MATERIALS AND METHODS

Chapter 2. Materials and Methods

Materials

Acrylamide (Sigma, USA)

Agarose (ICN Biochemicals, USA)

Ammonium Molybdate (Hi-Media, India)

Ammonium Per Sulfate (Sigma, USA)

Ascorbic Acid (Hi-Media, India)

ATP (Hi-Media, India)

Bis-Acrylamide (Sigma, USA)

Bradford Protein Assay Reagent (Biorad, USA)

Brewers Thioglycolate Medium (Hi-Media, India)

Bromophenol Blue (Sigma, USA)

BSA (Hi-Media, India)

Chemiluminescent Reagent Kit (Amersham, USA)

Coomassie Blue (Sigma, USA)

Dextran T-40 (Pharmacia, USA)

Dialysis Membrane (Hi-Media, India)

DMEM (Hi-Media, India)

E-64 (ICN Biochemicals, USA)

EDTA (ICN Biochemicals, USA)

Ferric Chloride (Sigma, USA; Hi-Media, India)

Ferrous Chloride (Sigma, USA; Merck, Germany)

Fetal Calf Serum (ICN Biochemicals, USA)

FITC-dextran (mol wt 70,000, Sigma, USA)

Formaldehyde (Qualigens, India)

Formvar Coated Grids (Ted Pella, USA)

Gelatin (Hi-Media, India)

Gentamycin (ICN Biochemicals, USA)

Glycerol (Sigma, USA)

Glycine (Hi-Media, India)

Hemoglobin (Hi-Media, India)

HEPES (Sigma, USA)

HPTLC Plates (12x10 cm, cat.1.05554.007, Merck, USA)

Imidazole (Sigma, USA)

Kodak MR™ film (Kodak, India)

Leupeptin (ICN Biochemicals, USA)

Lipid standards (Pierce, USA)

LPS (*Escherechia coli* LPS 0111:B4 Sigma, USA)

Mercaptoethanol (Sigma, USA)

NBD-Cl (ICN Biochemicals, USA)

NBT (ICN Biochemicals, USA)

Nigericin (Sigma, USA)

No.1 Thickness Cover Slips (Pierce, USA)

Perchloric Acid (Qualigens, India)

PMSF (ICN Biochemicals, USA)

pNPP (ICN Biochemicals, USA)

Polycarbonate filters, 5μm and 8μm (Poretics, USA)

Protease Inhibitor Cocktail (Roche Scientific, USA)

PVDF Sheets (Amersham, USA)

Rats (Sri Venkateshwara Enterprises, India)

RPMI 1640 (Hi-Media, India)

SDS (Sigma, USA)

Skimmed Milk (Hi-Media, India)

Sodium Succinate (Sigma, USA)

Stainless steel wire mesh (FE205110, 25μm diameter, Goodfellow Inc, USA)

Super Signal Dura™ Substrate (Pierce, USA)

TEMED (Sigma, USA)

Tris Buffer (Sigma, USA)

Triton-X-100 (Sigma, USA)

Trypan Blue (Hi-Media, India)

Uranyl Acetate (Merck , USA)

Primary antibodies

Goat anti human Cathepsin B (Santa Cruz Biotech, USA)

Goat anti rat Cathepsin D (Santa Cruz Biotech, USA)

Goat anti human Cathepsin H (Santa Cruz Biotech, USA)

Goat anti human LAMP-1 (Santa Cruz Biotech, USA)

Rabbit anti human Rab 5B (Santa Cruz Biotech, USA)

Rabbit anti human Rab 7 (Santa Cruz Biotech, USA)

Secondary antibodies

Rabbit anti goat IgG - HRP conjugate (Bangalore Genei, India)

Goat anti rabbit IgG - HRP conjugate (Bangalore Genei, India)

Goat anti rabbit IgG - Alexa Fluor-568 conjugate (Molecular Probes, USA)

All other reagents were of analytical grade.

Methods

2.1 Animal usage, primary cell culture and cell lines.

2.1.1 Animal protocol

Adult Sprague-Dawley rats (8 - 12 weeks old) maintained in PERD Centre Animal Core facility, were allowed to acclimatize for 1 week, prior to the start of experiments at which time they weighed approximately 300 ± 30 gm. Food and water were provided *ad libitum* and the animals were housed in rooms with 22°C temperature and 40 - 60% humidity. A 12:12 h light:dark cycle was maintained. Animals were randomly separated in three sets. The first set was injected with 10 ml 12% sterile brewers thioglycolate medium 72 h prior to euthanization, by carbon dioxide inhalation, through intraperitoneal (*i.p.*) route as described by Adams (2). In the second set, previously thioglycolate treated rats were injected *i.p.* with sublethal dose (7.5 mg/kg) of LPS 18 h before euthanization as described by Ghiselli *et al.* (71). Animals in the third set were similarly injected with 10 ml sterile physiological saline for isolation of resident (unstimulated) peritoneal macrophages.

2.1.2 Isolation of rat peritoneal macrophages

Rat peritoneal macrophages were isolated from the above mentioned three groups of animals by procedure described by Adams (2). Following appropriate treatment selected animals were euthanised by carbon dioxide inhalation. Subsequently 10 ml of sterile HBSS (Hanks Basal Salt Solution) was injected into the peritoneal cavity of individual animal. The distended abdomen was massaged briefly and the injected HBSS was retrieved with a sterile hypodermic syringe. The resulting lavage was centrifuged at 700 x g for 10 min at 4°C in a refrigerated tabletop centrifuge (32R, Hettich, Germany). The supernatant was gently decanted; the pellet was aseptically suspended in DMEM. Cell viability and number was determined using trypan blue exclusion (86) and manual counting in Neubauer chamber. Finally, the cells were re-suspended in fresh DMEM at $2x10^7$ /ml concentration.

2.1.3 Maintenance of cell line

Lung macrophage cell line U937, a gift from Prof. S. Banerjee of Indian Institute of Chemical Biology India (IICB), was maintained in the cell culture facility of IICB. The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and gentamycin (50 mg/ml) at 37°C in a cell culture incubator, under similar conditions described above.

2.2 Isolation of endosomes

2.2.1 Endocytic probes

A. FITC-dextran

Fresh suspension of FITC-dextran (10 mg/ml in DMEM) was used as stock and appropriate volume was added to achieve a final concentration 0.1 mg - 1.0 mg/ml to the experimental plates when required.

B. Colloidal iron-dextran

Iron particles were prepared as described by Rodriguez-Paris *et al.* (174). This involved mixing 5 ml of 1.2 M ferrous chloride with 5 ml of 1.8 M ferric chloride. To this mixture, 5 ml concentrated ammonium hydroxide (30%, v/v) was added while gentle stirring at room temperature. The iron rich precipitated product of the reaction was sequestered using a large permanent magnet and washed batchwise, once with 5% (v/v) ammonium hydroxide and twice with deionized water. The residue was resuspended in 40 ml of 0.3 M hydrochloric acid and stirred for 30 min. Dextran T-40 was added (2 gm) and the colloidal suspension stirred. The suspension was dialyzed extensively against cold de-ionized water for 2 days. Large aggregates were removed by centrifugation at 20,000 x g, for 10 min in a Sorvall SS-34 rotor (RC5C, Sorvall, USA). The iron concentration of the resultant suspension was estimated chemically (66) and the stability of the colloidal suspension at various temperature and pH ranges was monitored.

C. Assessment of the effect of endocytic probes on cell viability.

Pinocytic uptake of both endosomal probes (iron-dextran and FITC-dextran) by rat primary macrophage cultures was carried out as described by Perrin-Cocon *et al.* and Rodriguez-Paris *et al.* (160, 174), after necessary standardization. Primary cultures of rat peritoneal macrophages were exposed to iron-dextran at 1 mg, 2 mg or 3 mg/ml with or without FITC-dextran at 0.5 mg, 1 mg or 2 mg/ml. Individual sets were incubated for 2 h (pulse) at 37°C, 70% humidity, 5% CO_2 in a cell culture incubator. The cells were then scrapped off with a rubber policeman, washed three times with 5 ml of ice cold buffer H (250 mM sucrose, 1 mM EDTA, 1 mM PMSF, 5 mM HEPES, pH 7.4) at 1000 x g, 4°C, 5 min and checked for viability using the trypan blue exclusion method (86). The efficiency of endocytic vesicle isolation and quantity of endocytosed probe (FITC-dextran) in each set was determined by methods described in section 2.3.2.

2.2.2 Fluid-phase pinocytosis of endosomal probes

Peritoneal macrophages from three different sets of animals (described in detail in section C) were used for experiments: *(i) thioglycolate elicited rat peritoneal macrophages, (ii) macrophages from thioglycolate treated animals under inflammatory (LPS) stimulus,* and *(iii) resident peritoneal macrophages.*

Peritoneal macrophages isolated as in section 2.1.1 and 2.1.2 were re-suspended in fresh DMEM at $2x10^7$ cells/ml concentration, transferred (total 5 ml, 100 µl/well) in 24 well plastic cell culture plates or 25 ml cell culture flasks (Tarson, India) and incubated in cell culture incubator set at 37°C, 70% humidity, 5% CO_2, 95% air for 1 h. Non-adherent cells were removed by washing thrice with HBSS medium (5 ml, 5 min each wash). The primary cultures from each set were then pulsed with a constant final concentration of 1 mg/ml FITC-dextran and 2 mg/ml iron-dextran in DMEM at 37°C, 70% humidity, 5% CO_2 in a cell culture incubator. Following pulse and chase, cells were immediately kept on ice for 5 min to halt intracellular traffic and then washed thrice in ice cold HBSS to remove excess probe(s). Chasing was carried out in probe free DMEM when indicated at 37°C for specified time.

A. Assays of pinocytic uptake with time

Primary macrophage cultures ($2x10^7$ cells/ml) from set *(i)* were continuously pulsed from 0 - 90 min with either FITC-dextran or FITC-dextran and iron-dextran in DMEM at 37°C, 70% humidity, 5% CO_2 in a cell culture incubator. Similarly, identical sets of primary macrophage cultures ($2x10^7$ cells/ml) from all three sets were continuously pulsed from 0 - 90 min with FITC-dextran and iron-dextran. At every 10 min interval between 0 - 60 min, individual sets were put on ice to halt intracellular traffic, washed three times (1000 x g, 4°C, 5 min) in ice cold HBSS to remove excess probe(s) and suspended in buffer H containing 0.2% Triton-X-100 to permeabilise cells. The total internalized fluorescence was determined as described in section 2.3.2 using excitation at 495 nm and emission at 520 nm, and the data is plotted against time.

B. Assays of pinocytic uptake at different temperatures

The effect of temperature on pinocytic uptake was determined by subjecting identical sets of thioglycolate elicited primary macrophage culture ($2x10^7$ cells/ml) to a constant 45 min pulse (using both probes simultaneously) at temperatures between 0°C - 55°C with 5°C intervals respectively. Following pulse, cells were immediately kept on ice for 5 min to halt intracellular traffic and treated in the same way as described above. The total internalized fluorescence is plotted against temperature.

C. Pulse and chase conditions

For *(i)* and *(ii)* an initial pulse feeding of 60 min without any chase was generally used except where specified. Subsequently, as indicated below, various pulse/chase regiments were developed for the respective sets.

(i) *thioglycolate elicited rat peritoneal macrophages: (a) 5 min pulse with, or without 30 min chase (5'/0', 5'/30'); (b) 15 min pulse with or without 30 min and 60 min chase (15'/0', 15'/30',15'/60').*

(ii) *macrophages from thioglycolate-LPS treated animals: (a) 15 min pulse with, or without 30 min. chase (15'/0', 15'/30')*

(iii) *resident macrophages: (a) 5 min pulse with no chase (5'/0')*

Subsequent to pulse and chase, cells of each set were re-suspended in buffer H at a concentration of $2x10^7$/ml, homogenized and subjected to endosome purification using electromagnetic chromatography as described in section 2.2.3 and 2.2.4 respectively.

2.2.3 Subcellular fractionation

A. Cell disruption

The efficiency of the three methods (as mentioned below), to homogenize primary cultures of rat peritoneal macrophages was compared. In each case, cells were washed (1000 x g, 4°C, 5 min) in HBSS medium and then suspended in buffer H at a cell concentration of $2x10^7$/ml. After homogenization, the homogenate was viewed under a 40x lens (400x total magnification) of an Olympus epifluorescence microscope (BX41TF, Olympus, Japan), equipped for phase-contrast optics, to assess cell disruption. Following this, the homogenate was centrifuged at 14000 x g, 10 min, 4°C to pellet down intact cells, cellular debris, nuclei, intracellular organelles and membrane fractions. Efficiency of disruption was estimated by determining both the relative number of disrupted cells following homogenization and the relative levels of lysosomal marker enzyme acid phosphatase, in the pelleted intact lysosome like vesicles, following centrifugation.

(i) Membrane filtration: Macrophage cells were subjected to forced passage through series of 5 μm and 8 μm polycarbonate filters attached to a syringe (5 ml) assembly following the protocol described by Padh *et al.* (155) with slight modification. Cell suspension, at a concentration of $2x10^7$/ml was forced through the filters.

(ii) Dounce homogenization: Similar cell suspension described above in buffer H was transferred into a Dounce homogenizer. Homogenization was carried out on ice for 1 min at medium rotational speed.

(iii) Sonication: Sonication was performed by a modification of the procedure of Lovdal *et al.* (123) using a MSE Soniprep 150 Ultrasonic disintegrator at minimum speed for 3 min (6 pulses, each pulse set for 30 sec for a 2 ml volume) on ice.

B. Preparation of post nuclear supernatant (PNS)

Following homogenization, the crude cell lysate was centrifuged at 760 x g, 4°C, 10 min. Nuclei and intact cells were removed and the post nuclear supernatant (PNS) was collected. Aliquots of PNS were immediately fractionated to isolate endosomal vesicles; other aliquots were subjected to biochemical analysis to estimate subcellular organelles or stored at -80°C for related experiments.

2.2.4 Electromagnetic purification of endosomes

Electromagnetic column chromatography was performed according to protocol described by Padh *et al.* (150,151) using an electromagnet operated at 0.8 Tesla with two poles kept 1.5 cm apart. A fine stainless steel wire mesh was placed at the bottom of a water jacketed glass column (0.5 cm internal diameter, 10 cm long). The column was equilibrated with buffer H and mounted between the two electromagnetic poles. The circulating cold water in the jacket around column maintained the temperature in the column at ~4°C. PNS was added on to the top of the column at a flow rate of 1 ml/min and the flow-through (FT), which was not retained in the column was collected. The column was subsequently washed with ten volumes of buffer H to remove unbound material. The retained fraction (RF) in the column, which was magnetically purified following affinity chromatography was eluted by withdrawing (switching off) the magnetic field and eluting the column with two volumes of buffer H at 1 ml/min. The eluate (RF) was subjected to various analysis outlined in subsequent sections.

2.3 Characterization of endosomes

2.3.1 Protein and lipid quantitation

Total protein was quantitated by the method of Bradford (24), with the help of Bradford protein assay reagent. A fixed volume of the dye (200 µl) was mixed with 800 µl of sample and incubated at room temperature for 10 min. Spectrophotometric reading at 595 nm was quantitated against calibration curve using known amount of BSA as standard.

The total phospholipid content of the samples was measured by the method of Rouser *et al.* by measuring the total inorganic phosphate in lipid samples (176). Vacuum dried samples were reconstituted in 0.3 ml (70%) perchloric acid and heated for 20 min at 180°C. Subsequently 1.0 ml de-ionized water, 0.4 ml ammonium molybdate (1.25% w/v) and 0.4 ml ascorbic acid (5% w/v) were added sequentially. The samples were then heated for 5 min at 100°C. Optical absorbance was read at 810 nm in a spectrophotometer (RS 232C, Elico, India) and the inorganic phosphate content was estimated against calibration curve using potassium di-hydrogen phosphate as standard.

2.3.2 Fluorescence analysis

Analysis of internalization of probe (FITC-dextran) was performed as described by Padh *et al.* (151). The RF, PNS or appropriate controls were adjusted to 1.5 ml in buffer H containing 0.2% of Triton-X-100. The fluorescence was measured in a spectrofluorimeter (SL174, Elico, India). Excitation wavelength was set at 450 nm or 495 nm and emission wavelength was 520 nm. The specific activity of respective fractions was expressed as relative fluorescence/ total protein (μg). The yield of purified endosome internalized (FITC) fluorescence was expressed as the percentage of intravesicular fluorescence in the PNS that is present in the purified (RF) fraction. Relative fluorescence intensity was measured with excitation wavelength set at 495 nm and emission wavelength set at 520 nm while relative fluorescence ratio was measured with excitation wavelengths set at 450 nm and 495 nm and emission wavelength set at 520 nm.

2.3.3 Analysis of intravesicular pH of endosomes

A. Development of pH dependent fluorescence calibration curve for FITC

Calibration curve for pH dependent fluorescence intensity of FITC was prepared as follows. FITC-dextran (0.01 mg/ml) was prepared in 10 mM HEPES, pH 7.4. The pH of different aliquots were adjusted between 3.5 - 8.0 (1.5 pH unit increments) using dilute hydrochloric acid or sodium hydroxide. Fluorescence intensities were measured

with emission wavelength set at 520 nm and excitation wavelengths set at 450 and 495 nm. The ratio of the fluorescence intensity with excitation at 450 and 495 nm was plotted as a function of pH.

Similar curves were also prepared for intracellular FITC. Macrophage primary cultures were pulsed for 15 min and subsequently washed thrice in ice cold buffer H to remove excess probes. Cells were then permealibized by incubating aliquots in buffer H of different pH ranges (pH 3.5 - 8.0), containing 0.1% formaldehyde, for 10 min at 37°C. The ratio of the fluorescence intensity was determined as described above.

B. Intravesicular pH of endosomes

Primary cultures of macrophage cells were pulsed for 15 min and kept immediately on ice to stop intracellular traffic. The cells were then washed with ice cold buffer H to remove excess FITC-dextran and aliquoted in two sets. One set was stored at 0° - 4°C (15'/0') and the other was incubated in DMEM at 37°C for 60 min (15'/60'). Both sets (15'/0' and 15'/60') were washed and resuspended in cold buffer H. The relative fluorescence ratio was compared with standard curve and the pH of the system was determined.

In yet another set of experiments vesicular pH of magnetically purified endosome rich fractions (15'/0' and 15'/60') was determined.

2.3.4 *In vitro* acidification of endosomes

In vitro acidification of endocytic vesicles was initiated by ATP as described by Padh *et al.* and Perrin-Cocon *et al.* (152, 160) with slight modification. Affinity purified endosome rich fractions (15'/0') were incubated with 1 mM ATP in 125 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 10 mM HEPES, pH 7.4 at 37°C. Samples were analyzed at every 30 sec interval for relative fluorescence using excitation wavelengths set at 450 and 495 nm and emission wavelength set at 520 nm. After 210 sec, 10 μM nigericin was added to the system to disrupt the ATP induced proton gradient. In yet another set of experiment only 10 μM nigericin was added to the endosome rich fractions

after 210 sec, without any ATP addition. Control sets were not exposed to either reagent.

2.3.5 Biochemical characterization of isolated endosomes

A. Assay for succinate dehydrogenase

Succinate Dehydrogenase was assayed according to the protocol of Padh *et al.* (153) with slight modifications. RF, PNS or appropriate controls adjusted to 0.5 ml in buffer H, were diluted with 0.2 ml buffer B (200 mM sodium phosphate buffer, pH 7.4). To this, 0.1 ml of 2.5 mg/ml NBT was added, followed by 0.1 ml substrate B (100 mM sodium succinate, pH 7). The tubes were incubated at 37°C for 30 min. The reaction was stopped by adding 2.0 ml of 2% SDS to each tube. The absorbance was measured at 630 nm in a spectrophotometer calibrated against reagent blanks.

B. Assay for acid phosphatase

Acid Phosphatase was assayed according to the protocol of Padh *et al.* (153) with slight modification. RF, PNS or appropriate controls adjusted to 0.1ml in buffer H, were diluted with 0.1 ml buffer C (0.25 M glycine-HCl, pH 3.0, containing 0.5% Triton X-100). To this, 50 µl substrate C (50 mM pNPP in water) was added. The tubes were incubated at 37°C for 1 h. The reaction was stopped by adding 2.75 ml of ice cold 0.2 M tri-sodium phosphate (pH 12). The absorbance was measured at 405 nm in a spectrophotometer calibrated against reagent blanks.

C. Assay for vacuolar H^+-ATPase

Vacuolar H^+-ATPase was assayed according to the procedure of Padh *et al.* (154). RF, PNS or appropriate controls were incubated in 35 µl imidazole hydrochloride (250 mM) pH 7.0; 5 µl magnesium chloride (350 mM); 20 µl potassium chloride (350 mM). The reaction volume was adjusted to 260 µl with water. To this 10 µl of water or NBD-Cl (0.88 mM) was added. The reaction mixtures were preincubated at 30°C for 5 min before the addition of 20 µl of (35 mM ATP) pH 7.0. After 10 min incubation at 30°C, the reaction was terminated by the addition of 0.7 ml of 2%

sulphuric acid containing 0.5% SDS and 0.5% ammonium molybdate, followed by 10 µl of 10% ascorbic acid. Inorganic phosphate (P*i*) generated by ATPase activity was determined as the molybdate complex, measured at 750 nm using potassium di-hydrogen phosphate as standard. Vacuolar H^+-ATPase activity was defined operationally as the difference in the rate of ATP hydrolysis in the presence and absence of 25 µM NBD-CI.

D. RNA estimation

RNA was quantified by a procedure of Reinhart *et al.* (169) with slight modification. 0.1 ml of RF, PNS or appropriate controls were added in duplicates (a and b) to 1 ml of 6% perchloric acid contained in 1.5 ml microfuge tubes and kept on ice for at least 3 h. The precipitate was collected by centrifugation for 7 min at 15,000 x g and was washed once with 1 ml of 3% perchloric acid. Following recentrifugation, the pellets were dissolved in either 0.2 ml of 1 N NaOH (a) or 0.2 ml of 3% perchloric acid (b) and allowed to stand for 1 h at 37°C (for a) or for 0 h at 37°C (for b). To the hydrolysate was added 0.8 ml of ice-cold 3% perchloric acid and the precipitate was removed by centrifugation for 7 min at 15,000 x g. The absorbance of the resultant supernatant was measured at 260 nm in a spectrophotometer calibrated against 3% perchloric acid.

2.3.6 Fluorescence microscopy of endosomes

Epifluorescence microscopy was performed on FITC-dextran fed macrophages, respective PNS fractions, RF and FT fractions using protocol described by Racoosin *et al.* with slight modification (167). The PNS, RF or FT fraction(s) following chromatography were mounted on individual glass slides and covered with 2 cm, square No.1 thickness cover slips. The edges of the coverslips were sealed with nail polish and the slides were viewed under a 100x oil immersion lens (total magnification 1000x) using fluorescein filters set in an Olympus epifluorescence microscope (BX41TF, Olympus, Japan). Typically phase bright vesicles were compared with those exhibiting bright fluorescence for FITC. Photographs were taken with an Olympus Digital Camera (Japan) using 10 - 40x zoom.

2.3.7 Electron microscopy of endosomes

Electron microscopy of vesicles was performed according to the protocol described by Perrin-Cocon *et al.* (160) with slight modification. Briefly, colloidal iron-dextran and purified endosomal vesicles were adsorbed on to formvar coated grids and washed once with buffer H. The grids were then fixed in 1% (v/v) paraformaldehyde. Negative staining of the vesicles was performed by incubating the grids in 1% (w/v) uranyl acetate. The grids were subsequently analysed with a ZEISS EM 900 Transmission Microscope (West Germany) operated at 50 keV.

2.3.8 Confocal microscopy

Macrophage cell line was cultured as described in section 2.1.3. Two identical sets were treated with 0.1 μg/ml (experimental) or without (control) LPS for 18 h before the experiment. Cells were then washed with fresh DMEM. Fluid phase pinocytic uptake of FITC-dextran was performed by the same protocol used for primary macrophage culture described in section 2.2.2. Briefly, cells at $2x10^7$/ml density were kept on ice for 5 min to arrest intracellular traffic. Cells were immediately transferred to fresh RPMI 1640 media kept at 37°C containing 1 mg/ml FITC-dextran. The cells were continuously pulsed for 10 min and subsequently chased for 40 min in the absence of FITC-dextran. Aliquots taken at 10 min pulse and after 40 min chase, were immediately transferred on ice.

Cells were subsequently analyzed by confocal microscopy using protocol described by Welter *et al.* (229) with slight modification. Briefly, cells were washed in cold HBSS media twice (5 ml each wash) to remove excess probe(s) and fixed in 2% paraformaldehyde in PBS for 15 min at 4°C. After fixation, cells were washed with cold PBS and permeabilized with 0.2% Triton-X-100 in PBS for 10 min at room temperature. Cells were blocked with 3% BSA in PBS and incubated with primary or secondary antibodies in blocking solution for 1 h at 37°C. The concentration of primary antibodies were: 1:500 for both the rabbit polyclonal anti human Rab 5B and anti human Rab 7 l respectively. Goat anti-rabbit Alexa Fluor-568 tagged secondary antibody was used at a dilution of 1:500. After incubation with antibodies, cells were washed four times with 5 ml PBS for 5 min each wash, at room

temperature. Confocal microscopy was performed on Leica SP-2 Confocal Laser Scanning Microscope equipped with argon 488 and krypton 568/647 lasers using a 40x PlanApo 1.4 NA oil objective, with a total magnification of 250x. Adobe Photoshop version 7 (Adobe Systems, USA) software was used for digital image superimposition and enhancement.

2.3.9 Analysis of endosomal proteins

A. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (112) as described by Sambrook *et al.* (182) and Harlow *et al.* (86).

Reagents for stock solutions of SDS gel:

Gel running buffer: 0.025 M Tris (pH 8.3), 192 mM glycine, 0.1% SDS

Resolving buffer: 1.5 M Tris-Cl (pH 8.8)

Stacking buffer: 1.0 M Tris-Cl (pH 6.8)

30% Acrylamide: bis-Acrylamide solution.

10% SDS

10X Running Buffer: 0.25 M Tris, 1.92 M glycine, 1% SDS, pH 8.3

Loading dye: 60 mM Tris-Cl (pH 6.8), 24% glycerol, 2% SDS, 14.4 mM mercaptoethanol, 1% bromophenol blue.

Preparation of SDS gels

Resolving Gel (10%)		**Resolving Gel (12%)**		**Stacking Gel (5%)**	
Water	5.90 ml	Water	5.00 ml	Water	3.40 ml
1.5 M Tris-Cl (pH 8.8)	3.80 ml	1.5 M Tris-Cl (pH 8.8)	3.80 ml	1.0 M Tris-Cl (pH 6.8)	0.63 ml
10% SDS	0.15 ml	10% SDS	0.15 ml	10% SDS	0.05 ml
30% Acrylamide mix	5.00 ml	30% Acrylamide mix	6.00 ml	30% Acrylamide	0.83 ml
10% ammonium persulfate	0.15 ml	10% ammonium persulfate	0.15 ml	10% ammonium persulfate	0.05 ml
TEMED	0.006ml	TEMED	0.006ml	TEMED	0.005ml
Total Volume	**15 ml**	**Total Volume**	**15 ml**	**Total Volume**	**5 ml**

Staining of SDS gels

a. *Coomassie Brilliant Blue:*

Reagents:

0.2% Coomassie brilliant blue in 10% acetic acid, 40% methanol in water.

Destaining solution: 10% acetic acid, 40% methanol in water.

Procedure: Gels were incubated for 1 h in staining solution with shaking and destained as required.

b. *Silver Staining:*

Reagents:

1. Fixing solution I: 50% methanol, 12% glacial acetic acid in water.
2. Fixing solution II: 10% ethanol, 5% glacial acetic acid.
3. Fixing solution III: 10% ethanol.
4. Solution A: 37 µl of formaldehyde (37% w/v), 42 µl of sodium hydrogen sulphide (1M) in water.
5. Solution B: 0.2% silver nitrate in water.
6. Solution C: 0.6% sodium carbonate, 1 µl sodium hydrogen sulphide.
7. Stop solution: 5% glacial acetic acid.

Procedure: Glassware cleaned with deionized water was used for staining. The gel was fixed overnight in solution I at 4°C. This was followed by incubation in solution II for 30 min and then in solution III for 15 min. The fixed gel was then incubated in solution A for 60 sec. After three washes with water the gel was incubated in solution B for 6 - 10 min with gentle shaking. The gel was then washed three times with water and incubated in solution C till bands were visible. Further darkening of bands was blocked by incubating the gel in stop solution.

Analysis of SDS-PAGE

Appropriately stained SDS-PAGE gels were digitally analyzed by a Kodak 1D Proteomic Analysis Workstation using manufacture's instructions and software. Band detection setting was set at highest sensitivity and automatic detection mode. Following identification/demarcation of bands, each set of RF was compared between

(a) their corresponding PNS fraction, and (b) other RF, for unique differences and assigned unique colour codes as follows:

a. Bands unique to any one type of purified fraction (RF): yellow
b. Bands common to any two type of purified fraction (RF): Blue
c. Bands in purified fraction (RF) distinct from the corresponding PNS: white, and
d. Bands common in all purified fractions (RF): none

B. Western blotting

Western blotting was carried out by protocol described by Harlow *et al.* (86) with slight modification. Following electrophoresis the gel was equilibrated in transfer buffer {10 mM Tris, 20 mM glycine, 20% methanol (v/v)}. PVDF sheets were wetted in methanol followed by water and equilibrated in transfer buffer for 10 - 15 min. The gel was placed on top of the PVDF membrane and sandwiched between presoaked whatman paper pads. The system was placed on a semi-dry blotting apparatus (Owl Systems, USA). Transfer was carried out at 120 mA constant current for 30 min. After transfer, the membrane was air dried, rewetted and equilibrated in PBS for 5 min. It was then blocked using nonfat milk for 1 h, washed with PBS once (5 min and 50 ml each wash) and incubated in appropriate primary antibody for 1 h at room temperature or overnight at 4°C. The membrane was washed three times (5 min and 50 ml each wash) with PBST (PBS supplemented with 0.1% Tween-20) and then incubated in appropriate HRP conjugated secondary antibody. The membrane was again washed three times with PBST (5 min and 50 ml each wash), excess buffer allowed to drip off. Equal volume of solution A and B from the chemiluminescent reagent kit was mixed and the membrane was incubated in this solution following manufacturer's instructions. Excess reagent was dripped off; the membrane was enclosed in a transparent plastic jacket and exposed to Kodak MR™ film. Membranes with insufficient signal intensity were treated with Super Signal Dura™ substrate. Whenever required, membranes were stripped according to manufacturer's instruction and re-probed with appropriate antibodies. Bands were scanned and analyzed using the Kodak 1D Image Analysis Software™.

C. Immuno dot blot analysis of endosome fractions

Total protein from macrophage whole cell lysates, the corresponding PNS fraction, and RF were estimated. From each set 10, 5 and 3 μg protein was spotted on a PVDF membrane. The membrane was fixed in 3% non fat (w/v) milk, and then probed with an anti Rab 5 antibody. Immunodetection was performed as described in section 2.3.7(B).

D. Antibodies and conditions

Primary antibodies

Rabbit anti human Rab 5B, 1:1000

Rabbit anti human Rab 7, 1:1500

Goat anti human LAMP-1, 1:1000

Goat anti human Cathepsin B, 1:1000

Goat anti rat Cathepsin D, 1:1000

Goat anti human Cathepsin H, 1:1000

Secondary antibodies

Rabbit anti goat IgG - HRP conjugate, 1:7000

Goat anti rabbit IgG - HRP conjugate, 1:7000

E. Analysis of endosomal proteases by zymography

Zymography was performed in 10% non-reducing SDS-PAGE containing 1.6% hemoglobin or 1 mg/ml gelatin as substrates. The samples were prepared from endosomes isolated from macrophages that were not exposed to protease inhibitors during subcellular fractionation. In order to detect acid protease activity, gels were analysed using conditions described by Ikuzawa *et al.* (95). Samples were incubated with electrophoresis sample buffer without reducing agents at 30°C for 3 h unless otherwise indicated. After electrophoresis, the gel was soaked in 2.5% Triton X-100 in water for 15 min. Following this, gels containing hemoglobin were incubated in

100 mM sodium acetate buffer, pH 4.5, containing 100 mM KCl and 0.1% Triton X-100 at 37°C for 4 h. For gelatin zymography, gels were incubated overnight in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM calcium chloride at 37°C. Following incubation, the gels were rinsed in water, stained with coomassie blue and destained until pale bands of protease activity could be seen on a dark blue background.

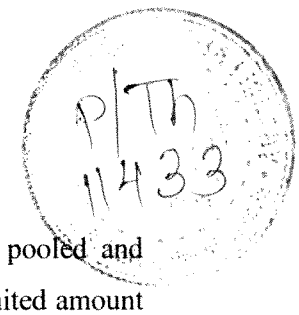
F. Radial Agar Diffusion Assay for total proteolytic activity

Agar based assays for detecting total proteolytic activity of samples were carried out by loading 30 μg (protein) from purified endosome fractions in all wells except B2 (15 μg protein) dug into 2.5% agarose containing 1.6% hemoglobin, 100 mM KCl and 0.1% Triton X-100 in 100 mM sodium acetate buffer, pH 4.5. Affinity purified samples were incubated at 37°C for 1 h, in the presence of broad spectrum protease inhibitor cocktail (well A1), or without addition of inhibitors (well B1 and B2: 30 μg and 15 μg total protein respectively); or incubated independently with 10 mM PMSF (A4), 15 mM EDTA (B3), 50 μM leupeptin (A2), 10 μM E-64 (B4) and 1 μM pepstatin (A3). Following incubation the gel was stained with 1% amido black solution made in 10% acetic acid.

2.3.10 Analysis of endosomal lipids

A. Extraction of lipids

Endosomal lipids were extracted as described by Bligh and Dyer (20) with slight modification suggested by Soldati (74). RF or PNS proteins at a concentration of 100 μg/ml and 525 μg/ml respectively in aqueous phase, was mixed with freshly prepared solution B (50 part chloroform, 100 part methanol, 1.5 part concentrated hydrochloric acid) at 1:3.75 ratio (v/v). The system was vortexed and mixed with 1:1 (v/v) chloroform. After vortexing the mixture again, an equal volume of water was added; samples were vortexed and centrifuged to increase phase separation. The chloroform phase was transferred to a second tube for a second cycle of phase separation. When required, the remaining methanol/water phase from the first tube was back extracted



with chloroform. The total chloroform solubilised lipid samples were pooled and dried completely under nitrogen. Total lipids were then solubilised in limited amount of chloroform:methanol (1:1) mixture and subjected to analysis.

B. HPTLC

HPTLC analysis of endosomal lipids was performed using protocol described by Rodriguez-Paris *et al.* with slight modification (174). HPTLC plates were prewashed in chloroform:methanol:water (60:40:10, v/v/v), dried in air, and activated at 150° - 200°C for 30 min. PNS and corresponding RF samples (10 µg/lane) and lipid standards (1 µg/lane, each standard) were spotted using an automated HPTLC system (Camag HPTLC System, Switzerland). Plates were developed to 9 cm with ethyl acetate/1-propanol/chloroform/methanol/O.25% KCl (25:25:15:20:9, v/v/v/v/v), dried in air, and then developed to the end with hexane/diethyl ether/acetic acid (75:21:4, v/v/v). Bands were developed by spraying 10% cupric sulfate (w/v) made in 8% phosphoric acid (v/v) and then heating at 110°C for 10 min in an oven (177). Plates were scanned and analyzed using the Camag TLC Evaluation Software™.