expenditure of energy (*20*). More specifically, they may be limited by the availability of abundant ant nests and termitaria. Presumably more equable climates and year-round availability of ants and termites existed at El Golfo in the early Pleistocene.

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Steroidogenesis-Activator Polypeptide Isolated from a Rat Leydig Cell Tumor

ROBERT C. PEDERSEN AND ALEXANDER C. BROWNIE

A cycloheximide-sensitive protein responsive to adenosine 3′,5′-monophosphate has been postulated to participate in the regulation of cholesterol side-chain cleavage activity in steroidogenic tissues. Such a steroidogenesis activator polypeptide (SAP) had been isolated from rat adrenocortical tissue and partially characterized. Now a polypeptide with comparable chromatographic behavior and biological activity has been purified from the rat H-540 Leydig cell tumor in quantities sufficient for amino acid sequencing. The activator contains 30 amino acid residues and has a molecular weight of 3215. The synthetic construct based on this sequence is virtually equipotent with native H-540 tumor SAP in an adrenal mitochondrial cholesterol side-chain cleavage assay. Hormonal regulation of the intracellular concentration of this activator may control the rate of cholesterol metabolism in steroidogenic organs.

THE HORMONALLY REGULATED, committed reaction in steroid formation—the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage (cholesterol scc) cytochrome P-450 complex (*1*)—is acutely sensitive to inhibitors of protein synthesis (*2, 3*). Considered with the rapid metabolic response of steroidogenic tissues to appropriate hormonal stimuli, this observation suggested the existence of a labile intracellular protein mediator of adrenocorticotropin (ACTH) and gonadotropin action on pregnenolone formation (*3, 4*) that is increased in activity by adenosine 3′,5′-monophosphate (cAMP) (*5, 6*).

A factor in the adrenal cortex of rats implanted with an ACTH-secreting tumor was identified (*7*) as a steroidogenesis activator polypeptide (SAP) that exhibits many of the characteristics imputed to the hypothetical modulator. However, the quantities of material available were inadequate for a successful determination of its primary structure. Therefore, we turned to the H-540 rat Leydig cell tumor as a source that might be enriched in the activator. This approach was suggested by two observations. First, a material that is chromatographically similar to adrenal SAP and that enhances cholesterol scc activity in the adrenocortical mitochondrial assay can be detected in the testis of the normal postpubertal rat (*8*). Second, the H-540 Leydig cell tumor contains substantial cholesterol scc activity that is responsive to gonadotropins or cAMP and sensitive to cycloheximide (*9*).

The isolation of H-540 tumor SAP was accomplished by minor modifications of the protocol described for adrenocortical SAP (*7*). Material eluting in a low molecular-weight range (1500 to 6000) during size-exclusion high-performance liquid chromatography (HPLC) was collected, neutralized, and subjected to reversed-phase HPLC (Figs. 1 and 2A). Chromatography fractions were monitored for stimulation of cholesterol scc activity (Fig. 1), and the material in the region of interest was rechromatographed twice to obtain a preparation (Fig. 2A) that was homogeneous by NH_2-terminal amino acid analysis (isoleucine) (*10*).

A comparison of adrenocortical SAP (*7*) and Leydig cell tumor SAP suggested that each has a dose-dependent effect on cholesterol scc activity when measured with either adrenocortical or H-540 tumor mitochondria (Table 1). In the adrenocortical assay system, $10^{-8}M$ or $10^{-7}M$ concentrations of each activator stimulated cholesterol conversion to about 1.5 or 6 times, respectively, the activity of control incubations. At the higher concentration of polypeptides, this increase is comparable to that observed in the adrenal mitochondria of stressed rats (*11*). With mitochondria from the Leydig cell tumor, the overall levels of enzyme activity were predictably (*9*) lower (Table 1), but both of the polypeptides produced effects qualitatively similar to those seen in the adrenocortical assay system.

From a tryptic digest of 4.4 nmol of H-540 SAP (25 tumors), three fragments were resolved by reversed-phase HPLC (Fig. 3) and sequenced manually by Edman degradation (*10*). The phenylthiohydantoin (PTH) amino acids were identified by reversed-phase HPLC. Vapor-phase microsequencing (*12*) of 0.8 nmol of intact H-540 tumor SAP, carried out through residue 28, was used to confirm these sequence data and to establish fragment order. The 3215-dalton

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Table 1. Effect of rat adrenocortical SAP and H-540 Leydig cell tumor SAP on mitochondrial cholesterol scc activity. The cholesterol scc assay measures the rate of pregnenolone formation from endogenous cholesterol (nanomoles of pregnenolone per minute per milligram of mitochondrial protein) when mitochondria are provided with a means (10 m*M* isocitrate) for generating reducing equivalents. The mitochondrial preparations were poised for optimal response by treating hypophysectomized (24 hours) female rats (Sprague-Dawley and Holtzman) with cycloheximide (15 mg) and either $ACTH_{1-24}$ (Cortrosyn, Organon; 100 ng) or human chorionic gonadotropin (Sigma; 10 IU). Cycloheximide was given intraperitoneally and the pituitary hormones intravenously at 45 and 10 minutes, respectively, before the collection of adrenal and H-540 tumor tissue. The transport of cholesterol to the outer mitochondrial membrane was thereby stimulated, while the level of endogenous SAP and the rate of steroidogenesis were greatly diminished (*7*). For each assay with adrenocortical mitochondria, two pairs of adrenals were harvested by enucleation in situ; for each assay with H-540 mitochondria, only a fraction of one tumor implant (~0.1 g) was needed. Mitochondria were prepared by differential centrifugation as described (*21*). The preparation was resuspended [1 mg of protein per milliliter (*22*)] in reaction buffer (*8*) containing 10 μ*M* cyanoketone (Upjohn) and (for H-540 tumor mitochondria) 20 μ*M* spironolactone (Sigma) to inhibit pregnenolone metabolism; it was then apportioned by 20-μl aliquots into tubes containing 50 μl of sample or buffer. The various controls have been described (*8*). Tubes were warmed at 37°C for 10 minutes, and the reaction was started by the addition of 10 μl of isocitrate solution to each tube. After 60 seconds the tubes were plunged into liquid nitrogen and subjected to two freeze-thaw cycles. Samples were diluted with radioimmunoassay buffer and assayed for pregnenolone (*23*). Data (mean ± SEM) are derived from three such experiments.

Addition	Cholesterol scc activity (nmol min^{-1} mg^{-1})	
	Adrenal	H-540 tumor
Buffer only	0.26 ± 0.03	0.03 ± 0.01
+ Adrenal SAP (10^{-8}*M*)	0.42 ± 0.05	0.10 ± 0.03
(10^{-7}*M*)	1.80 ± 0.25	0.38 ± 0.03
+ H-540 SAP (10^{-8}*M*)	0.31 ± 0.04	0.12 ± 0.01
(10^{-7}*M*)	1.54 ± 0.21	0.45 ± 0.05

activator consists of 30 residues and has unblocked termini, as follows:
NH_2-Ile-Val-Gln-Pro-Ile[5]-Ile-Ser-Lys-Leu-Tyr[10]-Gly-Ser-Gly-Gly-Pro[15]-Pro-Pro-Thr-Gly-Glu[20]-Glu-Asp-Thr-Asp-Glu[25]-Lys-Lys-Asp-Glu-Leu-COOH.

A search of the Protein Identification Resource of the National Biomedical Research Foundation failed to identify any significant overall homology with proteins in that database. Notably, there is no apparent structural relatedness with the mature form of bovine sterol carrier $protein_2$ (*13*). The largest partial sequence identity (60%) exists between the NH_2-terminal region of SAP (SAP_{1-15}) and residues 603 to 617 of the frog heat-shock protein hsp70 (*14*), but that relation is not sustained into the COOH-terminal portion of SAP, and its significance is therefore unclear.

We synthesized the polypeptide on the basis of the assigned sequence of H-540 tumor SAP (*15*), and its chromatographic behavior (Fig. 2B) was indistinguishable from that of the native material (Fig. 2A). Moreover, in the adrenocortical cholesterol scc assay, synthetic SAP displayed a dose-dependent effect that was virtually identical to that of the tumor-derived preparation (Fig. 4). The polypeptide does not stimulate adrenocortical 11β- or 18-hydroxylase activity in vitro.

An identity between the polypeptide described here and adrenocortical SAP has not yet been established. The amino acid composition for the adrenal material (*7*) differs somewhat from that of Leydig cell tumor SAP, but the former analysis is now known to contain several inaccuracies. The two factors exhibit chromatographic, biological, and immunological properties that suggest they are at least very similar. It will be interesting to evaluate an analogous factor in the normal rat testis and the superovulated rat ovary (*8*), for our studies to date suggest that this polypeptide may be common to all of the steroidogenic organs.

Rat adrenal SAP seems to facilitate the association of cholesterol with cytochrome $P\text{-}450_{scc}$ when examined spectrophotometrically (*7*), but it does not bind cholesterol.

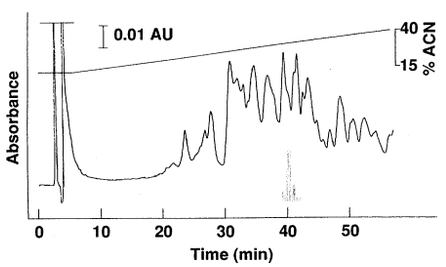

Fig. 1. Reversed-phase HPLC profile of the low molecular weight fraction of H-540 tumor homogenate. Tumor fragments were implanted by subcutaneous injection into the left hindlimbs of castrate Fischer 344 adult male rats (Hilltop Lab Animals) and allowed to grow for 4 weeks (*9*). Tumors were harvested 10 minutes after intravenous administration of 10 IU of human chorionic gonadotropin, freed of connective tissue, and homogenized in an acidic extraction medium (2 ml of medium per gram of tissue) (*7, 24*). After centrifugation (9000*g* for 30 minutes), the supernatant was concentrated on pre-equilibrated C_{18}-silica SepPak cartridges (Millipore) and eluted with a gradient of acetonitrile (15% to 75%) in 0.1% aqueous trifluoroacetic acid (TFA). [In a recent modification of this protocol, we found that the subsequent HPLC chromatography is significantly cleaner if the acid extract is first adjusted to *p*H 5.5 with triethylamine and subjected to batch anion-exchange fractionation (*25*) on a SepPak cartridge of Accell QMA (Millipore) before the C_{18}-silica SepPak step.] The SepPak eluate was then reduced under a stream of nitrogen, loaded onto a Superose 12 column (Pharmacia) calibrated with appropriate molecular weight standards, and subjected to size-exclusion HPLC (Varian 5500) under denaturing conditions (20% acetonitrile in 0.1*N* HCl, 0.5 ml/min). Absorbance of the stream was monitored at 278 nm (Varian Polychrome diode array detector) (AU, absorbance unit). Fractions of interest (*7*), eluting between 26 and 30 minutes and corresponding to the 1500 to 6000 molecular size range, were pooled, syringe-loaded onto a C_{18}-silica guard cartridge (Brownlee) mounted in the place of the injection loop of a Rheodyne 7125 valve, and resolved by reversed-phase HPLC on a MicroPak SP-C_{18} column (0.4 by 15 cm, 3-μm packing; Varian) with a 60-minute linear gradient of acetonitrile (15 to 40%) in 0.1% aqueous TFA (0.5 ml/min). Absorbance of the stream was monitored at 215 nm, 1-minute fractions were collected, and each was split into two sets. One set was dried in a Speed Vac concentrator (Savant), resuspended in assay buffer, and examined for cholesterol scc–stimulating activity (hatched area) with adrenocortical mitochondria, as described in the legend to Table 1. Active fractions were pooled and rechromatographed under the same conditions (Fig. 2A).

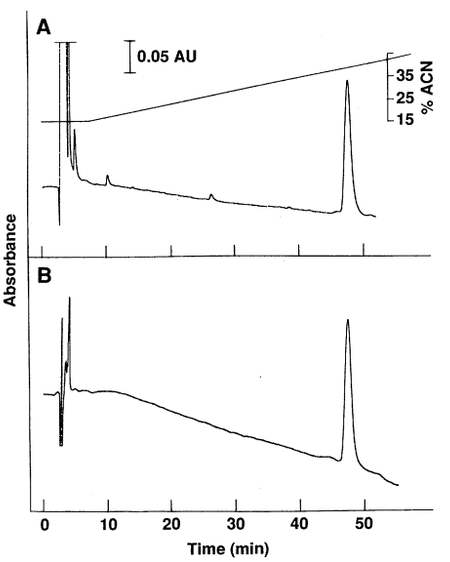

Fig. 2. Reversed-phase HPLC of (**A**) native and (**B**) synthetic tumor H-540 SAP. Chromatography was carried out with ~1.0 nmol of each polypeptide according to the conditions described in Fig. 1. The homogeneity of native SAP (A) was confirmed by NH_2-terminal analysis of fractions representing the leading, apical, and trailing portions of ultraviolet-absorbing material. The polypeptide is stable for at least 4 months when stored in chromatography eluant at 4°C.

However, the mechanism by which SAP activates pregnenolone formation remains undefined. The polypeptide appears primarily in the high-speed supernatant after subcellular fractionation, although a loose association with mitochondria in situ cannot be ruled out. It is difficult to envision how such a relatively hydrophilic molecule could physically interact with elements of a reaction complex which, as characterized in the adrenal cortex, reside within the bilayer of the inner mitochondrial membrane (*16*). It seems more likely that SAP facilitates intramitochondrial cholesterol redistribution in some indirect manner, as, for example, by influencing events impinging on membrane morphology (*17*) or lipid remodeling (*18*). Moreover, the groups of vicinal acidic residues in the COOH-terminal portion of the molecule could define a functional Ca^{2+}-binding domain.

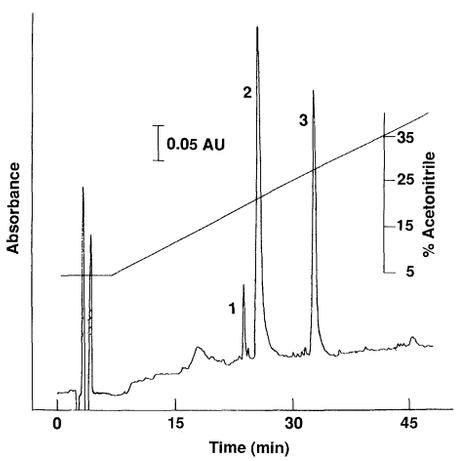

Fig. 3. Peptide mapping of a tryptic digest of 4.4 nmol of H-540 tumor SAP by reversed-phase HPLC. Purified SAP from 25 tumors was digested with trypsin treated with tosyl-phenylethyl-chloromethyl-ketone (Sigma) (30:1 by weight) for 48 hours at 37°C in 200 μl of 0.1*M* tris-HCl, *p*H 8.0. The digest was acidified with TFA and subjected to reversed-phase HPLC with the system configuration described in Fig. 1 and a 48-minute linear gradient of acetonitrile (5 to 41%) in 0.1% aqueous TFA. The stream was monitored at 205 nm, and the three resolved fragments plus material eluting in the injection front (3 to 6 minutes) were collected and dried under vacuum. Fifteen percent of each sample was subjected to conventional acid hydrolysis (6*N* HCl; 24 hours at 105°C) and resolution of the component phenylthiocarbamyl–amino acid derivatives (*26*) by reversed-phase HPLC (50-pmol detection limit). No protein was evident in the injection front. The remainders of fragments 1, 2, and 3 were sequenced by manual Edman degradation (*10*) and the PTH–amino acid adducts were identified by reversed-phase HPLC (*27*) (20-pmol detection limit). Fragment order was assigned as follows, on the basis of the sequence of intact SAP derived by vapor-phase technology: (peptide 3, SAP_{1-8}; peptide 2, SAP_{9-26}; and peptide 1, SAP_{28-30}). The broad peak eluting at 18 minutes is present in the blank, and the small peak at 46 minutes coelutes with undigested SAP.

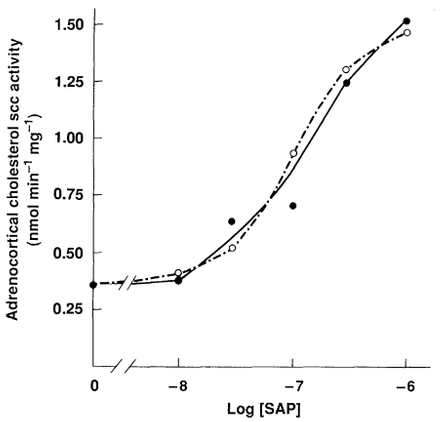

Fig. 4. Dose-dependent stimulatory effect of native (○) and synthetic (●) H-540 tumor SAP on adrenocortical cholesterol scc activity. The assays were carried out as described in Table 1.

Finally, in a model for the hormonal regulation of SAP that borrows from the work of others (*3, 4*), we have suggested (*19*) that the intracellular concentration of this activator may be controlled by its rate of formation from some larger, unstable precursor. We have preliminary data in support of a higher molecular weight form of immunoreactive SAP in the adrenal cortex, but the precise relation between these proteins has not yet been resolved. It is also uncertain whether there is any connection between SAP and the ACTH- and cAMP-responsive sets of proteins identified by Orme-Johnson and colleagues (*20*). Clearly, however, with the structure of the polypeptide activator now in hand, it should be possible to address these questions expeditiously.

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