Membrane-enclosed Crystals in Dictyostelium discoideum Cells, Consisting of Developmentally Regulated Proteins with Sequence Similarities to Known Esterases

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Abstract. Developing cells of Dictyostelium discoideum contain crystalline inclusion bodies. The interlattice spaces of the crystals are ~11 nm, and their edge dimensions vary in aggregating cells from 0.1 to 0.5 μm. The crystals are enclosed by a membrane with the characteristics of RER. To unravel the nature of the crystals we isolated them under electron microscopical control and purified the two major proteins that cofractionate with the crystals, one of an apparent molecular mass of 69 kD, the other of 56 kD. This latter protein proved to be identical with the protein encoded by the developmentally regulated D2 gene of D. discoideum, as shown by its reactivity with antibodies raised against the bacterially expressed product of a D2 fusion gene. The D2 gene is known to be strictly regulated at the transcript level and to be controlled by cAMP signals. Accordingly, very little of the 56-kD protein was detected in growth phase cells, maximal expression was observed at the aggregation stage, and the expression was stimulated by cAMP pulses.

The 69-kD protein is the major constituent of the crystals and is therefore called "crystal protein." This protein is developmentally regulated and accumulates in aggregating cells similar to the D2 protein, but is not, or is only slightly regulated by cAMP pulses. mAbs specific for either the crystal protein or the D2 protein, labeled the intracellular crystals as demonstrated by the use of immunoelectron microscopy.

The complete cDNA-derived amino acid sequence of the crystal protein indicates a hydrophobic leader and shows a high degree of sequence similarity with Torpedo acetylcholinesterase and rat lysophospholipase. Because the D2 protein also shows sequence similarities with various esterases, the vesicles filled with crystals of these proteins are named esterosomes.

During growth, cells of Dictyostelium discoideum live as single amebae. They enter a social stage after their nutrient supply is exhausted. By cell aggregation a multicellular body is formed which gives rise to a fruiting body, whereby the major portion of cells differentiates into spores and a minor one into stalk cells.

The development of D. discoideum is driven and accompanied by stage and cell-type specific gene expression. The expression of one group of genes is induced or enhanced between growth and the aggregation stage. Among them are genes whose expression is strongly stimulated by periodic pulses of cAMP, as they are generated by the cells in the course of their development. Examples are the gene encoding the contact site A protein, a cell adhesion molecule of aggregating cells (Müller and Gerisch, 1978), the gene encoding the cell-surface cAMP receptor (Chisholm et al., 1987), and the D2 gene probably encoding an esterase (Mann and Firtel, 1987). Other members in this group of early expressed genes are not controlled by external cAMP signals or are even suppressed (Williams et al., 1980).

Electron microscopic studies have revealed membrane-enclosed protein crystals within the cells that accumulate between growth and the aggregation stage (Gezelius, 1959, 1961; Maeda and Takeuchi, 1969), suggesting that the proteins constituting these crystals are encoded by developmentally regulated, early expressed genes. The crystals remain present throughout the stages after aggregation. They are even found in the mature spores and disappear only after their germination (Gezelius, 1961; Maeda and Takeuchi, 1969; Cotter et al., 1969). To eventually identify fate and function of the proteins that are stored during multicellular development within the crystals, we have characterized the two main proteins associated with a purified crystal fraction. One protein proved to be the product of the D2 gene, whose regulation has been extensively studied (Mann et al., 1988). The other protein, denoted "crystal protein," is the most prominent constituent of the crystals and will be the main topic of this study.

Materials and Methods
Cultivation of D. discoideum

Cells of D. discoideum strains AX2-214 or AX3 were cultivated in axenic...
medium and harvested at a density of not more than 5 × 10^6 cells/ml (Malchow et al., 1972). AX2 cells were used for all experiments except that shown in Fig. 11 A. Washed cells were examined immediately as growth phase cells, or starved in 17 mM Sorensen K⁺/Na⁺ phosphate buffer, pH 6.0, at a density of 1 × 10^7 cells per ml. Aggregation-competent cells were harvested at 6 h of starvation. To monitor the two proteins by immunoblotting throughout the entire developmental cycle, cells were plated onto non-nutrient agar (2 % Bacto-agar) (Difco Laboratories Inc., Detroit, MI), in 17 mM phosphate buffer, pH 6.0, and allowed to develop for various times.

mAbs

Antibodies designated as 130-80-2, 129-202-6, and 83-418-1 will be abbreviated as mAb 80, 202, and 418 in this paper. After immunizing BALB/c mice by intraperitoneal injections of a fusion product of the COOH-terminal region of the D2 protein, which was a gift of Dr. W. Riverkamp (Heidelberg), mAb 80 was obtained. Immunization with partially purified crystal protein in 0.1 % SDS gave rise to mAbs 80 and 202. The adjuvant was either Alxgel S (Serva Fine Biochemicals Inc., Garden City Park, NY) or an alternative with Bordetella pertussis antigen for mAb 202, or Freund's complete adjuvant followed by a boost with incomplete Freund's adjuvant for mAbs 80 and 418. The antibodies were purified from hybridoma culture supernatants by ammonium sulfate precipitation and protein A-Sepharose chromatography. Using subtype specific antibodies (Meloy Laboratories Inc., Springfield, VA), mAbs 202 and 418 were identified as IgG1, and mAb 80 as IgG2A.

Fluorescence Microscopy

Growth phase or aggregation-competent cells were seeded on 12-mm-diam coverslips and allowed to attach and move for 15 min. Then the cells were immersed on the coverslip into methanol at ~30°C and air dried. After washing with PBS, pH 7.4, containing 100 mM glycine, specimens were treated for 20 min with PBS containing 0.05 % fish gelatine and 0.5 % BSA (van Bergen en Henegouwen and Leunissen, 1986; Birrell et al., 1987), and subsequently incubated with 2-3 µg of mAb 80 or 418 per ml of PBS supplemented with 0.05 % BSA and 0.05 % fish gelatine, washed, and labeled with 8 µg/ml of FITC-conjugated, affinity-purified goat anti-mouse IgG (Jackson Immuno Research Laboratories, Avondale, PA). The coverslips were mounted on semisolid medium (Lennette, 1978) containing 25 mg per ml of 1,4-diazabicyclo-(2,2,2)-octane (Langanger et al., 1983) to reduce fading during fluorescence microscopy.

EM

Purified crystals were negatively stained with uranyl acetate. Sections of cells were obtained from suspended aggregation-competent cells. The cells were fixed for 15 min per step in 0.5, 1, 2, 4, and finally 1 h in 8 % formamide made of freshly depolymerized paraformaldehyde. Then the cells were dehydrated and embedded in 10 % gelatine, and specimens of ~1 µm^3 were fixed for 15 min per step in 0.5, 1, 2, 4, and finally for 1 h in 8 % formaldehyde. Then the cells were dehydrated in a graded series of ethanol and embedded in Lowicryl K4M by progressively lowering the temperature (Carlemalm et al., 1982). Cells shown in Fig. 2 were cryofixed by immersion into liquid propane at ~185°C, freeze substituted and low temperature embedded in Lowicryl HM20 according to Humbel and Müller (1986). Sections were obtained on an ultratome (model III, LKB Instruments, Inc., Gaithersburg, MD) and collected on piezof orm and carbon-coated copper grids (G 200 hex, Science Services, München, FRG). Serial Lowicryl K4M sections were indirectly labeled using 5 nm colloidal gold-conjugated goat anti-mouse IgG as second antibody, which was a gift of Dr. J. Chandler (BioCell Research Laboratories, Cardiif, UK). Alternatively, Lowicryl K4M sections were double labeled with mAb 202 directly conjugated to 4-nm gold particles and with mAb 418 conjugated to 12-nm gold particles (De Mey and Moeremans, 1986). The labeled sections were stained with aqueous solutions of 2 % uranyl acetate and 0.4 % lead citrate (Venable and Coggeshall, 1964), and photographed in a microscope (100CC; JEOL USA, Analytical Instruments Div., Cranford, NJ) at 100 kV.

Protein Crystal Purification

Washed aggregation-competent AX2 cells were pelleted and resuspended in 2 vol of cold homogenization buffer (30 mM Tris–HCl, pH 7.8, 2 mM DTT, 2 mM EDTA, 4 mM EGTA, 0.2 mM ATP, 5 mM benzamidine, and 30 % wt/vol of sucrose) and homogenized at 0–4°C by nitrogen evacuation in a Parr bomb after incubation for 10 min at 800 psi. The homogenate was centrifuged for 20 min at 100,000 g, the pellet washed twice in cold TEDABA buffer (10 mM Tris–HCl, pH 8.0, 1 mM EGTA, 1 mM DTT, 0.1 mM ATP, 1 mM benzamidine, and 0.02 % NaN₃), resuspended in 1/4 of the pellet vol of cold TEDABA buffer and layered onto a 55–85 % continuous sucrose gradient in TEDABA using ultra-clear tubes (Beckman Instruments, Inc., Palo Alto, CA). After centrifugation for 15 h at 170,000 g in a rotator (VTI; 50; Beckman Instruments, Inc.) the material of the faint, white band at a density of 1.30 g/cm^3 was collected with a needle. Purity of the crystals in this fraction I of the sucrose gradient was checked by negative staining and SDS-PAGE.

Isolation and Sequencing of cDNA Clones Coding for the Crystal Protein

A AgtI cDNA library of strain AX3, provided to us by Dr. R. Kessin (Columbia University) (Lacombé et al., 1986), was screened with mAb 202 as described by Noegel et al. (1985). Eco RI fragments of two clones, λDCP172 and λDCP174, harboring inserts of 1.8 and 1.75 kb were separated in 0.7 % agarose gels in Tris–borate buffer, pH 8.3 (Maniatis et al., 1982). The inserts were eluted from the gel as described by Dretzen et al. (1981), and cloned into dephosphorylated, Eco RI-digested pUC19 (Yanisch-Perron et al., 1985). The resulting plasmids pDCP172 and pDCP174 were used for deletion subcloning with the erase-a-base kit from Promega Biotech (Madison, WI). Enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and used according to the manufacturer's recommendation.

Both strands of the entire coding region were sequenced as outlined in Fig. 1, using the chain termination method (Sanger et al., 1977; Chen and Seeburg, 1983) and buffer gradient gels for resolving the reaction products (Bigin et al., 1983).

Isolation and Sequencing of a Genomic DNA Clone Coding for the D2 Protein

A genomic library of Eco RI-digested strain AX3 DNA in lambda phage charon 14 (Williams and Blattner, 1979) was probed with a nick-translated D2 fragment, the insert of plasmid pCDM142 (Riverkamp and Firtel, 1980). The 7-kb Eco RI D2 fragment was recloned into pUC8 (Vieira and Messing, 1982). Initial mapping data indicated that the whole D2 gene was contained within an internal 2.5-kb Ava II fragment of this 7-kb insert. The Ava II fragment was isolated and recloned after SI nuclease treatment into the Sma I site of pUC8, resulting in plasmid pUD2XK. The entire insert of this plasmid was sequenced in both directions using the chain termination method (Sanger et al., 1977) as well as the chemical degradation method (Maxam and Gilbert, 1977).

Southern and Northern Blots

DNA and RNA were prepared from D. discoideum cells as described by André et al. (1988). For Southern blot analysis, restriction fragments of genomic DNA were separated on 0.7 % agarose gels in Tris-phosphate buffer, pH 7.8 (Maniatis et al., 1982). For Northern blotting, total cellular RNA was separated in 1.2 % agarose gels containing 6 % formaldehyde. Southern and Northern blots were labeled with cDCP172 encoding the crystal protein or A2C5 (Gerisch et al., 1985), a 1.7-kb fragment from the

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1. Abbreviation used in this paper. TEDABA, 10 mM Tris–HCl, pH 8.0, 1 mM EGTA, 1 mM DTT, 0.1 mM ATP, 1 mM benzamidine, and 0.02 % NaN₃.
Figure 2. Sections of aggregation competent *D. discoideum* cells. Enclosure of crystals by ribosome-coated membranes is particularly clear in the inset. The cells were freeze substituted and low temperature embedded in Lowicryl HM20. Bar in main figure, 500 nm; in the inset, 100 nm.

3' portion of D2 cDNA. Filters were incubated with nick-translated probes for 15–20 h at 37°C in 2× SSC, 50% formamide, 4 mM EDTA, 1% sarcosyl, 0.1% SDS, 4× Denhardt's solution, and 0.12 M sodium phosphate buffer, pH 6.8 (Mehdy et al., 1983).

**Protein Analysis and Sequencing**

Proteins were separated by SDS-PAGE in 10% acrylamide gels (Laemmli, 1970) and either stained with Coomassie blue or transferred electrophoretically to BA85 nitrocellulose (Schleicher & Schuell, Keene, NH) according to Towbin et al. (1979). The blots were labeled with 125I-mAbs or, for Fig. 10 A, indirectly with mAb and alkaline phosphatase–coupled goat anti-mouse IgG (Jackson Immunological Research Laboratory Inc., Avondale, PA) as described by Knecht and Dimond (1984). Protein concentrations were determined according to Bradford (1976) using BSA (Sigma Chemical Co., Poole, UK) as standard.

For sequencing of the NH2 terminus, ~90% pure crystal protein was separated by SDS–PAGE on a 7% acrylamide gel, electroblotted onto a siliconized sheet (Glassy-bond; Biometra, Göttingen, FRG), excised after Coomassie blue staining and subjected to Edman degradation using a gas-phase sequencer (Applied Biosystems Inc., Foster City, CA) (Eckerskorn et al., 1988).
D2 protein, is developmentally regulated. Small amounts of these two proteins were found in growth phase cells, and substantially higher amounts in aggregation competent cells (Fig. 5). Both mAb 80 and 202 were used in parallel with mAb 418 for in situ localization of the crystal and D2 proteins. Fluorescent labeling of permeabilized cells with each of the three antibodies showed a punctate distribution of the label throughout the cytoplasm of aggregating cells, and very little label in growth phase cells (Fig. 6).

**Results**

**Intracellular Location and Isolation of Protein Crystals**

In confirmation of previous results, crystals enclosed into vesicles were found to be distributed throughout the cytoplasm of aggregating *D. discoideum* cells (Fig. 2). The membranes tightly surrounding the crystals were decorated on their cytoplasmic phase by ribosomes as described by George et al. (1972), indicating that the vesicles are derived from the RER. The interlattice space of the crystals was ~11 nm, and their edge dimensions varied between 0.1 and 0.5 μm.

The crystals were isolated from the 10,000 g pellet of cell homogenates on a continuous sucrose gradient. As shown by electron microscopic evaluation of the fractions, crystals were enriched in a thin colorless band at a density of 1.30 g/cm³ (Fraction I in Fig. 3). Proteins of this band and of other layers of the gradient were separated by SDS–PAGE and stained with Coomassie blue (Fig. 4 A). Two major proteins were enriched in fraction I, the most abundant one with an apparent molecular mass of 69 kD, the other of 56 kD. Three more proteins of ~63, 52, and 42 kD were detectable in much smaller amounts after two-dimensional electrophoresis (not shown). Because of its abundance in the crystal fraction, the 69-kD protein will be designated as "crystal protein."

The 56-kD protein was identified as the translation product of the D2 gene of *D. discoideum* by mAb 418, an mAb directed against this protein (Fig. 4 B). To characterize the crystal protein, mAbs were raised against the crystal fraction, and two antibodies, mAb 80 and 202, were chosen for further work. In immunoblots these antibodies recognized the 69-kD band of the crystal protein but not the 56-kD band of the D2 protein (Fig. 5).

Immunoblotting showed that the crystal protein, like the D2 protein, is developmentally regulated. Small amounts of these two proteins were found in growth phase cells, and substantially higher amounts in aggregation competent cells (Fig. 5). Both mAb 80 and 202 were used in parallel with mAb 418 for in situ localization of the crystal and D2 proteins. Fluorescent labeling of permeabilized cells with each of the three antibodies showed a punctate distribution of the label throughout the cytoplasm of aggregating cells, and very little label in growth phase cells (Fig. 6).
Developmental regulation and selective antibody labeling of the crystal and D2 protein. *D. discoideum* strain AX2 cells were either harvested during exponential growth (0 h) or after starvation at the aggregation-competent stage (6 h). Total cellular proteins were separated by SDS-PAGE, and blots labeled with mAb 202 for the crystal protein (anti-CP) or with mAb 418 for the D2 protein (anti-D2). Equivalents of $3 \times 10^5$ cells were loaded per lane. Molecular mass standards are indicated.

By examination of immunogold-labeled sections of aggregation competent cells in the electron microscope, it became evident that the labeled particles seen in the fluorescence microscope were the crystals shown in Fig. 2. In comparing serial sections it was found that mAb 202 against the crystal protein and mAb 418 against the D2 protein labeled the same crystals (Fig. 7, A and B). This result was confirmed by double labeling of sections with mAb 202 and mAb 418 conjugated to gold particles of different sizes. Mixed labeling of single crystals with gold particles of the two sizes was found (Fig. 7 C).

The cDNA-derived Sequence of the Crystal Protein Reveals Similarities with Various Esterases

Using mAb 202 for screening a λgt11 cDNA library, clones λcDCP172 and λcDCP174 were isolated from which the complete sequence of the crystal protein coding region was obtained (Fig. 8). Gas-phase sequencing of the NH$_2$-terminal region of the crystal protein purified from *D. discoideum* cells indicates that 19 amino acids constituting the hydrophobic leader are cleaved off in the course of transport at the aggregation-competent stage (6 h). Total cellular proteins were separated by SDS-PAGE, and blots labeled with mAb 202 for the crystal protein (anti-CP) or with mAb 418 for the D2 protein (anti-D2). Equivalents of $3 \times 10^5$ cells were loaded per lane. Molecular mass standards are indicated.

The calculated molecular mass of 59 kD for the crystal protein without leader differs considerably from 69 kD as it was determined by SDS–PAGE. The difference may be due to glycosylation. The sequence indicates five potential N-glycosylation sites NXS(T), two of them containing proline in the second position are unlikely to be used.

The cDNA sequence of the crystal protein showed similarities with that of D2 DNA (Fig. 8). Accordingly, 52% identity was found between the amino acid sequences, when the cDNA-derived sequence of the crystal protein was compared with the D2 protein sequence as derived from a genomic DNA (Fig. 9). Similarities between the two proteins are also reflected in hydrophobicity plots (Fig. 10). Hydrophobic or hydrophilic regions are distributed in similar patterns along the length of the sequences. But except of the leader, no strongly hydrophobic regions were detected, in accord with the finding that the proteins are secreted into the lumen of vesicles rather than associated with membranes. The D2 protein sequence derived from our DNA sequence is in 92% of the amino acid residues identical with the previously published one (Rubino et al., 1989). The differences include the cysteine residue in position 109, which is only present in the sequence shown in Fig. 9.

The crystal protein shows sequence similarities with several serine esterases and with thyroglobulin (Table I). Especially the region of the active site including the catalytically active serine of the esterases is very similar to the sequence between residues 213 and 221 of the crystal protein. Four cysteine residues that are conserved in several esterases and known to form disulfide bonds in acetylcholine-esterase (MacPhee-Quigley et al., 1986) and butyrylcholine-esterase (Lockridge et al., 1987) are also present in the crystal protein, and in the D2 protein as well (Fig. 9).

The Crystal Protein Is Encoded by a Single, Developmentally Regulated Gene

Eco RI and Hind III do not cleave within the coding region of the crystal protein gene, and Eco RV cleaves at a single site. In Southern blots probed under high stringency conditions with cDCP172, which includes the complete coding region, one band of 9.0 kb was labeled after Eco RI digestion, and one band of 23 kb after Hind III digestion. A 1.4- and a 6.3-kb band were both recognized by the probe in Eco RV digests (data not shown). These results indicate that the crystal protein is encoded by a single-copy gene.

Regulation of the crystal protein gene during early development and control by extracellular cAMP signals was examined in comparison to the D2 gene. These proteins were not completely absent from growth phase cells, but both accumulated to higher levels up to the aggregation stage during the development of starving AX2 cells on agar plates (Fig. 11 A). The cellular concentration of the D2 protein remained almost constant during the postaggregative period of development, whereas that of the crystal protein was slightly reduced.

The effect of cAMP pulses on expression of the crystal protein gene was studied in the AX3 strain. Expression of the contact site A gene and of other cAMP-controlled genes in suspension cultures of this strain is strongly dependent on externally applied cAMP pulses. This effect is much stronger than in similar cultures of the AX2 strain which autonomously generate cAMP pulses (Gerisch and Hess, 1974). AX3 cells were starved with or without stimulation by cAMP pulses, and Northern blots of RNA from cells harvested at 2-h intervals were assayed for transcripts of the crystal protein and D2 genes. While the accumulation of D2 mRNA was strongly enhanced by cAMP pulses as described before (Mann et al., 1988), there was for the crystal protein mRNA only a small increase detectable at 4 h of development (Fig. 11 B). These differences in cAMP regulation were also seen in immunoblots incubated with mAb 202 and mAb 418 for labeling of the crystal and the D2 protein. While the D2 pro-
Figure 6. In situ fluorescence labeling of the crystal and D2 protein. Permeabilized growth phase (A, B, E, F) or aggregation-competent (C, D, G, H) D. discoideum cells of strain AX2 were labeled with mAb 418 for the D2 protein (B, D) or with mAb 80 for the crystal protein (F, H). (Left) Phase-contrast images; (right) fluorescence images of corresponding groups of cells. Bar, 10 μm.

Figure 8. Comparison of the crystal protein cDNA sequence (CP) with genomic D2 DNA (D2). The alignment was made using the UWGCG program Bestfit (Devereux et al., 1984), omitting one 100-bp intron of the D2 sequence. Vertical lines indicate identical nucleotides. Start codons, stop codons, and putative polyadenylation signals are underlined.
Figure 7. Immunogold labeling of the crystal and D2 protein in sections of aggregation-competent cells. (A and B) Serial sections of an area containing a single crystal labeled indirectly with mAb 202 for the crystal protein (A) or with mAb 418 for the D2 protein (B). The secondary antibodies were coupled to 5 nm colloidal gold. (C) Double labeling of a section with mAb 202 coupled to 4 nm gold and with mAb 418 coupled to 12-nm gold particles. The bar represents 100 nm.
development of membrane-enclosed crystals that accumulate during the de-
tals, indicating that these proteins are exclusively located in
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Discussion

Esterosomes in Developing D. discoideum Cells

In this paper, we identified two proteins associated with
membrane-enclosed crystals that accumulate during the de-
velopment of D. discoideum cells. In sections of aggregating
cells, antibody-gold label was found specifically on the crys-
tals, indicating that these proteins are exclusively located in

Questions about the Origin and Fate of Esterosomes

The most interesting question concerning the esterosomes is
Figure 10. Analysis of the deduced amino acid sequences of the crystal protein (top) and the D2 protein (bottom) according to Kyte and Doolittle (1982) using a window of nine amino acids and the program PEPPLOT (UWGCG).

Table I. Sequence Identities of the Crystal Protein to Other Proteins

| Protein           | Source          | Residues in overlapping region | Identity in this region | Reference          |
|-------------------|-----------------|-------------------------------|-------------------------|--------------------|
| Acetylcholinesterase | *Torpedo californica* | 481                           | 30                      | Schumacher et al., 1986 |
| Butyrylcholinesterase | *Homo sapiens* | 493                           | 30                      | McTiernan et al., 1987 |
| Carboxylesterase   | *Rattus norvegicus* | 470                           | 30                      | Long et al., 1988   |
| Esterase-6         | *Drosophila melanogaster* | 331                           | 31                      | Oakeshott et al., 1987 |
| Lysophospholipase  | *Rattus norvegicus* | 389                           | 27                      | Han et al., 1987    |
| Thyroglobulin      | *Bos taurus*    | 329                           | 33                      | Mercken et al., 1985 |

The sequences were compared using the Protein Identification Resource (PIR) program FastP.

Figure 11. Expression of the crystal protein (CP), of the D2 protein (D2) during development, and different effects of cAMP pulses in regulating the transcript levels for these proteins. (A) Immunoblot of total cellular proteins separated by SDS-PAGE, probed with mAb 202 for the crystal protein and mAb 418 for the D2 protein. Strain AX2 cells were starved on nonnutrient agar plates. 0 h corresponds to growth phase, 6–9 h to aggregation, 9–12 h to the tipped aggregate and 15 h to the slug stage, 18–21 h to culmination, and 21–24 h to the final fruiting body stage. Equivalents of 1 x 10^6 cells were loaded per lane. (B) Northern blots of total cellular RNA probed for crystal protein and D2 transcripts. Strain AX3 cells were starved in suspension with (P) or without stimulation of development by 20-nM pulses of cAMP applied every 6 min. 10 μg of RNA were loaded per lane.

How the crystallized proteins are sorted from the RER. Each individual crystal is tightly enclosed by a membrane that does not leave much space for other, noncrystallized proteins to be present within the vesicles. There are two possible mechanisms of sorting, either one of which alone, or both together, may be responsible for targeting specific proteins into the esterosomes. First, synthesis is not localized, the proteins crystallize at any site in the cisternae, and a special mechanism is responsible for final budding and separation of those portions of the ER that embrace the crystals. The second possibility is that specialized areas of the ER membrane that carry recognition sites for the signal sequences of the crystal and D2 protein sort out to form separate vesicles, the attached ribosomes synthesize then specifically these two proteins to be transported into the vesicles. There are arguments in favor of each of these two possibilities. George et al. (1972) have depicted a crystal-containing vesicle that is connected through a tube-like extension with the ER, suggesting membrane budding to follow crystallization. But structures like this are rare. Usually separate, round vesicles are found that are filled with a crystal and decorated with ribosomes (Fig. 2). These structures indicate that synthesis and secretion of proteins into the lumen still occurs after the esterosomes have been formed as individual organelles. It is tempting to believe that the proteins synthesized at the membranes of the esterosomes are specifically the esterases, thus allowing the crystals to grow continually in the separate vesicles.

The fate of esterosomes determines the site and time of action of the proteins they contain, because as long as they are stored in the crystals, the proteins can not fulfill a function.
There are three possibilities of how the proteins are liberated: (a) the membranes of esteromes may fuse with the plasma membrane, releasing their contents into the extracellular or intercellular space; (b) they may fuse with the membranes of lysosomes thus contributing to the stock of hydrolyses in these organelles; and (c) the membranes may be removed to allow distribution of the crystallized proteins in the cytoplasm. An extracellular function during early development is not supported by experimental data because neither the crystal nor the D2 protein was yet detected in the medium in which cells had developed up to the aggregation competent stage (our unpublished results). Pulse-chase experiments in combination with cell fractionation and immuno-labeling will show into which compartment these proteins are released.

Pulse-chase experiments will also precisely define the time at which the stored proteins are released from the esteromes. Maeda and Takeuchi (1969) reported that the crystals disappear during spore germination. Treatment of germinating spores with cycloheximide prevents degradation of the crystals and arrests germination at a stage in which the outer and middle spore walls are ruptured but the inner spore wall stays intact ( Cotter et al., 1969). One might assume therefore that the crystal and D2 proteins are required for lysing the innermost spore wall. Another view has been presented by Rubino et al. (1989) who suppressed expression of the D2 transcript by antisense RNA. From the finding that aggregation was reduced and development delayed, it was concluded that the D2 protein is important for development to proceed normally. This would require a portion of the protein to exist in a noncrystallized state during development. Inactivation by homologous recombination of both the D2 and crystal protein gene will be the method of choice to identify the possibly overlapping functions of these two related proteins.

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