

Etoposide Phosphate Enhances the Acetylation Level of Translation Elongation Factor 1A in PLC5 Cells

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Translation elongation factor 1A (eEF1A) is a factor critically involved in the process of protein synthesis. The activity of eEF1A has been shown by several studies to be regulated by post-translational modifications such as phosphorylation and dephosphorylation. However, until now less research has focused on other post-translational modifications of eEF1A, especially acetylation. In this report, we provide new evidence for the existence of eEF1A acetylation in PLC5 cells by immunoprecipitation and Western blotting. Using the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), we found that the deacetylation of eEF1A is mainly attributable to classes I and II HDAC rather than class III HDAC, and, furthermore, that the antitumour agent etoposide phosphate (VP 16) enhances the acetylation of eEF1A in a synergistic way with TSA. Our data suggest the possibility that the increased acetylation of eEF1A could be a new mechanism for the antitumour effect of etoposide.

Key words: eEF1A, Acetylation

Introduction

Protein synthesis in eukaryotic cells is a complicated process which requires hundreds of proteins and RNAs. The translation process includes three phases: initiation, elongation, and termination. Many studies indicated that the regulation of the elongation phase can be important during the cell cycle (Moldave, 1985), aging (Cavallius *et al.*, 1986), and oxidative stress (Ayala *et al.*, 1996). The eukaryotic translation elongation factor 1A (eEF1A), whose primary function is to shuttle aminoacyl-tRNA during protein translation, plays a key role in the elongation process (Kaziro *et al.*, 1991). Apart from its central role in translation, eEF1A also is involved in other cellular processes, including nuclear export processes, proteolysis, oncogenic transformation, apoptosis, cell proliferation, and organization of the cytoskeleton (Mateyak and Kinzy, 2010).

A protein participating in a certain metabolic pathway in a cell can influence the efficiency of the pathway through the change of the expression of its proteins, or through alterations occurring at the post-translation modification (PTM) level. PTM of proteins, characterized by a revers-

ible covalent modification process, is a common mode of regulation in cell metabolism. To date, dozens of PTMs have been reported, among which phosphorylation, methylation, and acetylation modifications have received much attention. In the case of eEF1A, modification by phosphorylation, methylation, and ethanolamine incorporation have been found (Rosenberry *et al.*, 1989). Phosphorylation of eEF1A has been reported to increase its activity (Peters *et al.*, 1995), while the function of methylated eEF1A is not clear yet. To date, few reports have elucidated whether or not eEF1A is subject to modification by acetylation. In the present study, we provide evidence that eEF1A is partly acetylated in PLC5 cells, and, furthermore, that the level of acetyl-eEF1A can be enhanced by the antitumour agent etoposide phosphate (VP 16).

Materials and Methods

PLC5, a hepatocellular carcinoma cell line obtained from ATCC, was maintained in 10-cm dishes with Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS)

(Invitrogen) and 1% penicillin-streptomycin (P-S) (Invitrogen) at 37 °C and under 5% CO₂. Nicotinamide (NAM) (Sigma, St. Louis, MO, USA) and trichostatin A (TSA) (Sigma) were added at final concentrations of 2.5 μ M (NAM) and 0.5 μ M (TSA), respectively. Four h after the addition of NAM and TSA, cells were lysed with 500 μ l lysis buffer [containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin] for 15 min on ice. The cell lysate was transferred to a 1.5-ml tube and centrifuged at 15000 $\times$ g for 10 min at 4 °C, and the supernatant was transferred to a new tube. Concentration of proteins in the supernatant was determined spectrophotometrically by the BCA method (Bio-Rad, Hercules, CA, USA). The supernatant containing 500 μ g protein was used to perform immunoprecipitation as follows. Cell protein was diluted with lysis buffer to a final volume of 1 ml. Twenty μ l protein G beads (GE Healthcare, Little Chalfont, UK) were added to the protein solution and shaken at 4 °C for 1 h to bind non-specific endogenous IgG. The protein G beads were removed by centrifugation. Then, 40 μ l protein G beads and 5 μ g mouse monoclonal anti-eEF1A antibody were added to the supernatant and the suspension was shaken at 4 °C overnight. Protein G beads were harvested by centrifugation and the beads washed three times with lysis buffer. The beads were then subjected to polyacrylamide gel electrophoresis (PAGE) and assayed by Western blotting. A mouse monoclonal anti-eEF1A antibody was used to detect total eEF1A in the immunoprecipitated material and a goat polyclonal antibody against ϵ -N-acetyllysine to probe acetyl-eEF1A after stripping the same PVDF membrane. All antibodies used were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The experiments were conducted in triplicate.

For mass spectrometric analysis, total cell proteins were prepared from the cells treated with 0.5 μ M TSA alone or 25 μ M VP 16 (Sigma) plus 0.5 μ M TSA or phosphate-buffered saline (PBS) by the method described above. Immunoprecipitation of eEF1A was performed from 2 mg of protein for each sample. The protein G beads coupled with proteins were directly subjected to sodium dodecyl sulfate (SDS)-PAGE and protein bands were visualized by a silver staining kit

(Invitrogen). Gel slices at the position of 53 kDa were cut and sent to BGI Corporation (Shenzhen, China) for mass spectrometric analysis.

Results and Discussion

Direct detection of acetylated proteins can be difficult because of their low abundance and rapid turnover in cells. To probe acetyl-eEF1A, we utilized the deacetylase inhibitors NAM and TSA to inhibit deacetylation in cells. As shown in Fig. 1, Western blotting of the immunoprecipitated proteins presented a similar quantity of eEF1A in the three samples, indicating that NAM and TSA treatment did not alter the overall level of eEF1A expression significantly in our experiment. When probed with an acetylation-specific antibody, the TSA-treated sample showing a clearly visible band at the same position like eEF1A could be detected. The NAM-treated sample, however, like the mock sample, showed no obvious signal at the corresponding position. These results prove the specificity of the acetylation-specific antibody and indicate that eEF1A is, at least partly, acetylated in PLC5 cells. There may be a dynamic acetylation-deacetylation balance for eEF1A, since no apparent acetyl-eEF1A was detected in the absence of a deacetylase inhibitor. Furthermore, only treatment with the inhibitor against classes I and II histone deacetylase (HDAC) (TSA) rather

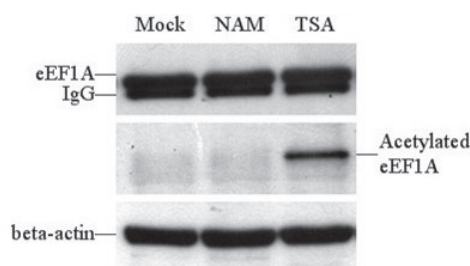


Fig. 1. Acetylation of eEF1A in PLC5 cells. PLC5 cells were treated with nicotinamide (NAM; 2.5 μ M), trichostatin A (TSA; 0.5 μ M) or PBS (mock) for 4 h, respectively. Total cell proteins were harvested after lysis. A total of 500 μ g protein for each sample was used for the following assay. Seventy five μ g protein of each sample first was analysed by PAGE and Western blotting with beta-actin antibody to normalize the quantity of immunoprecipitate. Then eEF1A present in each remaining sample was precipitated using an anti-eEF1A antibody. The product of immunoprecipitation was analysed by PAGE and Western blotting with the anti-eEF1A antibody and antibody directed against acetyllysine successively.

than that against class III HDAC (NAM) allowed detection of acetyl-eEF1A.

Next we assessed the influence of VP 16, an antitumour agent which has been reported to increase the acetylation of p53 (Luo *et al.*, 2004), pRb (Markham *et al.*, 2006), and SV40 large T-antigen (Shimazu *et al.*, 2006), on the acetylation of eEF1A. PLC5 cells were exposed to VP 16 (25 μ M) alone or VP 16 (25 μ M) plus TSA (0.5 μ M) for 4 h. eEF1A and acetyl-eEF1A were immunoprecipitated and detected by Western blotting. As shown in Fig. 2, no significant change in the overall levels of eEF1A expression was detected in the VP 16-treated samples, neither at 2 h nor 4 h of VP 16 treatment. The level of acetyl-eEF1A, however, presented a different picture: (i) Treatment with VP 16 alone did not make acetyl-eEF1A detectable; (ii) TSA alone, just as seen in Fig. 1, made acetyl-eEF1A detectable again, and an average of a 35% higher amount of acetyl-eEF1A 4 h post treatment was observed compared to that at 2 h post treatment; (iii) VP 16 plus TSA also enhanced eEF1A acetylation, with an amount higher at 4 h post treatment than at 2 h post treatment; (IV) compared to TSA alone, VP 16 plus TSA increased eEF1A acetylation 1.6-fold (mean value) 4 h post treatment. From the increased amount of acetyl-eEF1A at 4 h relative to that at 2 h, we can postulate that a dynamic effect of eEF1A acetylation may exist in the situation of both TSA alone and VP 16 plus TSA treatment. It seems that VP 16 plus TSA cause more acetylation of eEF1A than either TSA or VP 16 alone, suggesting that VP 16 actually enhances eEF1A

acetylation. In addition, there appears to be a synergy between VP 16 and TSA, since the amount of acetyl-eEF1A at 4 h induced by VP 16 plus TSA was higher than the sum of acetyl-eEF1A induced by either TSA alone or VP 16 alone. In fact, VP 16 treatment alone hardly led to any detectable acetyl-eEF1A in our experiments.

To identify the positions of the acetyl moieties in the eEF1A protein after VP 16 treatment, we carried out a mass spectrometric analysis. Specific acetyl signals were identified in 219K (RK*DGNASGTT) and 255K (QDVYK*IGGIG) of eEF1A from cells treated with VP 16 plus TSA. However, the acetyl signals were weak when cells were exposed to TSA alone and in the control cells.

Reversible acetylation of lysine residues has been found in both nuclear proteins, such as histones, and cytoplasmic proteins, such as p53 (Luo *et al.*, 2004) and sp1 (Torigoe *et al.*, 2005). Histone acetylation and deacetylation are catalyzed by two enzyme families, histone acetyltransferases (HATs) and HDACs, respectively. Recently, more and more non-histone proteins have been shown to be modified by acetylation. Using high-resolution mass spectrometry, Choudhary *et al.* (2009) performed a global analysis of lysine-acetylated cellular proteins. They identified 3600 lysine acetylation sites in 1750 proteins, one of which is eEF1A. Here we identified acetyl-eEF1A in PLC5 cells by Western blotting and mass spectrometry, thus providing independent evidence for the modification of eEF1A by acetylation. Direct detection of acetyl-eEF1A by Western blotting is difficult because of its rapid deacetylation reaction. Only when a deacetylase inhibitor was used, acetyl-eEF1A could be easily detected. Our data also revealed that the deacetylation of acetyl-eEF1A is mainly, if not entirely, attributable to the class I or/and II mammalian HDAC pathway. Which roles the classes I and II HDACs play in the eEF1A acetylation process is still an open question. Discrimination between the functions of classes I and II HDACs in this process requires the use of classes I or II HDAC-specific inhibitors. Recently a selective class II HDAC inhibitor, MC1568, has been reported (Nebbioso *et al.*, 2009). This specific HDAC inhibitor may help us to make a detailed elucidation of the functions of classes I and II HDACs in the process of eEF1A acetylation.

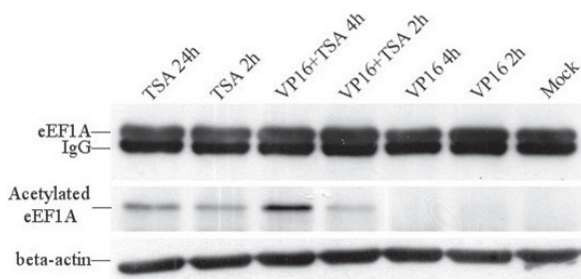


Fig. 2. Effect of etoposide phosphate (VP 16) on acetylation of eEF1A in PLC5 cells. PLC5 cells were exposed to VP 16 (25 μ M) alone or VP 16 (25 μ M) plus trichostatin A (TSA; 0.5 μ M) for 4 h. eEF1A and acetyl-eEF1A were immunoprecipitated, subjected to PAGE, and detected by Western blotting. Normalization of proteins was done like in Fig. 1.

VP 16 is an anticancer agent that causes topoisomerase II-mediated DNA damage by increasing the steady-state concentration of covalent DNA cleavage complexes (Froelich-Ammon and Osheroff, 1995). VP 16-induced DNA damage has been shown to increase the acetylation of several proteins. In H460 cells treated with VP 16 approximately 20% of total p53 was found to be acetylated, and, furthermore, acetylation of the C-terminal domain of p53 can dramatically enhance its site-specific binding to DNA on both short oligonucleotide probes and long DNA fragments (Luo *et al.*, 2004). Acetylation of pRb can also occur in response to DNA damage induced by VP 16, and this modification regulates the interaction between the C-terminal E2F-1-specific domain of pRb and E2F-1 (Markham *et al.*, 2006). VP 16 treatment also enhances the acetylation of SV40 large T-antigen, thereby destabilizing it and regulating its transforming activity in NIH3T3 cells (Shimazu *et al.*, 2006). Here we report that VP 16

increases the acetylation of eEF1A in PLC5 cells. To our knowledge, this is the first report establishing a connection between VP 16 and eEF1A acetylation. Several interesting questions arising from this finding warrant further investigation: What is the mechanism by which VP 16 enhances eEF1A acetylation? Is the down-regulation of deacetylase activities or the up-regulation of acetylase activities responsible for the increased acetyl-eEF1A? How does acetylation affect the functions of eEF1A? Is the increased acetyl-eEF1A associated with the antitumour effect of VP 16? Answering these questions would help us to understand more deeply the regulation of the function eEF1A and the antitumour mechanism of VP 16.

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