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cDNA structure and expression of calpactin, a peptide involved in Ca^{2+}-dependent cell aggregation in sponges

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Aggregation of cells of the marine sponge *Geodia cydonium* is mediated by an aggregation factor (AF) particle of M_r 1.3×10^8. It is now reported that the AF particle is associated with calpactin, which was ascribed a role in the cell-adhesion process. In order to identify the sequence similarity to other members of the lipocortin family, the cDNA of sponge calpactin was cloned and found to display an 80% sequence similarity to vertebrate calpactin II but only a 47% similarity to calpactin I. The calpactin gene, which contains the consensus sequence coding for the amino acids G-T-D-E, was expressed in *Escherichia coli* and subsequently purified to a 37000-M_r polypeptide. Both the p32 and the p37 are provided with approximately two Ca^{2+} ions/molecule and the property to bind to phospholipids. The dissociation constant (calpactin–Ca^{2+}) was in the absence of phospholipids in the range 500–700 μM-Ca^{2+} but in their presence about 20–30 μM-Ca^{2+}. On the basis of (i) inhibition studies with antibodies to calelectrin and (ii) competition experiments with soluble phospholipids (both chemically defined as well as total homologous membrane lipids) we conclude that the AF-associated calpactin and plasma-membrane-bound phospholipid(s) are involved in cell–cell aggregation in sponges.

INTRODUCTION

Cell–cell aggregation in marine sponges is mediated by an aggregation factor (AF) and a plasma-membrane-associated aggregation receptor (AR) (Humphreys, 1963; Weinbaum & Burger, 1973; Müller, 1982). The sponge AFs represent particles of M_r larger than 10^7 (Müller *et al.*, 1979*a*; Burger & Misević, 1985). Additionally the particle characterizing the sponge *Geodia cydonium* does not only contain the p47 cell-binding fragment, which recognizes the plasma-membrane-associated AR (Gramzow *et al.*, 1986), but also a collagen-binding site (Gramzow *et al.*, 1988*a*). As recently established, the cell-binding fragment acts as a growth factor after binding to its corresponding AR through an activation of protein kinase C (Müller *et al.*, 1987; Gramzow *et al.*, 1988*b*). This event leads to the phosphorylation of DNA topoisomerase II (Rottmann *et al.*, 1987) followed by subsequent DNA replication and specific gene expression (Müller *et al.*, 1987; Schröder *et al.*, 1988).

In a previous study we also provided experimental evidence that a 32000-M_r polypeptide (p32), identified by immunological means as a calelectrin-like polypeptide, is associated with the extracellularly localized AF from *G. cydonium* (Gramzow *et al.*, 1989). For the detection an antiserum against calelectrin from *Torpedo marmorata* was used, which reacted with proteins of M_r 32000, 34000 and 68000 in rat brain (Stoll *et al.*, 1988). Sequence analyses revealed that calelectrin contains three regions exhibiting sequence similarity to lipocortin (Kretsinger & Creutz, 1986). Preceding research has emphasized anew the fundamental distinction between two types of lipocortin (I and II): type I (identical with calpactin II) is monomeric, type II (identical with calpactin I) is tetrameric. Since the sponge calelectrin-like polypeptide is apparently a monomer (M_r 32000) similar to the different calpactins II (lipocortins I), which are all in the M_r range 30000–40000 (see Crompton *et al.*, 1988), we decided to investigate the function of this sponge polypeptide in the cell–cell reaggregation process.

The *G. cydonium* cDNA for calpactin was cloned, and it displayed a high sequence similarity to vertebrate calpactin II. Moreover, the present study shows that the natural and recombinant sponge calpactin molecules are Ca^{2+}- and phospholipid-binding proteins. Moreover, novel data were obtained suggesting that AF-particle-mediated cell–cell recognition involves also an interaction between AF-associated calpactin and plasma-membrane-bound phospholipids.

MATERIALS AND METHODS

Chemicals and media

The following materials were used: L-α-phosphatidic acid (sodium salt; catalogue no. P9511), L-α-phosphatidyl-L-serine (from bovine brain; catalogue no. P6641), L-α-phosphatidylcholine (from bovine brain; catalogue no. P6638) and secondary anti-(rabbit IgG) antibody from goat (G3766) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); $^{45}CaCl_2$ (1.4 Ci/mmol) from Amersham International (Amersham, Bucks., U.K.); Protein A–gold complex (average size of the gold particles 12–15 mm) from E–Y Laboratories (San Mateo, CA, U.S.A.).

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

Abbreviations used: AF, aggregation factor; AR, aggregation receptor; ASW, artificial sea water; CMFSW, Ca^{2+}- and Mg^{2+}-free sea water; nt, nucleotide residue.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X16980.

Sponge and components from it

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

The AF particle was isolated and purified as described previously (Müller & Zahn, 1973). The purified AF with a protein content of 1.7 mg/ml had a specific aggregation-promoting activity of 3.1×10^6 aggregation units (Müller & Zahn, 1973)/mg.

Membrane lipids were extracted from homologous cells. Plasma membranes were treated in the same way (Cuello *et al.*, 1983), and the lipids subsequently were isolated by following the chloroform/methanol procedure described previously (von Kieckebusch *et al.*, 1988).

Cells and incubation assay

Cells were obtained by dissociation of sponge tissue in CMFSW (Müller & Zahn, 1973), with the exception that the trypsin step was omitted. The different cell types were extracted by separation through Ficoll-discontinuous-gradient centrifugation (Müller *et al.*, 1981). The cell viability was greater than 95% as checked by Trypan Blue (0.5%, w/v) exclusion.

In the standard incubation assay (3 ml volume) (Müller & Zahn, 1973) a suspension of $25 \times 10^6 \pm 5 \times 10^6$ archaeocytes/ml of ASW was placed into glass tubes and rolled at 35 rev./min at 20 °C. AF particles (40 μg of protein) were added as indicated. Where mentioned, antibodies to calelectrin (Fab fragments; 20 μg) were added to the AF particles 5 min before the addition to cells. In one series of experiments the antibodies to calelectrin (100 μg) were incubated for 12 h at 4 °C either with 30 μg of natural sponge p32 calpactin or with 30 μg of recombinant p37 calpactin in 100 μl of Ca^{2+}-containing ASW. Afterwards the samples were centrifuged at 100000 *g* for 1 h and the supernatants were assayed in the aggregation assay.

The sizes of the aggregates were determined optically (Müller *et al.*, 1979*b*).

Antibody

Antibodies to calelectrin purified from *Torpedo marmorata* (Fritsche *et al.*, 1988) were raised in rabbits. They were purified by affinity chromatography on Protein A–Sepharose (Ey *et al.*, 1978). The Fab fragments of the IgGs were prepared by papain digestion (Utsumi, 1969).

Isolation of p32 from *G. cydonium*

The method applied is based on the protocol described by Fritsche *et al.* (1988). Purified AF particles (300 mg with respect to protein) were suspended in 10 ml of CMFSW containing 20 mM-$CaCl_2$. After incubation for 5 h at 4 °C the AF was pelleted at 4000 *g* for 15 min and resuspended in 5 ml of CMFSW containing 10 mM-EGTA and 0.5% (v/v) Triton X-100. p32 was solubilized from the AF particles (during 24 h at 4 °C) and recovered in the supernatant after centrifugation at 100000 *g* for 60 min at 4 °C. The supernatant was dialysed against 1 litre of CMFSW containing 5 mM-2-mercaptoethanol.

Final purification was achieved by immunoaffinity chromatography essentially as described elsewhere (Bachmann *et al.*, 1986). Briefly, antibodies to calelectrin were coupled to Sepharose 4B. An anti-calelectrin–Sepharose column (0.3 × 1.0 cm) was prepared and equilibrated with CMFSW containing 5 mM-2-mercaptoethanol. The column was loaded with the p32-containing fraction. The purified p32 was obtained by elution with 200 mM-glycine/HCl buffer, pH 3.0, containing 500 mM-NaCl. The purified fractions were dialysed against CMFSW containing 5 mM-2-mercaptoethanol. Starting from 300 mg of AF particles, a yield of 510.7 μg of purified p32 was obtained.

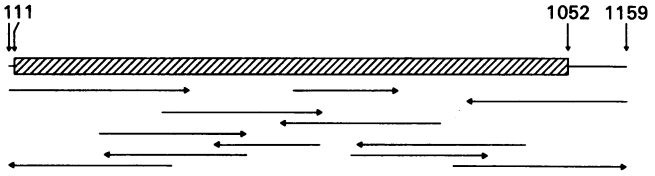

Fig. 1. Strategy for sequencing of calpactin cDNA

The Figure shows a diagrammatic representation of the calpactin cDNA clone encompassing the 5′ untranslated region (nt 1–10), sequence encoding the sponge calpactin (nt 11–1051) and the 3′ untranslated region (nt 1052–1159). A total of six specific oligonucleotides were used to sequence 100% of the sense strand, and six oligonucleotides were used to sequence the complete antisense strand.

Isolation of sponge calpactin cDNA

A cDNA library of *G. cydonium* was prepared and constructed in the λgt11 vector (Robitzki *et al.*, 1989). Radioactively labelled rat calpactin II cDNA (Shimizu *et al.*, 1988) was used as a hybridization probe in three replicas of the library (Figs. 1 and 2).

DNA sequencing

After cleavage of λgt11 cDNA with *Eco*RI, the cDNA was tailed with dCTP, and the dC-tailed cDNA was annealed to dG-tailed *Pst*I-digested pBR322. The resulting chimeric plasmids were used to transform *Escherichia coli* HB101. This cal-1 cDNA insert contains a 5′ untranslated region of ten nucleotide residues, the coding region of 1038 nucleotide residues and the 3′ untranslated region of 111 nucleotide residues (Fig. 2). The sequence was analysed by the dideoxy chain-termination method (Sanger *et al.*, 1977); the result was confirmed by application of the Maxam & Gilbert (1983) procedure. The strategy for sequencing is given in Fig. 1.

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

All oligonucleotides were synthesized on a Beckman DNA synthesizer.

Expression and purification of sponge calpactin in *E. coli*

An expression plasmid pKK233-2-cal-1 was constructed as follows. After cleavage of cal-1 cDNA with *Pvu*I (nt position 3735 of pBR322), linearized products were treated with *Bal*31 to reduce the length of the 3′ untranslated region. The 5′ untranslated region of the cDNA was removed by digestion with *Sph*I located at nt 45. The resulting shortened cDNA fragment (nt 46–1084) was subcloned between the *Sph*I and *Hinc*II sites of the polylinker region of pUC18 (Takara Shuzo, Kyoto, Japan) to introduce an *Hin*dIII site outside of the 3′ untranslated region of the cDNA. After digestion with *Sph*I and *Hin*dIII, the cDNA fragment was isolated and inserted between the *Nco*I and *Hin*dIII sites of the prokaryotic expression vector pKK233-2 (Amann & Brosius, 1985) together with the two complementary oligodeoxynucleotides 5′-dCATGGCAATGGTATCAGAATTTATCAATCAAGCATG-3′ and 5′-dGTAGCATGCTTGATTGATAAATTCTGATACCATTGC-3′. These two oligonucleotides replace the missing coding region (nt 11–45). The resultant plasmid pKK233-2-cal-1 contains the entire protein-coding region and the 36 bp 3′ untranslated region of sponge calpactin II cDNA (nt 11–1084), and expresses sponge calpactin II under the regulation of the *trc* promoter. *E. coli*

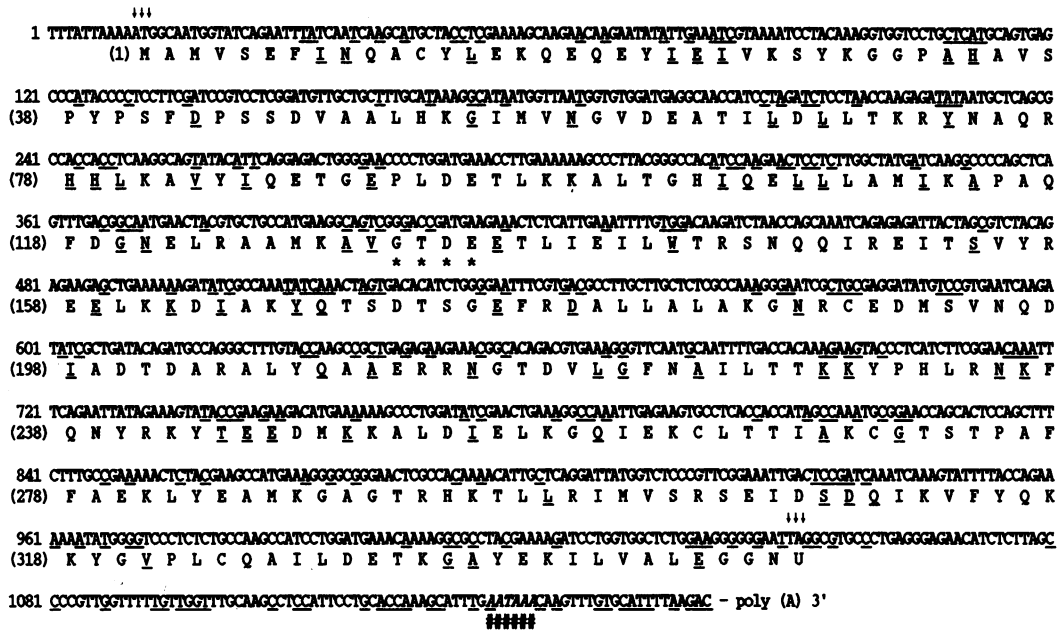

Fig. 2. Nucleotide sequence of calpactin cDNA of the sponge *G. cydonium* and the predicted polypeptide sequence

These sequences were compared with those of rat calpactin II (Shimizu *et al.*, 1988). The underlined sequences denote divergence from the rat sequence. The deduced amino acid sequence is numbered in parentheses. The Geisow motif is indicated by asterisks. # indicates the polyadenylation signal site. The sequence encoding calpactin is enclosed by arrows.

JM 105 harbouring either pKK233-2-cal-1 or pKK233-2 (the expression vector without the insert) were grown at 37 °C in LB broth containing 50 μg of ampicillin/ml to an absorbance of 0.6 at 600 nm; subsequently they were induced with 1 mM-isopropyl β-D-thiogalactoside for 10 h.

Recombinant sponge calpactin was purified from sonicated lysate of *E. coli* JM 105 (pKK233-2-cal-1) in 200 mM-Tris/HCl buffer, pH 7.7, containing 5 mM-EGTA and 0.5% (v/v) Triton X-100, as given below. The sonicated lysate was centrifuged at 10000 *g* for 15 min, and the supernatant was dialysed against CMFSW containing 20 mM-$CaCl_2$ for 12 h at 4 °C. The pellet formed was collected by centrifugation at 100000 *g* for 1 h and dialysed against CMFSW containing 5 mM-EGTA and 5 mM-2-mercaptoethanol. Final purification of the sponge recombinant p37 calpactin was achieved by immunoaffinity chromatography as described above.

One-dimensional SDS/PAGE was performed as described by Laemmli (1970); the resolving gel contained 15% acrylamide. The gels either were stained with Coomassie Brilliant Blue or the proteins were transferred from the gels to nitrocellulose sheets (Towbin *et al.*, 1979). To identify sponge calpactin, the blots were sequentially treated with anti-calelectrin antibody and anti-(rabbit IgG) antibody (peroxidase-conjugated) (Bachmann *et al.*, 1986).

Ca^{2+}-binding measurements

The binding of Ca^{2+} to both natural and recombinant sponge calpactins was estimated by gel filtration (Hummel & Dreyer, 1962). A Bio-Gel P-6DG (Bio-Rad Laboratories) column (0.8 cm × 50 cm) was equilibrated and eluted with CMFSW [previously passed over a column of Chelex-100 (Bio-Rad Laboratories)] containing 10 μM-$^{45}Ca^{2+}$. Natural (p32) or recombinant (p37) calpactin (0.2 mg; 6.3 or 5.4 nmol respectively) was applied in 0.5 ml of equilibration buffer, and 0.6 ml fractions were collected. Calpactin was monitored by reading the absorbance at 278 nm, and $^{45}Ca^{2+}$ by its radioactivity.

In a second approach, Ca^{2+}-binding was measured by equilibrium dialysis essentially as described elsewhere (Diehl-Seifert *et al.*, 1985). Briefly, deionized and freeze-dried p32 and p37 calpactin samples were reconstituted in CMFSW, and 1 ml portions (100 μg/ml) of calpactin were dialysed against 100 ml of CMFSW supplemented with 100 μCi of $^{45}Ca^{2+}$ and $CaCl_2$ to achieve a free Ca^{2+} concentration between 1 μM and 1 mM. To determine the amount of Ca^{2+} bound, portions of the equilibrated solutions outside as well as inside the dialysis bag were dried on filter-paper strips and subjected to scintillation counting. The dissociation constant was estimated by Scatchard analysis with the use of the LIGAND program (Munson & Rodbard, 1980).

The binding affinity of calpactin to different phospholipid fractions was determined by application of the flotation method essentially as previously described (von Kieckebusch *et al.*, 1988). For these experiments ^{125}I-labelled calpactin (labelled as described in Cuello *et al.*, 1983) (0.1 mg/ml; 500 nCi/μg) and sonicated lipid (1 mg/ml) in CMFSW were used.

For the determination of dissociation behaviour of calpactin from lipids the procedure previously described (von Kieckebusch *et al.*, 1988) was applied. The protein (10 μg) was bound to the respective lipid fraction (100 μg) in the presence of 5 mM-Ca^{2+} in CMFSW. The calpactin-lipid complex formed after 30 min at 20 °C was collected by centrifugation at 50000 *g* for 10 min. After being washed in CMFSW the precipitate was suspended in CMFSW at a defined Ca^{2+} concentration (adjusted with EGTA and $CaCl_2$). After a further incubation period for 30 min at 20 °C and an additional centrifugation of the assay mixtures the lipid-bound calpactin was obtained by EGTA treatment of the pellet. The amount of calpactin was semi-quantitatively measured after SDS/PAGE by scanning the Coomassie Blue-stained gels with an integrating densitometer (Shimadzu CS-910/C-R1A).

RESULTS AND DISCUSSION

Inhibition of cell–cell aggregation by antibodies to calelectrin

Antibodies to calelectrin (Fab fragments) strongly suppress AF-mediated cell–cell aggregation (Fig. 3). Under the incubation

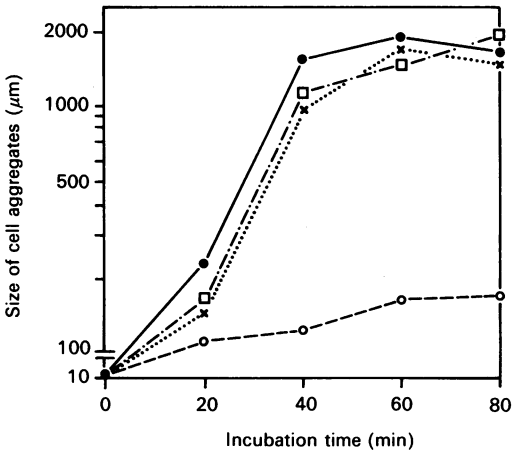

Fig. 3. Inhibition of cell–cell aggregation by anti-calelectrin antibodies

A single-cell suspension was incubated with the AF particles in the absence (●) and in the presence (○) of antibodies to calelectrin in the standard assay mixture containing ASW. In two further experiments the AF-containing standard incubation assay mixture was supplemented with 20 μg of antibodies to calelectrin adsorbed either with natural p32 calpactin (×) or with recombinant p37 calpactin (□) as described in the Materials and methods section. Results are from five parallel determinations; the mean values are presented, and the S.D. was less than 20%.

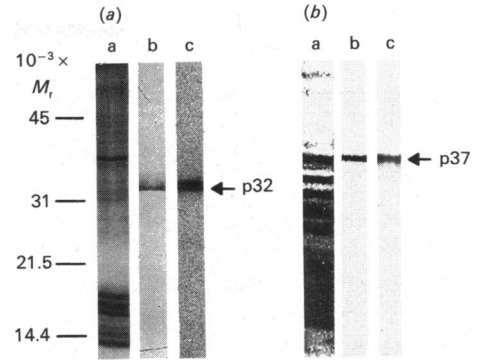

Fig. 4. PAGE of natural (*a*) and recombinant (*b*) sponge calpactin

(*a*) Purified AF particles (lane a) or purified natural calpactin (lane b) was size-separated on a 15% polyacrylamide gel under denaturing conditions and stained with Coomassie Brilliant Blue. The separated purified calpactin was transferred to nitrocellulose sheets and incubated first with anti-calelectrin antibodies (rabbit) and secondly with anti-(rabbit IgG) antibody (peroxidase-conjugated) (lane c). (*b*) Lysate from induced *E. coli* JM105 (pKK233-2-cal-1) (lane a) or purified recombinant calpactin (lane b) was electrophoresed and stained in the same way as in (*a*). The purified recombinant calpactin was subjected to Western-blot analysis as for lane c in (*a*). The arrows indicate the positions of natural p32 and recombinant p37 protein. M_r standards were BSA (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 31000), soya-bean trypsin inhibitor (M_r 21500) and lysozyme (M_r 14400).

conditions used, the aggregation process reached a plateau after 40 min. Addition of antibodies to the assay almost totally blocked the aggregation process. The specificity of this inhibition was supported by experiments with antibody preparations that had been adsorbed both with natural p32 calpactin or with recombinant p37 calpactin. In both assays the inhibitory activity of the antibodies could be abolished (Fig. 3).

Sequence similarity of sponge p32 to calpactin II

As a probe to screen the cDNA library of the sponge *G. cydonium* we used the cDNA coding for rat calpactin II (Shimizu *et al.*, 1988). The corresponding cDNA clone for sponge calpactin was sequenced on both strands with the use of specific oligonucleotide primers as outlined in Fig. 1. The analyses revealed that the nucleotide sequence of sponge calpactin (Fig. 2) had a high degree of sequence similarity to the rat sequence. The sponge 5′ untranslated region consists of 10 nt and the coding region of 1038 nt (83% sequence similarity compared with the rat sequence); the 3′ untranslated region consists of only 111 nt, in contrast with the rat sequence with 291 nt. Concerning the coding region the sponge A+T proportion was 0.55 (rat: 0.58) and the G+C proportion 0.45 (rat: 0.42). The predicted polypeptide of the sponge calpactin cDNA consists of 346 amino acid residues with an M_r of 37769. In contrast with the high sequence similarity of the cDNAs of sponge calpactin and rat calpactin II, the sequence similarity to mouse calpactin I (heavy chain) cDNA (Saris *et al.*, 1986) was only 47%. Similarly to the other members of the lipocortin family (e.g. lipocortin, calpactin, calelectrin, endonexin), the sponge predicted amino acid sequence also contains the Geisow motif G-T-D-E (Geisow *et al.*, 1986; Weber & Johnsson, 1986) at position 131–134. The poly(A)-addition site was found to be 22 nt upstream of the poly(A) sequence.

Purification and physicochemical properties of natural p32 and recombinant p37 calpactin

By application of the antibodies to calelectrin, a p32 polypeptide was isolated from purified AF particles as described in the Materials and methods section. This preparation was homogeneous, as checked by PAGE (Fig. 4*a*, lane b). Western-blot analysis (Fig. 4*a*, lane c) revealed that the isolated p32 material reacted with antibodies to calelectrin. Starting from 500 mg of purified AF particles, 510.7 μg of purified p32 was obtained. In a first approach to estimate the number of p32 molecules associated with one purified AF particle (M_r 1.3×10^8; Müller *et al.*, 1979*a*), 4.2 molecules of p32 were estimated to be bound per AF particle.

For expression of rat calpactin we used the inducible *trc* promoter carried by pKK233-2 plasmid. The cDNA fragment containing the complete protein-coding region and a portion of the 3′ untranslated region of sponge calpactin were inserted. The resulting plasmid pKK233-2-cal-1 directed the synthesis of sponge calpactin II (Fig. 4*b*). The extract of *E. coli* JM 105 (pKK233-2-cal-1) contained a prominent polypeptide with an M_r 37000 (Fig. 4*b*, lane a). This p37 was absent from extracts from *E. coli* JM 105 (pKK233-2). p37 polypeptide was purified and found to cross-react with antisera raised against *Torpedo* calelectrin (Fig. 4*b*, lanes b and c).

Both the natural p32 and the recombinant p37 calpactin are Ca^{2+}-binding proteins. The number of Ca^{2+}-binding sites was calculated by the gel-filtration method (Hummel & Dreyer, 1962; see the Materials and methods section). Having reached a concentration of 10 μM-$^{45}Ca^{2+}$, 2.1 mol of Ca^{2+} was estimated to be bound per mol of p32 and 1.7 mol of Ca^{2+} per mol of p37. The dissociation constants were determined by equilibrium dialysis. Evaluation of the binding data by Scatchard analysis with the use of the LIGAND program revealed straight lines, irrespectively of the calpactin sample used. The dissociation constant for the natural p32 was estimated to be 483 ± 91 μM and that for the recombinant p37 to be 759 ± 152 μM.

Phospholipid-binding property of calpactin

To determine whether sponge calpactin binds to and dissociates from phospholipids, three pure lipid fractions (L-α-phosphatidic acid, L-α-phosphatidyl-L-serine and L-α-phosphatidylcholine) and homologous plasma-membrane lipids from *G. cydonium*

Table 1. Association of natural and recombinant calpactin with lipids and dependence on Ca^{2+} concentration

For binding studies the flotation method was used (von Kieckebusch *et al.*, 1988). The dissociation behaviour was investigated by applying the sedimentation (centrifugation) procedure. The values are means of five parallel experiments; the S.D. was less than 25% (binding) and 40% (dissociation) respectively.

	[Ca^{2+}] for half-maximal binding (μM)		[Ca^{2+}] for half-maximal dissociation (μM)	
Phospholipid	p32 (natural)	p37 (recombinant)	p32 (natural)	p37 (recombinant)
Phosphatidic acid	425	387	17	8
Phosphatidylserine	390	315	13	24
Phosphatidylcholine	715	590	5	12
Total membrane lipids	683	612	38	45

were selected. Natural as well as recombinant calpactin binds to phospholipids at a Ca^{2+} concentration between 390 and 715 μM (Table 1). After having established the lipid–calpactin complex at 2 mM-Ca^{2+} only low Ca^{2+} concentrations (< 50 μM) are required for a subsequent dissociation. These findings indicate that 20-fold lower Ca^{2+} concentrations are required to stabilize the lipid–calpactin complex than are necessary for its initial formation.

Conclusions

In a previous report experimental evidence was presented indicating that calpactin is associated with the *G. cydonium* AF particle (Gramzow *et al.*, 1989). Calpactin (type II) could also be detected extracellularly in some vertebrate systems (Pepinsky *et al.*, 1986). Calculating from the yield achieved during the purification of calpactin, approximately 4 molecules of calpactin are associated with one AF particle. Interesting was the fact that neutralizing antibodies to calpactin abolish the AF-particle-mediated cell–cell aggregation.

The sponge calpactin cDNA was analysed and found to possess a more than 80% sequence similarity to monomeric mammalian calpactin II (= lipocortin I) but only a 47% sequence similarity to the tetrameric calpactin I (= lipocortin II). Similarly to other members of the lipocortin family (Geisow *et al.*, 1986; Weber & Johnsson, 1986), the sponge calpactin also contains the G-T-D-E Geisow motif. This tetrapeptide represents the consensus sequence of phospholipase A2-inhibitor proteins (Kretsinger & Creutz, 1986; Geisow *et al.*, 1986). The sponge calpactin does not contain the R-G-D-S tetrapeptide that represents the cell-attachment site of fibronectin and vitronectin (Buck & Horwitz, 1987). Taking the suggestions of Rice & Humphreys (1983) into account that the amino acids D and E are binding sites for Ca^{2+}, it is likely that Ca^{2+} binds to the Geisow motif in the D- and E-rich sponge calpactin (overall presence of D and E, 5.8% and 8.7% respectively).

In order to understand further the function of the sponge calpactin, we isolated this molecule from the animal and also prepared it as a recombinant molecule. The M_r of the natural material was 32000 and that of the recombinant material was 37000. At present we do not know whether the p32 is a result of a degradation occurring during purification or rather of a physiological post-translational processing step. The latter possibility has not yet been observed with other calpactins. Both sponge calpactin preparations behave almost identically with other known calpactin II preparations from vertebrates (reviewed in von Kieckebusch *et al.*, 1988) with respect to their function. They bind Ca^{2+} ions with low affinities (K_d 500–750 μM) in the absence of phospholipids, but with high affinities in the presence of phospholipids (K_d about 20–30 μM). The number of binding sites for Ca^{2+} at the p32 calpactin was 2.1 and in the case of p37 calpactin 1.7, and hence only half of the number determined for bovine calpactin II (Glenney & Zokas, 1988). Furthermore it was noted that sponge calpactin, unlike *Torpedo* calelectrin, requires a 20-fold higher Ca^{2+} concentration to bind to phospholipids than for its dissociation, indicating that calpactin in association with phospholipids is present in a state of lower potential energy.

On the basis of (i) inhibition studies with anti-calelectrin antibodies as well as (ii) the presented data that homologous total plasma-membrane lipids increase the binding affinity to calpactin in the presence of Ca^{2+}, we conclude that binding between plasma-membrane-associated phospholipids and calpactin at the AF complex is one process that facilitates cell aggregation in the sponge system. Hence the cell-binding fragment of the AF displays a dual role: (i) it functions as a mitogenic stimulus during the initial phase of cell–cell aggregation (Müller *et al.*, 1987) and (ii) it recognizes the AR in a species-specific manner (Gramzow *et al.*, 1986).

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