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Demonstration of an endocrine signaling circuit for insulin in the sponge *Geodia cydonium*

Andrea Robitzki[1], Heinz C.Schröder[1], Durdica Ugarkovic[1,2], Karin Pfeifer[1], Gerhard Uhlenbruck[3] and Werner E.G.Müller[1]

[1]Institut für Physiologische Chemie, Universität, Duesbergweg 6, 6500 Mainz, FRG, [2]Institute Ruder Bošković, Center for Organic Chemistry and Biochemistry, 41001 Zagreb; and [3]Abteilung für Immunbiologie, Medizinische Universitätsklinik, 5000 Köln 41, FRG

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The existence of an insulin-mediated cell-to-cell signaling in the sponge *Geodia cydonium* is demonstrated in this study by molecular biological and immunological techniques. The sequence of a sponge cDNA clone encoding preproinsulin was analyzed for the first time and determined to comprise a high homology to human preproinsulin (60-80% homology). The predicted polypeptide of preproinsulin from sponge contains two disulfide bridges which link the A- to the B-chain. The intra-A chain disulfide bridge is absent. Applying immunological and electron microscopical techniques it is shown that insulin is produced in specialized cells (spherulous cells). Experimental evidence is presented which indicates that the sponge preproinsulin (predicted M_r 11 850) is processed to insulin (M_r 5600; B-chain, M_r 3700 and A-chain, M_r 1900). Plasma membranes of sponge cells are shown to be provided with an insulin-binding receptor composed of two molecules (M_r 104 000 and M_r 98 000). Heterologous insulin (from bovine pancreas) was found to stimulate gene expression in *G.cydonium* cells. It is concluded that sponges are provided with an endocrine signaling circuit: signaling cells (spherulous cells), hormone (insulin), and hormone receptor bearing target cells which respond to the hormone stimulus.

Key words: endocrine signaling/*Geodia cydonium*/Insulin/preproinsulin cDNA/sponges

Introduction

Porifera, a phylum of animals which comprises 5000-5500 described species are first identifiable from the Paleozoic (1.6 billion years) (Termier and Termier, 1979). Since then they are isolated from other metazoans. Recently, sponges have gained high interest in developmental biology because they represent a suitable model system to study firstly the mechanism of cell adhesion (Burger *et al.*, 1975; Müller, 1982) and secondly, the signal transduction mechanism for the control of a variety of cellular processes (Rottmann *et al.*, 1987; Schröder *et al.*, 1988). Despite the low number of structural components, sponges have evolved a high degree of growth forms. Some extracellular proteins have been identified in the mesohyl compartment of sponges which act, in addition to their functions during cell adhesion, as morphogenetic factors; e.g. aggregation factor (Moscona, 1963; Müller and Zahn, 1973), collagen assembly factor (Diehl-Seifert *et al.*, 1986) and lectin (Schröder *et al.*, 1988). However until now, no hormones or hormone-like molecules which act as specific growth factors have been described in sponges.

In the present study we have used both immunological and gene technological methods to demonstrate that the genome of the sponge *G.cydonium* contains the preproinsulin gene. Moreover, we show that insulin is both secreted by specialized cells and causes gene activation.

Results

Isolation and characterization of the preproinsulin cDNA

A cDNA library of the sponge *G.cydonium* was prepared. Applying a cDNA probe coding for preproinsulin from a primate, the sponge preproinsulin cDNA was detected and cloned. Sequence analysis revealed a high homology of the human preproinsulin (Figure 1A). The degree of homology between the different regions was as follows: 5'-untranslated region: 60/36 (60%); pre-peptide: 72/55 (76%); B-chain 93/75 (81%); C-peptide 108/83 (77%) and A-chain 63/47 (75%). These surprisingly high homologies between the human cDNA of preproinsulin and the sponge preproinsulin are also reflected by the A+T and C+G ratios determined for the two DNAs; sponge A+T: 157, C+G: 225 and human A+T: 142, C+G: 250. The complete *G.cydonium* preproinsulin cDNA was 382 bp long. This size is almost identical to that of the human preproinsulin cDNA (392 bp).

The predicted polypeptide of the sponge preproinsulin cDNA consists of 109 amino acids with an M_r of 11 850 (human cDNA: 111 amino acids; M_r 11 966) (Figure 1B). The deduced amino acid sequence conforms to the typical feature of a vertebrate preproinsulin with two disulfide bridges between cysteine residues of the A and B chains (cys 5 to cys 7 and cys 18 to cys 20). However, the additional bridge connecting two cysteine residues of the A chain is not possible due to the deletion of the cysteine adjacent to the cys 5 in the A chain.

Identification of insulin in the sponge

Insulin was identified in a cell-free extract from *G.cydonium*. In one series of experiments this extract was not treated with 2-mercaptoethanol and not heated. The material was size-separated by 15% SDS-PAGE and the proteins were transferred to nitrocellulose sheets. The monoclonal anti-insulin antibodies reacted only with one species of polypeptide of M_r 5600 (Figure 2A,b). In a control, an insulin-adsorbed antibody preparation was used; this antibody gave no reaction (Figure 2A,c).

In a further assay the cell extract was treated with both 2-mercaptoethanol and heat and then subjected to Western

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A. NUCLEOTIDE SEQUENCE

Sponge 5' untranslated region
AGCCGT  AAGTGAGACTACATTCGGAAACCATCAGCAAGCAGGTCATTG   TTCCAAC
**** *  * *  ** ** ** ** ***     ** ******** *** **   ***   *
AGCCCTCCAGGACAGGCTGCA TCAGAAGAGGCCATCAAGCAGATCACTGTCCTTCTGCC
Human  5' untranslated region

Sponge Pre I
ATGGCCCTGTGGAT   CCTGCTGCCGCTACTAGCGCTGCTAATCCTCTGGGGGCCGGATCCGGCCCAAGCA
**************   *** ***** ** ** ********   ********* ** ** ** ***  ***
ATGGCCCTGTGGATGCGCCTCCTGCCCCTGCTGGCGCTGCTGGCCCTCTGGGGACCTGACCCAGCCGCAGCC
Human  Pre I

Sponge B 1
TTTGTAAACCAGCACTTGTGCGGCTCCCATCTAGTGGAAGCTCTCTACATTCTGGTGTGTGGGGAGCGTGGC
***** ***** *** ********** ** ** ***************   ** ***** ***** ** ***
TTTGTGAACCAACACCTGTGCGGCTCACACCTGGTGGAAGCTCTCTAC   CTAGTGTGCGGGGAACGAGGC
Human  B 1

TTTTTTTACACACCGATGTCC
** ** ******** * * **
TTCTTCTACACACCCAAGACC

Sponge C 1
CGCCGGGAAGTGGAGGACCCACAGGTGGGACAAGTGGAGCTGGGGGCGGGCCCTGGGGCAGGCAGCGAACAG
******** *  *******  ******** ** ********** * ********* *********   ***
CGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGCGGGGGCCCTGGTGCAGGCAGCCTGCAG
Human  C 1

ACGTTAGCGCTGGAGGTGGCCCGACAG  GC ACGC
 * ** ** ******* *****  ***  **   **
CCCTTGGCCCTGGAGGGGGCCCTGCAGAAGCGTGGC

Sponge A 1
ATCGTGCAGCAGTGTACCAGCGGCATCTGCAGCCTCTACCA   AGAGAACTACTGCAACTAG
** *** * ** **    * * ********  *********    ******************
ATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCAACTAG
Human  A 1


B. PREDICTED POLYPEPTIDE SEQUENCE

Sponge Pre I              B 1
MALWI LLPLLALLILWGPDPAQA    FVNQHLCGSHLVEALYILVCGERGFFYTPMS
****  ******** ******* *   ***************** ************
MALWMRLLPLLALLALWGPDPAAA    FVNQHLCGSHLVEALY LVCGERGFFYTPKT
Human  Pre I              B 1

Sponge C 1                          A 1
RREVEDPQVGQVELGAGPGAGSEQTLALEVARQAR   IVQQCTSGICSLYQ ENYCNU
*** ** ******** ****** * **** * * *   ** **   ****** ******
RREAEDLQVGQVELGGGPGAGSLQPLALEGALQKR  GIVEQCCTSICSLYQLENYCNU
Human  C 1                          A 1
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Fig. 1. (**A**) Nucleotide sequence of the preproinsulin cDNA of the sponge *G.cydonium*. This sequence was compared with human preproinsulin cDNA sequence published by Sures *et al.* (1980). The figure depicts the nucleotide sequences of the four regions of the preproinsulin together with the 5'-untranslated region. (**B**) Predicted polypeptide sequence. Asterisks denote homology.

blot analysis. Under these conditions one band with an M_r of 3700 was detectable (Figure 2B).

Distribution of insulin in tissue

Based on protein content insulin was determined to be present extracellularly in a 30-fold higher concentration than intracellularly. The only cell type which contained significant amounts of insulin were the spherulous cells (Table I), the free choanocyte clusters and the archaeocytes were free of insulin.

The intracellular storage of insulin in spherulous cells (Figure 3a) was confirmed by electron microscopic immunocytochemistry. The anti-insulin antibody was found to react strongly with the material in vesicles of spherulous cells (Figure 3b): the content of the vesicles appeared uniformly labeled. After adsorption of the antibody with insulin, no immunocomplexes could be detected (Figure 3c).

Insulin-binding membrane receptor

By using the technique of electron microscopic immunocytochemistry it could be visualized that insulin reacted specifically with cell surface-associated molecules (Figure 3d and e). These molecules were identified in the plasma membrane preparation. The polypeptides in this preparation were size-fractionated by SDS–PAGE. After blot transfer

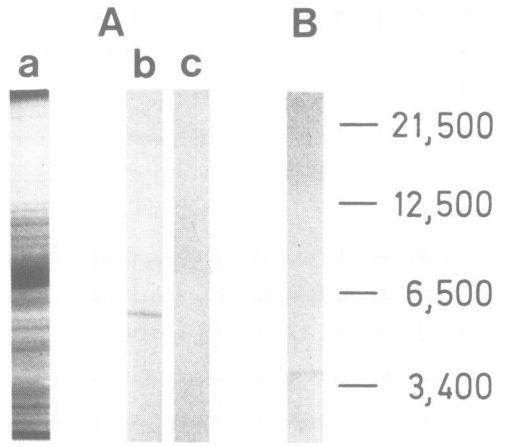

Fig. 2. Sponge insulin. (**A**) Extract (100 μg of protein per slot) from sponge cells was electrophoresed on a 15% polyacrylamide gel in the presence of SDS. The samples applied were not treated with 2-mercaptoethanol and were not heated. The gels were either stained with Coomassie brilliant blue (**a**) or the proteins were transferred to nitrocellulose filters and incubated either with nonadsorbed (**b**) or adsorbed anti-insulin antibodies (**c**). (**B**) In a separate assay, the sample was treated with both 2-mercaptoethanol and heat and then subjected to gel electrophoresis. After transfer, the filter was incubated with anti-insulin antibodies. Mol. wt standards are indicated.

Table I. Distribution of insulin in the sponge *G.cydonium*

	Insulin concentration	
Compartment	ng/μg protein	ng/μg DNA
Extracellular	75.7 ± 6.4	–
Intracellular	2.4 ± 0.2	0.004 ± 0.001
Cells:		
free choanocytes	<0.5	<0.001
choanocyte clusters	<0.5	<0.001
Archaeocytes	<0.5	<0.001
spherulous cells	43.1 ± 0.4	0.094 ± 0.008

The results are means of five independent experiments ± SD.

two protein species in the plasma membrane fraction with M_rs of 104 000 and 98 000 reacted specifically with insulin during the immunoblotting procedure (Figure 4).

Activation of lipocortin gene expression by insulin

Dot-blot analysis of sponge RNA using the lipocortin I specific probe revealed that in dissociated *G.cydonium* archaeocytes, lipocortin I gene is not expressed (Figure 5). After a 10 h incubation period in the presence of insulin the lipocortin I-related message was detectable. The expression is time- and concentration-dependent. At an insulin concentration of 1 μg/ml (or 10 μg/ml respectively) the extent of gene expression after an incubation period of 10 h amounted to 8% (25%) and after 20 h to 40% (100%) if the maximal amount of gene expression measured (10 μg of insulin/ml after a 20 h incubation period) was set at 100%.

Discussion

Insulin and insulin-like proteins are highly conserved proteins which have been identified not only in vertebrates but also in invertebrates (Conlon *et al.*, 1988), e.g. in insects (Maier *et al.*, 1988), echinodermata (De Pablo *et al.*, 1988), and

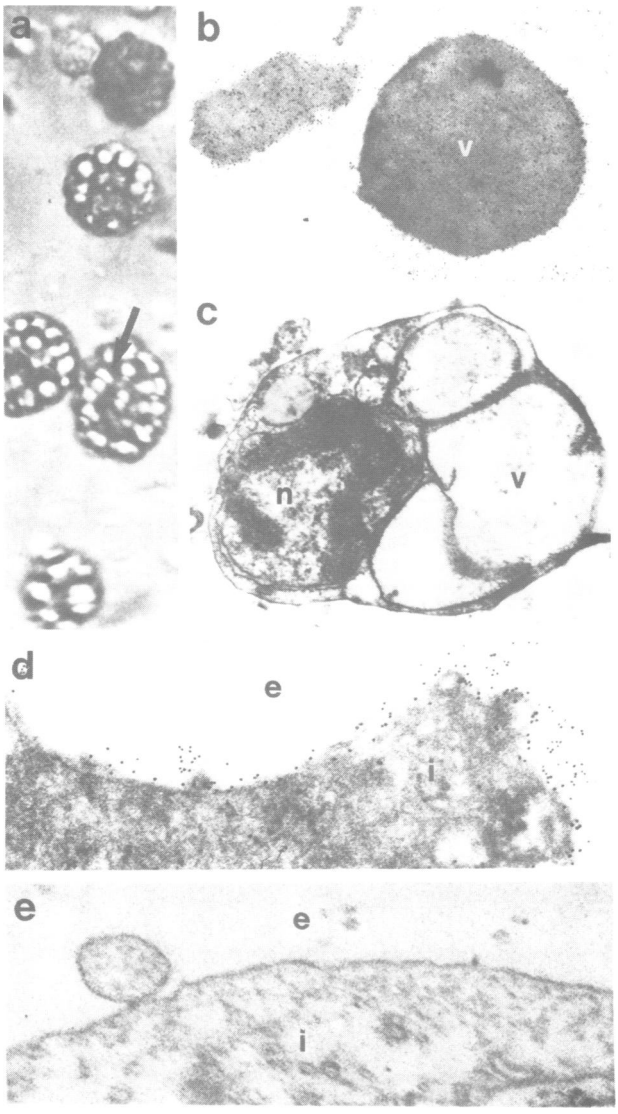

Fig. 3. Distribution of insulin and insulin receptor in *G.cydonium*. (**a**) Spherulous cells; phase-contrast picture. (**b** and **c**) Localization of insulin. The sections through vesicles of spherulous cells were treated with nonadsorbed (**b**) or adsorbed anti-insulin antibodies (**c**). Then the immunocomplexes were visualized by applying the double immunogold staining procedure. (**d** and **e**) Localization of insulin receptor. The sections were first treated with insulin and after washing, they were incubated with nonadsorbed (**d**) or adsorbed anti-insulin antibodies (**e**) and then with colloidal gold-labeled secondary IgG. Arrow, vesicle in a spherulous cell; v, vesicle; n, nucleus; i, intracellular space; e, extracellular space. a × 1000; b 10 000; c 4000; d and e 40 000.

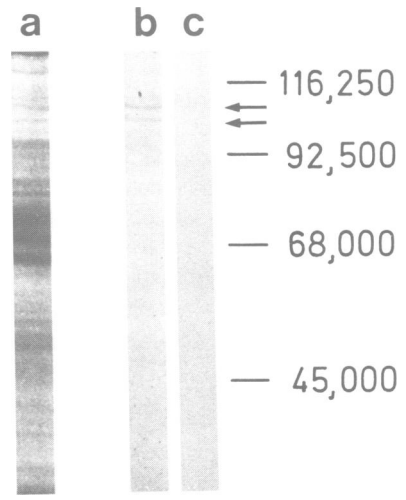

Fig. 4. Identification of the insulin-binding membrane receptor *Geodia* plasma membrane fraction (60 μg of protein per slot) was electrophoresed in polyacrylamide (8%) gels and either stained directly with Coomassie brilliant blue (**a**) or the proteins were transferred to nitrocellulose sheets (**b** and **c**). The sheets were incubated either with nonadsorbed (b) or with adsorbed anti-insulin antibodies (c). Mol. wt standards are given. Arrows: insulin-binding proteins.

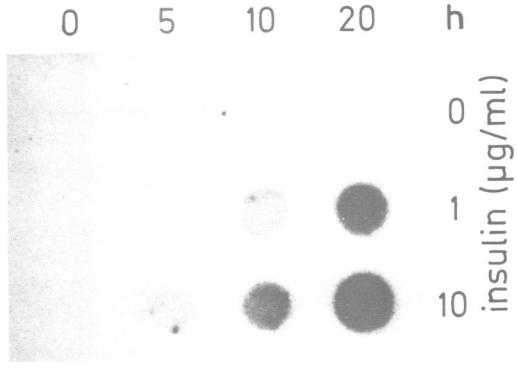

Fig. 5. Dot-blot analysis of RNA from archaeocytes incubated in the standard assay in the presence of 0, 1 or 10 μg of insulin/ml. After incubation for 0, 5, 10 or 20 h total RNA was isolated and 5 μg each were assayed with ^{32}P-labeled lipocortin probe.

perhaps in plants (Collier *et al.*, 1987). Now we show that the genome of the sponge *G.cydonium* also contains preproinsulin gene.

In the present study the sequence of the preproinsulin cDNA from a sponge is given for the first time. Very unexpectedly, the preproinsulin cDNA had a high homology to vertebrate preproinsulin cDNAs; e.g. the human (Sures *et al.*, 1980) and the primate cDNA (Wetekam *et al.*, 1982). The homologies of the different regions of the sponge preproinsulin cDNA to human preproinsulin varied between 60% (5′-untranslated region) and 81% (B-chain region). Due to this high homology of the sponge preproinsulin cDNA with the vertebrate cDNA, the sponge sequence must be considered to code for a true preproinsulin (Blundell and Humbel, 1980). The homologies in the predicted polypeptide sequence regions were estimated to be even higher: 73% (A-chain region) and 90% (B-chain region). This result shows that the sponges are provided with a different codon usage pattern than vertebrates. A characteristic feature of the sponge sequence is the lack of a cysteine in the putative amino acid A-chains, indicating that the intra-A chain disulfide bridge is lacking.

Until the work of Nagasawa *et al.* (1986) no amino acid sequence data of an insulin-related sequence in invertebrates were available. These authors described a neuropeptide, the prothoracicotrophic hormone, of the silkworm *Bombyx mori* as an insulin-related polypeptide which consists of two non-identical chains with a 47 and 30% homology to the A- and B-chain of human insulin respectively. Recently a similar insulin-related peptide has been identified in the mollusc *Lymnaea stagnalis* (Smit *et al.*, 1988); both polypeptides show the presence of cysteines at positions in the A- and B-chain typical for the insulin family. Moreover, Smit *et al.* (1988) and Adachi *et al.* (1989) provided experimental evidence that the mollusc insulin-related polypeptide displays a homology with vertebrate preproinsulin. From these data

we have to conclude that the sponge preproinsulin nucleotide sequence shows a much higher similarity to the mammalian counterpart than the other two invertebrate insulins described. At present we attribute this fact to convergent evolution.

Sponge insulin was determined to be produced in specialized cells, the spherulous cells, from which it was secreted into the extracellular space. The amount of insulin in spherulous cells (43 ng/μg of extracted protein) was one-half of the amount determined in human pancreatic islet cells (Ricordi *et al.*, 1989). The mol. wt of the material in sponge, reacted with anti-insulin antibodies was determined by Western blot analysis. Before treatment with 2-mercaptoethanol and heat, the M_r of the material reacting with anti-insulin antibodies was determined to be 5600 and after the treatment was 3700. Therefore, we have to assume that the sponge preproinsulin (predicted M_r 11 850) is processed to insulin (M_r 5600 consisting of a B-chain of M_r 3700 and a calculated A-chain of M_r 1900). Such a conversion is well described for the processing of vertebrate preproinsulin to insulin (Robbins *et al.*, 1984).

It is well established that the human insulin receptor is a tetramer consisting of two α-subunits (M_r 95 000) (Falcon *et al.*, 1988). We could show that two polypeptides from the plasma membrane of *Geodia* bind to insulin; their mol. wts were determined to be 104 000 and 98 000. At the present time we cannot decide if two insulin-binding polypeptides are present in the sponge plasma membrane or if the lower mol. wt insulin-binding species is a degradation fragment. Further information is necessary to clarify whether the sponge receptor is perhaps a dimer or a higher association form. Moreover, the establishment of a homology with the mammalian insulin receptor or with the tetrameric insulin-like growth factor I receptor (M_r 127 000−140 000 of the α-subunit and M_r 90 000 of the β-subunit) (Ocrant *et al.*, 1988; Ullrich *et al.*, 1986), or the monomeric insulin-like growth factor II receptor (M_r 250 000; Laburthe *et al.*, 1988) is only possible after knowing the sequence of the sponge receptor. It has been documented that insulin and insulin-like growth factor exhibit cross-reactivity to each others receptor, using immunological techniques (Banskota *et al.*, 1989).

Hence, *G.cydonium* is provided with the molecular prerequisites to establish an endocrine signaling circuit: signaling cells (spherulous cells), hormone (insulin), and hormone receptor bearing target cells. The function of this communication pathway was proven by the demonstration that insulin has a transcriptional effect. As marker for the insulin-caused induction, the increase in the transcription of calelectrin mRNA was measured. Recently it was shown that in sponges, calelectrin, which belongs to the lipocortin family, is present in the extracellular space where it modulates the activity of phospholipase A2 (Gramzow *et al.*, to be published). Now it is shown that after incubation of archaeocytes from *G.cydonium* in the presence of insulin the expression rate of the calelectrin gene is enhanced.

We are convinced that the insulin-mediated signaling system between cells is not the last endocrine circuit to be discovered in sponges. Moreover, the observations reported here for the first time provide the basis for the biochemical understanding of a variety of conflicting biological results, e.g. the differentiative potential of sponge cells.

Materials and methods

Materials

The following materials were used: monoclonal antibodies against human insulin (mouse) from Amersham Int., Amersham (UK); colloidal gold (10 nm)-labeled secondary anti-mouse IgG from goat from E and Y Laboratories, San Mateo, CA (USA); insulin (bovine pancreas) from Sigma, St Louis, MO (USA).

Live specimens of *G.cydonium* (Demospongiae) were collected near Rovinj (Yugoslavia).

Tris-buffered Ca^{2+}- and Mg^{2+}-free sea water (CMFSW) was made as described (Müller *et al.*, 1978). Ca^{2+}- and Mg^{2+}-containing artificial sea water (ASW) had in addition to the components in CMFSW 50 mM $MgCl_2$ and 10 mM $CaCl_2$.

Cells and incubation assay

Cells were obtained by dissociation of sponge tissue in CMFSW (Müller and Zahn, 1973) with the exception that the trypsin step was not included in the procedure. The different cell types were obtained by separation through Ficoll discontinuous gradient centrifugation (Müller *et al.*, 1981). The cell viability was >95% as checked by Trypan blue exclusion (0.5% w/v).

In the standard incubation assay (3 -ml vol.) (Müller and Zahn, 1973), a suspension of 25 ± 5 × 10^6 archaeocytes/ml of ASW was placed into glass tubes and rolled at 35 r.p.m. at 20°C. Where indicated 0−10 μg/ml of insulin were added. The suspension was incubated for a period of 0−60 min. Then the assays were incubated for a period of up to 20 h without moving them.

Cell extract and extracellular material

Cells were extracted as follows: 0.5 g of packed cells were suspended in 1 ml of CMFSW and homogenized with 10 strokes of a Dounce homogenizer. The homogenate was centrifuged at 20 000 *g* for 30 min at 2°C. The supernatant was collected. The cell-free extracellular material was isolated from sponge cubes as described (Reuter *et al.*, 1987). The DNA concentration was <1 μg/mg of protein.

The plasma membranes were prepared as described (Conrad *et al.*, 1984). Starting from 3.5 g of *Geodia* cells (~1.5 × 10^{11} cells) 130 mg of plasma membrane protein were obtained.

Construction of G.cydonium cDNA library

Total RNA was extracted from sponge material following the guanidinium method (Chirgwin *et al.*, 1979). A cDNA was prepared (Okayama and Berg, 1982) and the cDNA library was constructed in the λgt11 vector (Huynh *et al.*, 1984). Radioactively labeled primate preproinsulin cDNA (Wetekam *et al.*, 1982) was used as a hybridization probe (Paonessa *et al.*, 1988) in three replicas of the library.

DNA sequencing

The DNA segments were subcloned in the M13mp18 or M13mp19 vector and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). The sequence obtained was confirmed by application of the Maxam and Gilbert (1983) procedure.

Computer analysis was performed with the Microgenie (C.Queen and L.Korn, Beckman Inst.) and the HIBIO DNASIS (Hitachi) programs and searching for homologies in the EMBL and GenBank sequence data banks. The sequence of a sponge preproinsulin cDNA is not recorded in any of these data banks.

Electron microscopy

The material was fixed with glutaraldehyde, dehydrated with dimethyl-formamide and embedded in Lowicryl. After sectioning the specimens were incubated with mouse anti-insulin antibodies (1:2000 dilution) and then with the colloidal gold (10 nm)-labeled secondary anti-mouse IgG (1:100 dilution); finally the material was counterstained with uranyl acetate and lead citrate and then inspected by electron microscopy (Priestley and Cuello, 1983; Gramzow *et al.*, 1988). Controls were performed with an antibody preparation from a nonimmunized mouse; these antibodies gave no reaction with the sponge material.

For the localization of the insulin receptor the slices were incubated firstly with bovine insulin (5 μg/ml) for 2 h at 20°C, secondly, after washing with 20 mM Tris−HCl buffer (pH 8.2, 150 mM NaCl, 1 mg/ml of BSA), with anti-insulin antibodies and then with colloidal gold-labeled antimouse IgG as described above.

Where indicated, anti-insulin antibodies were adsorbed with insulin (from bovine pancreas) (Gramzow *et al.*, 1986).

Gel electrophoresis and protein blotting

One dimensional SDS–PAGE was performed under denaturing conditions as described by Laemmli (1970); the resolving gels contained 8 or 15% acrylamide. Either the gels were stained with Coomassie brilliant blue or their proteins were transferred to nitrocellulose sheets (Towbin *et al.*, 1979). For the identification of insulin the blots were directly incubated with anti-insulin antibodies. For the identification of the insulin-binding protein the blots were first incubated with insulin (5 μg/ml of CMFSW) for 1 h at 37°C and after washing in CMFSW with anti-insulin antibodies. The immunocomplexes formed were visualized as described (Nakane, 1968).

Dot-blot hybridization analysis

The isolation of the total RNA from cells was performed by the method of Cathala *et al.* (1983). The 1355 bp fragment containing the rat lipocortin I (calpactin II) segment inserted into pBR322 was used (Shimizu *et al.*, 1988). The lipocortin-containing fragment was labeled with [α-^{32}P]dTTP to a sp. act. of 2.5 × 10^7 c.p.m./μg DNA (Rigby *et al.*, 1977). The dot-blot assay was performed according to the method of White and Bancroft (1982) and the filters were exposed to Kodak XAR-5 X-ray film. The relative amounts of the transcripts were estimated by scanning densitometry.

Analytical methods

For protein determination the Fluoram method (Weigele *et al.*, 1973) was used (standard: BSA). DNA was determined according to the published procedure (Kissane and Robins, 1958). Insulin concentrations were determined according to Mosier *et al.* (1987) using the radioimmunoassay system (Amersham).

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The preproinsulin sequence is held at the EMBL Data Library under the accession number X15458 *G.cydonium* preproinsulin.

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