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THE EFFECT OF ONCOMODULIN ON cAMP PHOSPHODIESTERASE ACTIVITY

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SUMMARY: Oncomodulin was purified from Morris rat hepatoma according to the procedure of Durkin, J.P., Brewer, L.M. and MacManus, J.P. (1983) Cancer Res. 43, 5390-5394. The preparation, in general, had the properties and amino acid composition of the material which they described. However, we were unable to confirm the reported stimulation of cyclic nucleotide phosphodiesterase under conditions where calmodulin gave the usual stimulation.

Oncomodulin is an acidic calcium-binding protein of 108 amino acids which has been detected only in tumors; it was first observed in rat hepatomas by MacManus (1-3). Using a specific radioimmuno assay, MacManus and his colleagues noted the appearance of this protein in several types of mouse, rat and human tumors. These findings have recently been reviewed (4). Oncomodulin is not a fragment of calmodulin (4), but it is considered by MacManus and his associates to be a similar kind of modulator protein, since they found that it stimulated both a calmodulin-dependent cyclic-nucleotide phosphodiesterase (5) and a nuclear protamine kinase (Sikorska and MacManus, quoted in (6)). A specific antiserum against oncomodulin purified from Morris hepatoma 5123 tc was developed in a goat (3). It was reported not to cross-react with an excess of either calmodulin or parvalbumin. Oncomodulin in mouse tumors appeared to be similar to that in rat tumors as judged by both crossed polyacrylamide gel electrophoresis and crossed isoelectric focusing immunoelectrophoresis.

ABBREVIATIONS: HPLC, high pressure liquid chromatography; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid.

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Raising the calcium concentration, or adding oncomodulin, stimulated DNA synthesis in non-neoplastic rat liver cells whose DNA-synthetic ability had been reduced by incubation in medium containing only 0.02 mM Ca2+ (7). A calcium-oncomodulin complex was considered to be the active agent because the effect of oncomodulin was blocked by further reduction of the already low level of calcium by using EGTA. Oncomodulin was not only capable of re-initiating DNA synthesis, but was some 100-fold more potent than calmodulin (4). Skeletal muscle parvalbumin was reported to be inactive in stimulating DNA synthesis in these calcium-deficient cells (4,7).

In a recent paper (6), MacManus et al reported the complete amino acid sequence of oncomodulin purified from Morris hepatoma 5123 tc. The N-terminus was N-acetyl serine. They established that homology existed between the oncomodulin sequence and that of members of the troponin C superfamily of calcium-binding proteins. Even greater homology was seen between oncomodulin and the parvalbumins, including rat parvalbumin. Oncomodulin was therefore considered to be a parvalbumin-like calcium-binding protein.

Other parvalbumins have been investigated and reported not to stimulate cyclic nucleotide phosphodiesterase. For this reason we decided to purify oncomodulin and to re-investigate its effect on the phosphodiesterase.

MATERIALS AND METHODS:

cAMP (cyclic adenosine 3': 5'-monophosphate) was from Sigma. PD-10 columns were from Pharmacia Fine Chemicals. Calmodulin and calmodulin stimulated phosphodiesterase were prepared as described previously (8,9). cAMP-phosphodiesterase was assayed as described in the literature (8,10). [2,8-^{3}H] cAMP was from New England Nuclear. CAPP-AffiGel 10 was prepared as described (8). This is an affinity column in which norchlorpromazine (2-chloro-10-[3-aminopropyl] phenothiazine, or CAPP) is covalently linked to Affi-Gel 10 (Bio-Rad Laboratories).

Amino acid analyses were performed by a modification of the method of Spackman et al (11), using a Waters High Performance Liquid Chromatography Amino Acid Analysis System, equipped with a CATEX Resin column (0.4 cm x 25 cm). The ionic strength gradient system was used as described in the manufacturer's manual. At 58°C, trimethyllysine elutes at 59 min between phenylalanine (retention time 51 min) and lysine (retention time 63 min).

SDS-gel electrophoresis was performed in slabs of 15% acrylamide using the Laemmli system (12). Samples were made 2 mM $CaCl_2$ or 2 mM EDTA prior to loading. Oncomodulin was seen on Coomassie-blue-stained gels as a protein of apparent M_r 11,500 in the presence and absence of Ca^{2+}. Molecular weight standards were phosphorylase a (97,000), bovine serum albumin (68,000),

catalase (58,000), fumarase (48,000), actin (42,000), lactate dehydrogenase (35,000) and β-lactoglobulin (17,500).

UV-absorption spectra were measured with a Cary model 118C spectrophotometer.

RESULTS AND DISCUSSION:

Tumor calcium binding protein was purified from Morris hepatoma 5123 tc as described by MacManus (2,3) through the following steps: (a) homogenization (25% w/v) in buffer A (100 mM NaCl, 100 mM sodium acetate, 0.5 mM $MgCl_2$, 50 mM KCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% β-mercaptoethanol, pH 7.2); (b) centrifugation at 12,000 xg for 15 min; (c) heating of the resultant supernatant fraction at 80°C for 5 min, followed by centrifugation at 25,000 xg for 15 min; (d) addition of ammonium sulfate to the supernatant from step (c), to bring it to 65% saturation (4.3 g per 10 ml), followed by removal of precipitated proteins by centrifugation; (e) adjustment of the supernatant to pH 4.0 with glacial acetic acid to obtain a second precipitate, which was dissolved in 0.1 M NaCl, 0.04 M Tris Cl pH 8, 3 mM $MgCl_2$, 1.5 mM β-mercaptoethanol.

The precipitate obtained at pH 4.0 was dialyzed and passed through a CAPP-Affi-Gel 10 affinity column. The flow-through fractions were subjected to HPLC chromatography, using an alkyl phenyl column. Elution was with 20-65% CH_3CN in 10 mM potassium phosphate buffer, pH 6.1 - 1 mM EGTA. The flow rate was 1.5 ml per min. In SDS gel electrophoresis the protein had a greater mobility than calmodulin and showed no significant Ca^{2+} shift. Its absorption spectrum was similar to that of calmodulin.

The peak fractions were flash evaporated and dissolved in 0.05 ml NH_4HCO_3. The material was then passed through a PD-10 column equilibrated with 0.05 M NH_4HCO_3. Protein containing fractions were applied to a reverse phase HPLC column (RPP 18, 4.1 x 100 mm, 300 Å pore, 6.5 μ; Synchrom, Inc., Linden, Ind.). Operation of the RPP column was under conditions similar to those used by Durkin, Brewer and MacManus (13). The column was equilibrated with 10 mM KH_2PO_4 and eluted with a 0-40% gradient of N-1-proponol. Fractions of 0.5 ml were collected and the flow rate was 0.8 ml per min. A peak of

TABLE I
Amino acid composition of oncomodulin from rat hepatoma 5123 tc

	Residues/mol
Asp	16.7 (17)
Thr	5.0 (5)
Ser	10.2 (11)
Glu	18.0 (18)
Pro	2.8 (2)
Gly	8.2 (6)
Ala	9.2 (9)
Val	2.9 (2)
Met	2.4 (3)
Ile	5.4 (6)
Leu	8.3 (8)
Tyr	1.8 (2)
Phe	8.3 (8)
Lys	6.6 (7)
His	1.3 (1)
Arg	2.6 (2)

Rat hepatoma 5123 tc was carried through steps (a) to (e) of purification as described in the text. This was followed by affinity chromatography using a phenothiazine column. The final steps involved HPLC chromatography, first with an alkyl phenyl column and then with a Synchron C18 column. After passage through a PD10 column equilibrated with 0.05 M NH_4CO_3, the material was dissolved in 40 mM Tris HCl, pH 8 - 0.1 M NaCl. A sample was hydrolyzed in 6 N HCl with a norleucine standard. Recovery of norleucine was 103%. The results of two separate assays, which agreed closely, were averaged.

For the molar ratio, glutamic acid was assumed to be 18 residues/molecule. Figures in parentheses, included for comparison, are from reference (13).

protein with UV spectrum the same as that of calmodulin was applied to a PD10 column equilibrated with 0.05 M NH_4HCO_3. Protein containing fractions were lyophilized. This material was used for amino acid analysis and for assay as an activator of phosphodiesterase activity.

Table I shows the results of amino acid analysis. The data are similar to those reported by Durkin *et al* (13), which are shown in parentheses. As indicated in Fig 1, no activation of phosphodiesterase was observed under conditions where calmodulin gave the usual stimulation. Fractions obtained after the pH step (step e) were also inactive.

The possibility always exists, of course, that we lost activity during the course of purification of oncomodulin, even though we observed all of the precautions that we could think of. However, our results are consistent with the fact that other parvalbumins have been tested and also did not stimulate phosphodiesterase activity. It was reported that trifluoperazine inhibits cyclic nucleotide hydrolysis non-competitively when stimulated by

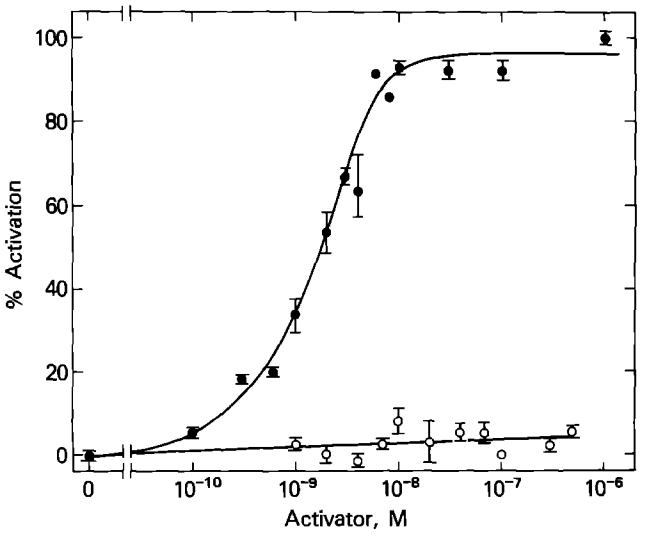

Figure 1. Oncomodulin did not activate phosphodiesterase under conditions where calmodulin gave the usual activation. cAMP phosphodiesterase was assayed as described in the literature (8,10). Upper curve: calmodulin. Lower curve: Oncomodulin, after the second HPLC step.

oncomodulin, and with a K_i double that seen with calmodulin (5). We therefore expected that oncomodulin would bind to a CAPP-Affi-Gel affinity column, but we found that no significant amount was retained.

Oncomodulin is an intriguing molecule and its biological role warrants further investigation. An attractive possibility is that it stimulates a particular nuclear kinase activity, especially since an effect on protamin kinase has already been found (5).

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