Cloning of the polyubiquitin cDNA from the marine sponge *Geodia cydonium* and its preferential expression during reaggregation of cells

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SUMMARY

Ubiquitination of proteins is a critical step in the controlled degradation process of many polypeptides. Here we show that sponges, the simplest multicellular group of eukaryotic organisms, are also equipped with the ubiquitin pathway. The polyubiquitin cDNA was isolated and characterized from the marine sponge *Geodia cydonium*. The open reading frame contains six ubiquitin moieties, which are lined up head to tail without spacers. A comparison of the predicted amino acid sequence of the six sponge ubiquitin-coding units with those from other organisms revealed a high degree of homology (> 93%). The ubiquitin gene is expressed to almost the same extent in the two main compartments of the sponge, the cortex and the medulla. However, only in the cortex are detectable amounts of the ubiquitin protein synthesized. The ubiquitin protein isolated from the sponge organism was found to initiate protein degradation in the heterologous reticulocyte system in the same manner as bovine ubiquitin. In vitro studies with dissociated sponge cells revealed that the homologous aggregation factor causes (i) a strong increase in the steady-state level of mRNA coding for ubiquitin and (ii) a drastic increase in ubiquitin protein synthesis, while the homologous lectin failed to display that effect in isolated cells. These data suggest that ubiquitin may play a role in sponge morphogenesis.

Key words: sponge, *Geodia cydonium*, ubiquitin, cell adhesion, aggregation factor

INTRODUCTION

All eukaryotes contain the small protein ubiquitin (76 amino acids (aa); \( M_r \), 8.5 kDa), whose sequence is extremely well conserved from protozoans to vertebrates (Jentsch et al., 1991). The ubiquitin genes are grouped into three classes. Classes I and II are expressed during the fusion of ubiquitin and ribosomal proteins. Class III ubiquitin genes code for polyubiquitin proteins with varying numbers of head to tail repeats (up to 12 units in the *Xenopus* polyubiquitin gene; Dworkin-Rastl et al., 1984). Ubiquitin plays an important role in protein catabolism both in the nucleus (e.g. degradation of nuclear oncoproteins, cyclins and histones) and in the cytoplasm (intermediate filaments and lysosomal system) (reviewed in Mayer et al., 1991). A series of enzymes is involved in the highly controlled, ATP-dependent and ubiquitin-mediated degradation process (reviewed in Finley and Chau, 1991; Hershko and Ciechanover, 1992).

Sponges, the simplest multicellular animal phylum (sponges have been known since the Proterozoic period, > 1 billion years ago; Orlov, 1971), have routinely been used as model systems for investigating the cellular and molecular basis for cell-cell adhesion (Moscona, 1963; Henkart et al., 1973; Weinbaum and Burger, 1973; Müller and Zahn, 1973) as well as self-nonself recognition phenomena. Studies have shown that well organized degradation processes proceed during allo- and xenograft rejection (Paris, 1961; Hildemann et al., 1980; Müller et al., 1981) and perhaps also during asexual development (Simpson, 1984).

In sponges two main cell adhesion systems (cell to cell and cell to matrix interaction) together with their associated intracellular signalling pathways (the protein kinase C and the arachidonic acid pathways), have been elucidated (Müller et al., 1987; Rottmann et al., 1987; Gramzow et al., 1989). The aggregation systems in sponge cells have been studied in *Microciona prolifera* (Moscona, 1963; Henkart et al., 1973; Weinbaum and Burger, 1973) and *Geodia cydonium* (Müller and Zahn, 1973; Müller et al., 1988). A sponge cell adhesion molecule, the aggregation factor (AF), was isolated from these organisms (Müller and Zahn, 1973; Henkart et al., 1973). The AF interacts with the membrane-associated aggregation receptor (AR). The cell-cell adhe-
sion system of both *M. prolifera* (Weinbaum and Burger, 1973) and *G. cydonium* (Müller et al., 1976) is heterophilic and of the third order (it is set up by two different species of molecules (AF and AR) and three molecules are necessary to form a cell-cell linkage: AR - AF - AR). In sponges, the cells of the outer epithelial layer (the cortex) interact via AF-AR binding, while the cells in the mesohyl (= medulla), which are embedded in a voluminous lectin matrix, bind to the lectin via the membrane-associated lectin receptor (Gramzow et al., 1989).

Both the AF and the lectin of sponges act not only as cell adhesion molecules but also function as mitogens (reviewed in Müller et al., 1990) modulating morphogen-induced differentiation of sponge cells (Biesalski et al., 1992). During the initial phase of reaggregation (until 8 hours) the cells adhere via AF-AR interaction and subsequently via the matrix (lectin)-lectin receptor binding process (Müller et al., 1990).

Here we demonstrate for the first time that the ubiquitin pathway exists in sponges. Moreover, the expression of this protein is regulated by the cell adhesion molecule (AF) during reaggregation of cells.

**MATERIALS AND METHODS**

**Materials**

Restriction endonucleases and other enzymes for recombinant DNA techniques, AZapTM Kit, Giga pack II Gold packaging extract and cloning vectors were obtained from Stratagene (La Jolla, CA); ubiquitin (bovine; U 6253), polyclonal anti-ubiquitin antibodies (U-5379), alkaline phosphatase-conjugated sheep anti-rabbit IgG, 5-bromo-4-chloro-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT) from Sigma (St. Louis, MO); sequencing kit (sequenase version 2.0) from USB (Cleveland, OH); Poly(A) tract from Promega (Madison, WI); disodium 3-(4-methoxyspirol(1,2-dioxetane-3,2-(5′-chloro)tricyclo-3.1.3.1.7-decan)-4-yl)phenylphosphate (CSPD) from Tropix (Bedford, MA); digoxygenin labelling and detection kit, Nylon 66 and isopropyl β-D-galactopyranoside (IPTG) from Boehringer (Mannheim); T3 and T7 sequencing primers from Pharmacia (Uppsala); PVDF Immobilon P from Millipore (Bedford, MA); Liquifiy No. 1 from Liquifiy Co., Dorking (UK).

**Buffers**

The description of the compositions of the Ca²⁺- and Mg²⁺-containing artificial seawater (ASW) (containing 500 mM NaCl), and of the Ca²⁺- and Mg²⁺-free seawater containing 20 mM EDTA (CMF-SW-E) were given earlier (Rottmann et al., 1987).

**Sponge and sponge components**

Live specimens of *Geodia cydonium* (Demospongiae) were collected near Rovinj (Croatia). The material was immediately frozen in liquid nitrogen until use. For the analysis of ubiquitin protein and RNA levels, the sponge tissue was dissected into two compartments, the cortex (2 mm outer epithelial layer of the sponge) and the medulla. For in vitro studies, sponge cells were isolated from fresh material, within 2-6 hours after collection.

AF particles were isolated and purified as described (Müller and Zahn, 1973). Purified AF (protein content of 1.7 mg/ml) had a specific aggregation-promoting activity of 3.1×10⁶ aggregation units/mg (Müller and Zahn, 1973). *G. cydonium* lectin was isolated and purified by affinity chromatography (Müller et al., 1983). We also used the monoclonal antibody 5D2-D11 against the sponge AF (Gramzow et al., 1986) for the neutralization experiments.

**Construction of *G. cydonium* cDNA library**

Total RNA was extracted from sponge tissue by the method described by Chirgwin et al. (1979), modified by Semsel et al. (1988). Polyadenylated mRNA was isolated from total RNA by Poly(A) Tract, according to the instructions of the manufacturer. cDNA synthesis was performed using the AZapTM-cDNA synthesis kit; the product was ligated into Uni-Zap vector arms (Short et al., 1988). This vector system allows directional insertion of cDNA into the *XhoI* and *EcoRI* site as well as expression of the β-galactosidase fusion protein of the cloned fragments. The ligated products were packaged by Giga pack II Gold packaging extracts (Kretz, 1989). Amplification of the primary library was done in *E. coli* PLK-1' (1×10⁶ independent plaques). The library was transfected into *E. coli* XL1-Blue and plated out for screening.

**Plaque screening**

Plaque lifts into nitrocellulose filters (1.6×10⁶ plaque-forming units) were carried out and screening of the library was performed under low stringency hybridization conditions. Filters were hybridized overnight at 38°C in 50% formamide, 5× SSC, 0.1% NaDodSO₄, 0.1% *N*-laurylsarcosine and 2.1% blocking reagent using the *MaeI* fragment (nt 1084-1956) from the human ubiquitin sequence (Baker and Board, 1987) as probe. The dsDNA restriction fragment, isolated by microelution (Ausubel et al., 1987) was labelled with digoxigenin-11-dUTP using the random primed labelling kit (Boehringer). Filters were washed in 2× SSC, 1% NaDodSO₄ at room temperature, followed by additional washing in 2× SSC, 0.1% NaDodSO₄ (50°C), 0.1× SSC, 0.1% NaDodSO₄ (50°C) and 0.1× SSC (room temperature). Positive clones were detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody using BCIP/NBT as substrate (Blake et al., 1984). Single plage plagues were obtained by three additional screening cycles. Following an in vivo excision procedure described by Stratagene, phagemids (pBluescript SK−) were excised from the phage using the filamentous helper phage R408 and *E. coli* strain XL-1-blue. The positive sponge ubiquitin clones were termed GCUBI.

**DNA sequencing**

The DNA for sequencing was isolated by alkaline lysis according to Sambrook et al. (1989). Double-stranded DNA was sequenced by the dideoxy chain termination method (Sanger et al., 1977). After analysis of the 5′ and 3′ ends of the sequence, subclones were constructed either by ligation of restriction fragments (EcoRI in the multiple cloning site - *XhoI* (nt 374); *BamHI* fragments nt 207-1119 and nt 1119-1347; *Cj101* nt 239-695) or by creating unidirectional deletions (Henikoff et al., 1987) and analysed by end over end sequencing. Computer analysis was performed by the PC/GENE 1991 programmes (IntelliGenetics, Mountain View, CA).

**Northern blotting**

Total sponge RNA was isolated as described before and subjected to denaturing agarose gel electrophoresis. After denaturation at 68°C for 10 minutes in electrophoresis buffer (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) containing 50% formamide and 18% formaldehyde, the samples were separated on 1.2% agarose gels containing 18% formaldehyde and blot-transferred to Nylon 66 membranes. Hybridization was performed with the complete digoxigenin-labelled *G. cydonium* ubiquitin cDNA probe at high stringency. The detection of the digoxigenin-labelled probe was performed using CSPD as substrate for alkaline phosphatase (Beck and Koestner, 1990). For quantitative analysis the chemoluminograms
were scanned with an integrating densitometer (Shimadzu CS-910/C-R1A).

Western blotting

Gel electrophoresis of the extracts was performed in 15% or 10% polyacrylamide gels containing 0.1% NaDodSO₄ (SDS-PAGE) according to Laemmli (1970).

Total cellular extracts or extracts from the medulla and cortex regions of the sponge were obtained as follows: frozen sponge was homogenized in liquid nitrogen and transferred to phosphate buffered saline, supplemented with 500 mM NaCl and 5 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation (10,000 g, 4°C, 30 minutes) the supernatant was subjected to gel electrophoresis. Semi-dry electrotransfer was performed according to Kyhse-Andersen (1984) onto PVDF-Immobilon P. Filters were processed (Towbin et al., 1979) and incubated with polyclonal anti-ubiquitin antibodies after blocking the membranes with 5% bovine serum albumin. The immune complexes were visualized by two methods; either after additional incubation with anti-rabbit IgG (peroxidase-conjugated), followed by staining with 4-chloro-1-naphthol, or with alkaline phosphatase-conjugated anti-rabbit IgG, applying the BCIP/NBT procedure (see above).

For dot blots, 1 × 10⁷ cells were extracted with 1 volume of the phosphate buffer saline (see above). After centrifugation the supernatant was collected and 5 µl of the extracts was spotted onto activated PVDF-Immobilon P membrane. After blocking with bovine serum albumin in 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl, the membranes were incubated with anti-ubiquitin antibodies; the immune complexes were detected by alkaline phosphatase-conjugated anti-rabbit antibodies using CSPD as substrate (see above).

Expression of cloned ubiquitin in E. coli

Fusion protein expression of the G. cydonium ubiquitin cDNA was performed in pBluescript SK±E. coli XL-1 Blue system by induction with 10 mM IPTG (until an OD₆₀₀ of 1 was reached), according to the instructions of the manufacturer (Stratagene). The suspension was centrifuged, resuspended in 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 1 mM PMSF and 10% sucrose, and lysed in the presence of 1 mg/ml lysozyme and 0.1% Triton X-100. After centrifugation (14,000 g, 1 hour, 4°C) the supernatant was analysed by gel electrophoresis (10% polyacrylamide; see above).

Protein degradation assay

The assays were performed by modification of a previously described procedure (Isoe et al., 1991). The 50 µl assay mixture contained 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 3 mM dithiothreitol, 5 mM ATP, 10 mM creatine phosphate, 0.5 units of creatine phosphokinase and 4 µg of ¹²⁵I-labeled bovine serum albumin (Ciechanover et al., 1980) (4 × 10⁵ cpm/µg) and 60 µg of ATP-depleted Fraction II protein, isolated from reticulocytes (Ciechanover et al., 1978). Where indicated, 10 µg of bovine ubiquitin or of the sponge extract was added to the assays. After incubation (37°C) the reaction was stopped by addition of albumin (100 µg) and trichloroacetic acid (19% w/v). The samples were centrifuged and radioactivity was determined. Control values (assays without Fraction II) were subtracted from the results. Where indicated, either ATP or ubiquitin was omitted from the assays. One unit of ubiquitin is defined (Ciechanover et al., 1980) as the amount required to achieve 50% of the maximal degradation.

Cells and incubation assay

Viable sponge cells were obtained as described (Müller and Zahn, 1973). The cells were used for the experiments within 6 hours after dissociation.

A suspension of 25 ± 5 × 10⁶ cells/ml of ASW, supplemented with Liquifry (Weissenfels and Langenbruch, 1985), was placed into glass tubes (3 ml) and rolled (35 rpm; 20°C) (Müller and Zahn, 1973). Where indicated, the cultures were supplemented with AF (30 µg/ml) or 1 µg/ml of G. cydonium lectin and incubated for 60 minutes. Then the cells were incubated for an additional period of up to 15 hours without moving them.

Partial purification of ubiquitin

For the enrichment of ubiquitin from G. cydonium, the protocols of Ciechanover et al. (1980) and Wilkinson et al. (1980) have been applied in a modified form:

Step 1: heat treatment

Fresh sponge material (100 g) was homogenized in 200 ml of CMF-SW-E. After centrifugation (20,000 g, 10 minutes, 4°C) the supernatant was heated to 90°C for 20 minutes. The suspension was centrifuged and the supernatant was brought to 90% saturation with respect to ammonium sulfate. The precipitated material was collected and dissolved in 10 ml of 10 mM potassium phosphate (pH 6.0) and dialyzed (dialysis tubing: Sigma no. 2272) against the same buffer.

Step 2: CM-Sepahex chromatography

The enriched material was applied to a column of CM-Sepahex C-50 (2 cm × 15 cm) equilibrated with the potassium phosphate buffer. The column was washed with this buffer, supplemented with 25 mM KCl, and then eluted by a 25 to 125 mM KCl gradient (in potassium phosphate buffer). Fractions of 3 ml were collected. The peak fractions were collected and concentrated by ammonium sulfate precipitation.

Step 3: DE chromatography

The precipitate obtained was taken up in 1 ml of 0.02 M ammonium carbonate (pH 9.0) dialyzed against this buffer and subjected to a DE52 cellulose column (2 cm × 10 cm), which was equilibrated with the same buffer. The ubiquitin-containing fractions were collected as described (Ciechanover et al., 1980). The enriched ubiquitin fraction had a specific activity of 2380 units/ml (212 units/ml) with a yield of 67%. The purification was 11-fold with respect to the heat-treated fraction; the overall purification could not be determined reliably because of the high protease content in the crude sponge extract.

Further analytical procedures

For protein determination the Fluoram method was used (Weigele et al., 1973); the standard was bovine serum albumin.

RESULTS

Primary structure of the sponge polyubiquitin cDNA

We have used the human cDNA to identify and isolate the corresponding cDNA clones from the marine sponge G. cydonium since the amino acid (aa) sequences of ubiquitin are highly conserved (Jentsch et al., 1991). Five independent clones, each resulting in the same sequence (also at the 5’ termini), were analyzed. All clones contained a 1.6 kb long cDNA insert. The nt sequence of sponge polyubiquitin cDNA (termed GCUBI) is shown in Fig. 1. The open reading frame (ORF) with the ATG-codon for methionine is 1374 bp long; this initiation codon conforms with the Kozak consensus (CAGC/ATG) sequence (Kozak,
1984). An inverted repeat GAAAGCTTTC close to the initiation codon (nt -13 to nt -4 ) is present. The stop codon is TAA (nt +1372 to +1374). The typical signal polyadenylation site AATAAA (Zarkower et al., 1986) is missing in GCUBI cDNA. One putative site for the polyadenylation process in GCUBI might be TAG.TAG..TT (nt +1419 to +1431) (Irniger et al., 1991); the same site (TAG....TAGT) was found recently in the cDNA clones for the sponge G. cyclodium lectin (Pfeifer et al., 1993).

The sponge GCUBI gene encodes a polyubiquitin precursor protein with six highly homologous sequences for ubiquitin, GCUBI-1 to GCUBI-6, joined head-to-tail, without spacers (Fig. 1). With respect to GCUBI-1 the identity of GCUBI-2 to GCUBI-6 was 90.4%, 96.5%, 89.5%, 94.7% and 90.8% at the nt level. Codon usage analysis revealed that for the following aa all possible codons have been used: Asn, Asp, Gln, Glu, Gly, His, Phe and Val.

### Amino acid sequence of sponge polyubiquitin

The aa sequence of sponge ubiquitin was deduced from the cDNA GCUBI (Fig. 2). The gene encodes a ~51.4 kDa primary translation product composed of six almost identical repeats of the ubiquitin sequence joined head-to-tail, with a deduced Mr of between 8,525 (GCUBI-4) and 8,539 (GCUBI-1, -2, -3 and -5) to 8,686 (GCUBI-6). The deduced aa sequences of GCUBI-1 to -6 display one potential proprotein kinase C phosphorylation site at Thr (aa 9), one casein kinase II phosphorylation site in the Thr moiety (aa 55) and no glycosylation site (Fig. 2). The deduced aa sequences of the six sponge ubiquitins are identical, with the exception of GCUBI-4; there Val replaces Leu) have the amino acid compositions of GCUBI-1 to GCUBI-6 are identical: 11 acidic, 11 basic, 3 aromatic and 24 hydrophobic residues. The calculated pI of the ubiquitins is 7.57. As in most other polyubiquitins (Finley and Varshavsky, 1985) the sponge sequence contains an extra amino acid residue at the last unit, which is identical with the one in Lupinus polyphyllus, Phe. All sponge ubiquitin units (with the exception of GCUBI-4; there Val replaces Leu) have the following typical C-terminal consensus sequence: Val-Leu-Arg-Leu-Arg-Gly-Gly (Finley and Varshavsky, 1985; Jentsch et al., 1991).

### Expression of cloned GCUBI in E. coli

GCUBI cDNA in pBluescript SK- was expressed in E. coli by induction of the β-galactoside promotor by IPTG, according to the manufacturer’s protocol (Stratagene).

The bacterial cultures remained either uninduced (Fig. 3, lane a) or have been induced with IPTG (lane d). Extracts were obtained and analyzed by western blot using anti-ubiquitin antibodies. In the extract from noninduced bacteria a low level of fusion protein is also detectable (lane a). After
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induction of the β-galactosidase promoter with IPTG four major protein bands (Mr 60,000, 51,800, 48,000 and 45,000) became visible in addition to minor expression products. The Mr 51,800 protein corresponds well to the calculated Mr of 51,500 for the cloned sponge ubiquitin cDNA.

In control experiments, using the vector without GCUBI cDNA, only one band with a Mr of 29,000 became visible (lanes b and c); this is considered to be due to nonspecific staining.

**ATP-dependent proteolysis mediated by sponge ubiquitin**

Proteolysis of 125I-labelled albumin by Fraction II of rabbit reticulocytes was measured in the presence of bovine and sponge ubiquitin. The DE-fraction of the enriched sponge ubiquitin was used for the experiments. Fig. 4 shows that both ubiquitin fractions caused ATP dependent proteolysis. In control experiments we established that the reaction was (i) ATP-dependent, (ii) mediated by ubiquitin and (iii) inhibited by antibodies directed against ubiquitin (Fig. 4).

**Identification of the sponge ubiquitin mRNA**

Sponge ubiquitin mRNA was identified on northern blots using the labelled HindIII fragment (nt −10 to +1,452) 1.6 kb GCUBI clone as a probe. As shown in Fig. 5, the 1.6 kb mRNA is present both in the medulla (lane a) and cortex (lane b) region of the sponge tissue. Even after overexpo-
sure no trace of more than one ubiquitin transcript species could be detected (not shown). Quantitative analysis revealed that the medulla contains approximately 20% less polyubiquitin mRNA than the cortex region.

**Differential synthesis of ubiquitin in the sponge tissue**

Both total extract and separate extracts from the medulla and the cortex were prepared. Identical tissue samples were used for the determination of the mRNA and protein levels in medulla and cortex. Ubiquitin protein (Mr 8,500) could be identified in extracts from the sponge (Fig. 6). However, it was present only in the extract from the cortex region (Fig. 6B, lane b) but was - under the conditions used - absent in the medulla when applying identical concentrations of total protein to gel electrophoresis (lane a). It is concluded that ubiquitin mRNA is not, or is only to a small extent, translated in the medulla region of the sponge.

**Effect of the AF on the level of sponge ubiquitin mRNA and protein**

Dissociated sponge cells were incubated for 0-8 hours in the presence of AF. Northern blot experiments revealed that the steady-state level of ubiquitin mRNA increased strongly even after an incubation period of 4 hours (Fig. 7A). The steady-state mRNA level did not change significantly during an additional 8-hour incubation in cells preincubated with AF for 8 hours following transfer to culture medium with lectin but without AF (Fig. 7B).

The total level of ubiquitin protein (and ubiquitin-protein conjugates) in the cells was determined by dot blotting using anti-ubiquitin antibodies. Cells (i) were not preincubated with AF (Fig. 8, upper panel, right half) or (ii) were pretreated with AF for 8 hours prior to incubation with either lectin or with an AF preparation that had been adsorbed with antibodies against AF (left half). The results show that in ‘non-preincubated cultures’ AF strongly increased the level of ubiquitin during a 12 hour-incubation period. During the first 4 hours AF induced the level of ubiquitin approximately 5-fold (Fig. 8, lower panel); this induction was prevented if the AF was pretreated with antibodies against AF. In contrast, the lectin displayed only a low induction potency during the same incubation period (Fig. 8, upper panel, right half). The level of ubiquitin was drastically reduced in cells that had been pretreated with AF (8 hours) and subsequently treated with either lectin or...
The cultures that had been pretreated with AF (8 monoclonal antibody 5D2-D11 directed against the AF (left half). preincubated (upper panel, right half) or preincubated for 8 hours assay for up to 12 hours with either conjugates) in extracts from Level of ubiquitin protein (and ubiquitin-protein Fig. 8. AF was pretreated with antibodies against AF (AF-AB).

preincubated with AF for 8 hours were the same and pretreated with AF were incubated in the standard incubation pathway exists in eukaryotes, which plays a central prepation that had been pretreated for 10 hours with an AF preparation. The preincubation studies with AF for 8 hours were performed to study cell-matrix interaction, which occurs 8 hours after addition of the AF (Müller et al., 1990), the cells synthesize ubiquitin differently after incubation of untreated cells for 0 or 4 hours with AF; in one experiments the AF was pretreated with antibodies against AF (AF-AB).

an AF preparation that had been treated with anti-AF antibodes for an additional 12 hours (left half). The preincubation studies with AF for 8 hours were performed to determine if, after the switch from cell-cell interaction to cell-matrix interaction, which occurs 8 hours after addition of the AF (Müller et al., 1990), the cells synthesize ubiquitin differently after incubation with one of the cell adhesion molecules.

No difference in the state of aggregation in the two series of experiments could be detected; the diameters of the aggregates in the assays in which cells had not been preincubated with AF or in those in which the cells had been preincubated with AF for 8 hours were the same and amounted to 2,200 ± 350 µm.

DISCUSSION

One prerequisite of controlled cellular functions and of the development of multicellular organisms is a regulated protein breakdown. Besides the nonspecific lysosomal protein breakdown, the energy (ATP)-dependent ubiquitin degra- tion pathway exists in eukaryotes, which plays a central role in protein catabolism (reviewed by Finley and Chau, 1991; Hershko and Ciechanover, 1992). Until now it was not known whether ubiquitination processes occur in the simplest group of multicellular organisms, the sponges. We isolated the cDNA coding for G. cydonium ubiquitin. It exhibits all the hallmarks of functional polyubiquitin. Until now no evidence for an additional ubiquitin gene was found in the sponge. This finding is interesting because it was claimed that all eukaryotes contain besides polyubiquitin (designated class III ubiquitin) class I and II ubiquitins (Jentsch et al., 1991). For example, Trypanosoma cells contain, under normal conditions, three size classes of ubiquitin-specific mRNAs (5.6, 1.8, 0.75 kb); they even respond to heat-stress with an induction of another 1.6 kb mRNA species (Neves et al., 1988).

The sponge polyubiquitin gene contains six highly homologous ubiquitin units lined up head to tail and containing (with the exception of GCUBI-4) at each C terminus the typical C-terminal consensus sequence: Val-Leu-Arg-Leu-Arg-Gly-Gly (Finley and Varshavsky, 1985; Jentsch et al., 1991). This ‘tail’ is essential for the function of ubiquitin; the terminal Gly residue can bind to the epsilon amino group of Lys residues in cytoplasmic, nuclear, and cell surface proteins (reviewed by Finley and Chau, 1991; Jentsch et al., 1991; Hershko and Ciechanover, 1992). Like in most other polyubiquitins the last (here, sixth) ubiquitin repeat is followed by a single Phe residue (Özkaynak et al., 1987). Also in sponges, the polyubiquitin precursor protein is processed by ubiquitin-C-terminal hydrolase to ubiquitin units (reviewed by Finley and Chau, 1991; Jentsch et al., 1991). At the nt level the percent identities among sponge ubiquitin units vary between 90 and 97% with respect to ubiquitin unit-I and around 80% with respect to other ubiquitins (e.g. human ubiquitin) (Fig. 1). Clustal analysis of the sponge ubiquitin units at the nt level support the conclusion that also in sponges ubiquitin repeats have evolved ‘in concert’ (Sharp and Li, 1987). At the aa level the homology within the sponge ubiquitin units is 100% (with the exception of unit 4) and to ubiquitins from other organisms between 99% (human) and 93% (the protozoa Euplotes) (Fig. 2). Like other ubiquitins the sponge ubiquitin units contain one phosphorylation site for protein kinase C and a second one for casein kinase II. However, there is no experimental evidence that ubiquitin itself undergoes phosphorylation in an intact cell system. It is known that other proteins involved in the ubiquitin process are phosphorylated and in response are activated (Kong and Chock, 1992).

Sponge ubiquitin has been enriched to an approximate purity of > 95%. This ubiquitin fraction initiated protein degradation if added to the heterologous system from reticulocytes (fraction II), which contains the ubiquitin-ATP-dependent protease and the other enzymes involved in the ubiquitin-protein ligation system (Ciechanover et al., 1978). The kinetics of ubiquitin-mediated ATP-dependent proteolysis was identical regardless of whether sponge or bovine ubiquitin were used. Sponge ubiquitin, both natural and recombinant, cross-reacted with antibodies against purified bovine ubiquitin.

As in other organisms the level of ubiquitin in sponges varies in accordance with physiological and pathophysiological conditions (Mayer et al., 1991). We found that detectable amounts of ubiquitin protein are present only in the cortex of the sponge colony and not in the medulla region while the level of ubiquitin mRNA was almost identical in both compartments. This finding might reflect a fast turnover of ubiquitin in the medulla region. The size of the G. cydonium mRNA was 1.6 to 1.7 kb; this value fits well with the length of the isolated sponge GCUBI clone (1.6
To study the molecular basis for the absence of ubiquitin in the medulla segment we performed experiments with isolated cells treated by one of the two adhesion molecules AF and lectin. The sponge cortex and medulla differ with respect to the adhesion mechanism since in the cells in the cortex layer adhere to each other by AF - aggregation receptor interaction, while in the medulla the cells are stabilized by lectin-lectin receptor interaction (Müller et al., 1988). Incubation of cells in the presence of AF resulted in a rapid elevation of the mRNA coding for ubiquitin; simultaneously the amount of ubiquitin protein increased as well. Incubation of the cultures with lectin did not lead to an increase in ubiquitin protein level while the amount of ubiquitin mRNA remained constant. This result is taken as a first indication that AF up-regulates while lectin down-regulates the translation efficiency of ubiquitin mRNA.

We plan to analyze the physiological role of ubiquitin during AF-mediated cell adhesion in future studies. Considering results obtained with other systems (Mayer et al., 1991; Glotzer et al., 1991; Paolini and Kinet, 1993) it can be hypothesized that ubiquitin is involved in the control of the following observed AF-mediated processes in the sponge system: proliferation control (Müller et al., 1988), downregulation of AR (Gramzow et al., 1988) or response to foreign invaders (Müller et al., 1981). In addition, it remains to be studied if oncoproteins, which have been described in sponges, such as ras (Schröder et al., 1988) or src (Ottillie et al., 1992) and which are assumed to be involved in differentiation, are also degraded by ubiquitination as has been described for higher eukaryotic systems (Ciechanover et al., 1991).

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