

Phagosome maturation in unicellular eukaryote *Paramecium*: the presence of RILP, Rab7 and LAMP-2 homologues

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Phagosome maturation is a complex process enabling degradation of internalised particles. Our data obtained at the gene, protein and cellular level indicate that the set of components involved in this process and known up to now in mammalian cells is functioning in unicellular eukaryote. Rab7-interacting partners: homologues of its effector RILP (*Rab-interacting lysosomal protein*) and LAMP-2 (*lysosomal membrane protein 2*) as well as $\alpha 7$ subunit of the 26S proteasome were revealed in *Paramecium* phagolysosomal compartment. We identified the gene/transcript fragments encoding RILP-related proteins (RILP1 and RILP2) in *Paramecium* by PCR/RT-PCR and sequencing. The deduced amino acid sequences of RILP1 and RILP2 show 60.5% and 58.3% similarity, respectively, to the region involved in regulating of lysosomal morphology and dynein-dynactin recruitment of human RILP. RILP colocalised with Rab7 in *Paramecium* lysosomes and at phagolysosomal membrane during phagocytosis of both the latex beads and bacteria. In the same compartment LAMP-2 was present and its expression during latex internalisation was 2.5-fold higher than in the control when P2 protein fractions (100 000 \times g) of equal load were quantified by immunoblotting. LAMP-2 cross-reacting polypeptide of ~106 kDa was glycosylated as shown by fluorescent and Western analysis of the same blot preceded by PNGase F treatment. The $\alpha 7$ subunit of 26S proteasome was detected close to the phagosomal membrane in the small vesicles, in some of which it colocalised with Rab7. Immunoblotting confirmed presence of RILP-related polypeptide and $\alpha 7$ subunit of 26S proteasome in *Paramecium* protein fractions. These results suggest that Rab7, RILP and LAMP-2 may be involved in phagosome maturation in *Paramecium*.

Key words: RILP, Rab7, LAMP-2, $\alpha 7$ proteasome, Phagolysosomes, *Paramecium*.

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Paper accepted on May 18, 2007

European Journal of Histochemistry
2007; vol. 51 issue 3 (July-September):163-172

During phagocytosis cells internalise large particles that are subsequently degraded in lysosomes that comprise the final stage of the endocytic pathways and intracellular traffic (Kornfeld and Mellman 1989; Gruenberg 2001; Luzio *et al.* 2003). Digestion capability of phagosomes is acquired in the process of maturation that culminates in the fusion of phagosomes with lysosomes leading to formation of the phagolysosomes (Desjardins *et al.* 1994).

Paramecium uses phagocytosis as a principal pathway to ingest bacteria and other food particles. Digestion process starts up upon fusion of phagosomes (i.e. digestive vacuoles at stage II; DV-II) with the primary lysosomes (Allen and Fok 2000) on which we focused our analysis looking for molecular machinery involved in phagolysosome formation in this cell.

The molecular mechanism of phagocytic maturation is not fully understood and majority of studies was performed on macrophages. There is evidence that the important role in this process plays Rab7-RILP complex (Harrison *et al.* 2003). Rab7 – small GTP-binding protein regulates membrane trafficking from early to late endosomal compartment and lysosomes in mammalian cells as well as in biogenesis of lysosomes and their acidification (Feng *et al.* 1995; Bucci *et al.* 2000; Coluci *et al.* 2005). We identified genes encoding Rab7 in *Paramecium* displaying 81.6-82.1% similarity to human, rat and mouse counterparts (Surmacz *et al.* 2006). We also showed that this protein was localised to the *Paramecium* phagosomal membrane (Surmacz *et al.* 2003) consistently with the data on mammalian macrophages and *Dictyostelium* (Rabinowitz *et al.* 1992; Rupper *et al.* 2001). RILP (*Rab-interacting lysosomal protein*) is the key effector of Rab7 and induces recruitment of dynein-dynactin motor to the late endosomes/lysosomes enabling their movement along the microtubules (Cantalupo *et al.* 2001; Jordens *et al.* 2001).

Rab7 interacts with RILP specifically via two distinct areas, with the first one involving the switch and interswitch regions and the second one consisting of Rab subfamily motifs (RabSF): RabSF1 and RabSF4 (Wu *et al.* 2005). These elements, RabSF1 (₃SQKKQLF₉) and RabSF4 (₁₇₂KAAASQEK DEEIFFP₁₈₆), exist in *Paramecium* genome (Surmacz *et al.* 2006).

We report here the presence of gene/mRNA encoding RILP-related proteins in *Paramecium* exhibiting a high homology to the regions involved in regulating of lysosomal morphology (Wang *et al.* 2004) and dynein-dynactin recruitment (Marsman *et al.* 2006) in human RILP. To our knowledge this is the first indication of expression of genes encoding RILP-related proteins in the lower eukaryotes.

Recently, a new specific interacting partner of Rab7 – α -subunit of the 26S proteasome – was identified (Dong *et al.* 2004). Proteasomes were shown to be recruited to phagosomes in mammalian cells (Houde *et al.* 2003) and therefore it was of interest whether in *Paramecium* – unicellular eukaryote that emerged early in evolution – a similar process may be observed.

Very recent data (Huynh *et al.* 2007) indicate that lysosome-associated membrane proteins (LAMPs) are required for fusion of phagosomes with lysosomes. LAMPs are very abundant and heavily glycosylated proteins in the lysosomal membranes (Eskelinen *et al.* 2005). LAMP-2 contains a large luminal domain with a number of N-linked oligosaccharide chains, a single transmembrane region and a short cytoplasmic tail (Gough and Fambrough 1997). Knockout of LAMP-2 in mice revealed its involvement in lysosomal biogenesis (Eskelinen *et al.* 2003). Defects in lysosomal function are associated with diverse storage diseases in human, including the Danon disease caused by mutations in the gene encoding LAMP-2 (Balmer *et al.* 2005). No data are available concerning presence of LAMP-2 in lower eukaryotes. We reported LAMP-2-homologous gene fragment in *Paramecium*, mapped its antigens to the phagolysosomal compartment, described a glycoprotein nature of the LAMP-2 immunoreactive polypeptide and observed its increased expression during process of phagocytosis.

Here we characterised the components of phagolysosomal compartments in *Paramecium* by variety of methods including PCR/RT-PCR and sequencing, quantitative immunoblotting, glycopro-

tein staining and double labelling in electron microscopy. These localisation and immunodetection studies were combined with an extensive search of available genomic databases and the respective *Paramecium* sequences homologous to the mammalian counterparts were described.

Materials and Methods

Cells

Paramecium octaurelia cells (stock 299s), 5-day-old axenic cultures were harvested as it was described in Wyroba (1987). Phagocytosis was induced by feeding with polystyrene monodispersed latex beads (0.95 μ m, Polysciences, Inc., Warrington, PA, USA) or bacteria *Aerobacter aerogenes* and aliquots of cells were withdrawn after various time periods of internalisation for studies described below. Efficiency of phagocytic activity was monitored prior to each experiment by digestive vacuole (DV) score (Wyroba 1987).

Fractionation and SDS-PAGE

Control (not treated) or latex-treated ciliates were fractionated as described previously (Wiejak *et al.* 2002; 2004a) yielding, after centrifugation at 100 000 x g, a high-speed pellet (P2) and supernatant (S2). Protein fractions (20 μ g per lane) were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to ECL nitrocellulose membrane by wet transfer method that was stained with 0.5% Ponceau Red (in 3% TCA) before Western blotting (Surmacz *et al.* 2001).

Western blot analysis

The membranes were blocked (45 min) at room temperature (RT) with 4% non-fat milk in TBS/0.1% Tween 20 as described previously (Surmacz *et al.* 2001). Immunodetection was performed (overnight at 4°C) using rabbit primary antibody (Ab) against LAMP-2 (1:150, H-207, Santa Cruz Biotechnology Inc., Santa Cruz, USA), rabbit Ab against human RILP (1:200) - kindly donated by Dr. J. Neefjes from The Netherlands Cancer Institute in Amsterdam (Jordens *et al.* 2001) and monoclonal Ab against proteasome 20S subunit α 7 (1:500; Affiniti, Exeter, UK). It was followed by incubation with the appropriate secondary HRP-conjugated antibodies (1:1000) for 1h (RT) and chemiluminescent detection with West Pico Trial Kit (Pierce, Rockford, USA). Control experi-

ments performed without the primary antibody produced no immunoreactive bands. Densitometric analysis was performed as described in Wiejak *et al.* (2003).

Glycoprotein detection

The glycoproteins were analysed using Pro-Q Emerald 300 Glycoprotein Gel and Blot Stain Kit (Molecular Probes, Eurogene, USA) enabling to detect as little as 0.5 ng of glycoprotein per band. After SDS-PAGE (8%) the proteins were transferred to PVDF membrane, air-dried and stained according to the manufacturer's protocol. The fluorescent signal was visualised and photographed under 300 nm UV illumination. The CandyCane™ (Molecular Probes, Eurogene, USA) molecular weight standards (1 µL per lane) containing a mixture of glycosylated and nonglycosylated proteins were used as positive and negative controls. The same blots (after wetting in methanol and three washes in PBS, 15 min each) were subsequently analysed by Western blotting with anti-LAMP-2 antibody.

N-Glycosidase F (PNGase F, G5166; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used to deglycosylate protein. Aliquots of protein fraction containing 100 µg of protein were denatured and next treated with N-Glycosidase F (0.0077 U per sample of 25 µL) at 37°C for 3 h according to manufacturer's procedure. Next the samples were subjected to electrophoresis (30 µg per lane), blotting, Pro-Q Emerald 300 staining followed by Western analysis as described above.

Electron microscopy

Paramecium cells induced to phagocytosis by feeding with latex beads (as described above) or with *Aerobacter aerogenes* for various time periods as well as the untreated ones were prepared using post-embedding method as in Wiejak *et al.* (2002). The following antibodies were used: anti-RILP (1:100), A-16 against Rab7 (1:250; Santa Cruz Biotechnology Inc., Santa Cruz, USA), anti-LAMP-2 (1:100) and against proteasome 20S subunit α 7 (1:100). The ultrathin sections were incubated with primary antibodies overnight at RT followed by the appropriate secondary Abs (1:20) conjugated with colloidal gold particles for 2h at RT. In the control experiments the primary antibodies were omitted and no labelling was detected. The sections were observed in a JEM 1200 EX electron microscope.

All the blots and images presented are representative of at least three experiments of each type.

PCR

PCR/RT-PCR amplifications were performed using DNA isolated from *Paramecium* (Wiejak *et al.* 2004b) or cDNA, respectively, as the templates. cDNA was obtained by reverse transcription of 1 µg of total RNA (Surmacz *et al.* 2006) with the Enhanced Avian RT-PCR Kit according to manufacturer's instruction (Sigma-Aldrich). The primers: forward 5'-TGATGCCTATTAATGAAG and reverse 5'-TCTTTGGGACCTTTGG were designed according to the sequence of scaffold 44 deriving from *Paramecium* database (<http://paramecium.cgm.cnrs-gif.fr>) that was homologous to the fragment of human *RILP* mRNA (NM_031430) important in lysosome morphology (Wang *et al.* 2004) and motor molecule recruitment (Marsman *et al.* 2006). PCR sample (25 µL) contained: 5 µL of 5 x PCR buffer, 1.3 µL of 2 mM dNTPs, 4 µL of 25 mM MgCl₂, 1 unit of GoTaq Flexi polymerase (Promega Corporation, Madison, US) and 0.4 µM of the primers. PCR setting (34 cycles) was: 93°C (1 min), 54°C (2 min) and at 72°C (2 min) in PTC-200 DNA Engine (MJ Research, Inc., Watertown, US). PCR products were separated on 1.5% agarose gel and ethidium bromide (EB) stained. The product of expected molecular size was excised from the gel, purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, US) and automatically sequenced. Control reactions without the template did not produce any products. Sequence searches were performed by the BLAST algorithm (Altschul *et al.* 1997) on NCBI databases and multiple alignments using the Clustal W method (Thompson *et al.* 1994).

Results

RILP homologues in *Paramecium*

Rab7 functions in directing late endosomal/lysosomal trafficking by association with its effector protein RILP (Jordens *et al.* 2001). We identified by PCR/RT-PCR and sequencing the gene fragment and transcript encoding RILP-related proteins in *Paramecium*.

We first looked for the most important regions in RILP involved in regulating of lysosome morphology (Wang *et al.* 2004) and dynein-dynactin recruitment (Marsman *et al.* 2006). Searching in

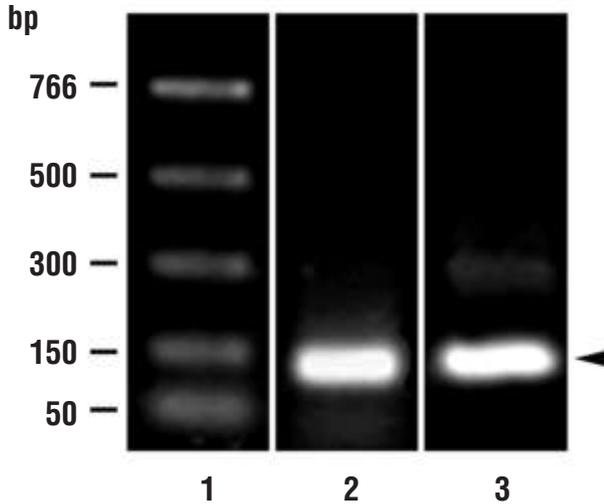


Figure 1. RILP-related PCR/RT-PCR products in *Paramecium*. Agarose gel separation followed by ethidium bromide staining. DNA (lane 2) and cDNA (lane 3) were used as templates. PCR Marker is shown in lane 1. DNA fragment of the predicted molecular size (arrowhead) was purified and sequenced.

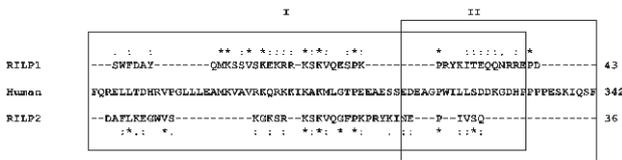


Figure 2. Alignment of the deduced amino acid sequences of *Paramecium* RILP1 (EF107050) and RILP2 (EF107051) with the region of human RILP (NP_113618) important in regulating lysosomal morphology (I) and dynein-dynactin recruitment (II).

Paramecium database (<http://paramecium.cgm.cnrs-gif.fr>) (Aury *et al.* 2006) of another species of the *P. aurelia* complex (Sonneborn 1975) – *P. tetraurelia* (strain d4-2), revealed that in scaffold 44 there is a gene fragment (193475-193929) of 455 nucleotides showing 42.6% of identity (*data not shown*) to human RILP mRNA (NM_031430). Within this gene fragment we mapped the sequence homologous to the above-described region of human RILP and designed primers for PCR/RT-PCR amplification. DNA fragment of the predicted molecular size of ~130 bp was obtained in PCR (Figure 1, lane 2) and RT-PCR (Figure 1, lane 3), purified and sequenced.

RILP1 gene fragment (EF107050) is 127 nucleotides in length and contains an open reading frame of 42 amino acids, whereas *RILP2* mRNA (EF107051) of 108 bp is an open reading frame of 36 amino acids. These sequences are 83% identical

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RILP1      SWFDAYQMKSSVSKKRRRSKVQE-SFKPKYKITEQQNRREP 42
RILP2      ---DAFLKEGWVSKGSRKRSKVQG-FKPKYKINEPIVSQ--- 36
sc44       IENDAYQMKTSVSKKRRRSKVQE-SFKPKYKITEQVESQKKK 397
sc18       NDNEAYQMKSSVSKKRRRSKVQDTSPLYKITEQVESQKKK 397
          *:* : *** * ***** *:* **:* *
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Figure 3. Homology of *Paramecium* RILP1 (ABL01487) and RILP2 (ABL01488) with amino acid sequences (GSPATP00015232001, scaffold 44 and GSPATP0007477001, scaffold 18) deriving from *Paramecium* database (<http://paramecium.cgm.cnrs-gif.fr>).

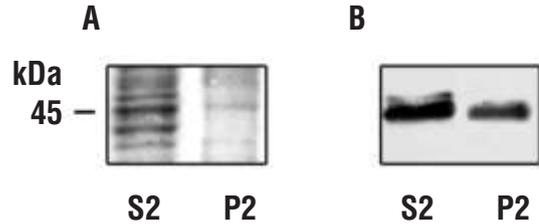


Figure 4. Immunodetection of RILP-related polypeptide(s) in *Paramecium*. (A) A high-speed supernatant (S2) and pellet (P2) protein fractions stained with Ponceau Red. (B) Western blot analysis performed with antibody against RILP.

to each other. The alignment of the deduced amino acid sequences of *Paramecium* RILP1 and RILP2 (Figure 2) with human RILP (NP_113618) revealed 60.5% and 58.3% similarity, respectively, to the region (272-342) essential for lysosomal morphology (Wang *et al.* 2004) and motor molecule recruitment (Marsman *et al.* 2006).

Comparison of *Paramecium* RILP1 and RILP2 nucleotide sequences with *Paramecium* database revealed the presence of two homologous DNA fragments: in scaffold 44 from 192272 to 193746 and in scaffold 18 from 364143 to 365623. The alignment of the amino acid sequences of these fragments (GSPATP00015232001 and GSPATP00007477001, respectively) with RILP1 and RILP2 shows 52% similarity (Figure 3). By use of antibody against RILP we could detect ~45 kDa cross-reacting polypeptide in Western blot analysis of S2 and P2 fractions (Figure 4B) isolated from *Paramecium* cells and separated by SDS-PAGE (Figure 4A).

The presence of RILP in phagolysosomal compartment was demonstrated by ultrastructural studies. During internalisation of either the latex beads (Figure 5A) or bacteria (Figure 5B-C), as a natural food of *Paramecium*, RILP colocalised with Rab7 in the primary lysosomes and on the membrane of the phagolysosomes (Figure 5A-C). A slight double labelling was also observed in the secondary lysosomes (Figure 5B).

The presence of the $\alpha 7$ subunit of 26S proteasome in *Paramecium* phagolysosomal compartment

The specific interacting partner of Rab7 – the $\alpha 7$ subunit of 26S proteasome was detected in the vicinity of phagosomal membrane in the tiny vesi-

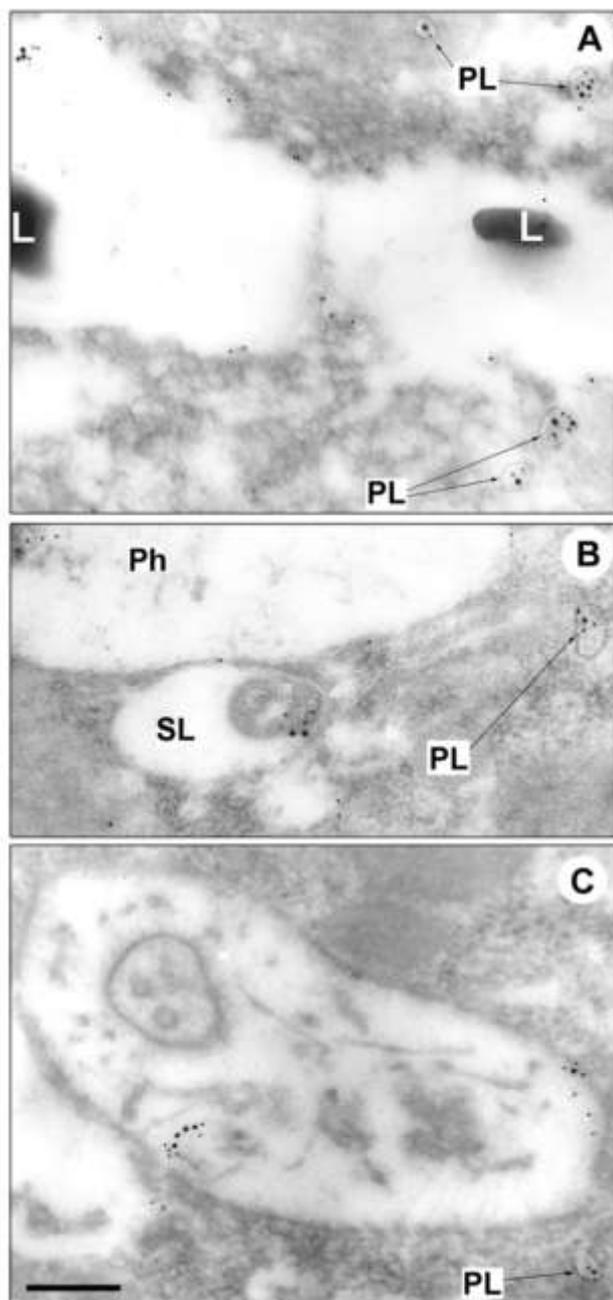


Figure 5. RILP-related protein in *Paramecium* phagolysosomal compartment during phagocytosis (A, B - 15 min, C - 25 min). The presence of Rab7 (5 nm gold particles) and RILP (10 nm gold particles) was observed in primary lysosomes (PL) (A-C) and on the membrane of phagolysosome (C) when latex beads (L) (A) or bacteria (B, C) were used to induce phagocytosis. A slight double labelling was also observed in secondary lysosomes (SL) located beneath phagosomal (Ph) membrane (B). Bar, 200 nm.

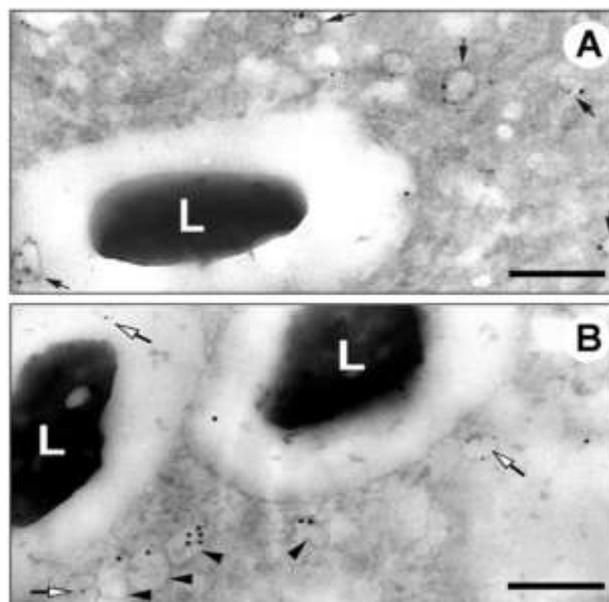


Figure 6. Localisation of $\alpha 7$ subunit of 20S proteasome to phagolysosomal compartment. Proteasomal subunit visualised with 5 nm immunogold particles in double labelling experiments with Rab7 (A) or with LAMP-2 (B), shown with 10 nm immunogold particles. (A) Small vesicles in which Rab7 colocalised with an $\alpha 7$ subunit of 20S proteasome are shown with black arrows. (B) Proteasome-positive vesicles were detected in vicinity of phagosomal membrane (white arrows) and did not colocalise with LAMP-2-positive primary lysosomes (black arrowhead). L – latex beads. Bar, 200 nm.

cles in some of which it colocalised with Rab7 (Figure 6A). In order to clarify the nature of these vesicles a double labelling was next performed using anti-LAMP2 antibody. However, $\alpha 7$ -proteasome antigen was not observed in LAMP-2-positive primary lysosomes (Figure 6B). Antibody against this proteasomal subunit revealed two immunoreactive bands of ~24 and ~22 kDa in P2 fraction (Figure 7).

Homologue of LAMP-2 in *Paramecium*

Western blot analysis of *Paramecium* P2 protein fraction (Figure 8A) using antibody against human LAMP-2 revealed the presence of ~106 kDa immunoreactive band (Figure 8B, lane 1). Interestingly, upon onset of phagocytosis we could detect significant increase in the amount of LAMP-2 associated with this fraction (Figure 8B, lanes 2, 3). Quantitative densitometric analysis showed that this increase reached 63% and 164% after 5 and 15 min of latex beads uptake, respectively, as compared to the non-phagocytosing cells (Figure 8C).

Pro-Q Emerald staining (Figure 9A, lane 1) and subsequent immunological detection of the same blot with anti-LAMP-2 antibody (Figure 9A, lane

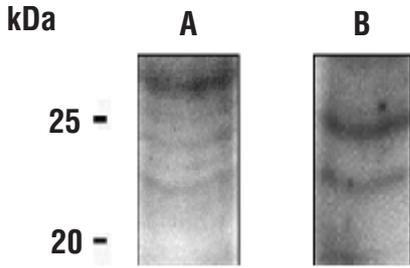


Figure 7. Presence of proteasome-related polypeptides in *Paramecium* cells. Detection by immunoblotting of SDS-PAGE separated and Ponceau Red stained P2 fraction (A) using antibody against human a7 of 20S proteasome (B).

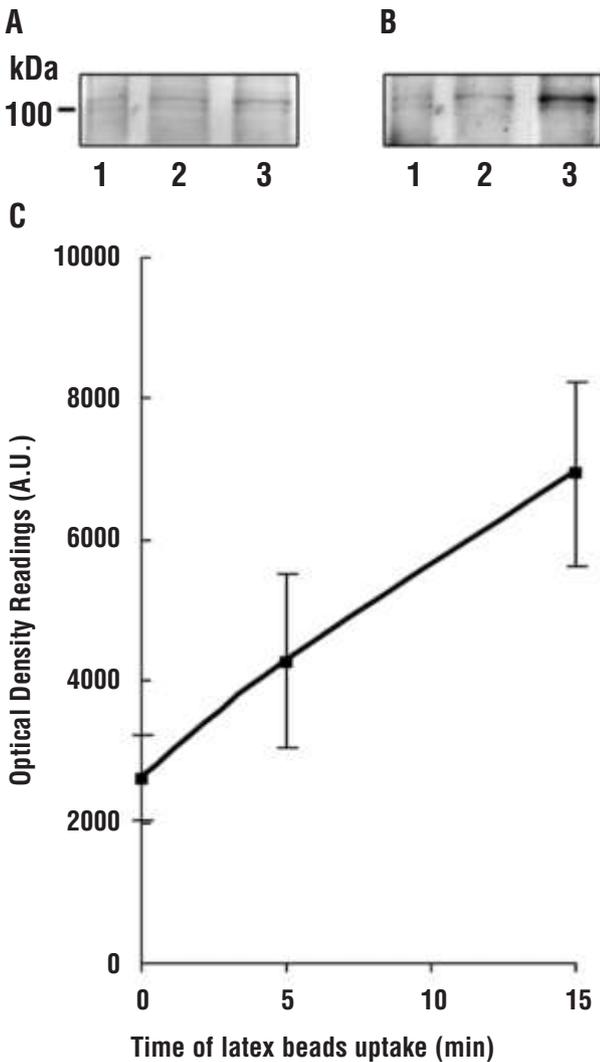


Figure 8. Expression levels of LAMP-2 homologue in *Paramecium* during phagocytosis of latex beads as analysed by quantitative Western blotting. The protein level was quantified after induction of phagocytosis for 5 min and 15 min as compared to non-phagocytosing cells. Lane 1 - 0 time, lane 2 - 5 min, lane 3 - 15 min. (A) SDS-PAGE of representative protein separations (P2 fractions) after staining with Ponceau Red. Each lane contained the same amount of protein (20 µg). Molecular mass markers (kDa) are shown at the left. (B) Western blot of corresponding electrophoretic separations shown in (A). (C) Quantitative densitometric analysis of LAMP-2 (■). Data were expressed in arbitrary units ± SD ($p < 0.05$), $n=3$.

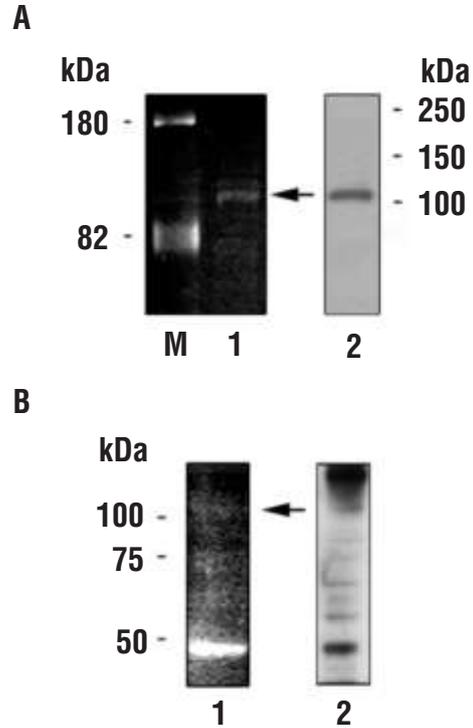


Figure 9. LAMP-2 homologue in *Paramecium* appears to be glycosylated. (A) Detection of glycoproteins with Pro-Q Emerald (lane 1) and subsequent Western analysis on the same blot with anti-LAMP-2 antibody (lane 2). The immunoreactive band of ~106 kDa in P2 fraction (30 µg of protein) is glycosylated (arrow). M - CandyCane molecular weight standards were used as positive or negative controls. After PNGase F digestion (B) and Pro-Q Emerald staining many fluorescent products of different size may be seen (lane 1) and a subsequent Western analysis reveals a ladder of immunoreactive bands (lane 2). Scale represents molecular mass.

2) demonstrated that LAMP-2-related polypeptide in *Paramecium* appears to be glycosylated. Following PNGase treatment – that cleaves a glycan from a glycoprotein – we could detect the partially digested fluorescent products (Figure 9B, lane 1) and a ladder of immunoreactive bands in Western blot analysis (Figure 9B, lane 2) with the most prominent polypeptide of ~48 kDa, i.e. the size corresponding to the polypeptide backbone of this glycoprotein (Eskelinen *et al.* 2005).

Analysing *Paramecium* database we could find the region homologous to mammalian LAMPs from different species as shown in Figure 10. This *Paramecium* amino acid sequence was deduced from the nucleotide sequence (722239-722541) of scaffold 1 and shows 35.4% similarity to mammalian LAMP-2.

Ultrastructural localisation of LAMP-2 and Rab7 was subsequently examined using immunola-

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mLAMP2      IFNINPATNFTGSCQPQSAQLRLNNSQIKVLOFIFAVKNEKREYLYKEVNVVYMLANGS 314
rLAMP2      IFNINPATNFTGSCQPQTAQLRLNNSQIKVLOFIFAVKNEKREYLYKEVNVVYMLANGS 310
hLAMP2a     VININFTNTHSTGSCRSHHTALLRLNNSQIKVLOFVFAVKNEKREYLYKEVNIISMYLVNGS 309
hLAMP2b     VININFTNTHSTGSCRSHHTALLRLNNSQIKVLOFVFAVKNEKREYLYKEVNIISMYLVNGS 309
Param       LLIFNFAQN-----IHLNSYLFENQSVSPSIIIRLNKFLIMLFQKSLLSL--- 44
           **  ***          ***  *  *  *  *  *  *  *  *  *  *  *  *  *  *
mLAMP2      AFNLSNKNLSFWDAPLGSSYMCNKEQVLSVSRAFQINTFNLRKQPFNVTKGQYSTAQ 371
rLAMP2      AFHVSNNLSFWDAPLGSSYMCNKEQVSVSRFQINTFNLRKQPFNVTKGQYSTAQ 367
hLAMP2a     VFSIANNLSYWDAPLGSSYMCNKEQVSVSGAFQINTFDLRVQPFNVTKGQYSTAQ 366
hLAMP2b     VFSIANNLSYWDAPLGSSYMCNKEQVSVSGAFQINTFDLRVQPFNVTKGQYSTAQ 366
Param       LTMFDNCSYPIQKVCIDGRYFLAYPIYIIRQANQKSSQNLEIKTEEHKQNDNTSLQ 101
           *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

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Figure 10. LAMP2-homologous *Paramecium* amino acid sequence was deduced from the nucleotide sequence (72239-722541) of scaffold 1 in *Paramecium* database (<http://paramecium.cgm.cnrs-gif.fr>). m – mouse LAMP2 (NM_001017959), r – rat LAMP2 (BC061990), h – human LAMP2a (NM_002294) and LAMP2b (NM_013995).

bellings with two types of gold particles: 5 nm for Rab7 and 10 nm for LAMP-2. A dense labelling of the primary lysosomes with LAMP-2 and Rab7 was observed (Figure 11A-B). Electron microscopic analysis revealed that LAMP-2 was also present in the secondary lysosomes (Figure 11 B-C). Gomori staining proved the presence of acid phosphatase in the primary and secondary lysosomes (*data not shown*).

During phagocytic process, after 5 min of latex particles uptake, LAMP-2 and Rab7 were found in the primary lysosomes (Figure 12A), some of which appeared to undergo fusion with phagosomes (Figure 12A, black arrowhead). Most of the primary lysosomes displayed both LAMP-2 and Rab7 label and very few were only Rab7 positive (Figure 12A-B, white arrow). Tubular membrane structures were also visible (Figure 12A, black arrow) indicative of remodelling of phagosomal membranes characteristic for progression of maturation process. After 15 min of latex internalisation, both the antigens were detected on the membrane of the forming phagolysosome (Figure 12B, white arrowhead).

Discussion

Phagosome maturation process culminates in the fusion of phagosomes with lysosomes and acquisition of the capability to digest vacuolar content (Desjardins *et al.* 1994). Based on our results we propose that Rab7-interacting partners: homologues of its effector RILP, LAMP-2 and $\alpha 7$ subunit of the 26S proteasome participate in this process in unicellular eukaryote *Paramecium*.

One of the important proteins involved in biogenesis of phagolysosomes is Rab7 (Harrison *et al.* 2003; Colucci *et al.* 2005). Vesicular trafficking by means of Rab proteins requires their interaction

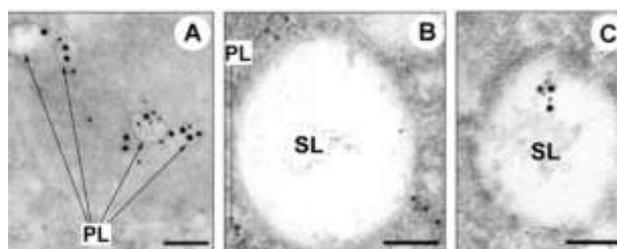


Figure 11. Ultrastructural localisation of Rab7 and LAMP-2 in *Paramecium* lysosomes using double immunogold labelling. Rab7 decorated with 5 nm gold particles and LAMP-2 – with 10 nm particles. Rab7 and LAMP-2 labelling was observed: (A, B) in the primary lysosomes (PL) and (B, C) secondary lysosomes (SL). Bar, 100 nm.

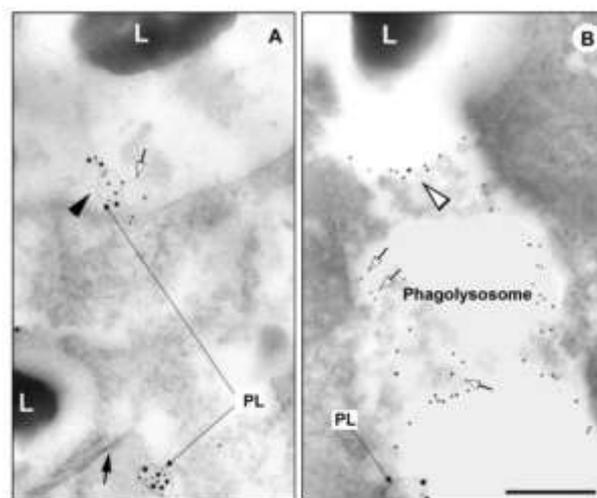


Figure 12. Electron microscopic study on Rab7 and LAMP-2 presence in *Paramecium* phagolysosomal compartment during latex beads (L) internalisation. (A) 5 min uptake and (B) 15 min. Double immunodetection with 5 nm gold particles for Rab7 and 10 nm gold particles for LAMP-2. Colocalisation of these proteins was observed in: (A) most of the primary lysosomes (PL), some of which appeared to fuse with phagosome (black arrowhead) containing latex beads and (B) on the membrane of forming phagolysosome (white arrowhead). A few primary lysosomes were only Rab7-positive (A, B, white arrow). Tubular membrane structures are visible in A (black arrow) indicative of rearrangement of phagosomal stages. Bar, 200 nm.

with a variety of effectors (Zerial and McBride 2001): RILP plays this role for Rab7 (Cantalupo *et al.* 2001; Jordens *et al.* 2001). Wang and coworkers (2004) localised a 62-residue region of RILP (272-333) that is important in regulating of lysosomal morphology whereas the region 315-342 is responsible for the ability of RILP to induce the recruitment of the dynein-dynactin complex (Marsman *et al.* 2006). In HeLa cells RILP was recruiting these motor molecules on Rab7-GTP-positive phagosomes thus enabling displacement of phagosomes along the microtubules (Colucci *et al.* 2005).

We identified gene and mRNA fragments encoding two RILP-related polypeptides (83% identical), indicative of the presence of two genes encoding these proteins in *Paramecium* consistently with a recent concept that most of *Paramecium* genes arose through successive whole-genome duplications (Aury et al. 2006). Paralogous genes in *Paramecium* show a very high identity (Perez-Castineira et al. 2002; Wiejak et al. 2004a; Surmacz et al. 2006). The deduced amino acid sequences of *Paramecium* RILP1 and RILP2 show 60.5% and 58.3% similarity, respectively, to the region essential for lysosomal morphology and dynein-dynactin recruitment (Wang et al. 2004; Marsman et al. 2006) in human RILP. This seems to be a quite interesting data taking into account that RILP was found to be poorly conserved in evolution (Wang et al. 2004). As far as we know this is the first indication of expression of genes encoding RILP-related proteins in the lower eukaryotes. In mammals there are two RILP-like proteins (RILP1 and RILP2) lacking a 62-residue region unique to RILP and not involved in regulating lysosomal morphology (Wang et al. 2004). No data are available on either RILP or RILP in lower eukaryotes.

At the protein level Western blot analysis revealed the presence of RILP-cross-reactive polypeptide of ~45 kDa in *Paramecium* consistently with the data on mammalian counterparts (Cantalupo et al. 2001; Jordens et al. 2001). In the ultrastructural analysis Rab7 and RILP were found in the primary lysosomes and phagolysosomes in the cells phagocytosing both latex beads and bacteria. Such a result may suggest that RILP is one of the proteins interacting with Rab7 in this cell that may link elements of endocytic machinery with molecular motor thus enabling transport of primary lysosomes and their fusion with phagosomes. This is consistent with the Rab7 role in phagosome maturation (Rupper et al. 2001; Vieira et al. 2002). In mammalian RAW 264.7 macrophages Rab7 recruitment to phagosomes was found to precede and to be essential for their fusion with late endosomes and/or lysosomes (Harrison et al. 2003). The presence of Rab7 in lower eukaryotes was first observed in *Dictyostelium discoideum* by Temesvari et al. (1994) who reported Rab7 association with lysosomal membrane. Buczynski and coworkers (1997) described that soon after formation, phagosomes in this amoeba fused with Rab7-positive lysosomes or postlysosomes and that Rab7 remained associated

with phagosomes for at least 4 h and regulated delivery of some of the lysosomal enzymes (Rupper et al. 2001). Orthologue of Rab7 was reported in *Trichomonas vaginalis* (Lal et al. 2005) but no data are available on its localisation and function. In *Entamoeba histolytica* EhRab7A, an amoebic homologue of human Rab7, was localised to uncharacterised vesicles that fuse with lysosomes to form prephagosomal vacuoles. Some other Rab7 isoforms in this parasite were associated with latex bead-containing phagosomes but RILP was not found in this cell (Okada and Nozaki 2006).

Dong and coworkers (2004) described another partner of Rab7 – XAPC7, an α -subunit of the 26S proteasome that is highly conserved and expressed in numerous organisms.

A central 20S degradative unit of this proteasome is composed of at least 14 subunits (seven α -subunits and seven β -subunits) of Mr 21 to 34 kDa with different isoforms identified (Huang and Burlingame 2005). Consistently with these data two cross-reacting polypeptides of ~22 and ~24 kDa were detected in *Paramecium* protein fractions. Several sequences encoding this subunit may be found in *Paramecium* database (data not shown). In macrophages proteasomes played a role at a precise point during phagolysosome biogenesis and in the processing of phagosomal membrane proteins (Houde et al. 2003; Lee et al. 2005). Most probably a similar function in phagosome maturation is fulfilled by proteasome in *Paramecium* detected in the small vesicles in the vicinity of the phagosomal membrane where it partially colocalised with Rab7, but it was not present in LAMP-2-positive primary lysosomes. Huynh and coworkers (2007) have recently shown that LAMPs were delivered to phagosomes during maturation process. LAMP-1 and LAMP-2 double deficient mice ingested particles but phagosome maturation was arrested. Such phagosomes failed to recruit Rab7 and did not fuse with the lysosomes.

No data are available concerning the presence of LAMP-2 in lower eukaryotes. It has been shown that there are many other high molecular weight integral membrane glycoproteins in purified lysosomal membranes from *D. discoideum* (Temesvari et al. 1994), one of which, LmpB was delivered to nascent phagosomes (Gotthardt et al. 2002).

We reported here the presence of LAMP-2-related protein in *Paramecium* and showed one of its gene fragments encoding the sequence displaying

35.4% similarity to mammalian LAMP-2. Quantitative immunoblotting revealed that during phagocytosis the expression level of LAMP-2 was ~2.5-fold increased. LAMP-2-related polypeptide in *Paramecium* was found to be glycosylated. The carbohydrate constitutes 55–65% of the total mass in the LAMP molecules and this posttranslational modification is essential for the stability of the proteins in the lysosomal membrane (Eskelinen *et al.* 2005). Lysosomes in *Paramecium* are present in two populations: the small primary lysosomes arising from *trans Golgi network* and the larger secondary lysosomes that bind to the DV-II. Primary lysosomes (loaded with hydrolases) may either fuse with the DV-II or with the secondary lysosomes (Allen and Fok 2000) that are characterised by thick membrane and prominent glycocalyx lining the luminal surface (Fok *et al.* 1987). We observed the colocalisation of LAMP-2 and Rab7 in both types of lysosomes and on the phagolysosome membranes. This is consistent with the data of Rabinowitz *et al.* (1992) who reported that phagolysosomes in mouse peritoneal macrophages were enriched in LAMPs and Rab7. Hmama and coworkers (2004) observed that the frequencies of phagosomes expressing LAMP and Rab7 increased with time.

Our studies showed the colocalisation of Rab7 with RILP and LAMP-2 in most of the *Paramecium* primary lysosomes, some of which apparently at the moment of fusion with phagosome, and on the membrane of thus forming phagolysosome. These observations seem to be consistent with recent model of Huynh *et al.* (2007) who reported that Rab7, RILP and LAMP are necessary for phagosome maturation and proposed that a tripartite complex involving these proteins is required for association with motor molecules. In *Paramecium* phagosome maturation process is initiated upon fusion of phagosomes (DV-II) with the primary lysosomes to form phagolysosome (digestive vacuole stage III; DV-III). This fusion is 'all or none' event and a specific, though unknown, trigger was suggested to evoke such a synchronous process (Allen and Fok 2000). Since Rab7 GTP-ases participate not only in maturation of phagosomes but also in coordination of their fusion with lysosomes (Rupper *et al.* 2001; Vieira *et al.* 2002; Harrison *et al.* 2003) it is reasonable to suppose that Rab7 and its effector(s)/partners in *Paramecium* may play a role of this trigger.

Acknowledgements

This study was supported by the statutory funds to the Nencki Institute of Experimental Biology and grant Nr 2 P04A 020 29 from the Ministry of Science and Higher Education. Authors are very grateful to Dr. J. Neefjes from The Netherlands Cancer Institute in Amsterdam for providing the anti-RILP antibody and Dr. C. Bucci from Università degli Studi di Lecce, Italy for sharing the C-19 antibody against Rab7 that has been used for pilot experiments.

References

- Allen RD, Fok A. Membrane trafficking and processing in *Paramecium*. *Int Rev Cytol* 2000; 198:277-318.
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; 25:3389-402.
- Aury J-M, Jaillon O, Duret L, Noel B, Jubin C, Porcel BM, et al. Global trends of whole-genome duplications revealed by the ciliate *Paramecium tetraurelia*. *Nature* 2006; 444:171-8.
- Balmer C, Ballhausen D, Bosshard NU, Steinmann B, Boltshauer E, Bauersfeld U, Superti-Furga A. Familial X-linked cardiomyopathy (Danon disease): diagnostic confirmation by mutation analysis of the LAMP2 gene. *Eur J Pediatr* 2005; 164:509-14.
- Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B. Rab7: a key to lysosome biogenesis. *Mol Biol Cell* 2000; 11:467-80.
- Buczynski G, Bush J, Zhang L, Rodriguez-Paris J, Cardelli J. Evidence for a recycling role for Rab7 in regulating a late step in endocytosis and in retention of lysosomal enzymes in *Dictyostelium discoideum*. *Mol Biol Cell* 1997; 8:1343-60.
- Cantalupo G, Alifano P, Roberti V, Bruni CB, Bucci C. Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. *EMBO J* 2001; 20:683-93.
- Colucci AM, Spinosa MR, Bucci C. Expression, assay, and functional properties of RILP. *Meth Enz* 2005; 403:664-75.
- Desjardins M, Huber LA, Parton RG, Griffiths G. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J Cell Biol* 1994; 124:677-88.
- Dong J, Chen W, Welford A, Wandinger-Ness A. The proteasome alpha-subunit XAPC7 interacts specifically with Rab7 and late endosomes. *J Biol Chem* 2004; 279:21334-42.
- Eskelinen EL, Tanaka Y, Saftig P. At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol* 2003; 13:137-45.
- Eskelinen EL, Cuervo AM, Taylor MR, Nishino I, Blum JS, Dice JF, et al. Unifying nomenclature for the isoforms of the lysosomal membrane protein LAMP-2. *Traffic* 2005; 6:1058-61.
- Feng Y, Press B, Wandinger-Ness A. Rab 7: An important regulator of late endocytic membrane traffic. *J Cell Biol* 1995; 131:1435-52.
- Fok AK, Ueno MS, Azada EA, Allen RD. Phagosomal acidification in *Paramecium*: effects on lysosomal fusion. *Eur J Cell Biol* 1987; 43:412-20.
- Gotthardt D, Warnatz HJ, Henschel O, Bruckert F, Schleicher M, Soldati T. High-resolution dissection of phagosome maturation reveals distinct membrane trafficking phases. *Mol Biol Cell* 2002; 13:3508-20.
- Gough NR, Fambrough DM. Different steady state subcellular distributions of the three splice variants of lysosome-associated membrane protein LAMP-2 are determined largely by the COOH-terminal amino acid residue. *J Cell Biol* 1997; 137:1161-9.
- Gruenberg J. The endocytic pathway: a mosaic of domains. *Nat Rev Mol Cell Biol* 2001; 2:721-30.
- Harrison RE, Bucci C, Vieira OV, Schroer TA, Grinstein S. Phagosomes fuse with late endosomes and/or lysosomes by extension of mem-

- brane protrusions along microtubules: role of Rab7 and RILP. *Mol Cell Biol* 2003; 23:6494-506.
- Hmama Z, Sendide K, Talal A, Garcia R, Dobos K, Reiner NE. Quantitative analysis of phagolysosome fusion in intact cells: inhibition by mycobacterial lipoarabinomannan and rescue by an α ,25-dihydroxyvitamin D₃-phosphoinositide 3-kinase pathway. *J Cell Sci* 2004; 117:2131-9.
- Houde M, Bertholet S, Gagnon E, Brunet S, Goyette G, Laplante A, et al. Phagosomes are competent organelles for antigen cross-presentation. *Nature* 2003; 425:402-6.
- Huang L, Burlingame AL. Comprehensive mass spectrometric analysis of the 20S proteasome complex. *Meth Enz* 2005; 405:187-236.
- Huynh KK, Eskelinen EL, Scott CC, Malevanets A, Saftig P, Grinstein S. LAMP proteins are required for fusion of lysosomes with phagosomes. *EMBO J* 2007; 26:313-24.
- Jordens I, Fernandez-Borja M, Marsman M, Dusseljee S, Janssen L, Calafat J, et al. The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr Biol* 2001; 11:1680-5.
- Kornfeld S, Mellman I. The biogenesis of lysosomes. *Annu Rev Cell Biol* 1989; 5:483-525.
- Lal K, Field MC, Carlton JM, Warwicker J, Hirt RP. Identification of a very large Rab GTPase family in the parasitic protozoan *Trichomonas vaginalis*. *Mol Biochem Parasitol* 2005; 143:226-35.
- Lee WL, Kim MK, Schreiber AD, Grinstein S. Role of ubiquitin and proteasomes in phagosome maturation. *Mol Biol Cell* 2005; 16:2077-90.
- Luzio JP, Poupon V, Lindsay MR, Mullock BM, Piper RC, Pryor PR. Membrane dynamics and the biogenesis of lysosomes. *Mol Membr Biol* 2003; 20:141-54.
- Marsman M, Jordens I, Rocha N, Kuijl C, Janssen L, Neeffjes J. A splice variant of RILP induces lysosomal clustering independent of dynein recruitment. *Biochem Biophys Res Commun* 2006; 344:747-56.
- Okada M, Nozaki T. New insights into molecular mechanisms of phagocytosis in *Entamoeba histolytica* by proteomic analysis. *Arch Med Res* 2006; 37:244-52.
- Perez-Castineira JR, Alvar J, Ruiz-Perez LM, Serrano A. Evidence for a wide occurrence of proton-translocating pyrophosphatase genes in parasitic and free living protozoa. *Biochem Biophys Res Commun* 2002; 294:567-73.
- Rabinowitz S, Horstmann H, Gordon S, Griffiths G. Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. *J Cell Biol* 1992; 116:95-112.
- Rupper A, Grove B, Cardelli J. Rab7 regulates phagosome maturation in *Dictyostelium*. *J Cell Sci* 2001; 114:2449-60.
- Sonneborn TM. The *Paramecium aurelia* complex of fourteen sibling species. *Trans Amer Micros Soc* 1975; 94:155-78.
- Surmacz L, Wiejak J, Wyroba E. Alteration in the protein pattern of subcellular fractions isolated from *Paramecium* cells suppressed in phagocytosis. *Folia Histochem Cytochem* 2001; 39:301-5.
- Surmacz L, Wiejak J, Wyroba E. Evolutionary conservancy of the endocytic machinery in the unicellular eukaryote *Paramecium*. *Biol Cell* 2003; 95:69-74.
- Surmacz L, Wiejak J, Wyroba E. Cloning of two genes encoding Rab7 in *Paramecium*. *Acta Biochim Pol* 2006; 53:149-56.
- Temesvari L, Rodriguez-Paris J, Bush J, Steck TL, Cardelli J. Characterization of lysosomal membrane proteins of *Dictyostelium discoideum*. A complex population of acidic integral membrane glycoproteins, Rab GTP-binding proteins and vacuolar ATPase subunits. *J Biol Chem* 1994; 269:25719-27.
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; 22:4673-80.
- Wang T, Wong KK, Hong W. A unique region of RILP distinguishes it from its related proteins in its regulation of lysosomal morphology and interaction with Rab7 and Rab34. *Mol Biol Cell* 2004; 15:815-26.
- Vieira OV, Botelho RJ, Grinstein S. Phagosome maturation: aging gracefully. *Biochem J* 2002; 366:689-704.
- Wiejak J, Surmacz L, Wyroba E. Immunoanalogue of vertebrate beta-adrenergic receptor in the unicellular eukaryote *Paramecium*. *Histochem J* 2002; 34:51-6.
- Wiejak J, Surmacz L, Wyroba E. Dynamin involvement in *Paramecium* phagocytosis. *Eur J Protistol* 2003; 39:416-22.
- Wiejak J, Surmacz L, Wyroba E. Dynamin- and clathrin-dependent endocytic pathway in unicellular eukaryote *Paramecium*. *Biochem Cell Biol* 2004a; 82:547-58.
- Wiejak J, Surmacz L, Wyroba E. Dynamin-association with agonist-mediated sequestration of beta-adrenergic receptor in single-cell eukaryote *Paramecium*. *J Exp Biol* 2004b; 207:1625-32.
- Wu M, Wang T, Loh E, Hong W, Song H. Structural basis for recruitment of RILP by small GTPase Rab7. *EMBO J* 2005; 24:1491-501.
- Wyroba E. Stimulation of *Paramecium* phagocytosis by phorbol ester and forskolin. *Cell Biol Int Rep* 1987; 11:657-64.
- Zerial M, McBride H. Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2001; 2:107-17.