Supplementary Subjects & Methods

Informed consent was obtained as part of a protocol approved by the Institutional Review Boards of the participating institutions. The index patients described in this study were referred for the investigation of CS; their relatives were recruited after the identification of the mutation (see supplementary Subjects and Methods). Index patients and available relatives were evaluated by a thorough history and physical examination. Briefly, the patients were studied for clinical signs of CS and CNC, including dermatological examination and thyroid palpation. Ovarian or testicular, thyroid, and cardiac ultrasound scans and pituitary magnetic resonance imaging were performed. Plasma concentrations of GH, PRL, and IGF-I were determined. In all patients with the mutation, including relatives, we screened for CS by standard testing; affectation status was determined using published diagnostic criteria (20). In Brief, no suppression of plasma cortisol after 1 mg oral dexamethasone, and a paradoxal rise in 24 h urinay free cortisol: mean 2.2 x ULN, increasing to a max of 6.2 x ULN after a 6 day Liddle test. After 100 μg CRH iv, there was no increase of plasma cortisol and ACTH concentrations remained undetectable.

DNA was extracted from peripheral blood leukocytes using the Wizard Genomic DNA Purification KIT (Promega) and the twelve exons and the flanking intronic sequences of the *PRKAR1A* gene were amplified using the primers and the conditions described previously (7,8).

Lymphocyte cell lines from patients or control subjects were established and treated as previously described with 100 μg/ml cycloheximide or vehicle for 6 hours as previously published (7,8). Total RNA was extracted and cDNA from lymphocytes was amplified; PCR fragments were run on polyacrylamide gel, purified and analysed by direct sequencing as previously published (7,8).

To study the functional effect of M1V on the PRKAR1A, a PCR - based cloning method was used to generate both the wild-type (WT) and mutant expression constructs, according to previously published methods (8). The effect of mutated RIα on PKA activity was determined as previously described (8).

PKA activity is defined as that amount of enzyme that transfers one pmole of 32P from [γ-32P]ATP to the recovered protein in min at 30oC in the standard assay system (Nesterova M, Sashchenko, Vasiliev V Yu, Severin ES A cyclic adenosine 3',5'-monophosphate-dependent hiostone kinase from pig brain. Purification and some properties of the enzyme. Biochem. Biophys. Acta, 1975, Feb 377 (2), 271-281)

cAMP-binding activity is represented as picomoles of bound cAMP per mg protein in the conditions when reaction was reched equilibrium (60 min incubation at 4oC) (Gilman AG A Protein Binding Assay for Adenosine 3":5"- Cyclic Monophosphate. PNAS 10970 Sept 67 (1) 305-312.

Supplementary Results

Significantly higher basal PKA activity was measured after transfection with the constructs bearing M1V compared to the wild type (WT) PRKAR1A constructs 0.76+0.02 vs 0.47±0.014 units/mg protein, $p<0.05$ (Supplementary Figure 1A). Addition of cAMP increased kinase activity in the M1V-bearing construct to 2.24 ± 0.14 vs 1.1±0.12 for the WT (fold increase 2.95 vs 2.38; $p=0.08$). Addition of a specific kinase inhibitor (PKI) reduced kinase activity in M1V to 0.63±0.018 vs 0.4±0.02 units/mg protein ($p=0.001$) in the WT. In line with above, binding to cAMP was significantly reduced for the M1V bearing protein compared to the WT (5.5±0.7 vs 15.75±1.06 pmoles cAMP/mg protein, $p= 0.001$, Supplementary Figure 1B).