Identifying the Estrogen Receptor Coactivator PELP1 in Autophagosomes

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Abstract

Resveratrol, a well-established phytoestrogen and chemopreventive agent, has gained much attention among oncologists because it can act as both estrogen receptor agonist and antagonist, depending on dosage and cell context. It is increasingly accepted that steroidal receptor coregulators may also function in the cytoplasmic compartment. Deregulation and altered localization of these coregulators could influence target gene expression and participate in the development of hormone-responsive cancers. Proline-, glutamic acid–, and leucine-rich protein-1 (PELP1), a novel estrogen receptor (ER) coactivator, plays an important role in the genomic and nongenomic actions of ER. Furthermore, recent studies have shown that differential compartmentalization of PELP1 could be crucial in modulating sensitivity to tamoxifen. In this study, we investigated the role of PELP1 in resveratrol-induced autophagy in lung cancer and salivary gland adenocarcinoma cell lines. Resveratrol reversibly inhibited the growth of these cancer cell lines and induced autophagy, as evidenced by microtubule-associated protein 1 light chain 3 (LC3) up-regulation in a time-dependent and 3-methyladenine–sensitive manner. Confocal microscopic analysis showed that resveratrol induced PELP1 accumulation in autophagosomes with green fluorescent protein-LC3. The intermediary molecule involved in PELP1 accumulation in resveratrol-induced autophagosomes is hepatocyte growth factor–regulated tyrosine kinase substrate (HRS), a trafficking molecule that binds to PELP1. These results identify PELP1 for the first time in autophagosomes, implying that both PELP1 and HRS reallocate to autophagosomes in response to resveratrol treatment, which might be important in the process of autophagy in the cancer cells. [Cancer Res 2007;67(17):8164–71]

Introduction

Proline-, glutamic acid–, and leucine-rich protein 1 (PELP1) is a novel estrogen receptor (ER) coregulatory protein that positively modulates ER transcriptional functions. PELP1 is overexpressed in breast tumors, and recent evidence indicates that it plays a permissive role in estradiol (E_2)-mediated cell cycle progression via its regulatory interaction with the retinoblastoma protein (pRb) pathway (1, 2). In particular, deregulation of PELP1 in breast cancer cells hypersensitizes tumor cells to E_2, leading to an increased G_1-S phase transition. Because PELP1 overexpression increases both basal and E_2-induced pRb phosphorylation and cyclin D1 transcription, this effect has been attributed to the ability of PELP1 to bind directly to pRb in response to E_2 and to affect cyclin D1 transcription. The intracellular localization of PELP1 accounts for its role as an ER coactivator (3, 4). PELP1 is mainly localized in the nucleus, although its nuclear abundance varies between different tissues. However, PELP1 can also be localized in the cytoplasm and in the proximity of the plasma membrane, suggesting that PELP1 may have a role in nongenomic ER signaling and possibly in selective ER modulator (SERM) resistance (2). More recently, it was shown that PELP1 interacts in the cytoplasm with hepatocyte growth factor–regulated tyrosine kinase substrate (HRS), a molecule that plays a role in endosomal membrane trafficking (5).

Resveratrol, a phytochemical found in grapes and red wine, has been categorized as a phytoestrogen because of its ability to bind and activate ERα. It has both estrogenic and antiestrogenic properties and therefore has characteristics similar to the SERMs (6). Resveratrol inhibits *in vitro* growth of cancer cells of diverse origin (breast carcinoma, ovarian cancer, hepatoblastoma, leukemia, colon cancer, and prostate cancer cell lines) via induction of apoptosis and/or other mechanisms, including autophagy (7). Besides this, it has been shown that resveratrol suppresses 7,12-dimethylbenz(*a*)anthracene–induced mammary carcinogenesis in rats (8).

Autophagy is a genetically programmed, evolutionarily conserved process that degrades long-lived cellular proteins and organelles. This process is important in normal development and response to changing environmental stimuli and is also important in numerous diseases, including bacterial and viral infections, neurodegenerative disorders, and cardiovascular disease (9). Autophagy involves the formation of large double-membrane vesicles, called autophagosomes, which encapsulate cytoplasm and organelles and then fuse with lysosomes, thus degrading the contents of the vesicles. This process is also thought to have an important role in tumor development. In cancer cells, autophagy acts as a tumor suppressor in the initial stages of tumorigenesis but as a promoter of tumor progression in the advanced stages (10, 11).

Various treatments induce autophagy in cancer cells (12). Tamoxifen, which targets the ER, induces autophagic cell death in breast cancer cells through down-regulation of the protein kinase B/Akt. Temozolomide, a DNA alkylating agent, induces autophagy in malignant glioma cells. Despite these examples, however, it is highly controversial whether autophagy ultimately plays a role in the protection from or the induction of cancer cell death (13). A better understanding of the molecular mechanism

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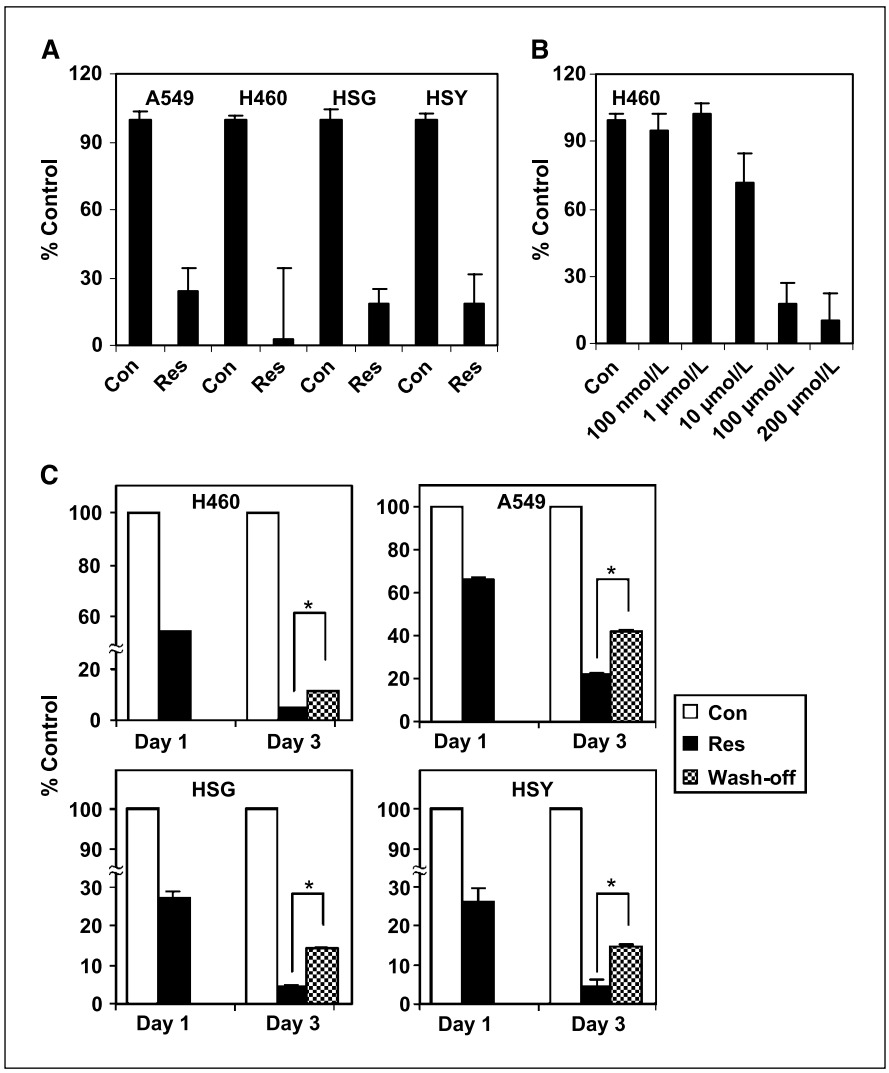

Figure 1. Resveratrol inhibits the growth of various cancer cells. *A,* lung cancer cell lines A549 and H460 and salivary gland adenocarcinoma cell lines HSG and HSY were treated with 100 μmol/L resveratrol (*Res*) for 3 d and cell numbers were counted. *Con,* control. *Bars,* SD. *B,* H460 cells were treated with various concentrations of resveratrol and then were counted. *C,* H460, A549, HSG, and HSY cells were treated with 100 μmol/L resveratrol for 24 h, washed with PBS thrice, and then cultured in the same medium without resveratrol for an additional 48 h; after that, cell numbers were counted.

and the regulatory pathways that control autophagy may provide a useful way to prevent cancer development, limit tumor progression, and increase the efficacy of cancer treatments.

In this study, we found that resveratrol reversibly inhibited the growth of lung cancer and salivary gland adenocarcinoma cell lines by autophagy, as evidenced by the up-regulation of microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagy, in a time-dependent and 3-methyladenine–sensitive manner. Confocal microscopic analysis showed that resveratrol induced the accumulation of PELP1 in autophagosomes and PELP1 colocalized with green fluorescent protein-LC3 (GFP-LC3). In addition, we identified HRS, a PELP1-binding protein, as an intermediary molecule involved in the accumulation of PELP1 in resveratrol-induced autophagosomes. Our results for the first time provided a glimpse of PELP1 and HRS together in autophagosomes in response to resveratrol, an association that may be important in the process of autophagy in cancer cells.

Materials and Methods

Cell cultures and reagents. Human lung cancer A549 and H460 and human salivary gland adenocarcinoma HSG and HSY cell lines were maintained in DMEM/F-12 (1:1) supplemented with 10% fetal bovine serum. Resveratrol was purchased from Tocris and 3-methyladenine from Sigma-Aldrich. The mitogen-activated protein kinase kinase (MEK) inhibitor U0126 was purchased from Promega. The cathepsin D inhibitor pepstatin A was purchased from U.S. Biochemical. Antibodies against PELP1 and HRS were purchased from Bethyl Laboratories and Alexis Biochemicals, respectively. Antibodies against Akt, phospho-Akt, and phosphorylation site–specific pRb were purchased from Cell Signaling Technology. Antibodies against pRb and p27 were purchased from NeoMarkers and those against vinculin and actin were purchased from Sigma-Aldrich. Anti–LC-3 antibody (14) was supplied by our Department of Neurosurgery. Anti-p21 antibody was purchased from Santa Cruz Biotechnology. GFP-LC3 expression vector was kindly provided by Dr. N. Mizushima (National Institute for Basic Biology, Okazaki, Japan).

Cell proliferation assay. For cell proliferation assays involving ligand treatment, equal numbers of cells were plated in triplicate and then treated with resveratrol. The proliferation rate of the cells was measured by counting them with a Coulter counter (Beckman Coulter).

Cell cycle analysis. For cell cycle experiments, equal numbers of cells were plated in triplicate, treated with 100 μmol/L resveratrol, and fixed in 70% ethanol. Cells were then treated with 500 μg/mL RNase, stained with 10 μg/mL propidium iodide (Sigma-Aldrich), and analyzed by flow cytometry.

Cell extracts and immunoblotting. At indicated places, U0126 or pepstatin A was added 1 h before resveratrol treatment. Cells were washed twice with PBS and then lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP40, 0.1% SDS,

0.1% sodium deoxycholate, 1× protease inhibitor cocktail (Roche Applied Science), and 1 mmol/L sodium vanadate] for 10 min on ice. The lysates were spun in an Eppendorf centrifuge at 4°C for 10 min. Cell lysates containing an equal amount of protein (50 μg) were then resolved on an SDS-polyacrylamide gel (12% acrylamide), transferred to a polyvinylidene difluoride membrane, probed with the appropriate antibodies, and developed using either the enhanced chemiluminescence or the alkaline phosphatase–based color reaction method.

Transfection, immunofluorescence labeling, and confocal microscopy. Transient transfection studies were done using a FuGENE 6 kit (Roche Molecular Biochemicals) in accordance with the manufacturer's instructions. The cellular localization of proteins was determined by indirect immunofluorescence (15). Briefly, the cells were grown on sterile glass coverslips, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 10% normal goat serum in PBS. Cells were incubated with primary antibodies, washed thrice in PBS, and then incubated with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated with Alexa 546 (red) or Alexa 488 (green) from Molecular Probes. The blue DNA dye Topro-3 (Molecular Probes) was used as a nuclear stain. Microscopic analyses were done with an Olympus FV300 laser-scanning confocal microscope (Olympus America, Inc.) using sequential laser excitation to minimize fluorescence emission bleed-through.

Small interfering RNA. HRS-specific and control nonspecific small interfering RNA (siRNA) were purchased from Dharmacon. siRNA transfections were done using 100 nmol/L pooled siRNA duplexes and 2 μL of Oligofectamine (Invitrogen) according to the manufacturer's protocol in six-well plates. After 48 h, cells were prepared for Western blotting and confocal microscopic analysis.

Results

Resveratrol inhibited the growth of several human cancer cell lines. Because previous studies have shown that resveratrol binds to ERα and ERβ and acts as a mixed agonist and antagonist (6), we explored the possibility of its effect on growth in lung cancer and salivary gland adenocarcinoma cell lines expressing ERα, ERβ, or both. Our results showed that resveratrol greatly inhibited the growth of the ERα- and ERβ-positive lung cancer cell lines A549 and H460 and the ERβ-positive salivary gland adenocarcinoma cell lines HSG and HSY (Fig. 1*A*). This growth inhibition was augmented with increasing resveratrol concentrations, although the physiologic concentration, 100 μmol/L (16), was the one at which maximum growth inhibition was achieved (Fig. 1*B*).

Next, to determine the reversibility of the growth inhibition caused by resveratrol, H460, A549, HSG, and HSY cells were treated with resveratrol for 24 h. Then, the medium was washed with PBS and replaced with medium without resveratrol, and the cells were cultured for another 48 h. As shown in Fig. 1*C*, resveratrol treatment during the first 24 h inhibited the growth of all four cell lines, whereas the removal of resveratrol improved the growth rate during the second 48-h period, indicating that the growth inhibition caused by resveratrol is reversible.

Resveratrol induced changes in cell cycle distribution in human cancer cell lines. Because several studies have shown that resveratrol inhibits cell growth by affecting cellular proliferation (17–19), we examined the effect of resveratrol on the cell cycle distribution of H460, A549, HSG, and HSY cells. Resveratrol treatment of lung cancer cell lines H460 and A549 resulted in a substantial arrest of the cell cycles at the G_1 phase, whereas salivary gland adenocarcinoma cell lines HSG and HSY accumulated in the S phase (Table 1; Fig. 2*A*). To examine the mechanism by which resveratrol arrested cells in the G_1 phase, we analyzed the status of several cell cycle regulatory proteins. Among all the cell cycle proteins analyzed, we observed the most profound change in the status of phosphorylated pRb. pRb plays an important role in cell proliferation and differentiation (20). It has also been shown to inactivate E2F through direct interaction and sequestration. However, phosphorylation of pRb by cell cycle–dependent kinases releases E2F, thus leading to G_1-S phase progression. Because we hypothesized that resveratrol-mediated cell cycle arrest could be due to dephosphorylation of activated pRb, we analyzed phosphorylation of pRb

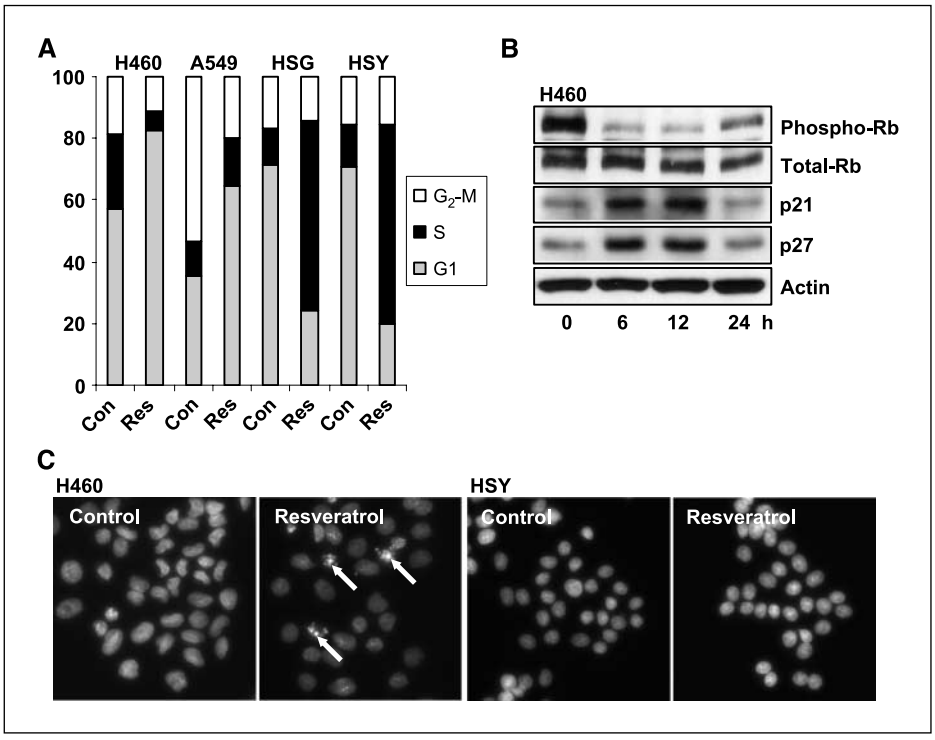

Figure 2. G_1- and S-phase arrest of cancer cell lines by resveratrol. *A,* H460 and A549 cells were arrested in the G_1 phase and HSG and HSY cells in the S phase after resveratrol treatment. Cells were fixed, treated with RNase, and then stained with propidium iodide. *B,* H460 cells were treated with 100 μmol/L resveratrol for 24 h and phosphorylation of pRb and expression of p21 and p27 were analyzed by Western blotting. *C,* H460 and HSY cells were treated with 100 μmol/L resveratrol and then stained with DAPI to examine whether nuclear fragmentation by apoptosis in these cells was induced by resveratrol treatment.

Table 1. Effect of resveratrol on cell cycle distribution of cancer cell lines

Phase	Percentage of cells in each phase (mean ± SD)							
	H460		A549		HSG		HSY	
	Control	Resveratrol	Control	Resveratrol	Control	Resveratrol	Control	Resveratrol
G_1	56.2 ± 0.03	81.0 ± 0.01	28.6 ± 0.01	60.5 ± 0.09	70.2 ± 0.06	22.9 ± 0.05	69.8 ± 0.05	19.4 ± 0.04
S	24.0 ± 0.07	6.3 ± 0.03	9.3 ± 0.01	13.9 ± 0.06	12.1 ± 0.03	57.8 ± 0.02	13.5 ± 0.01	61.8 ± 0.06
G_2 + M	18.0 ± 0.04	10.9 ± 0.02	43.3 ± 0.01	18.8 ± 0.01	16.3 ± 0.09	13.4 ± 0.02	15.5 ± 0.06	14.8 ± 0.07

in H460 cells by Western blotting. We found that phosphorylation of pRb decreased 6 h after resveratrol treatment, and this decrease continued until 24 h, indicating that inactivation of pRb was blocked by resveratrol (Fig. 2*B*). The status of the cyclin-dependent kinase inhibitors p21 and p27 were also investigated. The levels of both p21 and p27 were elevated 6 h after resveratrol treatment, and this increase continued further. Taken together, these results indicated that the cell cycle arrest at the G_1 phase of H460 could result from a series of steps caused by up-regulation of cyclin-dependent kinase inhibitors, hypophosphorylation of Rb, and arrest of the cell cycle in G_1 (Fig. 2*B*).

We found it interesting to note that in experiments using H460 cells, nuclear fragmentation was observed in a few cells treated with resveratrol for 24 h by 4′,6-diamidino-2-phenylindole (DAPI) staining, indicating that H460 cells undergo apoptosis (Fig. 2*C*). This process was not observed in salivary gland adenocarcinoma cells, indicating that resveratrol-induced apoptosis is cell line specific.

Resveratrol induced expression of LC3, a marker of autophagy. A previous study showed that resveratrol inhibited the growth of ovarian cancer cells by autophagy (18). Although the molecular pathways involved in the execution and regulation of apoptosis have been well defined, the mechanisms of autophagic cell death have not. In fact, aside from the finding that autophagic cell death is a caspase-independent process, investigations into autophagic cell death have been largely limited to discovering the morphologic characteristics of cells involved in the process, such as extensive autophagosomal formation and Atg-8/LC3 translocation to autophagic vesicles (21). Thus, to determine whether the cell growth inhibition and death caused by resveratrol is due to autophagy, and given the importance of the induction the typical autophagy marker LC3 as an indication of autophagy (10), we checked LC3 expression in lung and salivary gland cancer cells after treatment with resveratrol. LC3 expression was induced at 3 h and reached a maximum 24 h after resveratrol treatment in both H460 and HSY cells, indicating that the phytoestrogen resveratrol induced autophagy in both cell lines (Fig. 3*A*).

To validate our finding that the growth inhibition is reversible and due to autophagy, we analyzed the expression of LC3 in cells treated with resveratrol both before and after being washed with PBS. The expression of LC3 that was induced by resveratrol treatment decreased in H460 and HSY cells after it was washed off (Fig. 3*B*). This confirmed that the inhibitory effect of resveratrol on cell growth is due to autophagy and is reversible.

Resveratrol-induced LC3 expression is inhibited by 3-methyladenine, U0126, and pepstatin A. To further substantiate the role of autophagy in resveratrol inhibition of cell growth, we examined the effect of the autophagy inhibitor 3-methyladenine. 3-Methyladenine is a specific inhibitor of autophagy that acts at the sequestration stage when a double-membrane structure forms around a portion of the cytosol (22). We treated H460 cells with 3-methyladenine for 1 h before resveratrol treatment to determine whether resveratrol-induced LC3 expression was reduced by 3-methyladenine treatment. Indeed, LC3 expression stimulated by resveratrol was substantially blocked by 3-methyladenine (Fig. 4*A*). Then, because activation of the RAF1-MEK/extracellular signal–regulated kinase pathway by RAS stimulates autophagy (23), we examined whether a MEK inhibitor, U0126, inhibited resveratrol-induced LC3 expression. Treatment with U0126 at a concentration of 20 μmol/L effectively reduced LC3 expression in H460 cells (Fig. 4*B*). The aspartatic protease cathepsin D is abundant in lysosomes and plays an important role in autophagy in a various cells (24). Therefore, we also examined whether the cathepsin D inhibitor pepstatin A affected induction of LC3 expression caused by resveratrol. Treatment with 100 μmol/L pepstatin A for

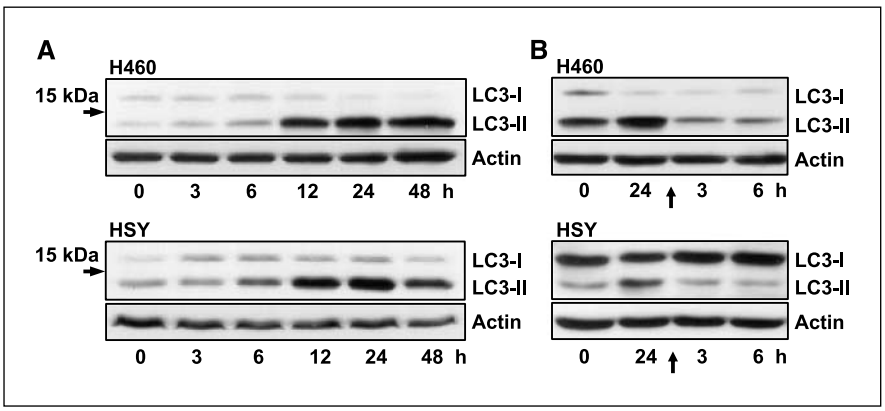

Figure 3. Resveratrol enhancement of autophagy marker LC3 is reversible. *A,* H460 and HSY cells were treated with 100 μmol/L resveratrol and the autophagy marker LC3 was analyzed by Western blotting. *B,* H460 and HSY cells were treated with 100 μmol/L resveratrol for 24 h, washed with PBS thrice (at time indicated by *arrow*), and then cultured in the same medium without resveratrol for 6 additional hours. The autophagy marker LC3 was analyzed by Western blotting.

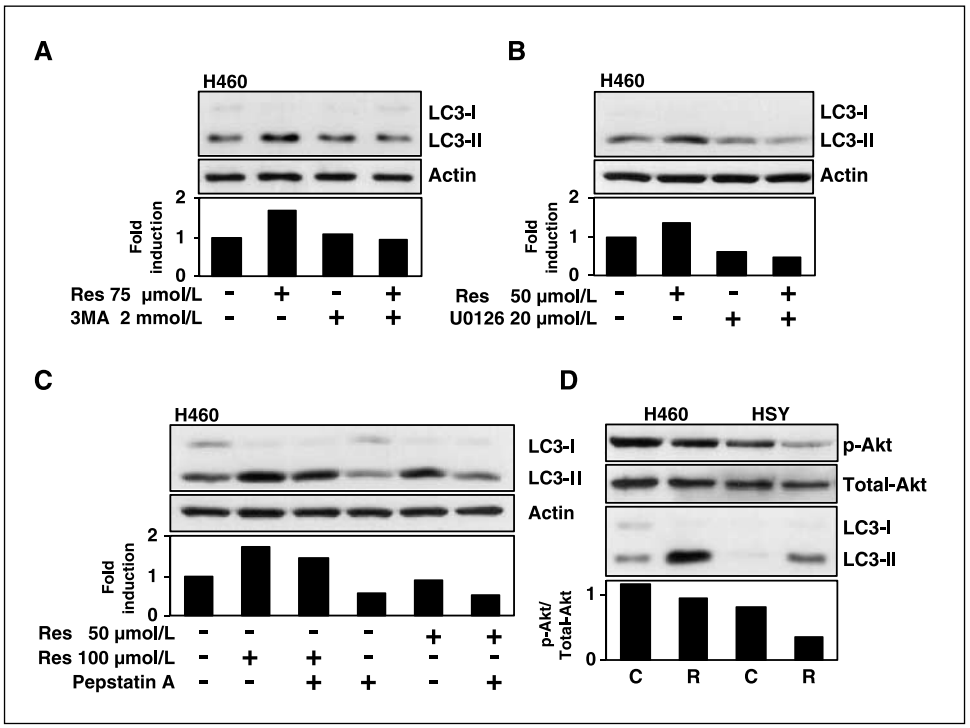

Figure 4. Inhibition of resveratrol-induced LC3 expression. H460 cells were left untreated or pretreated with 3-methyladenine (*3MA*; 2 mmol/L; *A*), U0126 (20 μmol/L; *B*), or pepstatin A (100 μmol/L; *C*) for 1 h before treatment with resveratrol (50, 75, or 100 μmol/L) for 24 h. LC3 expression was analyzed by Western blotting. *D,* H460 and HSY cells were treated with 100 μmol/L resveratrol for 24 h. Akt phosphorylation and LC3 expression were analyzed by Western blotting. *C,* control; *R,* resveratrol.

1 h before resveratrol treatment effectively blocked the induction of LC3 expression by resveratrol (Fig. 4*C*).

Because activation of Akt by class I phosphatidylinositol 3-kinase has been shown to inhibit autophagy via activation of mammalian target of rapamycin (9), we next analyzed the activation status of Akt in H460 and HSY cells after treatment with resveratrol. Although there was no change in the level of total Akt, phosphorylation of Akt was reduced in both cell lines (Fig. 4*D*). As expected, there was a considerable increase in the levels of LC3 expression after resveratrol treatment. Taken together, all these results confirmed that autophagy is the main cause of the antiproliferative effect of resveratrol in these cell lines.

PELP1 in resveratrol-induced autophagosomes. It is known that when autophagy is not activated, LC3 is localized homogeneously in the cytoplasm; however, on initiation of autophagy, LC3 associates with the isolation membrane and then remains associated with the autophagosomal membrane after these vacuoles are formed, indicating that LC3 can be used as a marker of autophagosomes (12). Because the presence of autophagic vacuoles in cancer cells after treatment with resveratrol indicates that they undergo autophagy and because GFP-LC3 localizes in the autophagosomes, we examined for cells transfected with GFP-LC3 and treated with resveratrol for autophagic vacuoles by immunofluorescence analysis. Autophagic H460 and HSY cells were identified by their characteristic GFP-LC3 dots on fluorescence microscopy (Fig. 5*A*).

Because resveratrol is known to bind to ERs as both agonist and antagonist (6) and it is known that, in addition to its direct ER binding, resveratrol has many non–ER-mediated cellular activities that may influence ER transcriptional activation through cross-talk mechanisms, we hypothesized that resveratrol induction of autophagy might have something to do with either the ER by itself or with one of its coactivators localized in the cytoplasm. This raised the possibility that PELP1, being able to bind to both ERα and ERβ (2) and a coactivator of both, owing to its localization in both cytoplasm and nucleus, plays a role in resveratrol autophagy induction. Another notable property of PELP1 is its ability to interact with HRS, an early endosomal protein that plays a role in regulating the trafficking of growth factor-receptor complexes through early endosomes (5). HRS sequesters PELP1 in the cytoplasm. Recently, an orthologue of HRS, CeVPS-27, was found to play a role in the endosomal and autophagic pathways in the nematode *Caenorhabditis elegans* (25). On the basis of all these findings, we examined the localization of endogenous PELP1 in both control and resveratrol-treated H460 and HSY cells. PELP1 was mostly present in the nuclei of H460 cells under control conditions, whereas resveratrol treatment induced the accumulation of PELP1 in vacuole-like compartments in the cytoplasm (Fig. 5*B*). These compartments were considered to be autophagosomes produced during autophagy. This finding provided a clue that PELP1 might be localized in autophagosomes. To substantiate this finding, we determined whether PELP1 is colocalized with GFP-LC3, a typical marker of autophagy in cells transfected with GFP-LC3 and treated with resveratrol. Confocal microscopic analysis showed that GFP-LC3 colocalized with endogenous PELP1 in autophagosomes in H460 and HSY cells treated with resveratrol (Fig. 5*B*). These findings suggest that PELP1 and LC3 are together in the autophagosomes.

The recent characterization of cytoplasmic PELP1 functions in our studies showed that it interacts in the cytoplasm with HRS, a trafficking molecule that activates mitogen-activated protein kinase in the presence of epidermal growth factor receptor, presumably by sequestering PELP1 in the cytoplasm. The fact that the general mechanism of autophagy involves the sequestration of cargo material into autophagosomes (11) raises the possibility that some sequestering molecule is involved in the localization of PELP1 in autophagosomes. Therefore, we next sought to determine whether HRS is what brings PELP1 to the autophagosomes. To verify this, we treated H460 cells with resveratrol and stained them for endogenous HRS and PELP1; we found that HRS colocalized

with PELP1 in the autophagosomes (Fig. 5*C*). Taken together, these results proved that HRS indeed brings PELP1 to the autophagosomes. To determine whether resveratrol-induced autophagosomes were reduced by 3-methyladenine treatment, H460 cells were transfected with GFP-LC3, left untreated or pretreated with 3-methyladenine for 1 h before treatment with resveratrol for 24 h, fixed, and then immunolabeled with anti-PELP1 antibody. The formation of autophagosomes induced by resveratrol in H460 cells was inhibited by 3-methyladenine (Fig. 5*D*).

To further confirm the HRS-dependent recruitment of PELP1 to autophagosomes on resveratrol treatment, we used HRS-specific siRNA to down-regulate HRS in cells treated with resveratrol, which were stained for HRS and PELP1. We found that in cells treated with resveratrol, HRS colocalized with PELP1 in the autophagosomes, whereas in HRS-knockdown cells, the number of PELP1-positive autophagosomes decreased drastically in resveratrol-treated cells (Fig. 6*A*). Together, these findings strengthen our hypothesis that HRS and PELP1 interact in the cytoplasm and move together to the autophagosomes on treatment with resveratrol. Recent studies from our group (26) showed that the cytoplasmic localization of PELP1 promotes resistance to SERMs. Because autophagy is a major intracellular pathway for the degradation and recycling of long-lived proteins and cytoplasmic organelles and because we found PELP1 in autophagosomes in response to resveratrol treatment, we speculated that this has some physiologic relevance to the protein levels of PELP1. To explore

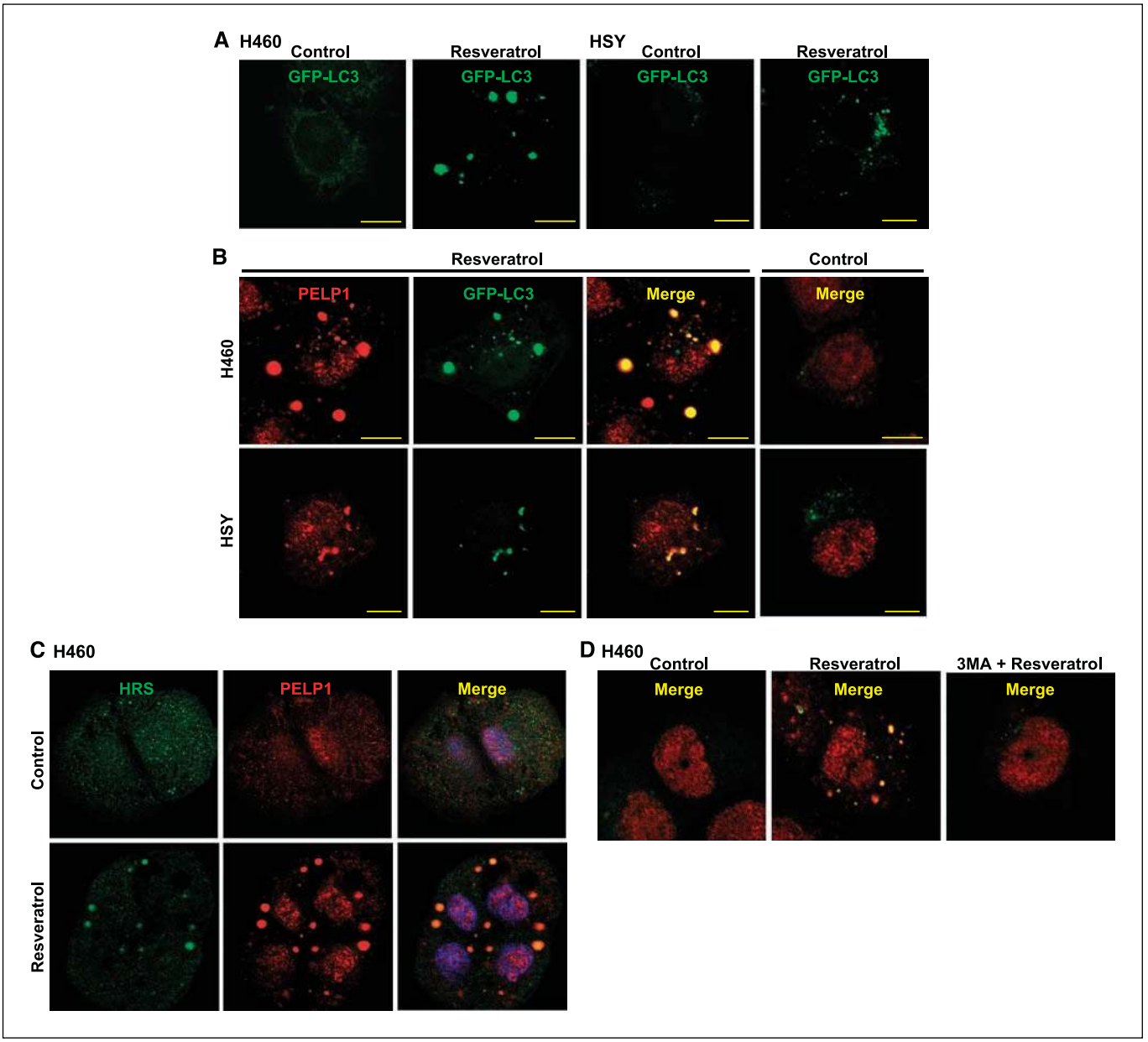

Figure 5. Recruitment of PELP1 into autophagosomes by resveratrol. *A,* H460 and HSY cells were transfected with GFP-LC3 and then treated with 100 μmol/L resveratrol for 24 h. *B,* H460 and HSY cells were transfected with GFP-LC3, treated with 100 μmol/L resveratrol for 24 h, fixed, and then immunolabeled with anti-PELP1 antibody. *C,* H460 cells were treated with 100 μmol/L resveratrol for 24 h, fixed, and then immunolabeled with anti-HRS and anti-PELP1 antibodies. *D,* H460 cells were transfected with GFP-LC3, left untreated or pretreated with 3-methyladenine (2 mmol/L) for 1 h before treatment with resveratrol (100 μmol/L) for 24 h, fixed, and then immunolabeled with anti-PELP1 antibody. *Bar,* 10 μm.

this possibility, we treated HSY and H460 cells with resveratrol and then checked their LC3 and PELP1 protein levels. The results of this experiment clearly showed that the PELP1 protein level was considerably lower in samples treated with resveratrol than it was in control samples (Fig. 6*B*). These differential changes in PELP1 levels in different cell lines could possibly be attributed to the effects of resveratrol on cell cycle progression, with a significant decrease when cells undergo G_1 arrest (H460) but not an S-phase arrest (HSY). There was also a clear increase in LC3 expression, indicating that these cells indeed undergo autophagy. Preliminary results from this experiment suggested that cells use autophagy as a means of degrading PELP1 in the cytoplasm in response to resveratrol treatment.

Discussion

The resveratrol-induced localization of the ER coactivator PELP1 in autophagosomes and its physiologic importance in cancer cells were the key issues being intensely investigated in this study. Our results showed that resveratrol inhibited cell growth and induced autophagy in various cancer cell lines in a reversible and 3-methyladenine–sensitive manner and that PELP1 is recruited to autophagosomes in response to resveratrol treatment.

The transcriptional activity of nuclear receptors is not only regulated by hormones but is also affected by several regulatory proteins called coactivators and corepressors (27). The novel ER coactivator PELP1 plays a role in both the genomic and nongenomic actions of ER. PELP1 is predominantly localized in the nucleus in hormonally responsive tissues, but recent findings suggest that PELP1 is localized exclusively in the cytoplasm in cancer cells, leading to excessive nongenomic signaling and possibly to SERM resistance (1).

The antiproliferative activity of the phytoestrogen resveratrol against tumor cell lines of different origin has been extensively characterized (7, 18). Opipari et al. (18) reported that resveratrol induces both apoptosis and autophagy in ovarian cancer cells. A number of studies showed cell cycle arrest of cancer cells induced by resveratrol and the ultimate induction of apoptosis and/or autophagy. Resveratrol treatment resulted in the S-phase arrest of ovarian cancer cell lines (28). Pozo-Guisado et al. (29) showed that MCF-7 breast cancer cells accumulated in the S phase after treatment with 50 μmol/L resveratrol, but at the 100 μmol/L concentration, they were blocked in the G_1 phase. The same was the case with ovarian cancer cells (18). However, in this study, we found that 100 μmol/L treatment with resveratrol resulted in the

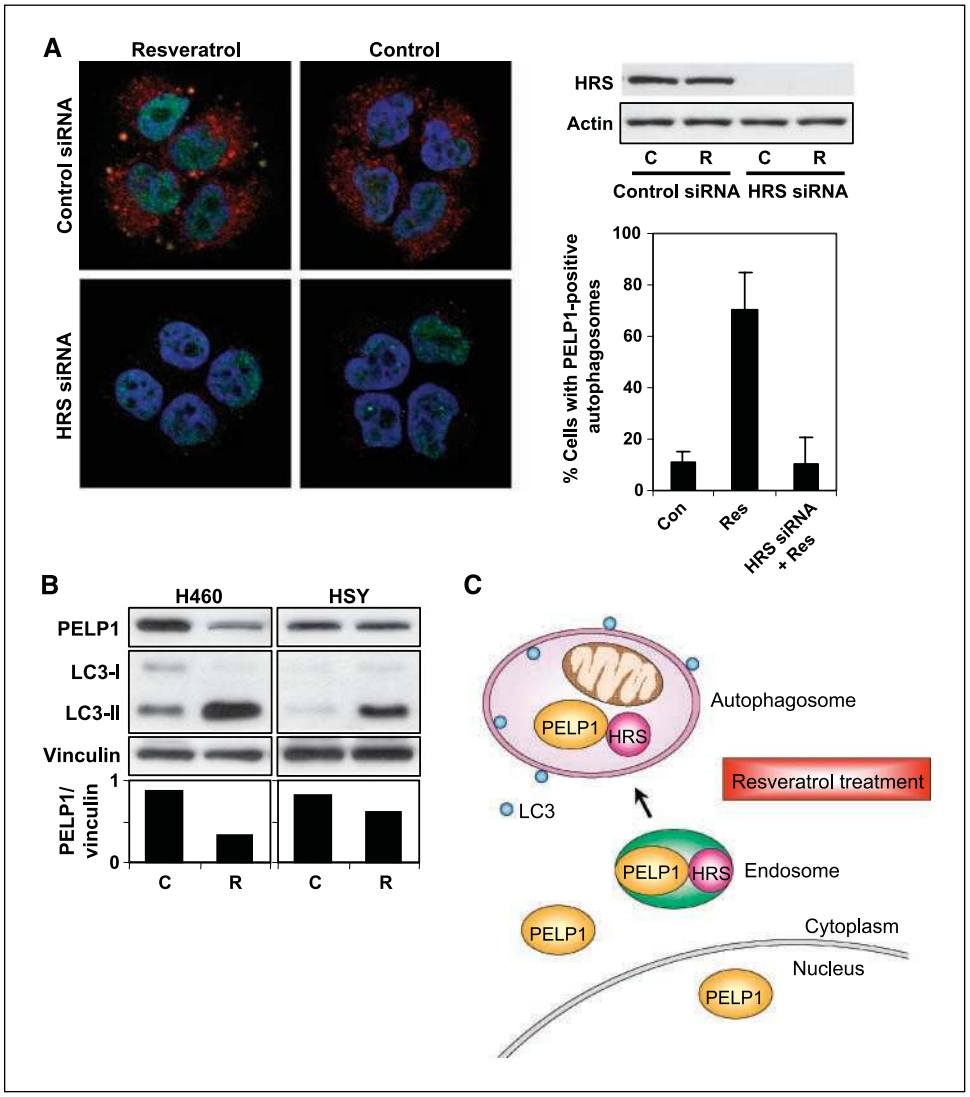

Figure 6. PELP1 recruitment to resveratrol-induced autophagosomes is HRS dependent. *A,* H460 cells were transfected with control or HRS siRNA and treated with resveratrol for 24 h. Cells were fixed and immunolabeled with anti-HRS (*red*) and anti-PELP1 (*green*) antibodies. After immunolabeling, PELP1-positive autophagosomes in the cells were counted by confocal microscopic observation. *Right, top,* efficiency of HRS knockdown by siRNA; *bottom,* quantitation of PELP1 positive autophagosomes (*bars,* SD). *B,* H460 and HSY cells were treated with 100 μmol/L resveratrol for 24 h and PELP1 and LC3 expression were analyzed by Western blotting. *C,* proposed working model for recruitment of PELP1 into autophagosomes on resveratrol treatment.

arrest of H460 and A549 lung cancer cells in the G_1 phase and of HSG and HSY salivary gland cancer cells in the S phase. This variation between different cell lines may be attributable to the difference in their basal proliferation rates. However, the overall outcome from these studies is that resveratrol provokes arrest of the cell cycle, preventing cancer cells from multiplying.

In our study, we found that resveratrol modulated cell cycle regulatory phosphorylated Rb and that the observed cell cycle arrest was associated with the accumulation of hypophosphorylated Rb. Recently, Kim et al. (17) showed that resveratrol inhibits proliferation of A431 human epidermoid carcinoma cells by blocking them in the G_1 phase and that this was associated with hypophosphorylated Rb. Our study showed that PELP1 was recruited to autophagosomes by resveratrol, and because PELP1 is known to regulate the cell cycle by interaction with pRb, we hypothesized that the recruitment of PELP1 to autophagosomes results in its degradation and might be related to the cell cycle arrest at the G_1 phase in H460 cells. Previous studies showed that E_2 induces up-regulation of cyclin D1 expression and that this increase is implicated in pRb phosphorylation (30). Furthermore, pRb is a coactivator and regulates cyclin D1 expression. Because our previous study showed that PELP1 overexpression resulted in persistent expression of cyclin D1 and hyperphosphorylation of pRb (3), it might be possible that resveratrol recruitment of PELP1 into autophagosomes causes down-regulation of cyclin D1, inducing the hypophosphorylation of pRb. It was reported earlier that cyclin D1 expression decreased with resveratrol treatment in SW480 colon carcinoma cells (16); Wolter et al. (19) also showed that finding in colon cancer cell lines.

In general, autophagy induces degradation of cytoplasmic proteins and organelles under both physiologic and pathologic conditions (9). The ER coactivator PELP1 has been shown to interact with HRS in the cytoplasm. Previously, an HRS orthologue, CeVPS-27, was found to play a role in the autophagic pathways in *C. elegans* (25). In this context, we hypothesized that during the process of autophagy induced by resveratrol, HRS clings to PELP1 as it is recruited into the autophagosomes, causing the degradation of PELP1 (Fig. 6*C*). This degraded PELP1 might be compensated for by the transition of PELP1 from the nuclear compartment to the cytoplasm. Till now, there are no reports about what regulates shuttling of PELP1 from nucleus to cytoplasm or vice versa. Because the cytoplasmic localization of PELP1 maybe linked with SERM resistance, another possibility is that cells might be using autophagy as a means of degrading PELP1 in the cytoplasm in response to resveratrol treatment. Further studies are needed to clearly understand the role of PELP1 in autophagy. It is quiet possible that other coactivators that bind to ERα and ERβ may have similar function as PELP1. In conclusion, these results give us a clue to the biological and physiologic mechanisms underlying autophagy in cancer cells.

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Announcements

Meeting of the Radiation Research Society

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

Erratum

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 821; substitute for the last paragraph:

The data in Table 3 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O_2 consumption, it is seen that the amount of glucose "cleavage products" *exceeds* the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and *is exceeded* by the glucose utilized by 16 per cent in CLL. If the assumption is made that, *in this respect*, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

$$0.65\ (+0.27) + 0.35\ (-0.16) = +0.12\ ,$$

a figure identical to the observed +0.12 for normal leukocytes.

Identifying the Estrogen Receptor Coactivator PELP1 in Autophagosomes

Kazufumi Ohshiro, Suresh K. Rayala, Seiji Kondo, et al.

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