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Emetine optimally facilitates nascent chain puromylation and potentiates the RiboPuromylation Method (RPM) applied to inert cells

Alexandre David^{1,2}, Jack R. Bennink¹, and Jonathan W. Yewdell¹

¹Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD 20892, USA

²Institut de Génomique Fonctionnelle, CNRS UMR5203 – INSERM U661, 34094 Montpellier, France

Abstract

We previously described the RiboPuromylation method (RPM) to visualize and quantitate translating ribosomes in fixed and permeabilized cells by standard immunofluorescence. RPM is based on puromylation of nascent chains bound to translating ribosomes followed by detection of puromylated nascent chains with a puromycin-specific mAb. We now demonstrate that emetine optimally enhances nascent chain puromylation, and describe a modified RPM protocol for identifying ribosome-bound nascent chains in metabolically inert permeabilized cells.

Keywords

ribopuromylation; puromycin; translation; ribosome

Introduction

Puromycin (PMY) is an aminonucleoside Tyr-tRNA mimetic antibiotic that enters the A site of prokaryotic and eukaryotic ribosomes, covalently incorporating into nascent chains, terminating translation [1]. Eggers *et al.* pioneered PMY as a nascent protein tag, using polyclonal Abs to PMY to immunoblot and immunoprecipitate nascent proteins [2]. To circumvent non-specific reactivity of polyclonal anti-PMY antibodies (Abs), Schmidt *et al.* generated anti-PMY monoclonal Abs (mAbs) and applied them in flow cytometry to measure relative translation rates in living cells exposed to PMY based on expression of PMY-terminated cell surface proteins [3]. More recently, we used these mAbs and generated several new mAbs to localize puromylated nascent chains on ribosomes immobilized by chain elongation inhibitors in living cells prior to processing for intracellular immunofluorescence [4,5]. We described this method, termed the RiboPuromylation Method (RPM), as a simple and generally applicable method to localize and quantitate actively translating ribosomes by immunofluorescence.

Here, we show that emetine, an irreversible translation elongation inhibitor, enhances *in situ* nascent chains puromylation, explaining our previous observation that emetine gives a brighter RPM-based signal than cycloheximide. We also describe a new RPM protocol to localize translation sites in permeabilized cells with a labeling efficiency similar to the regular RPM.

Material and Methods

Cells and treatments

We cultured HeLa cells in DMEM, 7.5% FBS, splitting cultures the day before each experiment to achieve log phase growth concomitant with high translation rate. We used drugs at the indicated concentrations: CHX (355 M, Sigma), emetine (208 M, Calbiochem), anisomycin (9.4 M, Calbiochem), pactamycin (0.5 M), sodium arsenite (500 M, Sigma), CCCP (10 M), deoxyglucose (50mM), sodium azide (10mM). We infected HeLa cells with VV at a multiplicity of 1 pfu/cell in saline supplemented with 0.1% BSA. After adsorption at 37°C for 1h, we overlaid infected monolayers with DMEM containing 7.5% FBS, and incubated for 6 more h.

RPM

RPM on ice—We transferred HeLa cells to coverslips the day before the experiment to be 60–80% confluent at the time of the experiment. Depending on the experiment, we pre-treated cells with different inhibitors. Optimal staining with this protocol is obtained by pre-treating cells with 208 M of emetine diluted in warm DMEM 7.5% FBS for 15 min at 37°C prior to PMY labeling. We transferred coverslips to 24 well plates and washed cells twice with cold PBS supplemented with CHX (355 M, Sigma). All extraction/labeling procedures are performed on ice, using reagents pre-chilled to ice temperature. We incubated cells for 2 min with 500 L/well of Permeabilization Buffer (50mM Tris-HCl pH 7.5, 5mM MgCl₂, 25mM KCl, 355 M CHX, EDTA-free protease inhibitors (Roche), 10U/ML RNase Out (Invitrogen) containing 0.015% digitonin (Wako)). Following this extraction step, we washed cells once with 500 L of polysome buffer, added 500 L of polysome buffer supplemented with 91 M of PMY, and incubated for 10 min. After washing with 500 L of cold polysome buffer, we fixed cells with 500 L of 3% paraformaldehyde (PFA) for 15 min at RT, aspirated the PFA, added PBS, and incubated cells in a 4 °C refrigerator prior to immunofluorescence staining.

RPM at 37°C—HeLa cells on coverslips were incubated with DMEM 7.5% FBS supplemented with 91 M of PMY and 355 M of CHX for 5 min at 37°C. We then performed the extraction procedure described for RPM on ice.

Abs

The mouse PMY-specific mAb (clone 2A4) has been described [4] Anti-ribosomal P antibody is a human polyclonal autoimmune antisera (Immunovision). As secondary Abs we used goat anti-mouse A488 (Molecular Probes), donkey anti-rabbit Texas Red and donkey anti-human Cy5 (Jackson ImmunoResearch). To label DNA we used Hoechst 3358 (Molecular Probes). For immunoblotting we used polyclonal rabbit anti-mouse Ig-HRP (Dako) and goat anti-human Ig-HRP (Jackson ImmunoResearch).

Immunofluorescence and Microscopy

All staining was performed using staining buffer (SB, 0.05% saponin, 10mM Glycine, 5% FBS, PBS), as previously described [4]. Coverslips were incubated in SB for 15 min, and then incubated with Abs diluted in SB for 1 h at RT. After washing 3 times with PBS coverslips were incubated with secondary Abs diluted in SB for 1 h at RT. Coverslips were washed twice and incubated with 1 g/mL of Hoechst 3358 diluted in SB for 5 min at RT. Coverslips were washed twice with distilled water and mounted on slides with Fluoromount-G (Southern Biotech). Images were acquired using a Leica TCS SP5 laser scanning confocal microscope with a HCX PL APO lambda blue 63.0×1.40 OIL UV objective, Type FF immersion liquid (Cargille), 152.4 m pinhole. Images were processed using LAS AF

software (Leica), Imaris (Bitplane), Huygens Essentials Software (Version 3.6, Scientific Volume Imaging BV), and Photoshop CS4 (Adobe) without manipulating the gamma function. Each set of images for a given experiment were processed identically to maintain the image intensity ratio. ImageJ and Prism software were used for quantitation and statistical analysis. Groups were analyzed for statistical significance with a two-tailed unpaired *t*-test; error bars represent the s.e.m.

Results and Discussion

Emetine optimizes puromycylation at 4 °C

The puromycylation reaction occurs spontaneously in cell free extracts [1]. To correlate this finding with RPM staining, we depleted cellular energy stores by treating cells with deoxyglucose to block glycolysis in combination with either CCCP or azide to block oxidative phosphorylation. We then incubated cells with PMY for 5 min at 37 °C and performed anti-PMY immunoblots using the 12D10 anti-PMY mAb. Using either combination of energy depleting drugs only slightly reduces puromycylation (Figure 1A), consistent with the energy-independence of the cell free puromycylation reaction. While *in vivo* puromycylation is energy independent, it is undetectable when live cells are incubated with PMY at 4 °C (Fig. 1A). This low temperature sensitivity is not due to the temperature dependence of puromycylation *per se*, but to the accessibility of PMY to ribosome bound-nascent chains probably caused by a general decrease of cell membrane passive diffusion [6]. 4°C puromycylation is clearly seen when cells are permeabilized with 0.015% digitonin prior to PMY addition (Fig. 1B), and strikingly enhanced when cells were pretreated with emetine prior to permeabilization, which increases puromycylation to levels obtained with live cells at 37°C. While the precise mechanism is uncertain, the enhancing effect of emetine on low temperature puromycylation clearly demonstrates that first, ribosomal catalysis is essential for protein puromycylation, and second confirms the energy-independence of nascent chain puromycylation in cells.

RiboPuromycylation Method for Permeabilized Cells

We adapted the permeabilized cells protocol to enable RPM staining cells cultured on coverslips. We permeabilized cells with digitonin, incubated with PMY on ice for 10 min, paraformaldehyde fixed, and stained with anti-PMY mAb. We obtained relatively weak PMY staining (Fig. 2A) under these conditions, which is consistent with the immunoblotting data (Fig. 1B). As predicted from immunoblotting, pre-treating living cells with emetine at 37°C greatly increases PMY staining, which extensively co-localizes with ribosomes, as expected (Fig. 2A).

We next treated live cells for 15 min at 37°C with various translation inhibitors prior to permeabilization and 4 °C PMY staining (Fig. 2B). Arsenite induced translation shutdown [7] reduces anti-PMY staining to near background levels, while anti-ribosome staining is unaffected. Pactamycin, a translation initiation inhibitor [8], completely abrogated staining. By competing with PMY, anisomycin [9] had the same effect. By contrast, emetine pre-treatment increased staining 4-fold. Emetine, anisomycin and pactamycin achieved near maximal effects within 5 min of pre-treatment, while arsenite required 15 min treatment, paralleling the known kinetics of translation inhibition (Fig. 2C).

In direct comparison, emetine is slightly but clearly superior to CHX at enhancing RPM staining at 4 °C (Figure 2D). Importantly, using emetine, inert cell staining equals standard live cell RPM staining performed at 37 °C (Fig. 2E)

Emetine irreversibly binds the ribosomal 40S subunit [10], inhibiting aminoacyl-tRNA transfer [11] but unlike CHX, does not prevent transpeptidation or translocation of peptidyl-

tRNA from acceptor to donor sites [1]. The superior properties of emetine vs. CHX may be related to its irreversibility or a differential effect on ribosome puromycylation activity. In any event, the ability to achieve a maximal RPM signal on inert cells following digitonin permeabilization is a twist on the RPM that reduces ambiguity regarding the potential release of nascent from ribosomes, which while unlikely based on our previous findings [4], cannot be unequivocally ruled out.

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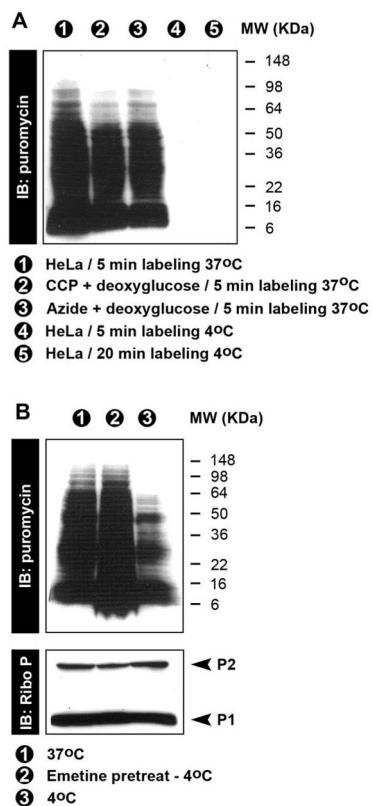
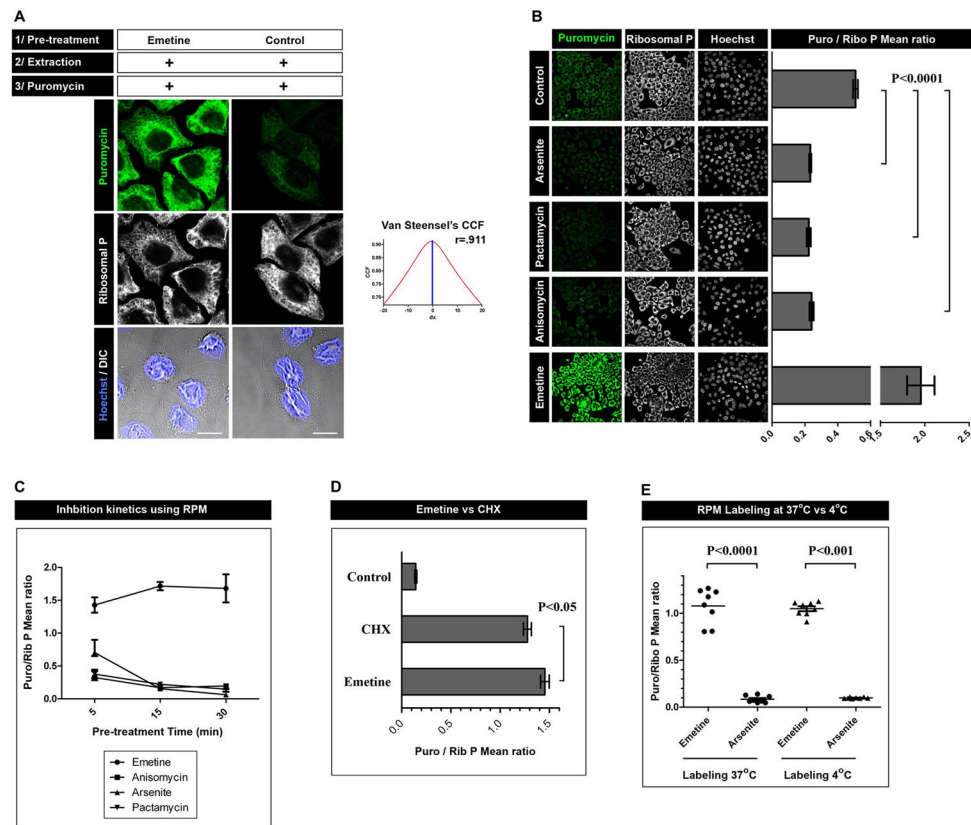


Figure 1.

A. Immunoblotting of total cell lysates demonstrates energy independence of puromylation and also the temperature dependence (37°C vs 4°C) when intact cells are labeled with PMY.

B. Immunoblotting of NP-40 extracts from HeLa labeled at 37 °C in culture or at 4 °C after digitonin treatment. Pre-incubation of cells with emetine prior to digitonin treatment greatly increases puromylation.

**Figure 2.**

A. HeLa cells were incubated or not for 15 min with emetine, were washed with PBS supplemented with CHX, permeabilized with digitonin and labeled with PMY in the presence of CHX. Cells were then fixed and stained for PMY (green) and large ribosomal subunit (white). Co-localization was quantitated using Van Steensel's cross-correlation coefficient (graph) and Pearson's coefficient (r ; 1=complete co-localization, -1 = complete non-colocalization). Bar scale: 10 μ m.

B. HeLa cells pre-treated for 15 min with the inhibitor indicated were labeled as in A. For each condition, multiple fields were acquired and the mean fluorescence ratio of PMY/ribosome staining for each field was quantitated using ImageJ. Values are plotted on the right. Statistical analyses, two-tailed unpaired t -test; ***, $P < 0.0001$.

C. As in B, but with the treatment times indicated

D. As in B with CHX and emetine pre-treatments. Statistical analyses, two-tailed unpaired t -test; *, $P < 0.05$.

E. Direct comparison of RPM labeling using the 4 °C or 37 °C protocol. Statistical analyses, two-tailed unpaired t -test; ***, $P < 0.0001$.