

A cAMP-triggered release of a hormone-like peptide

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Preparations of the catalytic subunit of cAMP-dependent protein kinase from rabbit skeletal muscle, which appear to be homogeneous by SDS-polyacrylamide gel electrophoresis, were often found to contain a hormone-like factor (HLF) which causes an immediate rise, then a decline of intracellular cAMP in a B-lymphoma cell line. Active HLF is released when the fractions that contain it in an inactive form are incubated with cAMP prior to chromatography, or passed through an immobilized cAMP column. HLF seems to be a peptide: it loses its cell-stimulating capability after proteolysis and has an apparent molecular mass of 2.2–2.5 kDa.

cyclic AMP; cyclic AMP-dependent protein kinase; Cell-cell signaling; Immobilized cyclic AMP; Peptide hormone; (B-lymphoma cell line)

1. INTRODUCTION

Following the isolation and characterization of a membranal proteinase which clips the catalytic subunit of cAMP-dependent protein kinase (PKA) with a unique biochemical specificity [1–3], and the finding that in cell membranes the proteinase faces the cell exterior [4], we considered a possible extracellular function for PKA [5,6]. While attempting to identify such a function, we observed that preparations of the catalytic subunit of PKA (purified from rabbit skeletal muscle [7]), which appeared to be homogeneous by SDS-PAGE and Coomassie blue staining, often contain a hormone-like factor (HLF) which brings about a rapid rise and a subsequent decline in the level of intracellular cAMP in a B-lymphoma cell line. This paper assigns the hormone-like activity to one (or

more) peptide(s) (2.2–2.5 kDa) and provides evidence that HLF may exist in an inactive complex which copurifies with the holoenzyme of type I PKA, and that cAMP triggers its release in an active form.

2. MATERIALS AND METHODS

2.1. *The B-lymphoma cell line (WEHI-231)*

The WEHI-231 cell line [8] was kindly provided by Professor Dov Zipori from the Weizmann Institute of Science, Rehovot. The cells were cultured at 37°C in tissue culture flasks in an RPMI-1640 medium containing heat-inactivated fetal calf serum (10%), 2-mercaptoethanol (50 µM), penicillin (200 U/ml), streptomycin (200 µg/ml) and neomycin (10 µg/ml). The culture atmosphere was composed of CO₂ (7%) and humid air (93%). Cell preparations used (<10⁶ cells/ml) were growing logarithmically and contained over 90% viable cells, as judged by standard trypan blue staining. Before challenging with HLF or a control hormone, the cells were 'washed' twice by centrifugation (5 min, 200 × g) at 22°C and resuspended in an RPMI-1640 medium (kept at 37°C) which contained no fetal calf serum, 2-mercaptoethanol or antibiotics. The final cell suspension had a cell density of 5 × 10⁷ cells/ml.

2.2. *Monitoring the cellular response to hormonal challenge [9]*

Aliquots of the cell suspensions described above (200 µl) were incubated at 37°C for 60 min [9,10] before challenge with HLF. Samples (50 µl) of the fractions assayed for HLF were added to these suspensions and cell stimulation was allowed to proceed at 37°C. At the indicated time of incubation, the cells were spun down (15 s, 12000 × g), the supernatant discarded and the

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Abbreviations: HLF, hormone-like factor; PKA, cAMP-dependent protein kinase; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS

pellets (10^7 cells) ruptured (to release their intracellular cAMP) by the addition of 50 μ l of 0.1 N HCl and boiling (3 min at 95°C) [11]. The boiled samples were cooled, neutralized, and their cAMP was determined [12,13], using the assay kit provided by Amersham. A control sample in which cells from the same cell suspension were challenged with DL-isoproterenol (final concentration 10^{-5} M) was routinely run alongside.

2.3. Assays

The following assays were carried out as described: PKA [14], cAMP [12,13], catecholamines [15].

2.4. Preparation of a crude extract of rabbit skeletal muscle and its fractionation on DEAE-cellulose

Rabbit skeletal muscle (5 kg) was homogenized in a Waring blender with 12.5 l of an extracting solution containing EDTA (4 mM) and 2-mercaptoethanol (15 mM), pH 7.0. The homogenate was centrifuged (10 min $11000 \times g$), and the supernatant filtered through glass wool, followed by dilution with 3 vols cold (4°C) deionized water containing 2-mercaptoethanol (15 mM). The conductivity of the resulting solution was ascertained to be 0.9–1.0 $m\Omega^{-1}$. DEAE-cellulose (DE-52) was washed and pre-equilibrated with a buffer composed of 4-morpholineethanesulfonic acid (5 mM), NaCl (9 mM), 2-mercaptoethanol (15 mM), pH 6.5 (buffer A), allowed to settle, then 5 l of the settled column material were added to the diluted supernatant mentioned above. The resulting suspension was stirred for 1 h (4°C) and then allowed to settle for another hour (4°C). The supernatant was discarded, the resin transferred to a sintered glass funnel and washed with buffer A till the absorbance of the washings at 280 nm was below 0.5. The resin (with the proteins adsorbed onto it) was packed in a column (10 \times 80 cm) and a linear gradient from 9 to 500 mM NaCl in buffer A (total volume 10 l) was applied. Fractions (20 ml each) were collected and assayed as described in the legend to fig.1.

2.5. Chromatography on CM-Sephadex (C50)

CM-Sephadex (C50) was pre-equilibrated with buffer B composed of phosphate (30 mM), EDTA (1 mM) and DTT (1 mM), pH 6.7. 10 ml of the settled column material was added to the sample chromatographed (100 ml) and swirled overnight at 4°C. The resin was then transferred to a sintered glass funnel and washed with buffer B until the absorbance at 280 and 260 nm was below 0.01. The resin was packed into a column (2 \times 50 cm) and a linear gradient of phosphate buffer (30–300 mM, total volume 80 ml) was applied. Fractions (2.2 ml) were collected and assayed as described in the legend to fig.2.

2.6. SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of SDS was carried out as in [16] using either 10%, or a gradient of 7–20% polyacrylamide. The gels were fixed, stained with 0.25% Coomassie blue and dried under vacuum.

2.7. Chemicals, radiochemicals, sera, culture media, assay kits and immobilized cAMP

These were purchased from the following commercial sources: cAMP, cGMP, ATP, DL-isoproterenol, *S. aureus* V8 protease (type XVII) and whole histone (Sigma); fetal calf serum and combined antibiotics (penicillin, streptomycin and

neomycin) (Bio-Lab, Jerusalem); 2-mercaptoethanol (Merck); trypan blue (Fluka); DEAE-52 (Whatman); CM-Sephadex (C-50), Sephadex G-25 (fine), Sepharose 4B and Seph-cAMP (agarose-ethane-adenosine 3',5'-cyclic phosphate AGcAMP, type 2, ethane spacer, 4.9 μ mol cAMP/ml) (Pharmacia); cAMP assay kit (TRK 432) and [γ - 32 P]ATP (2000–3000 Ci/mmol) (Radiochemical Centre, Amersham). RPMI-1640 was obtained from the Biological Services Unit of the Weizmann Institute of Science, Rehovot. All other chemicals were of the best available grade from commercial sources.

3. RESULTS AND DISCUSSION

The experiments described here stem from an unexpected observation that preparations of purified catalytic subunits of PKA, which were considered homogeneous on the basis of SDS-PAGE and Coomassie blue staining, were often found to stimulate WEHI-231 B-lymphoma cells, elevating and subsequently attenuating intracellular cAMP in a spike-like manner. Furthermore, the intensity and pattern of response obtained with these catalytic subunit preparations (0.7 – 1.0×10^{-6} M) was very similar to that obtained when the same cells were stimulated with a similar molar concentration of isoproterenol or with the peptide hormones β -MSH, ACTH and VIP (rise of cAMP to 15–20 pmol/ 10^7 cells within 0.5–1.0 min). Cell stimulation by the catalytic subunit preparations did not require ATP and Mg^{2+} , indicating that the PKA preparation was not acting as a kinase.

Now, the above-mentioned catalytic subunit of PKA was prepared from rabbit skeletal muscle following the method of Beavo et al. [7] which involves chromatography on DEAE-52 to resolve the type I and II holoenzymes (fig.1), dissociation of the holoenzymes with cAMP and isolation of the free catalytic subunit by preferential adsorption on carboxymethylcellulose or carboxymethyl-Sephadex (CM 50). Since the hormone-like activity described above was obtained with the catalytic subunit isolated from type I (but not type II) holoenzyme, and since it is known that the catalytic subunits of both holoenzyme types are identical, we suspected that the hormone-like activity is not an intrinsic property of the catalytic subunit itself but is rather due to a factor which copurifies with the catalytic subunit obtained from the type I holoenzyme.

We therefore returned to the previous purification step and attempted to identify the HLF by its

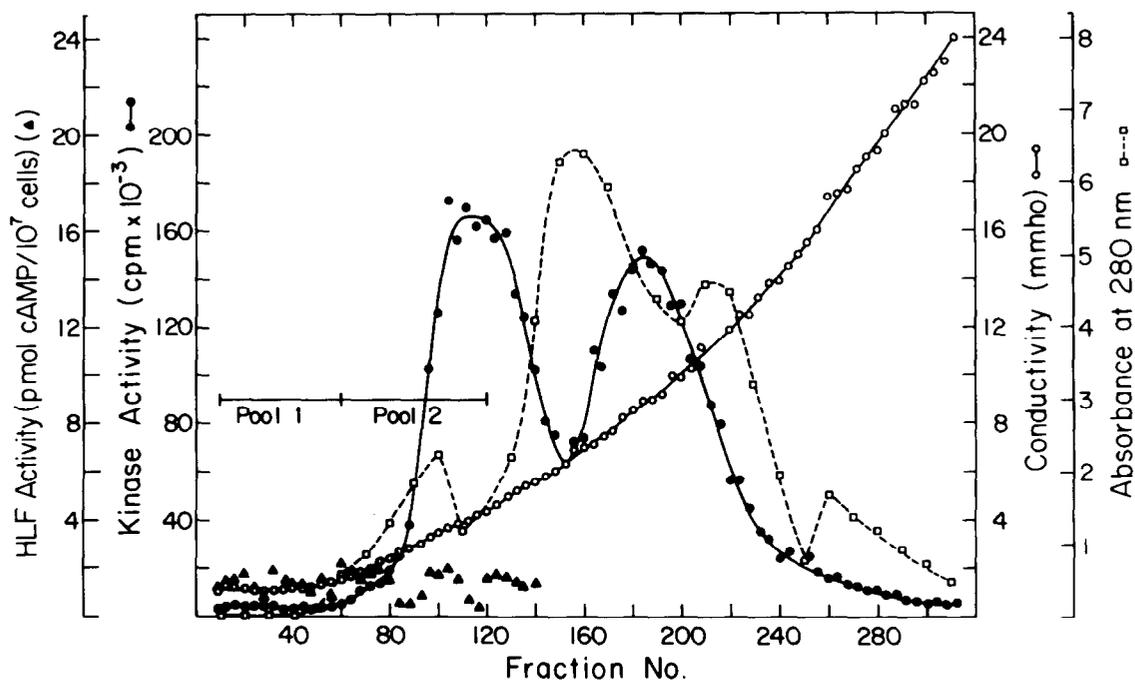


Fig.1. Chromatography of rabbit skeletal muscle extract on DEAE cellulose (DE-52). The following parameters were monitored: absorbance at 280 nm (□); conductivity (○); PKA activity (in the presence of 5×10^{-6} cAMP) (●); HLF activity (cell stimulation, 1 min) (▲).

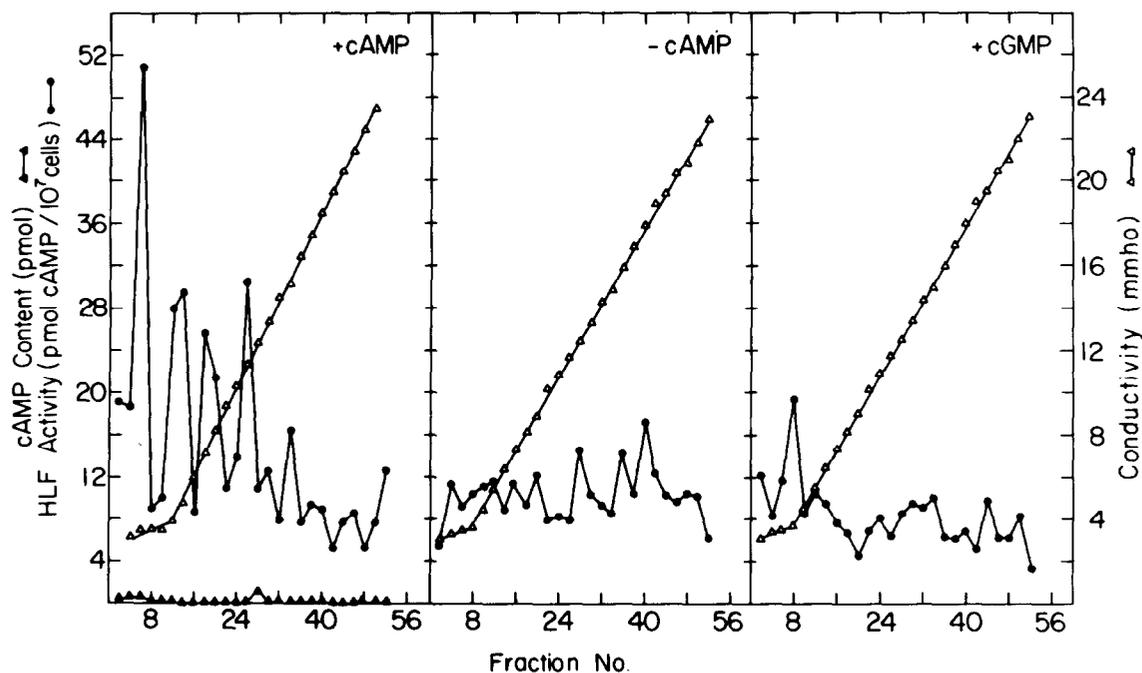


Fig.2. Chromatography of pool 1 (fig.1) on CM-Sephadex (C50). Three samples (100 ml each) of pool 1 were incubated and swirled (30 min, 4°C) with either cAMP (final concentration 10^{-5} M), cGMP (final concentration 10^{-5} M) or no addition. Chromatography on CM-Sephadex was carried out as described in section 2. The following parameters were monitored: conductivity (○); cAMP content (measured in 50- μ l samples) (▲); HLF activity (50- μ l samples used; cell stimulation: 1 min) (●).

cell-stimulating capability, assaying the various fractions within the type I holoenzyme peak and the fractions preceding it. As seen in fig.1, none of these fractions contained a significant level of active HLF. However, when the fractions were pooled as indicated in fig.1 and subjected to chromatography on CM-Sephadex after pretreatment with cAMP, we observed the release of several peaks of active HLF not only from pool 2 but also from pool 1 (fig.2, left panel) which had no PKA activity (cf. fig.1). To our surprise, no active HLF was observed when the CM-Sephadex chromatography was carried out without preincubation with cAMP (fig.2, center panel). In addition, when cAMP was replaced by cGMP, the amount of active HLF released was negligible (fig.2, right panel). This relationship between cAMP and cGMP is reminiscent of the low relative efficacy of cGMP in dissociating PKA [17]. It should be noted that the high cAMP levels obtained upon exposure of the cells to the various fractions could not be attributed to a carry-over of cAMP from the pre-incubation step. As shown in fig.2 (left panel), the fractions that contained a high level of HLF had a very low cAMP content (1 pmol/50 μ l) prior to their addition to cells for the HLF assay. We also excluded the possibility that the response of the cells was due to catecholamines: using a sensitive fluorometric assay [15] it was shown that these fractions do not contain a level of catecholamines that could account for the observed cellular stimulation.

On the basis of the results presented above, and by analogy to the activation of PKA by cAMP [17], we postulated that HLF emerges from the DEAE-52 column in the form of an inactive complex, which is dissociated upon binding of cAMP, and releases HLF in an active form. To test this hypothesis we attempted to remove the presumed cAMP-binding inhibitor of HLF with an immobilized cAMP column. Indeed, passage of pool 1 from fig.1 through such a column resulted in the release of active HLF (fig.3). A similar release of active HLF was not achieved with a control Sepharose 4B column (fig.3).

To assess the chemical nature of the HLF, we exposed it to a proteolytic enzyme. As shown in fig.4, when the HLF fractions of the first peak in the left panel of fig.2 were subjected to proteolysis (1 h, 37°C) by V8 protease type XVII from *S.*

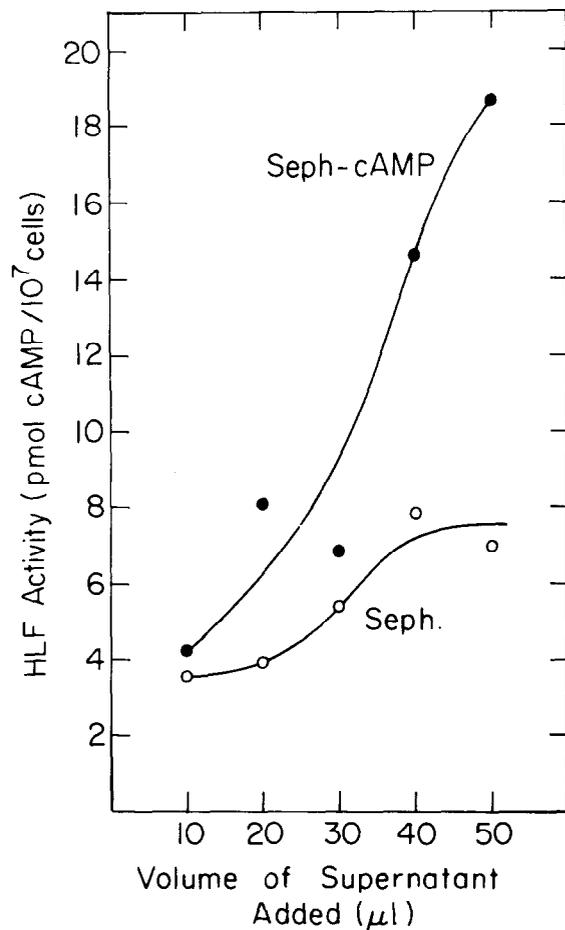


Fig.3. Release of active HLF by passage through an immobilized cAMP column. Two samples (10 ml each) of pool 1 in fig.1 were incubated (16 h, 4°C) with either Seph-cAMP (agarose-ethane-adenosine 3',5'-cyclic phosphate) or Seph (Sepharose 4B) (in each case the volume of settled gel used was 5 ml). The column material was then spun down, and the indicated volume of supernatant was assayed for HLF activity (cell stimulation, 1 min). Immobilized cAMP (●); Sepharose 4B (○).

aureus (final concentration 0.1 mg/ml), the HLF lost its activity and failed to stimulate WEHI-231 cells, suggesting that HLF is a polypeptide. When the same fractions were subjected to gel filtration on a pre-calibrated Sephadex G-25 (fine) column, two major activity peaks were obtained, with apparent molecular masses of 2.2 and 2.5 kDa (fig.5).

Until now, mammalian cells were shown to contain two targets for cAMP: (i) PKA [18], whose activity is directly controlled by the binding of cAMP

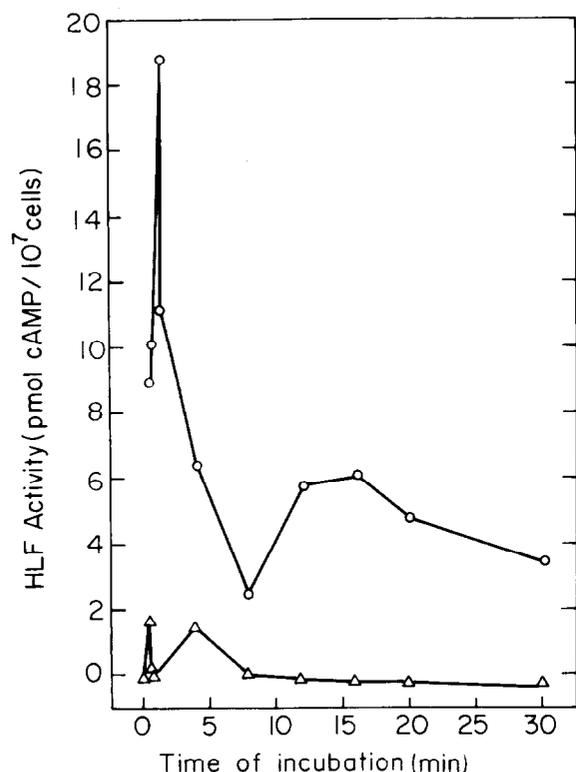


Fig. 4. Proteolytic degradation of HLF. A sample (1.0 ml) from the first peak of HLF activity (fig.2, left panel) was incubated (1 h, 37°C) either with V8 protease from *S. aureus* (final concentration 0.1 mg/ml) or with no addition. Aliquots (50 μ l) of both samples were assayed for HLF activity after the indicated cell stimulation times. Incubation mixture with V8 protease (Δ); incubation mixture with no addition (control) (\circ).

to implement the hormonal stimulation of cells, and (ii) cAMP phosphodiesterase, which is involved in the decomposition of cAMP to bring about the attenuation of such hormonal stimuli [19]. The results presented above raise the possibility that there may be an additional target for cAMP – a complex between HLF and an inhibitor which binds cAMP to release active HLF. If this complex is not the result of a fortuitous association between HLF and a cAMP-binding protein, and a fortuitous dissociation of this complex by a specific interaction with cAMP, then it might have a physiological assignment, for example in the amplification of a hormonal stimulus by an autocrine or paracrine mechanism [20]. In any

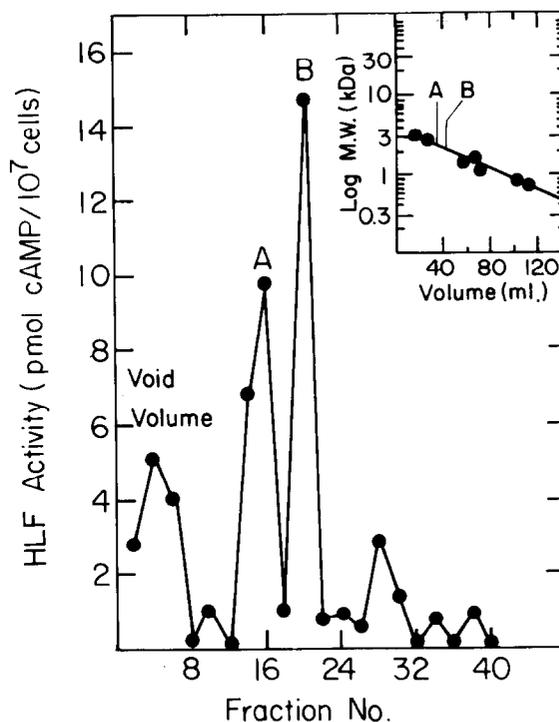


Fig. 5. Apparent molecular mass of active HLF by gel filtration. A sample (1.5 ml) of the first peak of HLF activity (fig.2, left panel) was applied on a column of Sephadex G-25 (fine), pre-equilibrated and run at 0.5 ml/min (4°C) with a buffer composed of phosphate (30 mM), EDTA (1 mM) and DTT (1 mM), pH 6.7. Fractions (2 ml) were collected and assayed for HLF activity (cell stimulation, 1 min). The column was pre-calibrated with synthetic peptides of known molecular mass (inset); VIP (3323 Da); sequence 1–20 of the α -subunit of the acetylcholine receptor (2940 Da); sequence 5–18 of the B-subunit of *Shigella* toxin (1546 Da); bacitracin (1500 Da); sequence 327–335 and 330–335 of the catalytic subunit of PKA (1205 and 810 Da, respectively), and sequence 12–18 of the B-subunit of *Shigella* toxin (768 Da).

event, the occurrence of a cAMP-triggered release of an active hormone-like factor is not only intriguing but should be kept in mind when cells are exposed to media that contain cAMP and partially purified cellular constituents.

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