Indian Journal of Experimental Biology
Vol. 52, April 2014, pp. 295-304

Analysis of rapamycin induced autophagy in *Dictyostelium discoideum*

Pynskhem Bok Swer, Rakhee Lohia[1] & Shweta Saran*
School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India
[1]Department of Genetics, University of Delhi, South Campus, New Delhi 110 021, India

Received 9 July 2013; revised 11 December 2013

Natural autophagy and autophagic cell death is being studied in the model system, *D. discoideum*, which has well known genetic and experimental advantages over the other known systems. There is no apoptotic machinery present in this organism which could interfere with the non-apoptotic cell death. The target of rapamycin (TOR) pathway is a major nutrient-sensing pathway which when inhibited by the drug rapamycin induces autophagy. Rapamycin was originally discovered as an anti-fungal agent but its use was abandoned when it was discovered to have potent immunosuppressive and anti-proliferative properties. It is a known drug used today for various cancer treatments and also for increasing longevity in many model organisms. It has a wide usage but its effects on other pathways or molecules are not known. This model system was used to study the action of rapamycin on autophagy induction. Using the GFP-Atg8, an autophagosome marker, it was shown that rapamycin treatment can induce autophagy by an accumulation of reactive oxygen species and intracellular free calcium. Rapamycin suppresses proliferation by induction of cell cycle arrest in the G1 phase. Taken together, the results suggest that the core machinery for autophagy is conserved in *D. discoideum* and it can serve as a good model system to delineate the action of rapamycin induced autophagy.

Keywords: Autophagy, Calcium, *Dictyostelium*, Rapamycin, ROS

Rapamycin is known to have profound effects on health and longevity in various model organisms[1] and is studied in humans as therapy for treating age-related diseases. In recent years, signaling through the target of rapamycin (TOR) kinase has emerged as a key pathway involved in lifespan extension. Reduction in TOR signaling is sufficient to increase lifespan in yeast[2], *Caenorabditis elegans*[3], *Drosophila*[4,5] and mice[6,7], probably by modulating the mRNA translation and enhanced degradation of damaged macromolecules. Mice with reduced TOR signaling in adipose tissue showed resistance to diet induced obesity and metabolic diseases. Rapamycin is now known to confer protection in model animals and cell based models of neurodegenerative diseases, cardiovascular disease and against a variety of cancers[8].

Rapamycin (sirolimus) is a natural product initially identified as a fungicide[9,10] and later found to be a potent immunosuppressant[11,12]. It has a wide usage but its effects on other pathways or molecules are not understood. Since the studies on humans cannot be performed easily, studies on a variety of model animals is required to understand the potential side effects of the long term use of this drug or its analog.

Dictyostelium discoideum, a protist is an eukaryote occupying a strategic phylogenetic position that represents a transition between unicellular eukaryote and multicellularity[13]. Under nutrient starvation conditions, the free living amoebae chemotax to common collecting points in response to cAMP to form the multicellular culminant having two terminally differentiated cell types, namely the spore and the stalk cells. Terminal differentiation into stalk cells is caspase independent[14] as inactivation of the single para-caspase gene did not show any alteration in the cell death program[15]. The dying stalk cells are marked by the presence of autophagic vacuoles[16]. Nutrient signaling plays an important role in the switch over from unicellular to multicellular forms and also help trigger the process of cell differentiation[17]. The autophagy machinery in *D. discoideum* overlaps considerably with mammals rather than yeast. Knockout mutant strains of *D. discoideum* autophagy genes are blocked in development but not growth[18]. This model system has been exploited to understand the process of autophagy upon treatment with rapamycin.

*Correspondent author
Telephone: (O) 91-11-26704157
E-mail: ssaran@mail.jnu.ac.in

Given the importance of autophagy in varied eukaryotic cellular processes and its possible role in host-pathogen interactions and longevity, two questions have been addressed in the present study: (i) is autophagy induced in *D. discoideum* by the drug rapamycin and (ii) if so, what is the mechanism governing this process of autophagy. The results show that rapamycin induces autophagy via accumulation of reactive oxygen species and intracellular free calcium and thus, *D. discoideum* serves as a good model system to delineate the pathways involved in autophagy.

Materials and Methods

Strain, growth and development—D. discoideum, Ax2 cells and the (*act15/gfp-atg8*)/Ax2 cells (plasmid was obtained from the Dicty stock centre at Northwestern University in Chicago, IL, USA) were used for all the experiments. The wild type and the transformed cells were grown in standard axenic culture medium, HL-5 with the required selection, and were developed on 2% non-nutrient agar plates[19].

Induction and measurement of autophagy—Autophagy was induced by treating the log-phase (~3-4×10^6 cells. mL^{-1}) vegetative cells of (*act15/gfp-atg8*)/Ax2 strain with various doses of rapamycin for varying times. Following induction, the cells were washed in KK_2 buffer[20] and the following parameters were obtained: fluorescence photograph was taken using Nikon microscope, the GFP fluorescence was measured (Ex/Em = 476/504 nm) and the GFP-Atg8 puncta/cell was counted. Cell viability was monitored using 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) reduction assay by the protocol of Mosmann[21].

Effect of autophagy inhibitors 3-methyladenine and wortmannin—To test the effect of the autophagy inhibitors, 3-methyladenine (3-MA) and wortmannin (W), cells were preincubated with of 0.5 mM 3-MA for 1.0 h prior to rapamycin or starvation treatments. Wortmannin (20 nM) was incubated along with rapamycin. The results shown here are an average of 4-6 individual experiments carried out in triplicates.

Measurement of free radicals, lipid peroxidation, intracellular calcium and mitochondrial membrane potential—ROS generation was measured[22] using the dye, 2', 7′-dichlorodihydrofluorescein diacetate (DCF-DA) which was used at a concentration of 15 µM and incubated for 15 min at 22 °C in HL-5 with shaking. After washing the fluorescence was measured at 525 nm after excitation at 495 nm. Untreated (C), rapamycin treated (R) and H_2O_2 treated cells were used for measurement of the ROS levels at different time periods as shown in the respective figures. Preincubations with inhibitors of ROS like N-acetylcysteine (at a concentration of 0.5 mM for 1 h) and diphenyliodonium (at a concentration of 1.0 µM for 30 min) was carried and then treated with either 50 nM rapamycin to induce autophagy or exogenous H_2O_2 (1.0 mM) before measuring the ROS levels over a period of 60 h. The ROS fluorescence data were then finally normalized to the DNA content by using propidium iodide (PI) at a concentration of 8 µM (Ex/Em = 535/617 nm) fluorescence representing DNA content per cell mass.

Lipid peroxidation was measured by determining the thiobarbituric acid reacting substances (TBARS) present in the cytosol[23]. TBARS was extracted into the organic layer by centrifugation at 4,000 *g* for 10 min and the absorbance was measured at 532 nm. The lipid peroxidation level is expressed in nmole MDA equated per mg protein by using the following formula:

$$\text{nmole of MDA} = (\text{V x OD})/(\text{mg protein} \times 0.152),$$

where, V is the final volume of the test solution, and

$$\text{Extinction coefficient, } \varepsilon = 1.52 \times 10^5\ M^{-1}cm^{-1}$$

Intracellular free calcium was measured using Fura 2-AM[24]. Incubation with 1.0 mM $CaCl_2$ in presence of calcium ionophore A23187 at a concentration of 7.0 µM was used to increase the levels of cellular calcium. EGTA (E), a calcium chelator at a concentration of 1.0 mM in presence of the ionophore was used. Fluorescence due to calcium was measured at Ex = 340/380 nm and Em = 510 nm by the Grynkiewicz[25] equation as shown below:

$$[Ca^{2+}]_i\ (nM) = K_d \times [(R - R\text{min})/R\text{max} - R)] \times \text{Sfb}$$

where K_d (for Ca^{2+} binding to fura-2 at 22 °C) = 225 n*M*, *R* = 340/380 ratio, *R*max = 340/380 ratio under Ca^{2+}-saturating conditions, *R*min = 340/380 ratio under Ca^{2+} -free conditions, and Sfb = ratio of baseline fluorescence (380 nm) under Ca^{2+}-free and -bound conditions.

The calcium fluorescence data were then normalized to the DNA content by using propidium iodide (PI) at a concentration of 8 µM (Ex/Em = 535/617 nm).

Mitochondrial membrane potential was measured using the cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1)[26]. JC1 was used at a concentration of 500 nM and incubated at 22 °C for 30 min. The fluorescence was measured at Ex = 514 nm and Em = 543/584 nm and calculated as a ratio of red:green fluorescence. To normalize the measurements, PI at a concentration of 8 µM was added and DNA fluorescence was measured (Ex/Em = 535/617 nm).

All the final concentrations used for the study was selected after individual standardization in the laboratory. The results shown are an average of 4-6 individual experiments carried out in triplicates.

Cell cycle analysis—Untreated (control) and 50 nM rapamycin treated cells at different time (0-60 h) points were collected for cell cycle analyses by flow cytometry (FACS caliber, Becton Dickinson). The samples were prepared and labeled with propidium iodide as per the protocol used by Chen *et al*[27]. The analyses were carried using the Cell Quest programme from Becton Dickinson.

Results

Rapamycin reduces cell viability in D. discoideum—To investigate the sensitivity of *D. discoideum* cells to rapamycin; we determined cell proliferation at various concentrations of the drug over a period of time (Fig. 1a). In comparison to the control, 48 h of incubation with 50 nM rapamycin inhibited cell proliferation by nearly 50%. Interestingly, the proliferation of treated cells did not get completely arrested but continued with a slow growth.

To assess the inhibitory role of rapamycin, treated cells were subjected to MTT cell viability assay which showed a decrease with increasing times of incubation (Fig. 1b) with nearly 65% viable cells after 48 h of treatment. There was a sharp decline in viability after 36 h of treatment.

The wild type control cells showed robust development and formed mounds by 8 h, slugs by 16 h, early culminants by 20 h and fruiting bodies by 24 h after starvation (Fig. 1c, panel Cn). Treatment with rapamycin caused a delay in development and showed aberrant phenotypes. Very large aggregates were formed (at 8 h) which developed multiple tips (at 16 h) that culminated into fruiting bodies having no distinct stalk or spore heads (at 38 h). The basal discs of the resulting culminants were in clusters (Fig. 1c, panel R). Taken together, these observations suggest the presence of a rapamycin dependent TOR signaling cascade in *D. discoideum* involved in both cell proliferation and differentiation.

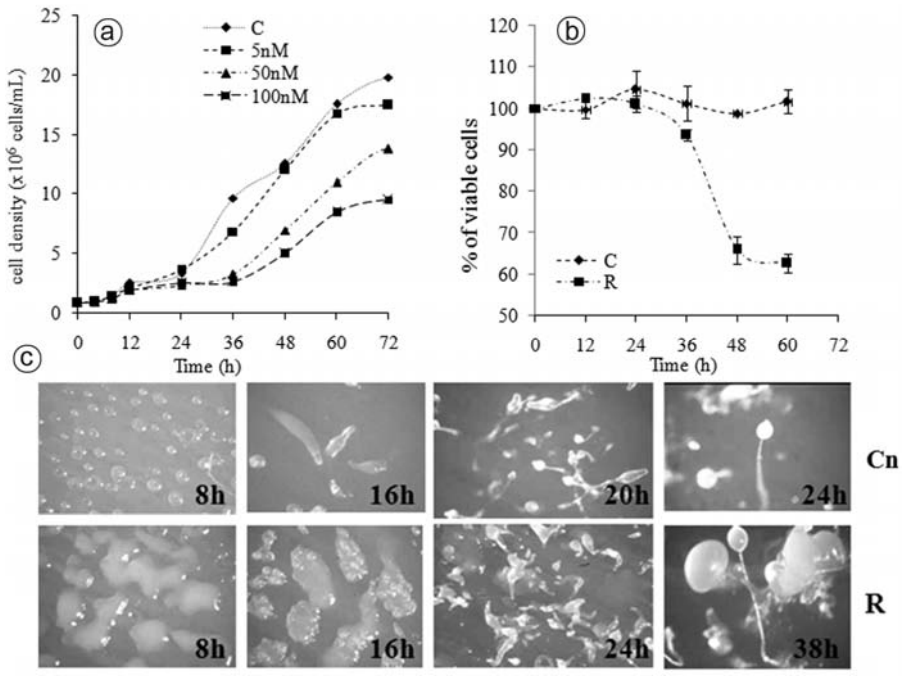

Fig. 1—Effect of rapamycin on growth, viability and development. (a) Dose dependent inhibition of A×2 cells by rapamycin. (b) Viability of untreated (C) and treated cells (R) were assayed by MTT (5 $mg.mL^{-1}$) and absorbance measured at 570 nm. It was expressed as MTT OD value of sample/MTT OD value of control. (c) Untreated (Cn) and cells treated with 50 nM rapamycin for 48 h (R) were developed synchronously at a density of 5×10^5cells.cm^{-2}. Few representative stages developed at the indicated times are shown.

Rapamycin induces autophagy in D. discoideum—The process of autophagy in *D. discoideum,* was analyzed using [*act15/gfp-atg8*]/Ax2 cells that express the autophagosome marker, Atg8[28] as a fusion protein with GFP driven under the constitutive promoter, *actin15*. The log phase cells were treated with 50 nM of rapamycin for various time intervals (0-60 h) and GFP fluorescence was visualized. By 60 h of treatment, the vacuoles fused to cover a large volume of the cell and probably induce cell death. A time dependent increase was observed in the number and size of puncta/cell which coalesced to form large sized vacuoles by t_{60} (Fig. 2a). The results clearly showed that treatment with 50 nM of rapamycin for 48 h could induce autophagy in *D. discoideum* without bringing death.

To evaluate whether the incorporations of GFP-Atg8 were indeed dependent on autophagy, the cells were pretreated with 3-methyladenine (3-MA) or wortmannin, the known inhibitors of autophagic sequestrations. 3-MA and wortmannin are phosphatidyl-inositol 3 kinase (PI3K) inhibitors, inhibiting both class I and III PI3K. Class I PI3K generates products that inhibit autophagic sequestrations while class III generates products that stimulate autophagic sequestration downstream of class I enzymes, the net result being blockage of autophagy[29,30]. After 48 h (Fig. 2c), there was an increase of nearly 1.5 folds in the level of GFP-Atg8 fluorescence in the rapamycin treated cells (R) as compared to the untreated control (C) cells. However, when the rapamycin treated cells were antagonized with 3-MA (RM) the GFP-Atg8 fluorescence decreased to almost control levels. Treatment with 3-MA alone (M) did not seem to increase the GFP-Atg8 levels and acts as an internal control (Fig. 2c). The inhibitory effect was also observed during fluorescence microscopy (Fig. 2biii). Fig. 2bi shows the untreated control cells having diffused fluorescence which upon treatment with rapamycin (Fig. 2bii) show an increased number of puncta.

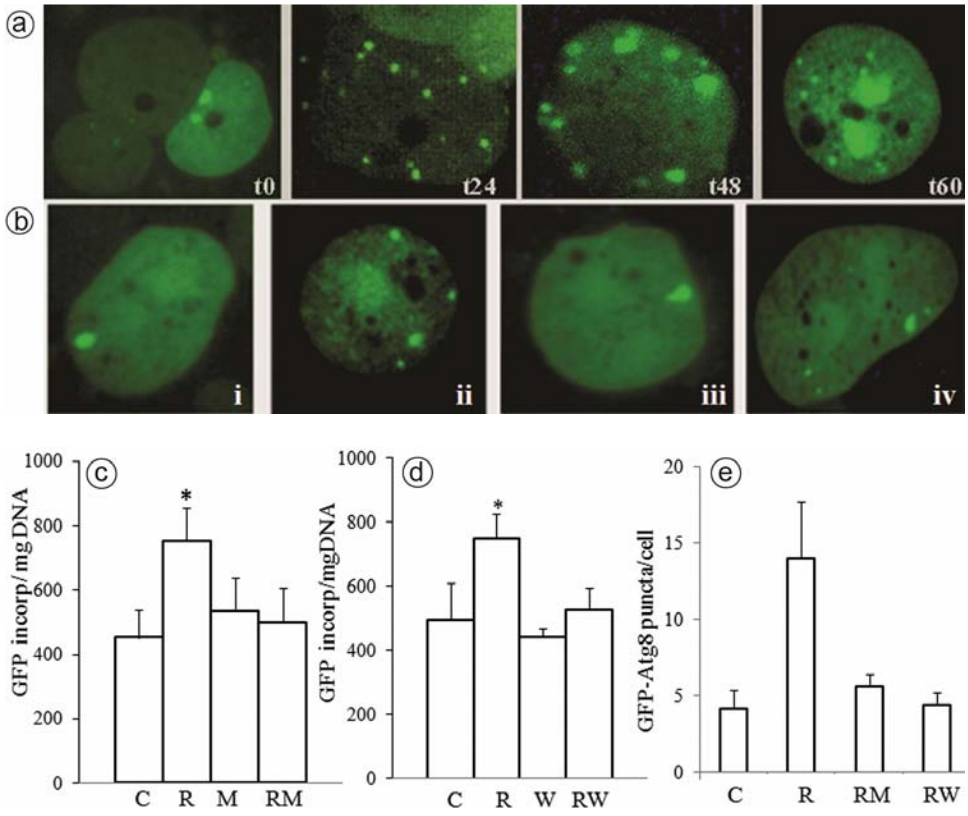

Fig. 2—Rapamycin induces autophagy. (a) Fluorescence images of (*act15/gfp-atg8*)/Ax2 cells after incubation with rapamycin for indicated time points. (b) Fluorescence images of (*act15/gfp-atg8*)/Ax2 cells: i-control, ii-rapamycin, iii-3-methyladenine and iv-wortmannin treatments. (c) and (d) GFP incorporations.mg^{-1} DNA in untreated (C), rapamycin (R), 3-methyladenine and wortmannin (W) treatment, RM and RW indicate effect of 3-MA and wortmannin after rapamycin treatment. (e) Puncta/cell in the (*act15/gfp-atg8*)/Ax2 cells was counted after treatments with various drugs. Results shown in the figures are an average of 4-6 individual experiments performed in triplicates.

Similarly, when the rapamycin treated cells were antagonized with wortmannin (RM) the GFP-Atg8 fluorescence decreased to almost control levels (Fig. 2d). Wortmannin substantially inhibited the accumulation of GFP-Atg8 labeled vacuoles (Fig. 2biv) in the treated cells (RW) confirming the involvement of a PI3 kinase.

In the above experiments, untreated control cells showed the basal autophagy levels, the rapamycin treated cells showed induced autophagy, the treatment with the drug alone acted as an internal control while the effect of the drug after induction of autophagy was also monitored and 5 individual experiments in triplicates were performed. A Student's *t* test was used to analyze the significance of the differences for each sample with their respective controls. A statistically significant difference observed is marked with an * where the P value was <0.05.

Further, the formation of autophagosomes (GFP-Atg8 puncta) was counted under the fluorescence microscope after treatment with rapamycin and other drugs (Fig. 2e). Rapamycin treatment showed almost 3 fold increase in the puncta/cell.

Taken together, these results indicate that the accumulation of GFP-Atg8 vacuoles (both fluorescence incorporations and puncta counts/cell) correlates with the induction of autophagy by rapamycin in *D. discoideum* which is abrogated by proven inhibitors of the autophagic pathway such as 3-MA and wortmannin. Therefore, autophagy can be followed both biochemically and morphologically in this system.

Accumulation of ROS correlates with induction of autophagy—What could be the possible mechanism of rapamycin induced autophagy in case of *D. discoideum*? Since, oxidative conditions are essential and treatment with antioxidative agents abolishes the formation of autophagosomes the role of ROS[31] was also explored. Simultaneous measurements of both ROS levels using DCF-DA which reacts mainly with H_2O_2 to form a fluorescent compound (Fig. 3a) and autophagy by the GFP-Atg8 fluorescence Fig. 3b was carried. Cellular ROS levels were increased by treatment with exogenous H_2O_2 (H) and decreased by treatment with specific inhibitors like N-acetylcysteine (N, inducer of intracellular glutathione) and diphenyliodonium (D, a NADPH oxidase)[32,33]. Nearly 2 and 1.5 fold increase in the ROS levels was observed after H_2O_2 and rapamycin treatments respectively over the control cells at 48 h while both N and D treatments decreased the fluorescence levels to near control levels (Fig. 3a). Increase in GFP-Atg8 fluorescence was observed after H_2O_2 and rapamycin treatments while N and D treatments decreased the fluorescence levels at 48 h (Fig. 3b). The studies showed a more pronounced decrease in the ROS levels by the inhibitor N-acetylcysteine. It can be concluded that the level of autophagy was positively correlated to the levels of ROS as the GFP-Atg8 fluorescence increased with an increase in the ROS levels both after rapamycin or H_2O_2 treatments. Conversely, an increase in ROS levels also showed increased autophagy.

Activity of antioxidant enzymes—Involvement of ROS prompted the measurement of the activity of important stress regulatory enzymes like superoxide dismutase (SOD) and glutathione S-transferase (GST) in both the untreated and rapamycin treated cells. Nearly 3 and 2 folds increase in the levels of SOD

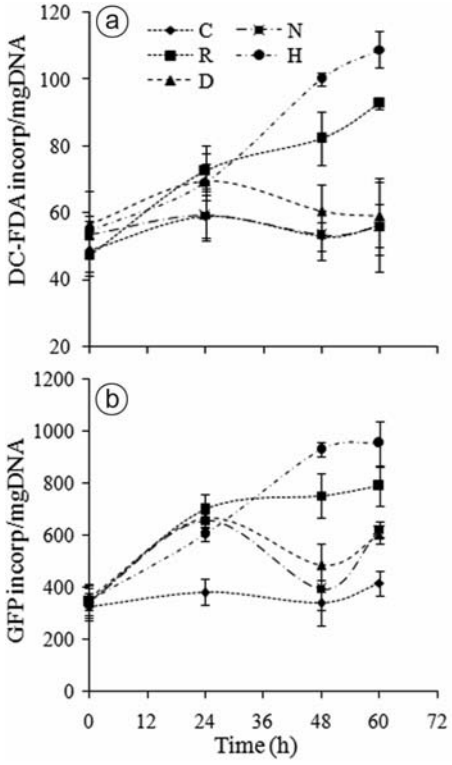

Fig. 3—Accumulation of ROS after autophagy induction and vice versa. Simultaneous measurements of ROS levels (a) and induction of autophagy (b) was carried and expressed as DC-FDA incorporations.mg^{-1} DNA and as GFP fluorescence.mg^{-1} DNA respectively under varying conditions: untreated (C), rapamycin (R), exogenous H_2O_2 (H), N-acetylcysteine (N) and diphenyliodonium (D) treatments. ROS levels was measured using DC-FDA (Ex/Em = 495/525 nm). Data are means $\pm$ SD of 5 independent experiments performed in triplicates.

(Fig. 4a) and GST (Fig. 4b) respectively were observed after the treatment.

So where does this redox signal arise? It has been reported that class III PI3K accumulates H_2O_2 in the mitochondria which is essential for autophagy. Level of mitochondrial membrane depolarization was measured using the dye JC-1 which exists as a monomer in the cytosol (green in colour); while in the mitochondria they form aggregates (red in colour). In the dying cell population the dye appears green in colour and fails to form aggregates. The ratio of red:green fluorescence decreased in rapamycin treated cells as compared to the control cells which remains more or less constant suggesting occurrence of depolarization of mitochondrial membrane during autophagy (Fig. 4c).

Increase in the oxidative stress due to accumulation of ROS prompted the measurement of its effects on lipid peroxidation as well as intracellular calcium levels. Oxidative damage of polyunsaturated fatty acids (membrane integrity) was measured by determining TBARS in the control as well as rapamycin treated cells. There was no significant change observed in the lipid peroxidation after treatment (Fig. 4d). These results show that the membrane remains almost intact during the whole period of incubation.

Increase in calcium induces accumulation of GFP-Atg8 fluorescence—Simultaneous measurements of both the $(Ca^{2+})_i$ and the GFP-Atg8 fluorescence (Fig. 5a and b) in the untreated and rapamycin treated cells were carried out. Measurements were done after perturbing the calcium homeostasis by the addition of exogenous calcium or by the calcium chelator, EGTA. The above experiments showed a dose dependent increase in the $(Ca^{2+})_i$ levels along with an increase in GFP-Atg8 fluorescence. Levels of both $(Ca^{2+})_i$ and GFP-Atg8 decreased upon treatment with exogenous EGTA. The results indicate an increase in endogenous $(Ca^{2+})_i$ levels upon rapamycin treatment and *vice versa*. Induction of autophagy was similar in both

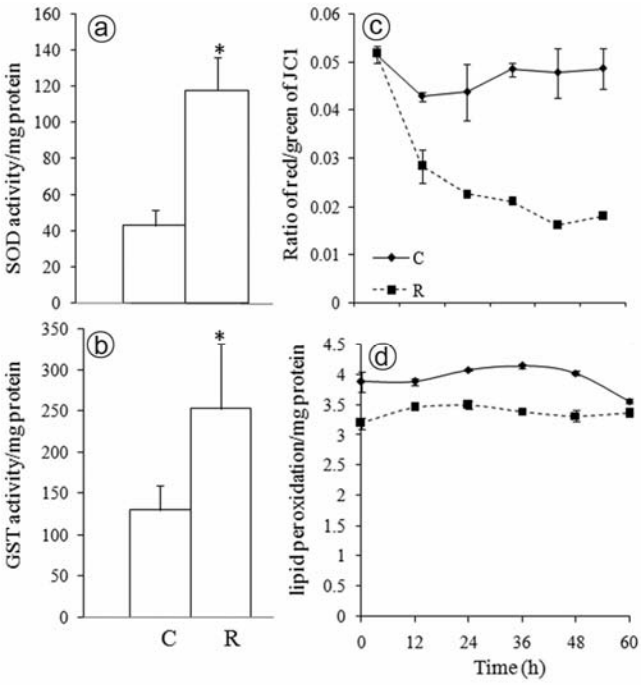

Fig. 4—Effect of autophagy on various parameters. Both the control (C) and rapamycin (R) treated cells were measured for the following parameters: (a) SOD enzyme activity; (b) GST enzyme activity; (c) Mitochondrial membrane potential (MMP) was measured by JC-1 (Ex = 514 nm and Em = 543/584 nm). A decrease in the red/green fluorescence measures an increase in the MMP. (d) Lipid peroxidation was measured at 532 nm by TBARS.

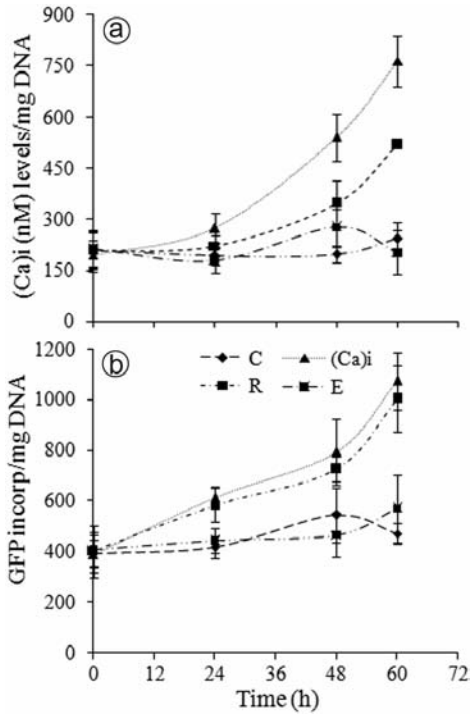

Fig. 5—Accumulation of intracellular free calcium after autophagy induction and vice versa. Simultaneous measurements of intracellular calcium levels (a) and induction of autophagy (b) was carried under varying conditions. Intracellular free calcium measured over a period of time as indicated in the untreated (C), rapamycin treated (R), cells treated with $CaCl_2$ and A23187 (Ca^{2+}) and cells treated with EGTA (E), a calcium chelator. GFP-Atg8 fluorescence measurements under similar conditions. Intracellular free calcium levels was measured using Fura2 AM (Ex = 340/380 nm and Em = 510 nm). Data are means $\pm$ SD of 5 independent experiments performed in triplicates.

rapamycin and calcium treated cells but the levels of $(Ca^{2+})_i$ was not similar in them.

Rapamycin blocks cells in G1 phase of cell cycle—Cell cycle analyses of cultures grown in presence and absence of rapamycin for varying time was carried by FACs analysis. Forward scatter measurements (Fig. 6a) showed a decrease in size by t_{24} after which they increased to resume near normal size by t_{60} after rapamycin treatment. The sizes of control cells with time did not show any changes. An absolute difference in the percentage of cells in each phase with respect to time from the control is shown in Fig. 6b. The results show a gradual increase in the percentage of cell number in the G1 phase till t_{24} after which they maintained themselves in the same phase till t_{48}. Correspondingly, percentage of cells in S phase shows a decrease from the control till almost t_{48} hours of treatment. Increase in the percentage of cells in the S phase of the treated sample at t_{60} suggests a transition from G1 to S phase. A corresponding increase in the percentage of cells was seen in the G2 phase. Taken together, the data suggests a blockage in the G1 phase till almost 60 h of treatment after which they progress in cell cycle. Interestingly, the transition appears at 60 h of treatment when the cells acquire a size similar to that of the wild type. This suggests that TOR signaling plays an important role in both cell cycle progression and growth of cells.

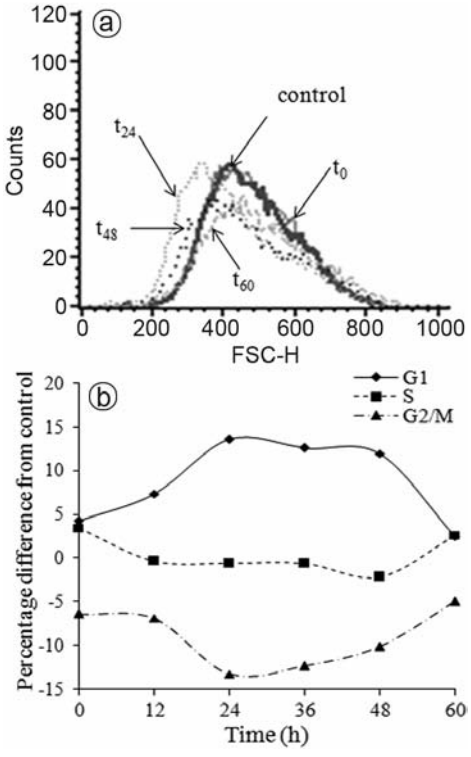

Fig. 6—Effect of rapamycin on cell cycle profile. (a) Forward scatter of the control cells and rapamycin treated cells with time. (b) Plot showing the relative percentage of different phases of cell cycle with respect to time for the rapamycin treated over control cells.

Discussion

Rapamycin and its derivatives are promising therapeutic agents having both anti-tumor and immunosuppressant properties. Rapamycin and its derivatives block multiple cellular functions linked to TOR signaling, therefore it is essential to gain a better understanding of the molecular links between TOR and cellular functions.

In the present study, *Dictyostelium discoideum* was used to understand the molecular and cellular functions of a complex process like autophagy. Orthologs of many proteins involved in autophagy found in yeast have been found in humans but many proteins found in humans are not present in them but are present in *D. discoideum*, which are known to undergo caspase independent developmental cell death.

The results demonstrate that autophagy which occurs naturally in this organism can also be studied by treatment with rapamycin which utilizes the well conserved autophagic machinery and help answer questions regarding TOR signaling. Autophagy was monitored both by counting the GFP-Atg8 puncta and the fluorescence levels. Rapamycin treatment for 48 h at 50 nM concentration induced autophagy that was inhibited by PI3 kinase inhibitors 3-MA and wortmannin.

The results reveal that rapamycin induced autophagy involves an accumulation of ROS and in turn, an increase in ROS could induce autophagy. Autophagy degrades and recycles the macromolecules and plays an important role in cellular response to oxidative stress indicating an existing relationship between mitochondria and autophagosomes. As a result of oxidative metabolism, mitochondria produce low levels of ROS which normally get reduced by enzymatic and non-enzymatic antioxidizing agents. This accumulation within the mitochondria results in loss of function of the organelle owing to lipid peroxidation, DNA damage and a decrease in mitochondrial membrane potential. Rapamycin induced autophagy is accompanied by early production of ROS which results in depolarization of mitochondria. The generation of ROS mainly takes

place in three cellular compartments i.e., in the ER, mitochondria or cytosol. However, all the three locations of ROS generating processes mutually affect each other and get influenced by exogenous ROS. Though mitochondrial respiration is the main source of ROS, it is also a target of ROS produced at other cellular locations. From the present study, it is not clear as to which ROS is the active species leading to the induction of autophagy. The use of exogenous hydrogen peroxide in this study suggests its involvement in this process. Chen *et al.*[33] used both catalase and N-acetylcysteine (NAC) extensively, as antioxidants which act specifically against hydrogen peroxide. This seems to corroborate with the present study because the level of H_2O_2 induced autophagy was more reduced when NAC was used as the antioxidant in comparison to diphenyliodonium (DPI). In addition, the level of SOD was found to be increased in the cells treated with rapamycin. Chen *et al.*[33] have measured in parallel the cellular content of H_2O_2 and superoxide and have shown that the level of superoxide is correlated with extent of autophagy induction. This does not however mean that H_2O_2 is not important in autophagy as its generation due to ER stress affects the function of mitochondria. Loss of membrane potential is the cause of superoxide production whereby hydrogen peroxide is converted to superoxide. Additionally, ER stress causes a release of calcium from the ER which leads to an increase in the concentration of cytosolic calcium.

Studies in yeast show that mutants defective in autophagy proceeds towards cell death as a result of mitochondrial dysfunction[34]. In addition, ROS was also found to induce autophagy by disruption of mitochondrial function in human glioma cell lines[35]. Mitochondrial ROS can also act as a signaling molecule able to switch cells between proliferation and differentiation. The preliminary results show that during normal development starvation triggers accumulation of ROS resulting in induction of autophagy and this signal was PI3K dependent suggesting that ROS starts accumulating from the time of starvation which could be a possible mechanism to induce autophagy (unpublished). Cells develop a very powerful defense mechanism to maintain a balance between the production and degradation of the ROS produced by the cells. The role of antioxidant enzymes is of fine tuning the levels of ROS required in the redox regulation of the cell cycle and PCD. Based on the present study and the current knowledge one can conclude that autophagy can be regulated by the mitochondria. ROS generated by mitochondria include superoxides, hydrogen peroxides and other free radicals which can induce autophagy. Specific ROS can also be converted to other forms of ROS but how they are regulated during autophagy is not known. It is still not clear whether one or more species of ROS is involved in triggering autophagy. For cell survival, either high defense mechanisms or low ROS levels are required. The present results showed that induction of autophagy led to increased levels of these antioxidizing enzymes but probably further accumulation of ROS degrades these enzymes and lead the cell towards autophagic cell death. Autophagy can disrupt the balance between the production and degradation of ROS by modulating the mechanism responsible for survival to become an efficient death promoter.

The prestalk cells which are destined to die during its differentiation into stalk cells have high $(Ca^{2+})_i$ as compared to the prespore cells and also an increase in intracellular calcium can bias the cells towards the prestalk pathway. The result showed $(Ca^{2+})_i$ to be involved in mediating autophagy. The first insights into the molecular mechanism of signaling pathway leading to autophagy in *D. discoideum* came from the studies of Lam *et al.*[36] where they have shown the requirement of IP3R and Ca^{2+}. The IP3R is a ligand gated channel governing calcium efflux from the endoplasmic reticulum stores into the cytosol.

A detailed study to identify a signaling cascade that would mediate autophagy in response to elevated calcium is still awaited. In this study it has been shown that an alteration of $(Ca^{2+})_i$ homeostasis is involved in the regulation of autophagy. Whether increased calcium levels are a prerequisite for the depolarization is not known in this system. It has been earlier reported that negative mitochondrial membrane potential draws calcium ions into the matrix of the mitochondria and releases calcium which may trigger calcium dependent signaling. Lam *et al.*[36] have shown the role of calcium flux in autophagy. It was suggested that increased $(Ca^{2+})_i$ as well as depletion of Ca^{2+} from the ER triggers an autophagic response[37]. It can be concluded that although calcium has long been known to play a role in autophagy, whether it promotes or inhibits autophagy may depend on actual cellular context.

Cell growth and proliferation depends on the nutritional environment of the cell. Nutrients provide materials for macromolecular synthesis as well as initiate the TOR signaling cascade. The present results show that rapamycin treatment blocks the cells in G1 phase of the cell cycle for 48 h after which they progress towards the S phase of the cell cycle. Interestingly, the cells resume division only when they achieve a size similar to that of the control cells. This observation supports the fact that cell size achievement is important for the cells to enter division. This is also true with the results from growth curve where cells resume division slowly by 60 h of treatment. It is assumed that TOR signaling remains active during whole cell cycle, acting not only to up regulate the macromolecular synthesis but also transmitting nutrients to the factors promoting cell division. It has been reported earlier that dysfunction of TOR is mainly attributed to the impairment of protein synthesis[38]. Increase in cell size as well as proliferation depends on the availability of the nutrients. As a result of increase in the intracellular concentration of calcium during the course of rapamycin treatment, it can be speculated that some role is played by calcium as it has been found to regulate the cell cycle progression at the G2/M checkpoint in hepatic stellate cells[39] and also in *D. discoideum*[24].

In conclusion, the results of the present studies show that rapamycin drug induces autophagy in *D. discoideum* via an accumulation of ROS and intracellular calcium. Autophagy is one of the chief mechanism by which longevity is achieved. Understanding the mechanism by which ROS production by mitochondria leads to an extension of life is still awaited.

Acknowledgement

Thanks are due to the Central Instrumentation facility of SLS for the use of FACS machine. SS thanks CSIR, New Delhi, India for financial support. PBS and RL thank UGC and CSIR New Delhi, respectively for fellowships.

References

1 Kaeberlein M, Resveratrol and rapamycin: are they anti-aging drugs, *Bioessays,* 32 (2010) 96.

2 Orozco H, Matallana E & Aranda A, Oxidative stress tolerance, adenylate cyclase and autophagy are key players in yeast chronological life span during winemaking, *Appl Environ Microbiol,* 78 (2012) 2748.

3 Hansen M, Taubert S, Crawford D, Libina N, Lee S J & Kenyon C, Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*, *Aging Cell,* 6 (2007) 95.

4 Bjedov I, Toivonen J M, Kerr F, Slack C, Jacobson J, Foley A & Partridge L, Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster, Cell Metab*, 11 (2010) 35.

5 Katewa S D & Kapahi P, Role of TOR signaling in aging and related biological processes in *Drosophila melanogaster*, *Exp Gerontol,* 46 (2011) 382.

6 Harrison D E, Strong R, Sharp Z D, Nelson J F, Astle C M, Flurkey K, Nadon N L, Wilkinson J E, Frenkel K, Carter C S, Pahor M, Javors M A, Fernandez E & Miller R A, Rapamycin fed late in life extends lifespan in genetically heterogeneous mice, *Nature,* 460 (2009) 392.

7 Miller R A, Williams J B, Kiklevich J V, Austad S & Harper J M, Comparative cellular biogerontology: primer and prospectus, *Ageing Res Rev,* 10 (2010) 181.

8 Anisimov V N, Piskunova T S, Popovich I G, Zabezhinski M A, Tyndyk M L, Egormin P A, Yurova M V, Rosenfeld S V, Semenchenko A V, Kovalenko I G, Poroshina T E & Berstein L M, Gender differences in metformin effect on aging, life span and spontaneous tumorigenesis in 129/Sv mice, *Aging (Albany NY),* 2 (2010) 945.

9 Sehgal S N, Baker H & Vézina C J, Rapamycin (AY-22,989), a new antifungal antibiotic II Fermentation, isolation and characterization, *Antibiot (Tokyo),* 28 (1975) 27.

10 Vézina C, Kudelski A & Sehgal S N, Rapamycin (AY-22,989), a new antifungal antibiotic I Taxonomy of the producing streptomycete and isolation of the active principle, *J Antibiot (Tokyo),* 28 (1975) 721.

11 Calne R Y, Collier D S, Lim S, Pollard S G, Samaan A, White D J & Thiru S, Rapamycin for immunosuppression in organ allo-grafting, *Lancet*, 2 (1989) 227.

12 Schreiber S L, Chemistry and biology of the immunophilins and their immunosuppressive ligands, *Science,* 251 (1991) 283.

13 Eichinger L, Pachebat J A, Glöckner G, Rajandream M A, Sucgang R, Berriman M, Song J, Olsen R, Szafranski K, Xu Q, Tunggal B, Kummerfeld S, Madera M, Konfortov B A, Rivero F, Bankier A T, Lehmann R, Hamlin N, Davies R, Gaudet P, Fey P, Pilcher K, Chen G, Saunders D, Sodergren E, Davis P, Kerhornou A, Nie X, Hall N, Anjard C, Hemphill L, Bason N, Farbrother P, Desany B, Just E, Morio T, Rost R, Churcher C, Cooper J, Haydock S, van Driessche N, Cronin A, Goodhead I, Muzny D, Mourier T, Pain A, Lu M, Harper D, Lindsay R, Hauser H, James K, Quiles M, Madan Babu M, Saito T, Buchrieser C, Wardroper A, Felder M, Thangavelu M, Johnson D, Knights A, Loulseged H, Mungall K, Oliver K, Price C, Quail M A, Urushihara H, Hernandez J, Rabbinowitsch E, Steffen D, Sanders M, Ma J, Kohara Y, Sharp S, Simmonds M, Spiegler S, Tivey A, Sugano S, White B, Walker D, Woodward J, Winckler T, Tanaka Y, Shaulsky G, Schleicher M, Weinstock G, Rosenthal A, Cox E C, Chisholm R L, Gibbs R, Loomis W F, Platzer M, Kay R R, Williams J, Dear P H, Noegel A A, Barrell B & Kuspa A, The genome of the social amoeba *Dictyostelium discoideum, Nature,* 435 (2005) 43.

14 Olie R A, Durrieu F, Cornilon S, Loughron G, Gross J, Earnshaw W C & Golstein P, Apparent caspase independence of programmed cell death in *Dictyostelium*, *Curr Biol,* 8 (1998) 955.

15 Roisin-Bouffay C, Luciani M F, Klein G, Levraud J P, Myriam Adam M & Golstein P, Developmental cell death in *Dictyostelium* does not require paracaspase, *J Biol Chem*, 279 (2004) 11489.

16 Tresse E, Kosta A, Luciani M F & Golstein P, From autophagic to necrotic cell death in *Dictyostelium*, *Seminars Cancer Biol,* 17 (2007) 94.

17 Brown J M & Firtel R A, Regulation of cell-fate determination in *Dictyostelium*, *Dev Biol,* 216 (1999) 426.

18 Otto G P, Wu M Y, Kazgan N, Anderson O R & Kessin R H, Macroautophagy is required for multicellular development of the social amoeba *Dictyostelium discoideum*, *J Biol Chem,* 278 (2003) 17636.

19 Saran S & Schaap P, Adenylyl cyclase G is activated by an intramolecular osmosensor, *Mol Biol Cell,* 15 (2004) 1479.

20 Fey P, Kowal A S, Gaudet P, Pilcher K E & Chisholm R L, Protocols for growth and development of *Dictyostelium discoideum*, *Nature Protocols,* 2 (2007) 1307.

21 Mosmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J Immunol Methods,* 65 (1983) 55.

22 Armstrong J S & Whiteman M, Measurement of reactive oxygen species in cells and mitochondria, *Methods Cell Biol*, 80 (2007) 355.

23 Slater T F, Overview of methods for detecting lipid peroxidation, *Methods Enzymol,* 105 (1984) 283.

24 Saran S, Calcium levels during cell cycle correlate with cell fate of *Dictyostelium discoideum*, *Cell Biol International,* 23 (1999) 399.

25 Grynkiewicz G, Poenie M & Tsien R Y, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, *J Biol Chem,* 260 (1985) 3440.

26 Salvioli S, Ardizzoni A, Franceschi C & Cossarizza A, JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis, *FEBS Lett*, 411 (1997) 77.

27 Chen G, Shaulsky G & Kuspa A, Tissue-specific G1-phase cell-cycle arrest prior to terminal differentiation in *Dictyostelium*, *Development,* 131 (2004) 2619.

28 Tekinay T, Wu M Y, Otto G P, Anderson O R & Kessin R H, Function of the *Dictyostelium discoideum* Atg1 kinase during autophagy and development, *Eukaryot Cell,* 5 (2006) 1797.

29 Blommart E F, Krause U, Schellens J P, Vreeling-Sindelarova H & Meijer A J, The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes, *European J Biochem,* 243 (1997) 240.

30 Petiot A, Ogier-Denis E, Blommaart E F, Meijer A J & Codogno P, Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells, *J Biol Chem,* 275 (2000) 992.

31 Scherz-Shouval R & Elazar Z, Regulation of autophagy by ROS: physiology and pathology, *Trends Biochem Sci,* 36 (2011) 30.

32 Qian L, Gao X, Pei Z, Wu X, Block M, Wilson B, Hong J S & Flood P M, NADPH oxidase inhibitor DPI is neuroprotective at femtomolar concentrations through inhibition of microglia over-activation, *Parkinsonism Relat Disord,* 3 (2007) S316.

33 Chen Y, McMillan-Ward E, Kong J, Israels S J & Gibson S B, Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Differentiation,* 15 (2008) 171.

34 Suzuki S W, Onodera J & Ohsumi Y, Starvation induced cell death in autophagy-defective yeast mutants is caused by mitochondria dysfunction, *PLOS ONE,* 6 (2011) 1.

35 Zhang H, Kong X, Kang J, Su J, Li Y, Zhong J & Sun L, Oxidative stress induces parallel autophagy and mitochondria dysfunction in human glioma U251 cells, *Toxicological Sciences,* 110 (2009) 376.

36 Lam D, Kosta A, Luciani M F & Golstein P, The inositol 1,4,5-trisphosphate receptor is required to signal autophagic cell death, *Mol Biol Cell,* 19 (2008) 691.

37 Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, Bianchi K, Fehrenbacher N, Elling F, Rizzuto R, Mathiasen IS & Jaattela M, Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2, *Mol Cell,* 25 (2007) 193.

38 Terada N, takase K, Paps P, Nairn A C & Gelfand E W, Rapamycin inhibits ribosomal protein synthesis nd induces G1 prolongation in mitogen-activated T lymphocytes, *J Immunol,* 155 (1995) 3418.

39 Soliman G A, Acosta-Jaquez H A, Dunlop E A, Ekim B, Maj N E, Tee A R & Fingar D C, mTOR Ser-2481 autophosphorylation monitors mTORC-specific catalytic activity and clarifies rapamycin mechanism of action, *J Biol Chem,* 285 (2010) 7866.