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Identification of major proteins associated with *Dictyostelium discoideum* endocytic vesicles

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SUMMARY

Magnetic isolation of endocytic vesicles from *Dictyostelium discoideum* was accomplished after feeding the amoebae with iron oxide particles. Proteins associated with the endocytic vesicles were resolved by SDS-PAGE and digested 'in-gel' with endoproteinase Lys-C or Asp-N to generate peptides for amino acid sequencing. This strategy allowed the identification of the major protein constituents of the vesicles: namely, the A, B, D, E and 110 kDa subunits of a vacuolar type H^+-ATPase, actin, a Rab 7-like GTPase, a p34 protein corresponding to a new cysteine proteinase and the 25 kDa product of a recently sequenced *D. discoideum* open reading frame.

Key words: V-ATPase, Rab, cysteine proteinase, *Dictyostelium discoideum*, endocytosis, microsequence analysis

INTRODUCTION

Endocytosis is the generic name for mechanisms by which eukaryotic cells pick up material from the extracellular medium and internalize it in plasma membrane-derived vesicles. Progress in understanding vesicle formation and transport into the cells has been achieved by the convergence of biochemical and genetic approaches (for a review, see Pryer et al., 1992). Important issues are, for example, how the vesicles bud from the surface of the cell, how they mature, and how they are correctly targeted to the appropriate membrane compartments. The endocytic pathway in axenic *Dictyostelium discoideum* amoebae has been well characterized (Thilo and Vogel, 1980; Klein and Satre, 1986). It fulfills primarily a nutritive function. Internalization of fluid (pinocytosis) mainly occurs via clathrin-coated vesicles (O'Halloran and Anderson, 1992; Ruscetti et al., 1994). Then, the endocytic cargo is channeled from acidified endosomes to a lysosome-like compartment where digestion of nutriments takes place (Cardelli, 1993). Undigested material is returned to the cell surface through near-neutral post-lysosomal vacuoles (Aubry et al., 1993; Padh et al., 1993). Here, we report a biochemical study of the protein components of endocytic vesicles in *D. discoideum*. This approach combines the preparation of highly purified endocytic vesicles by magnetic fractionation with the identification of proteins by peptide sequence analysis performed after 'in-gel' digestion of the SDS-PAGE-resolved vesicle proteins with proteolytic enzymes. Amoebae were allowed to internalize a suspension of iron oxide particles (pulse time), washed, and resuspended for predetermined periods (chase time) in iron oxide-free medium. Subsequently, the cells were homogenized and iron oxide-containing vesicles were separated magnetically. The use of colloidal iron oxide particles coated with dextran as a true fluid-phase marker in *D. discoideum* was demonstrated in a previous study (Rodriguez-Paris et al., 1993). Magnetic isolation of lysosomes was performed, and partial characterization of lysosomal membrane proteins was initiated by western blot and N-terminal sequence analyses (Temesvari et al., 1994; Nolta et al., 1994). Using this magnetic fractionation approach, we decided to further identify proteins associated with endocytic vesicles by a new strategy. 'In-gel' protein digestions were performed with endoproteinase Lys-C or Asp-N after one-dimensional gel electrophoresis. Proteolytic peptides were extracted from the polyacrylamide matrix, purified by microbore reverse-phase HPLC and sequenced. Hence, four *D. discoideum* proteins present in the prelysosomal compartment were unambiguously identified by showing perfect identity between sequences of proteolytic peptides and sequences of *D. discoideum* proteins present in the databases: the D subunit of a V-ATPase, actin, the small GTP-binding protein Rab 7, and the 25 kDa product of a recently sequenced open reading frame. Proteolytic peptides exhibiting strong homologies with the A, B, E and 110 kDa subunits of V-ATPases were also identified as well as peptides derived from a 34 kDa protein, which exhibit significant homologies with amino acid sequences present in cysteine proteinases.

MATERIALS AND METHODS

Materials

Polyvinylidene difluoride (PVDF) membranes and all sequencer reagents were from Applied Biosystems. T40 dextran was from Pharmacia. Endoproteinases Lys-C and Asp-N were purchased from Boehringer. Phenyl isothiocyanate (PITC) and trifluoroacetic acid (TFA) were obtained from Pierce. Hydrogenated Triton X-100 was from Sigma and acetonitrile from Merck. For magnetic purification, a permanent magnet (0.2 Tesla) was custom-made by UGIMAG (Saint Pierre d'Allevard, France); the plastic columns (50 ml) were home-filled with stainless steel wool (Spontex, Nanterre, France). The cell cracker (Balch and Rothman, 1985) was purchased from the EMBL-Heidelberg workshop (Heidelberg, Germany). Magnetite-dextran was synthesized as described by Rodriguez-Paris et al. (1993).

Biological preparations

D. discoideum strain AX2 (ATCC 24397) was grown in axenic medium at 21°C (Watts and Ashworth, 1970). Suspension culture was swirled on a rotary shaker at 180 rpm. The cells were harvested at a density of 10^7 per ml. Magnetic purification of endocytic vesicles was essentially conducted as described by Rodriguez-Paris et al. (1993). Briefly, the cells (100 ml; 1×10^7 cells per ml) were incubated in a colloidal iron suspension of an iron oxide (γ-Fe_2O_3) coated with dextran T40 (final concentration of 1 mg iron per ml) for a given pulse period. The endocytic activity was then stopped by the addition of an equal volume of ice-cold 5 mM glycine buffer, 100 mM sucrose, pH 8.5 (GS buffer), supplemented with 0.5% bovine serum albumin (BSA). The cells were pelleted (800 ***g***, 4 minutes, 4°C) and washed three times with the same buffer. When a chase time was required, the cells were washed three times with a large volume of ice-cold 17.5 mM phosphate buffer (pH 6.0) at the end of the pulse time, and then reincubated in axenic medium at 21°C for the chase period. Finally, the cells, suspended at a concentration of 10^8 cells per ml in GS buffer supplemented with 1% BSA and protease inhibitors (2 mM EDTA, 1 mM Pefabloc, 1 mM *N*-ethylmaleimide (NEM), 5 μg/ml leupeptin and pepstatin), were broken with a cell cracker. The postnuclear supernatant was loaded onto the column that was in the magnetic field. The column was washed with 10 volumes of ice-cold GS buffer supplemented with 2 mM EDTA (GSE buffer), and then removed from the magnetic field. The retained fraction was eluted with four volumes of GSE buffer and pelleted by centrifugation (15,000 ***g***, 40 minutes). Typically, for a 10-minute pulse experiment, about 80 μg of total vesicle protein was recovered. The overall yield of vesicle purification was determined by adding FITC-dextran together with the colloidal iron in the cell incubation medium and measuring the fluorescence associated with the magnet-retained fraction. It was reproducibly in the 50% to 60% range. The same value was found when the iron content of the purified vesicles was compared with that of the cell homogenate. It was found that the 40% to 50% loss was mainly due to breakage of vesicles during the cell cracking step. No vesicles were recovered when the magnetic fractionation was performed either on cells never exposed to colloidal iron or on cells fed with colloidal iron for 10 minutes and then chased for 15 hours. It was therefore concluded that: (1) no colloidal iron had access to a 'dead-end' type compartment; and (2) non-magnetic material did not contaminate the magnetically purified vesicles.

The amount of protein present in the purified endocytic vesicles was measured by quantitative amino acid analysis. The vesicles were solubilized in formic acid, and norleucine was added to each sample as an internal standard. Acid hydrolysis of the peptide bonds was performed in constant boiling HCl. After PTC precolumn derivatization, standard chemical, chromatographic and integration/quantitation protocols were used.

To estimate the iron content of the biological material, a protocol adapted from Beinert (1978) was used.

Electron microscopy

Endocytic vesicles originating from *D. discoideum* cells fed with colloidal iron for 10 minutes were negatively stained for 3 minutes with a 1% uranyl acetate solution, spread on a Formvar-coated grid, and viewed with a JEOL 1200 EX II TEM microscope. The diameters of all vesicles ranged between 0.4 and 1.1 μm. By looking carefully at the purified vesicles, we observed that the iron probe was clearly visible in the lumen of all vesicles, pointing to the reliability of the magnetic fractionation approach (Fig. 1).

SDS-PAGE

The purified vesicles were incubated at 50°C for 3 minutes in lysis buffer containing 2% SDS, 50 mM Tris-HCl, pH 6.8, 4% 2-mercaptoethanol (Laemmli, 1970). Then the dextran-coated iron oxide was pelleted by centrifugation (200,000 ***g***, 15 minutes, Beckman TL100 centrifuge) and the supernatant was loaded onto a 12% polyacrylamide gel (20 cm × 16 cm × 0.1 cm) overlaid with a 5% stacking gel. Proteins were vizualized by colloidal Coomassie Brilliant Blue staining (Neuhoff et al., 1988). It was noticed that the p110 vesicle protein was progressively lost on the SDS-PAGE profile when the purified vesicles were kept at −20°C in lysis buffer.

N-terminal sequencing

For N-terminal sequence analysis, proteins resolved by SDS-PAGE were electroblotted to a PVDF membrane for 3 hours at 60 V in a 10 mM (3-(cyclohexylamino)-1-propanesulfonic acid)/NaOH buffer (pH 11.0) containing 20% methanol. After staining for 1 minute with 0.1% Coomassie Brilliant blue R-250 (w/v) in 40% ethanol, 0.5% acetic acid, and destaining in 30% ethanol, the areas of the membrane containing the protein material were excised, destained with 70% ethanol, and loaded in a vertical cross-flow reaction cartridge (Blott™ cartridge, Applied Biosystems). Edman degradation was performed on an automated sequenator (Applied Biosystems 477A) connected with an on-line phenylthiohydantoin amino acid (PTH-aa) analyzer (Applied Biosystems, model 120A). The PTH-aa analyzer injection loop was a 100-μl loop (Tempst and Riviere, 1989). Blast (Altschul

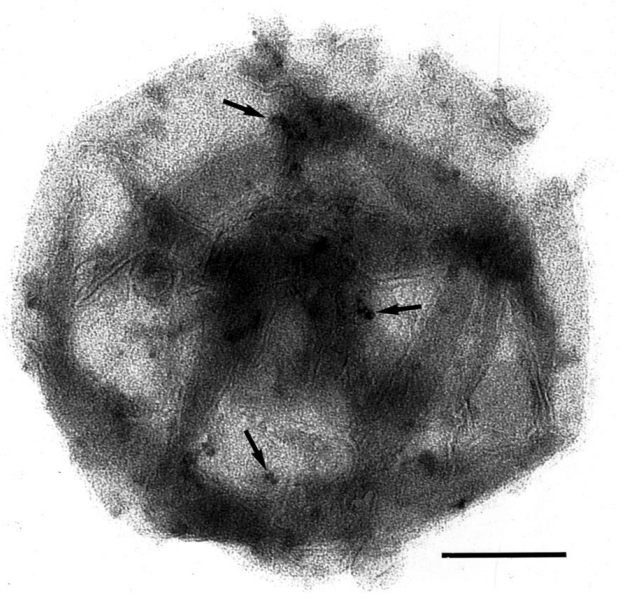

Fig. 1. Electron microscopy of *D. discoideum* endocytic vesicles. Endocytic vesicles were magnetically isolated from cells fed with colloidal iron for 10 minutes, and stained with 1% uranyl acetate. Arrows indicate iron oxide particles. The thin filaments of about 8 nm in diameter visible on the vesicle surface could be actin filaments. Bar, 200 nm.

et al., 1990) and Blitz (Sturrock and Collins, 1993) servers were used to search for sequence homologies in PDB, Swiss-Prot, PIR, and GenPept protein databases.

'In-gel' cleavage of vesicle proteins

After SDS-PAGE, the separated proteins were lightly fixed with 30% ethanol, 0.5% acetic acid for 15 minutes, and stained with 0.2% Coomassie Brilliant Blue R-250 in 30% ethanol and 0.5% acetic acid for 30 minutes. The stained bands were excised, destained with 30% ethanol for 1 hour at room temperature, washed twice with 10 ml of 100 mM NH_4HCO_3 in 50% acetonitrile for 15 minutes at 30°C, washed again twice with 10 ml of the cleavage buffer (25 mM Tris-HCl, 1 mM EDTA, 10% acetonitrile, pH 8.5, for endoproteinase Lys-C; and 50 mM NaH_2PO_4, 10% acetonitrile, pH 8.0, for endoproteinase Asp-N) and partially dehydrated in a Speed Vac concentrator. The gel slices were then rehydrated with 10 μl of the endoproteinase solution (0.1 μg/μl). The digestion was carried out for 36 hours at 37°C. Peptides were extracted from the acrylamide matrix in a two-step procedure: first, with 200 μl of 100 mM NH_4HCO_3 containing 10% acetonitrile and 0.1% hydrogenated Triton X-100; and second, with 200 μl of 100 mM NH_4HCO_3 containing 60% acetonitrile and 0.1% hydrogenated Triton X-100 (adapted from Fernandez et al., 1992). Both extracts were pooled and then partially dehydrated in a Speed Vac concentrator. Peptides were separated by reverse-phase microbore HPLC (column VYDAC C4, 2 mm × 150 mm). The fractions corresponding to the recorded peaks were collected manually, and the peptides present in these peaks were sequenced.

RESULTS

Characterization of the magnetically isolated endocytic vesicles

The amount of protein associated with the purified endocytic vesicles as a function of the pulse time was measured by quantitative amino acid analysis (Table 1). When the whole endocytic pathway was loaded for 90 minutes, this amount was found to be 0.6% ± 0.1% of the cell protein content. This value is in agreement with previous estimations (1.3%) (Rodriguez-Paris et al., 1993). The iron content of the purified endocytic vesicles was measured as a function of the pulse time. As expected for a fluid-phase marker, it increased with time. The strong increase in the iron/protein ratio observed between pulse periods of 15 minutes and 90 minutes might be explained by a significative concentration of the colloidal iron in the endocytic vesicle. Such a concentration had been previously shown using fluorescein isothiocyanate-dextran as a fluid-phase marker (Klein and Satre, 1986).

Table 1. Protein and iron contents of the endocytic fractions

Pulse length (min)	Vesicle protein content (μg)	Vesicle protein content (% of total)	Iron/protein (mass ratio)
3	70±30	0.05±0.02	0.02
15	170±55	0.3±0.1	0.035
90	280±45	0.6±0.1	0.14

A total of 10^9 cells were fed with colloidal iron for predetermined pulse periods, washed, broken, magnetically fractionated, and analyzed as described in Materials and Methods. The values (mean ± s.d., n=3) were corrected, taking into account the yield of vesicle purification (50-60%) determined in each experiment (see Materials and Methods).

The SDS-PAGE profile of vesicles purified after a 10-minute pulse showed about 20 major protein bands (Fig. 2, lane 4). When the cells were fed with the colloidal iron for a 1-minute pulse, a 10-minute pulse, or a 3-minute pulse followed by a 20-minute chase, only minor modifications of the SDS-PAGE profiles were observed, indicating that the most abundant endocytic vesicle proteins were already present in 1-minute-old vesicles and were maintained throughout the pathway to lysosomes (Fig. 2, lanes 2, 3, 4).

Identification of V-ATPase subunits, Rab 7, and actin as major proteins of *D. discoideum* endocytic vesicles

Amino acid sequences were determined on proteins associated with endocytic vesicles isolated after a 10-minute pulse with colloidal iron. In order to obtain internal sequences of the proteins and because of possible N terminus blockage, 'in-gel' digestions with endoproteinase Lys-C or Asp-N were systematically run in addition to N-terminal sequence analysis. The p59 protein, which was the most intense band on the SDS-PAGE profiles (Fig. 2, lane 4), was digested 'in-gel' with endoproteinase Asp-N. Proteolytic peptides were extracted from the gel and purified by microbore reverse-phase HPLC (Fig. 3). The amino acid sequences of six peptides were determined and found to be highly homologous to sequences present in the B subunits of the *Bos taurus*, *Arabidopsis thaliana*, and *Neurospora crassa* V-ATPases (Fig. 4), making the p59 protein a very good candidate for being the B subunit of the *D. discoideum* V-ATPase. This hypothesis was further supported by

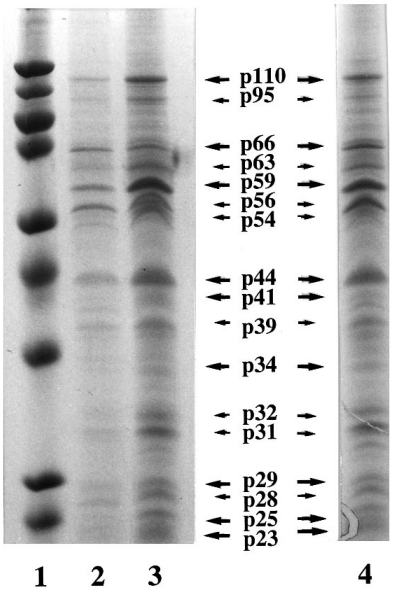

Fig. 2. Separation of endocytic vesicle proteins by one-dimensional gel electrophoresis. Endocytic vesicles were isolated from cells fed with colloidal iron for a 1-minute pulse (lane 2), a 3-minute pulse followed by a 20-minute chase (lane 3), a 10-minute pulse (lane 4). Vesicle proteins were subjected to SDS-PAGE and visualized by colloidal Coomassie Brilliant Blue G-250 staining (Neuhoff et al., 1988). Proteins that have been identified during the course of this work and unknown proteins are indicated by large arrows and small arrows, respectively. Lane 1: molecular mass markers (116, 97, 84, 66, 55, 45, 36, 29 and 24 kDa).

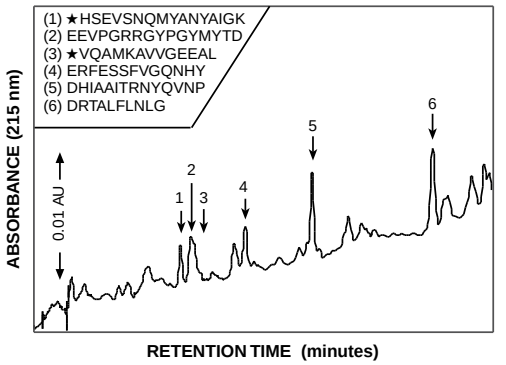

Fig. 3. Microbore HPLC purification of peptides obtained from an in-gel digestion of the p59 vesicle protein with endoproteinase Asp-N. Peptides were separated on a reverse-phase column (Vydac C4, 2 mm × 150 mm) eluted with a 0 to 40% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid for 40 minutes at a flow rate of 0.3 ml/minute. Amino acid sequences corresponding to the tagged UV peaks are given in the insert. The specificity of endoproteinase Asp-N is not absolute. Cleavages on the N side of both aspartic and glutamic acids were observed under our experimental conditions. Thus, where the identification of the first amino acid residue is uncertain, a ★ symbol, standing for Asp or Glu, is given in the peptide sequence.

N-terminal sequence analysis of p59. The first 15 amino acid residues were identified, and a significant homology (39% identity, 61% analogy) was found with a sequence present in the N-terminal region of the B subunit of a bovine V-ATPase kidney isoform (Fig. 4). We thus looked for the presence of other V-ATPase subunits in our vesicle fractions at their expected molecular mass positions on SDS-PAGE profiles. We obtained two endoproteinase Lys-C peptides of the p110 protein whose amino acid sequences exhibited strong homologies with sequences of peptides present in the 116 kDa subunit of the nematode *Caenorhabditis elegans* V-ATPase and in the 116 kDa subunit of the rat synaptic vesicle proton pump (Fig. 4). With respect to proteins p66 and p29, the sequence of one proteolytic peptide was determined for each. Their respective homologies with sequences present in the A and E subunits of V-ATPases from different organisms (Fig. 4) allowed the identification of the endocytic vesicle proteins p66 and p29 as the A and E subunits of the *D. discoideum* V-ATPase. Finally, successful N-terminal amino acid sequencing was performed on the p41 protein after electrotransfer onto a PVDF membrane. The first 15 amino acid residues were identified and found to be 100% identical to the N terminus of the recently published D subunit of the *D. discoideum* V-ATPase deduced from the nucleotide sequence of the *DVA41* gene (Temesvari et al., 1994) (Fig. 4) although the N-terminal amino acid obtained by Edman degradation of the vesicle p41 protein was reported to be an alanine instead of a glycine residue (Temesvari et al., 1994).

The 'in-gel' digestion strategy was also applied to identify the N-terminal blocked p44 protein. The amino acid sequences of three endoproteinase Asp-N peptides were determined to be EAQSKRGILTLKYPIEHGIV, DMEKIWHHTFYN, and DGVSHTVPIY, respectively. They are 100% identical to peptides 57-76, 81-92, and 157-166, respectively, derived from the *D. discoideum* major actin isoform sequence (Vandekerckhove and Weber, 1980).

Among the low molecular mass proteins resolved by SDS-PAGE in vesicles isolated after a 10-minute loading with colloidal iron, the N-terminal blocked p23 protein was digested with endoproteinase Lys-C. The amino acid sequences of four peptides were VIILGDSGVGK, TSLMNQYVNK, ATIGADFLTK, and GNIPYFETSAK, respectively. These sequences are 100% identical to sequences of peptides 11-21, 22-31, 39-48, and 148-158, respectively, deduced from the *D. discoideum* Rab 7-like GTPase gene (GenBank accession U02928). Interestingly, these Rab7 peptides could also be identified by the same strategy in vesicles isolated after a short pulse period (3 minutes) with colloidal iron.

A 34 kDa cysteine proteinase and an uncharacterized 25 kDa protein are present in *D. discoideum* endocytic vesicles

N-terminal amino acid sequencing was performed on the p34 vesicle protein. The first 14 amino acid residues of the protein were identified. A search in the protein data base revealed that this amino acid sequence shared significant homology with cysteine proteinases from different origins, and particularly with the product of the previously characterized *D. discoideum* cysteine proteinase CP1 gene (50% identity, 64% analogy) (Williams et al., 1985). A weaker but nevertheless significant homology to the *D. discoideum* cysteine proteinase CP2 gene product was also found (28% identity, 50% analogy) (Datta and Firtel, 1987) (Fig. 5). Digestion of the p34 protein with endoproteinase Lys-C yielded one major peptide, whose sequence was determined and found to be highly homologous to the sequence of a peptide that is part of the catalytic site of *D. discoideum* CP1 (66% identity, 78% analogy) and CP2 (55% identity, 78% analogy) (Fig. 5). Multiple cysteine proteinase forms were previously characterized during the life cycle of *D. discoideum* (North et al., 1988). The endocytic vesicle p34 protein could represent a new cysteine proteinase present in vegetative *D. discoideum* amoebae. In contrast, *D. discoideum* CP1 and CP2 are absent from growing cells and appear only after a few hours in the developmental phase (Williams et al., 1985; Datta and Firtel, 1987).

After an endoproteinase Asp-N digest of the p25 vesicle protein, two peptides were purified. Their amino acid sequences were found to be EYNKGETVRFNN and DTPTPGKFQELAQG. In both cases, 100% identity was found with sequences 19-30 and 58-71, respectively, of a recently sequenced *D. discoideum* open reading frame (GenBank accession U20997), indicating that this open reading frame is a gene that is indeed expressed in vegetative amoebae.

DISCUSSION

In the last few years, noteworthy progress has been made in the understanding of intracellular traffic mechanisms by both biochemical and genetic approaches. In this paper, we describe a biochemical approach that combines the capacity of magnetic fractionation to prepare highly purified endocytic vesicles of defined ages (Rodriguez-Paris et al., 1993) with the potency of recently introduced analytical methods that allow access to internal amino acid sequences from any protein present in a 50 to 100 picomole amount on acrylamide gels (for a review, see

Patterson, 1994). *D. discoideum* was chosen as a model for this study because of its high endocytic activity. After 'in-gel' digestion of the SDS-PAGE-resolved vesicle proteins, peptides were extracted from the acrylamide matrix and purified by microbore HPLC. Optimized protein sequence analysis was then conducted in order to determine sequences in the 2 to 10 picomole range. Following this strategy, we were able to get an insight into the protein composition of *D. discoideum* endocytic vesicles without any 'a priori' knowledge about the nature of the proteins expected to be present in the vesicle preparations.

Our results are complementary to those from a previous study published by Cardelli's group (Temesvari et al., 1994), in which *D. discoideum* lysosomes were purified by magnetic fractionation after a 15-minute pulse and a 15-minute chase. Cardelli and coworkers identified the presence of the V-ATPase D subunit in the lysosomes by N-terminal sequence analysis. They also demonstrated by western blot analysis that a Rab 4-like GTPase, a Rab 7-like GTPase, and the A and 110 kDa subunits of a V-ATPase were members of the *D. discoideum* lysosomal membrane protein population (Temesvari et al., 1994). We have extended their results here by a different approach and have clearly identified in vesicles isolated from a 10-minute pulse with colloidal iron, which correspond biochemically to a prelysosomal compartment (Aubry et al., 1993; Padh et al., 1993), some of the major proteins associated with those vesicles: namely, actin, a Rab 7-like GTP binding protein, the 110 kDa, A, B, D, and E subunits of a V-ATPase, a 34 kDa new cysteine proteinase, and the 25 kDa product of a recently sequenced *D. discoideum* open reading frame. The partial amino acid sequences that have been determined on the cysteine proteinase and the 110 kDa, A, B and E subunits of the V-ATPase are the first published sequences of these proteins.

One facet of our results is that, in *D. discoideum*, the major proteins associated with the endocytic vesicles are both present in prelysosomal (this work) and lysosomal (Temesvari et al., 1994) compartments. This is consistent with the finding that after Coomassie Blue staining the SDS-PAGE patterns of prelysosomes and lysosomes are very similar (Fig. 2). It is also consistent with the identification and localization of a new cysteine proteinase in the prelysosomal compartment (this work) when this kind of protein is generally involved in the breakdown of nutrient proteins within digestive vacuoles or lysosomes.

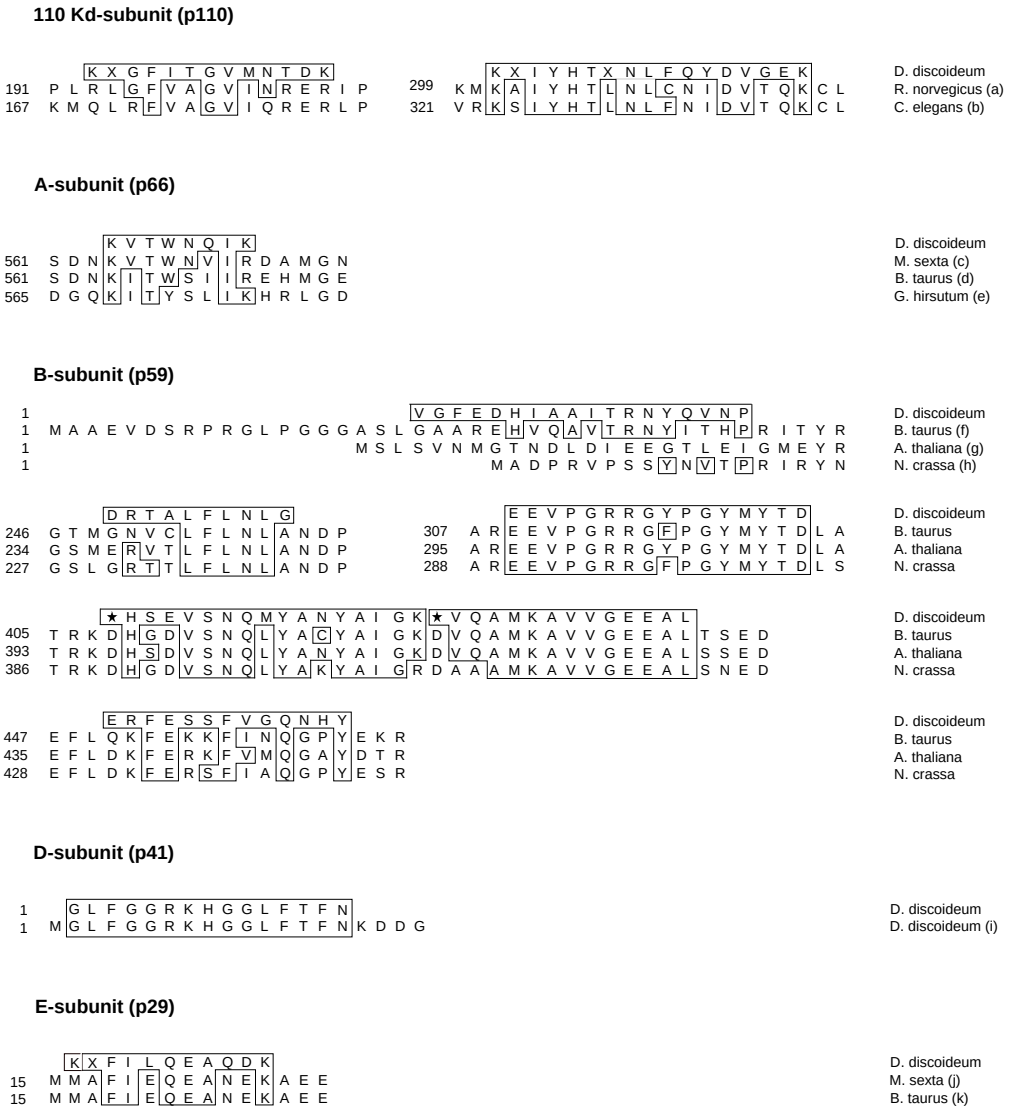

Fig. 4. Identification of the p110, p66, p59, p41, and p29 vesicle proteins as the 110 kDa, A, B, D, and E subunits of *D. discoideum* V-ATPase. The amino acid sequences of proteolytic peptides originating from the vesicle proteins p110, p66, p59, p41, and p29 are given. Sequences of the proteolytic peptides were aligned with sequences of the 110 kDa, A, B, D, and E subunits of V-ATPases from various organisms. Residues that are conserved between the homologous sequences and the proteolytic peptides are boxed. For the endoproteinase Lys-C peptides, a lysine residue was assumed at position (–1) on the basis of the protease selectivity. The protein sequence sources are as follows. (a) Perin et al. (1991), SwissProt P25286; (b) Sulston et al. (1992), SwissProt P30628; (c) Gräf et al. (1992), SwissProt P31400; (d) Puopolo et al. (1991), SwissProt P31404; (e) SwissProt P31405; (f) Nelson et al. (1992), SwissProt P31407; (g) Manolson et al. (1988), SwissProt P11574; (h) Bowman et al. (1988), SwissProt P11593; (i) Temesvari et al. (1994), PIR A55016; (j) Gräf et al. (1992), SwissProt P31402; (k) Hirsch et al. (1988), SwissProt P11019. ★ Asp or Glu (see legend to Fig. 3).

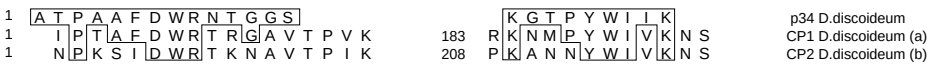

Fig. 5. Identification of the p34 vesicle protein as a new *D. discoideum* cysteine proteinase. In addition to N-terminal sequence analysis, the p34 vesicle protein was digested with endoproteinase Lys-C. One peptide was purified and sequenced. The amino acid sequences were aligned with homologous regions of *D. discoideum* cysteine proteinases CP1 and CP2. Residues that are conserved between the homologous sequences and the proteolytic peptides are boxed. The residue numbers are those of the mature enzymes. A lysine residue was assumed at position (−1) of the endoproteinase Lys-C peptide on the basis of the protease selectivity. (a) Williams et al. (1985), SwissProt P04988; (b) Datta and Firtel (1987), SwissProt P04989.

The structure of V-ATPases from different sources is remarkably conserved. V-ATPase is a multimeric enzyme composed of two distinct structures: a peripheral sector containing five different subunits (A to E in order of decreasing molecular mass) and a membrane sector probably containing two different subunits named a and c (Nelson et al., 1992). In *D. discoideum* cells, as in mammalian cells, an additional 110 kDa subunit was observed (Fok et al., 1993). The entire complex contains three copies each of the A and B subunits, six copies of the c subunit, and one copy of each of the remaining polypeptides (Forgac, 1992; Nolta et al., 1991). V-ATPase plays a pivotal role in endocytosis, as it is responsible for the acidification of the endocytic vesicles. *D. discoideum* mutants deficient in V-ATPase no longer exhibit acidification of their endocytic compartment (Bof et al., 1992). Elevation of the pH of intracellular acidic compartments has dramatic effects. It was shown to interfere with endocytosis, receptor recycling, protein targeting, development, and transport processes (Mellman et al., 1986; Ohya et al., 1991; Davies et al., 1994). We have been able to identify in the endocytic vesicle fraction the 110 kDa subunit and all the *D. discoideum* subunits of the V-ATPase peripheral sector except for the C subunit, which might be the p56 or p54 protein, as we did not succeed in digesting those two proteins with endoproteinases. Thus, our data, in agreement with previous results on lysosomes (Temesvari et al., 1994), favor the idea that *D. discoideum* endocytic vesicles are acidified by their own functional V-ATPase and not by interactions with the V-ATPase-rich acidosomes, as previously suggested (Padh, 1995). Under our experimental conditions, i.e. in the absence of magnesium ions, acidosomes are not retained on the column in the presence of the magnet whereas endocytic vesicles are (Padh, 1995). The precise role of acidosomes remains to be clarified.

In vegetative *D. discoideum* cells, actin amounts to 8% of total cellular protein (Romans and Firtel, 1985). Therefore, data showing the association of actin with endocytic vesicles should be considered with care. However, since the protein content of the vesicles was highly reproducible, we believe that the association of actin with *D. discoideum* endocytic vesicles is meaningful. The thin filaments of about 8 nm in diameter visible on the surface of the vesicles (Fig. 1) could be actin filaments. In yeast (Kübler and Riezman, 1993) and in polarized epithelial cells (Gottlieb et al., 1993), it was shown that actin plays a direct role in receptor-mediated internalization and pinocytosis, respectively. We believe that the presence of actin on *D. discoideum* endocytic vesicles reflects tight interactions between the vesicles and the actin cytoskeleton. These interactions might occur via a vesicle-associated actin binding protein, which remains to be identified. In *D. discoideum*, actin is encoded by a multigene family of 17 members (Romans and Firtel, 1985). The question of whether a specific actin isoform is associated with the endocytic function was addressed. From our peptide sequences, it appears that the isolated actin isoform is the major isoform expressed in *D. discoideum*.

Amino acid sequence analysis of four endoproteinase Lys-C peptides of the p23 protein identifies this protein as a Rab7-like GTP-binding protein. To our knowledge, this is the first GTP-binding protein isolated from a purified cellular compartment and identified by protein sequence analysis. Previous identifications relied on western blot or immunofluorescence analyses using specific antibodies often raised against fusion proteins. Rab proteins are small GTPases of the Ras superfamily that have been shown to play a pivotal role in intracellular membrane trafficking (for reviews, see Zerial and Stenmark, 1993; Nuoffer and Balch, 1994). In mammalian cells, several proteins belonging to the Rab family have been localized to distinct subcellular compartments of both the exocytic and endocytic pathways. Rab4, Rab5, Rab7 and Rab9 are associated specifically with the endocytic membrane system. The exact role of Rab7 is still unknown. The *Saccharomyces cerevisiae* homolog of mammalian Rab7 protein is Ypt7p. Data obtained from studies on a Ypt7 mutant suggest that Ypt7p is involved in the regulation of transport steps from late endosomes to the yeast vacuole (Schimmöller and Riezman, 1993). Interestingly, we were able to demonstrate that, in *D. discoideum*, the Rab7-like protein is already present in a very early endocytic compartment isolated after a 3-minute pulse with colloidal iron. These data would favor the idea that, in *D. discoideum*, in comparison to yeast, the endocytic cargo reaches a late endosome-like compartment in the very first minutes of the endocytic cycle.

Of particular interest is the finding that what was until now a putative *D. discoideum* 25 kDa protein is in fact a major protein associated with the endocytic vesicles. Its role and precise localization in the cell (only in the endocytic vesicles?) and in vesicles (outside or inside?) is currently being studied.

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