assigned to the GluC fragments with the closest calculated mass and ionization state.

nant et al., 1996). In either event, it is reasonable to expect that both crystal forms contain forskolin-bound dimers. Functional dimers of enzymes typically correspond to crystallographic or non-crystallographic two-fold axes of rotation. There is no evidence in the self-rotation functions of forms I or II for non-crystallographic rotation axes, although this does not rule out axes parallel to a crystallographic axis. Therefore is most likely that the functional dimer is related either by a crystallographic two-fold or by a local axis parallel to a crystallographic axis. The structure of the dimer is likely to lead to new insights into how forskolin and other adenylyl cyclase regulators alter enzyme activity by controlling subdomain interactions.

Methods: *Limited proteolysis and mass spectrometry:* Purified IIC_2-Δ3 prepared following Yan et al. (1996) was treated separately with chymotrypsin and endoproteinases LysC and GluC for 2 h each at room temperature at mass ratios of 1:150, 1:50, 1:25 protease to substrate. Digests were analyzed by SDS-PAGE on 16% Tris-glycine gels (Novex) and Coomasie stained. The unclipped IIC_2-Δ3 and its 1:150 GluC digest were further analyzed on a matrix-assisted laser desorption/ionization mass spectrometer (PerSeptive Biosystems, Voyager-DE). α-Cyano-4-hydroxycinnamic acid was used as the matrix. Masses were calibrated externally.

Protein expression and purification: DNA coding for IIC_2-Δ3 was mutagenized to convert residues 858-863 (VLPAHV) to the thrombin site LVPRGS. To construct pProEX-HAII6-IIC2-Δ4, a polymerase chain reaction was performed on the pProEX-HAII6-IIC2-Δ3 using the primers 5′-ggaattctggagaaattggttcctcgaggctcgg ctgaacacttcc-3′ and 5′-agcggataacaatttcacacagg-3′ with native Pfu polymerase (Stratagene). The 0.7 kb product was digested with EcoRI and HindIII and purified from an agarose gel with the Geneclean kit (Bio 101) and ligated into pProEX-HAII6-IIC2-Δ3 that had been digested with the same enzymes and purified from an agarose gel as described above. This construct was then transformed into BL21 (DE3) and the *met* auxotroph B834 (DE3).

"Pro"-IIC_2-Δ4 was purified on immobilized metal ion chelating chromatography (Yan et al., 1996) and desalted by dialysis. Thrombin cleavage (1.0 U per mg substrate) was carried out overnight concurrently with dialysis in the presence of 2 mM $CaCl_2$. "Mature" IIC_2-Δ4 was further purified on a FPLC Mono-Q HR10/10 column using a gradient of 0 to 0.5 M NaCl at pH 8.0. Following FPLC, protein was exchanged into crystallization buffer and concentrated to 15 to 20 mg/mL. Selenomethionyl IIC_2-Δ4 was prepared by transforming *E. coli* B834 (DE3), expressed in a defined medium with methionine replaced by selenomethionine (Hendrickson et al., 1990; Doublie & Carter, 1992), and purified by the same procedure. Incorporation of 5–6 mole selenomethionine per mole protein, as compared to 6 methionines in the amino acid sequence, was confirmed by mass spectrometry (not shown).

Enzyme assays: Adenylyl cyclase assays were carried out in a 100 μL final volume for 10 min (Fig. 3A) or 40 min (Fig. 3B) at 30 °C in 20 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA (pH 7.8), 0.5 mM ATP, and 0.1 mM 3-isobutyl-1-methylxanthine. Production of cAMP was measured by the method of Salomon et al. (1974) and with the cAMP assay kit (Amersham; Dhanasekaran et al., 1991; Voss & Wallner, 1992).

Crystallization: Both crystal forms were obtained in the presence of forskolin at 21–24 °C by hanging drop vapor diffusion. Forskolin was dissolved at 20 mg/mL in DMSO and diluted into concentrated protein at a volume ratio of 1:50. Initial conditions for crystallization were screened by the incomplete factorial method (Jancarik & Kim, 1991). Form I was grown by mixing 2 μL of 15 to 20 mg/mL IIC_2-Δ3 protein, 50 mM Tris HCl pH 8.0, 80 mM NaCl, 10 mM DTT, 1 mM forskolin, 2% DMSO with an equal volume of well solution composed of 1.5 M $(NH_4)_2SO_4$ and 0.1 M sodium citrate/acetate buffer pH 5.6. Form II was grown by mixing 2 μL of 17.5 mg/ml IIC_2-Δ4 protein, 50 mM Tris HCl pH 8.0, 80 mM NaCl, 10 mM DTT, 1 mM forskolin, 2% DMSO with an equal volume of well solution containing 1.1 to 1.2 M $(NH_4)_2SO_4$ and 0.1 M sodium phosphate buffer pH 5.80 to 5.90.

Data collection: Diffraction data were collected in the laboratory using a R-Axis IIC detector mounted on a Rigaku RU-200 rotating anode generator equipped with focusing mirrors and operating at 5 kW. Oscillation patterns from form I crystals were collected at room temperature for 10 min and 1° each. The space group was deduced by auto-indexing with the HKL package (Otwinowski & Minor, 1997) and manual inspection of integrated intensities for systematic absences. Still and 1° oscillation images of form I crystals obtained at room temperature on a CCD detector at the CHESS A1 beamline (6 sec exposure with the detector 80 mM from the crystal) diffracted to the same resolution limit as in the laboratory. Form II data were collected from crystals flash-frozen in mother

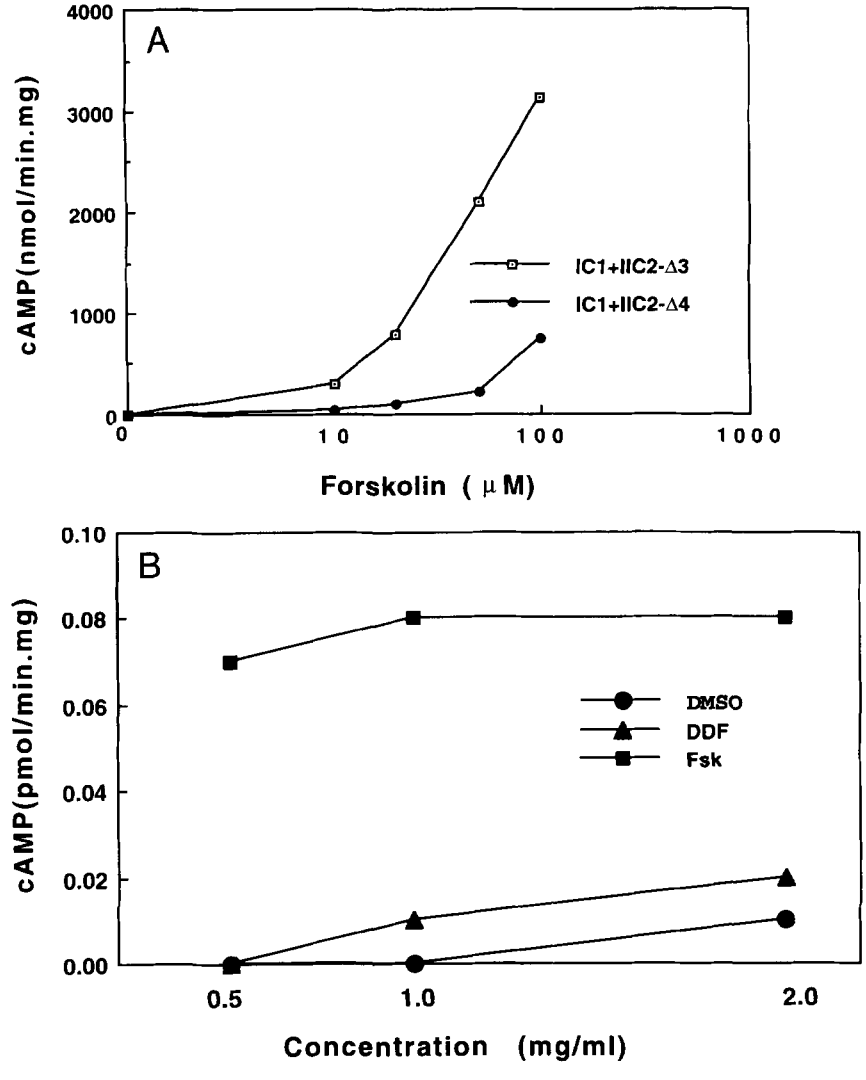

Fig. 3. A: Forskolin-dependent enzyme activity of 0.5 μg IIC_2-Δ3 or IIC_2-Δ4 in the presence of 0.5 μg IC_1. **B:** Concentration dependence of IIC_2-Δ4 activity in the absence of IC_1. Abbreviations: Fsk is 100 μM forskolin and 1% DMSO, DMSO is 1% DMSO, and DDF is 100 μM 1,9 dideoxyforskolin and 1% DMSO.

liquor supplemented with 15% glycerol. Form II data were collected on imaging plates in the laboratory (30 min exposures) and at the CHESS A1 beamline (20 sec exposures recorded on an image plate 200 mM from the crystal). Patterson syntheses were calculated for both crystal forms using the Phases package (Furey & Swaminathan, 1997), and self-rotation functions were calculated with Polarrfn (CCP4, 1994). Native Patterson syntheses were computed with data from 40 to 4 Å resolution and manually interpreted using mapview (Furey & Swaminathan, 1997).

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