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Localization of cyclic-AMP receptors with acidosomes in *Dictyostelium discoideum*

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Abstract Earlier studies have shown that in *Dictyostelium discoideum*, a buoyant membrane fraction contained ~90% of the vacuolar proton pump (V-H$^+$-ATPase) activity, leading to its designation *acidosomes*. It was proposed that acidosomes may be involved in endocytosis, specially in the acidification of endosomes. In this study we further investigated the putative function(s) of acidosomes.The findings suggest that acidosomes contain abundant receptors for cyclic AMP (CAR1) and that it may be the site for recycling of internalized receptors. Acidosomes also contain an abundance of Rab4 (Bush et al. 1994), a marker for early endosomes. By these criteria, we suggest that the acidosomes are analogous to early or recycling endosome present in mammalian cells. These findings suggest that the structure earlier defined biochemically, morphologically and immunologically as acidosomes may represent early and/or recycling endosomes in this protist.

Key words: Dictyostelium discoideum; Acidosome; Endosome; Cyclic-AMP receptor (CAR1)

1. Introduction

Endolytic cargo traverses a number of intracellular compartments which maintain their unique composition and identity in spite of the continuous exchange among these compartments. Many of these compartments, acidified by V-H$^+$-ATPase, have an acidic interior [1–3]. The endocytic circuit in mammalian cells has been characterized primarily by morphological and kinetic analysis. Attempts to rigorously purify the endosomal compartments have met with limited success, thus limiting biochemical characterization.

Recently it has been possible to isolate the lysosomal population from *Dictyostelium discoideum* using colloidal iron particles as pinocytic cargo and magnetic fractionation [4,5]. The preparations provide >20-fold purification, 50–70% yield and <1% contamination with other organelles. Our studies of *Dictyostelium* suggested that most of the V-H$^+$-ATPase does not reside on the lysosomal membrane but instead resides in a novel organelle, which we termed acidosomes, since it contained an abundance of V-H$^+$-ATPase. This structure was biochemically, morphologically, and immunologically characterized [6–9]. Efficient procedures were used to purify acidosomes to a high degree of homogeneity with minimal contamination from other organelles [5,8]. We suggested that the acidosome is an endocytic organelle and functions in the acidification of the endocytic circuit [10,11]. Others have suggested its role in contractile vacuole function [12,13].

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During development, unicellular cells of *D. discoideum* undergo chemotaxis and aggregate in a highly organized fashion to form a multicellular pseudoplasmodium which differentiates into either spore cells or stalk cells. The process of aggregation during early development is orchestrated by a pulsative release of cyclic AMP detected by cell surface receptors [14]. Multiple isoforms of cAMP receptors (CAR1, CAR2, CAR3) have been identified and characterized in this organism; CAR1 being a more prominent member is expressed during early stages of development [15]. Here we demonstrate that the acidosome is the subcellular site for accumulation of receptors indicating that the acidosome is a prelysosomal compartment in this ameba, perhaps akin to early/recycling endosomes.

2. Materials and methods

2.1. Materials and cells

D. discoideum strain Ax-3 was cultured axenically as described [6,10]. Colloidal iron particles were prepared as described earlier [5] and the preparations had about 10 mg iron/ml. Rabbit antiserum to cAMP receptor (CAR1) was a generous gift from Dr. Peter Devreotes of Johns Hopkins School of Medicine.

2.2. Endocytosis

Cells were harvested in mid-exponential phase, washed, and used immediately [6,16]. Prior to feeding probes, washed cells were allowed to recover at room temperature for 10 min. Incubations were conducted at room temperature (22 ± 1°C) with cells swirling continuously at 200 rpm.

2.3. Fluorescence

Fluorescence was quantified in a Hitachi F-2000 spectrofluorimeter. Total fluorescence was determined in 5 mM sodium glycinate (pH 8.5) containing 0.2% Triton X-100. Excitation and emission wavelengths for FITC-dextran were 495 and 520 nm, respectively.

2.4. Assays for marker enzymes

Assays for V-H$^+$-ATPase and other organelle markers have been described [7,8].

2.5. Electron microscopy

For negative staining, isolated fractions were spotted on carbon-coated grids, stained with 1% uranyl acetate, and viewed by Jeol 100 electron microscope operated at 80 kev.

2.6. Electromagnetic column chromatography

Electromagnet column chromatography was run as described [5]. Briefly, the dextran coated colloidal iron probe (1 mg/ml) was fed to the cells. Cells were washed and homogenized in glycinate buffer (5 mM Na-glycinate, pH 8.5, containing 100 mM sucrose). Homogenate from such cells was prepared as described [7] and passed through a glass column (0.7 × 7 cm), loosely packed with 190 mg of steel wire (25 μm diameter) mesh, and placed between two magnet poles 1.3 cm apart. The electromagnet was operated at 0.8 Tesla. As described elsewhere [5] the magnet columns were run in glycinate buffer with either 0 or 1.5 mM $MgCl_2$. The preparations provide excellent purity (20- to 25-fold), yield (60–70%) with <1% contamination from other organelles.

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2.7. Developed cells

Vegetative cells were washed and resuspended at 5×10^7 cells/ml in 16 mM MES-KOH buffer at pH 6.8 containing 2 mM $CaCl_2$. Cells were swirled at 200 rpm at room temperature for 6 h. cAMP at a final concentration of 100 μM was added at 05:50 and 06:00 to maximize downregulation and internalization of cAMP receptor (CAR1). Cells were spun, washed, and homogenized as described earlier.

2.8. Sucrose density gradient

Cell homogenate was spun at 1000 rpm for 1 min in Sorvall SS34 rotor to remove unlysed cells and larger undispersed cell fragments. The supernatant was loaded on a gradient of 45–25% sucrose (w/w) prepared on a cushion of 55% sucrose. After spinning in a Beckman SW28 rotor at 25,000 rpm (80,000 × *g*) for 3 h at 1°C, fractions were collected and assayed for marker enzymes as described earlier [7]. Since most of the markers (mitochondria, lysosomes, plasma membranes, ER) equilibrate in the dense region, the preparations provide pure acidosomes in the buoyant region of the gradient [8]. The only other organelle which is even more buoyant than the acidosome but clearly separable (see Fig. 1) is the contractile vacuole.

2.9. Western blot analysis

Sample proteins were separated in a 10% polyacrylamide gel by electrophoresis and electrotransferred to a nitrocellulose paper. The paper was blocked with shaking in 1% milk protein for 30 min. Blots were then incubated for 30 min at room temperature with an appropriate dilution of primary antibodies (usually 1:5000), followed by three washes with TTBS buffer (100 mM Tris–HCl, 0.9% NaCl, 0.1% Tween-20 at pH 7.5) for 10 min each. Blots were then incubated with shaking for 30 min at room temperature with HRP-labeled anti-rabbit antibodies (usually 1:10,000), then washed three times with TTBS buffer. Color was developed by incubating blots in a staining solution (5 ml containing 100 mM Tris–HCl, pH 7.5, 2.5 mg DAB (diaminobenzidine), 0.005% H_2O_2 and 0.04% $NiCl_2$).

2.10. Dot blot analysis

SDS was added to the samples to a final concentration of 0.1% and incubated at room temperature for 5 min. Samples (≤5 μl) were spotted on a nitrocellulose paper strip and air-dried. The paper strip was first wetted in TTBS buffer (100 mM Tris–HCl, 0.9% NaCl, 0.1% Tween-20 at pH 7.5) and then blocked with shaking in 1% milk protein for 30 min. Blots were then incubated with primary antibodies (usually 1:2000), washed, incubated with HRP-labeled anti-rabbit antibodies (usually 1:4000), washed again and developed by ECL reaction (Amersham) and detected by radiography.

3. Results and discussion

Earlier studies established markers for plasma membranes, lysosomes, mitochondria, acidosomes, and contractile vacuoles from vegetative cells of *Dictyostelium* [7,8]. These membranes can be fractionated on a sucrose density equilibrium gradient. The mitochondria (d = 1.22 g/ml), lysosomes (d = 1.21 g/ml) and plasma membrane (d = 1.2 g/ml) equilibrate in the dense region of the gradient and the acidosomes equilibrate in the buoyant region (d = 1.16 g/ml) of the gradient. The markers for contractile vacuoles equilibrate at even lower density (d = 1.14) than that of the acidosomes [7]. Since the equilibrium density of acidosomes is significantly different from that of other organelles, the procedure provides fairly pure preparation of acidosomes [7,8].

The subcellular markers from 6 h developed cells were separated on a sucrose density gradient as shown in Fig. 1. The marker profile is similar to the one observed for vegetative cells [7]. The subcellular distribution of CAR1 was determined by Dot blot analysis of the fractions from the gradient shown in Fig. 1. In dot blots (not shown) the fraction 1 (d = 1.22 g/ml) contained anti-CAR1 reactivity but the major peak of anti-CAR1 reactivity was observed at d = 1.16 g/ml, completely coinciding with V-H^+-ATPase activity peak (not shown). The data indicated that most of the CAR1 in developed cells is present in the acidosome fraction. CAR1 is a surface receptor which could be downregulated and internalized. The distribution of CAR1 on the cell surface and endomembrane is governed by its downregulated state [14,17]. The antiserum to CAR1 used here was developed in the laboratory of Dr. Devreotes. The antiserum is specific for the receptor and has been well characterized [17].

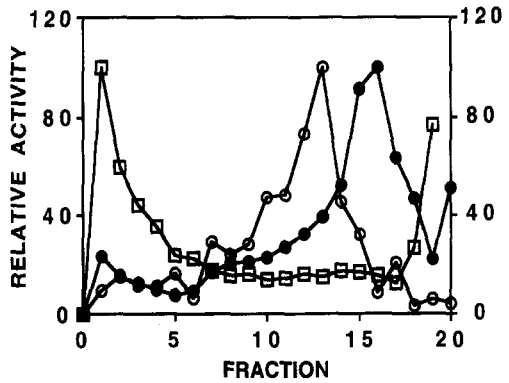

Fig. 1. Marker enzyme profile of developed cells on a sucrose density equilibrium gradient. Cells were developed for 6 h and homogenized as described in Section 2. After removing cell debris, the organelles from homogenate were separated by a density gradient centrifugation as described in Section 2. The homogenate from 6×10^8 cell equivalent was loaded on a 35-ml gradient. Marker enzyme profiles are plotted to indicate selected markers of interest. ○, V-H^+-ATPase; ●, alkaline phosphodiesterase (a marker for contractile vacuole); □, acid phosphatase.

This indication that acidosomes could be the site for accumulation of CAR1 was further examined by the Western blot analysis. Proteins from the selected fractions containing peaks of known organelle markers shown in Fig. 1 were separated by gel electrophoresis, transferred to a nitrocellulose filter, and stained with CAR1 antibodies as described in Section 2. Fig. 2 shows the Western blot analysis of the selected gradient fractions from Fig. 1. Fraction 1 showed two major reactive bands, with an apparent M_r of 40,000 and 30,000. The latter may be a degradation product, since the lysosomal peak is also in this fraction. The reactive band at M_r of 40,000 may have been derived from the lysosomes or the plasma membranes which are found in fractions 1–2. Fraction 2 contained a reactive band at M_r of 40,000 and no band at 30,000. Fractions 5 and 8 contained mildly reactive band at 40,000. Fractions 11–13, which contained the peak of V-H^+-ATPase activity, the marker for acidosomes, showed a band reactive with CAR1 antibodies. However, the band has an apparent M_r of 45,000 with less mobility than the band in fractions 1–2. We interpret the band at an apparent M_r of 45,000 to be a phosphorylated form of CAR1. Downregulated and internalized receptor for CAR1 is known to be in a phosphorylated form with an apparent M_r 3000–4000 larger than the one on the cell surface [14,18]. The data presented in Figs. 1 and 2 strongly suggest that the receptors for cAMP in aggregating cells of *Dictyostelium* reside in the acidosomes. Therefore, the acidosomes may be an equivalent of the early endosomal compartment involved in recycling of internalized receptors. The CAR1 reactivity did not coincide with the marker for the contractile vacuole (d = 1.14).

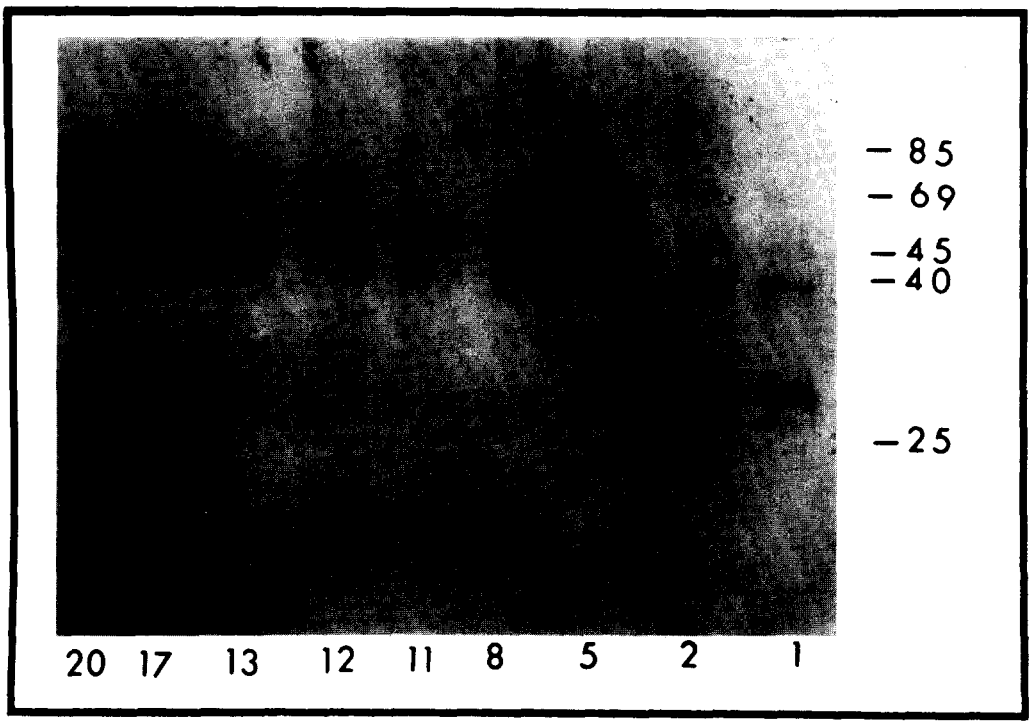

Fig. 2. Distribution of CAR1 in the sucrose density gradient fractions analyzed by Western blots. Selected fractions from the gradient shown in Fig. 1 were diluted and pelleted at 25,000 rpm for 1 h. The fractions were separated by 10% Polyacrylamide gel electrophoresis, transferred to a nitrocellulose paper and analyzed for CAR1 as described in Section 2.

To further analyze the distribution of CAR1 between acidosomes and lysosomes, acidosomes were isolated by sucrose density gradient as described earlier [8] and the lysosomal fraction was purified by chromatography on an electromagnetic column [5]. The lysosomal preparation had over 20-fold purification, $\geq 60\%$ yield and $<1\%$ contamination by other organelles. Similarly the procedure for purification of acidosomes also provided excellent purification with minimal contamination from other organelles [5,8]. An equal amount of protein from each preparation was spotted on a nitrocellulose paper and analyzed by dot blot analysis for CAR1 content. Fig. 3 clearly shows that the acidosome fraction was enriched in CAR1 while isolated lysosomes had very low levels of the reactive protein, suggesting that the internalized receptors may accumulate in acidosomes before being recycled to the cell surface. The data also indicate that very little of CAR1 is found in the lysosomes. The observation that CAR1 is highly enriched in the purified acidosomes compared to the purified lysosome was independently confirmed using ELISA (not shown).

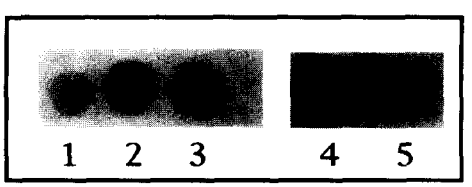

Fig. 3. Distribution of CAR1 in acidosome and lysosome fractions. Acidosomes were purified by density gradient centrifugation as described earlier [8] and the lysosomal fraction was isolated by an electromagnetic column in the absence of Mg^{2+} [5]. An equal amount of protein from each fraction was analyzed by dot blot analysis as outlined in Section 2. 1, homogenate; 2, acidosomes; 3, pooled fractions 15–17 from Fig. 1 containing membranes more buyoant than acidosomes; 4, membranes not retained on the electromagnetic column; 5, lysosomal fraction retained on the electromagnetic column. The blot analysis indicated that compared to the lysosomal fraction (No. 5), the acidosome fraction (No. 2) is heavily enriched in CAR1 reactivity.

Early endosomes (also known as CURL, prelysosomes) in higher eukaryotes have been described as a tubulo-vesicular system [19]. This compartment has been analyzed and characterized mainly through microscopy and kinetic analysis. Biochemical separation and isolation have been attempted with limited success. Recently Fuchs et al. [20] have partially purified endosomes and viewed negatively stained endosomes by an electron microscopy. The stained structure resembles the conceptualized tubulo-vesicular endosomes. Well characterized acidosomes also have a tubulo-vesicular morphology in vitro (Fig. 4; also see [8,9]) and in vivo [9,12,21], further supporting their putative function as an early endosomal compartment. Also note the smaller vesicles accumulated inside acidosomes (Fig. 4). The origin and function(s) of these vesicles remain unclear. Perhaps they represent the process of formation of multivesicular bodies, commonly found at the late endosomal stage. Available rapid and convenient isolation procedures for acidosomes will aid in biochemical characterization of an early endosomal compartment.

We have shown earlier that the acidosome is an endocytic organelle involved in the acidification of endocytic vacuoles. Using immunoblotting, we report here that the acidosome seems to be an intracellular site of CAR1 accumulation. The presence of Rab4 on acidosomes demonstrated recently [21] supports its function as an early endocytic compartment. If the acidosome is an early endosomal compartment, then it might receive pinocytic cargo and/or internalized receptors through

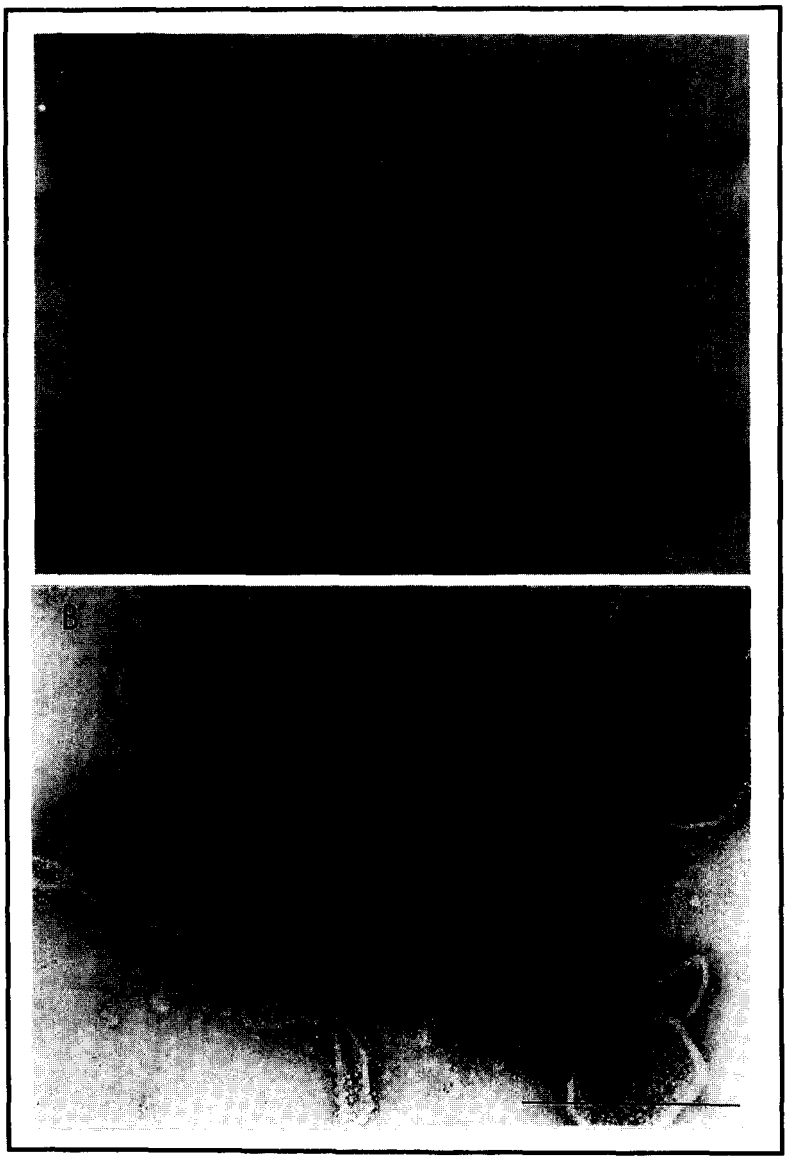

Fig. 4. Tubulo-vesicular reticulum of acidosomes. Acidosomes were isolated by an electromagnetic column chromatography in the presence of 1.5 mM $MgCl_2$ as described [5]. Acidosomes carry abundance of V-H^+-ATPase particles which can be easily identified in a negatively stained preparation like these [5,8]. Panel A shows an extensive tubulo-vesicular structure at lower magnification. Panel B shows many tubules emanating from a single vesicle. At this higher magnification, V-H^+-ATPase particles are easily visible covering almost entire surface area of the acidosomes. Also note the smaller vesicles accumulated inside acidosomes. Bar = 0.5 μm.

clathrin coated vesicles. It will be of interest to see, using quick-freeze deep-etch analysis, if clathrin coated vesicles interact with acidosomes.

It has been recently suggested that the tubulo-vesicular structure of the acidosome [22] in *Dictyostelium* is the contractile vacuole [12]. For the following reasons, we believe that contractile vacuoles and acidosomes are in fact two distinct organelles. Alkaline phosphatase is a well characterized marker for the contractile vacuoles [7,23–25] and V-H^+-ATPase is a marker for the acidosomes [6–8]. These two markers are easily separable from each other, indicating two different membrane systems [7,8]. Furthermore, these two organelles, when extensively purified, showed clearly distinct density, morphology, protein and lipid compositions [8,13]. Moreover, a cell has one or two contractile vacuoles while it has several dozen acidosomes spread throughout the cytoplasm ([9] and Figs. 3–5 in [12]). Finally, Bush et al. [21] recently immunolocalized Rab4 protein with the acidosome reticulum. The authors did not see any staining of the contractile vacuoles indicating that these two are distinctly separate membrane compartments. Although it is an interesting proposition that pumped protons may be a driving force to pump out water, it remains unsubstantiated at the moment.

Recently Nolta and Steck [13] described contractile vacuole system in *Dictyostelium*. They described three components of the contractile system: (a) main bladders-rich in alkaline phosphatase but lacking in V-H^+-ATPase; (b) the spongiome which contains 5–10% of cellular V-H^+-ATPase but is clearly separable from the acidosomes and the main bladders; (c) acidosomes which, they argue, are severed spongiome. The basis for this argument is not clear. The acidosome vacuoles are not found

clustered around the contractile vacuole bladder. Instead, the acidosome reticulum is found evenly well-spread throughout the cytoplasm in immunofluorescence as well as electronmicrographic images [9,12,21]. As discussed above, the relation, either structural or functional, of V-H^+-ATPase rich acidosome reticulum to the alkaline phosphatase rich contractile vacuole bladder is yet to be established.

In conclusion, it is shown here that acidosomes contain internalized receptors for cAMP. This finding and the localization of Rab4 protein on acidosomes indicate that well characterized buoyant membrane fraction, the acidosomes, is an early endosomal compartment containing internalized receptors.

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