

Assessment of HDACi-Induced Protein Cleavage by Caspases

Fabian Treude, Tobias Gladbach, Jacqueline Plaster, and Jörg Hartkamp

Abstract

Aberrant histone deacetylase (HDAC) activity often correlates with neoplastic transformation and inhibition of HDACs by small molecules has emerged as a promising strategy to treat hematological malignancies in particular. Treatment with HDAC inhibitors (HDACis) often prompts tumor cells to undergo apoptosis, thereby causing a caspase-dependent cleavage of target proteins. An unexpectedly large number of proteins are in vivo caspase substrates and defining caspase-mediated substrate specificity is a major challenge. In this chapter we demonstrate that the hematopoietic transcription factor PU.1 becomes cleaved after treatment of acute myeloid leukemia (AML) cells with the HDACis LBH589 (panobinostat) or MS-275 (entinostat). To define caspase specificity for PU.1, an in vitro caspase assay including caspases 1–10 with in vitro-translated PU.1 is described in detail.

Key words Caspase-8, PU.1, HDACi, LBH589, MS-275, In vitro translation

1 Introduction

Reversible acetylation regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs) plays a key role in the epigenetic regulation of gene expression [1]. Abnormal epigenetic changes are frequent and early events in tumorigenesis, and deregulated acetylation in particular, has been associated to tumor progression [2]. HDACs regulate acetylation by deacetylating lysine residues of nuclear histones as well as a large number of non-histone proteins that are involved in tumor progression, cell cycle control, and apoptosis [2–4]. 18 HDAC family members are divided into Zn²⁺-dependent class I (HDAC 1, 2, 3, 8), class II (HDAC 4, 5, 6, 7, 9, 10), class IV (HDAC 11), and the NAD⁺-dependent class III HDACs, which are termed sirtuins (SIRT 1–7) [4, 5].

Most HDAC inhibitors (HDACis) developed so far target the Zn²⁺-dependent class I, II, and IV enzymes and many of these have entered clinical trials [4, 6]. In cell-based studies HDACis are powerful antiproliferative agents that can promote a broad

spectrum of cellular changes, including apoptosis in cancer cells [1]. Furthermore, therapeutic efficacy and HDACi-induced tumor cell death have been shown to be directly connected in preclinical model systems [6]. Transformed cells are up to ten times more sensitive to HDACi treatment than their normal cellular counterparts, but why tumor cells are more sensitive to HDACi-induced apoptosis is not entirely understood [6].

Most morphological changes triggered by the induction of apoptosis are associated to the activation of cysteine-dependent aspartate-specific proteases (caspases) [7]. Human caspases are typically divided into inflammatory caspases (caspases 1, 2, 4, 5), apoptotic effector caspases (caspases 3, 6, 7), and apoptotic initiator caspases (caspases 8, 9, 10) [8]. Catalysis of caspases is executed by a conserved cysteine side chain in the catalytic domain and a highly stringent selectivity for cleaving substrate proteins or peptides on the C-terminal side of aspartate residues [7, 9]. Their biochemical properties are tightly regulated to ensure that the irreversible proteolysis catalyzed by caspases occurs specifically and efficiently. Caspase substrate recognition depends on primary, secondary, tertiary, and quaternary structure effects, posttranslational modifications, as well as spatial and temporal localization [7]. Recent advances in the field of proteome-scale studies of proteases and their corresponding substrates have led to the identification of almost 1000 human proteins that are cleaved by caspases [7]. In order to analyze caspase substrate specificity under certain physiological conditions, two different types of experiments, which complement each other, can be employed. Initially, cell culture or in vivo experiments can reveal which proteins are cleaved during a biologically relevant process. Following the discovery of a potential caspase substrate in vitro experiments can illustrate which caspase is capable of cleaving the identified protein. After the initial examination of a functionally relevant cleavage event the specificity of the caspase substrate interaction should be validated. In some cases, a specific caspase may cleave a target protein in vitro but may not have access to it in the cell. Therefore, overexpression of the identified caspase and substrate protein in cells should verify direct cleavage of the target protein. Furthermore, the same caspase should be active under the cell culture conditions under which the protein of interest was identified as a caspase target in the first place.

Here, we analyzed how the clinically tested HDACis MS-275 (inhibits HDAC 1–3) [10] and LBH589 (inhibits HDAC 1–11) [11] affect the transcription factor purine-rich box1 (PU.1) in the promyelocytic leukemia cell line HL-60. PU.1 belongs to the ETS transcription factor family and plays a key role in the development of most hematopoietic cell lineages [12, 13]. Downregulation of PU.1 expression is observed in human acute myeloid leukemia (AML) patients [13] and a reduction of PU.1 expression leads to AML in mouse models [14, 15], suggesting that PU.1 functions as

a tumor suppressor. PU.1 contains a C-terminal DNA-binding domain and an N-terminal activation domain followed by a domain enriched in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) (Fig. 1a). Treatment of HL-60 cells with LBH589 and MS-275 leads to cleavage of the caspase-3 substrate PARP-1 in a dose-dependent manner, indicating that both HDACis induced apoptosis under these conditions (Fig. 1b). Under apoptotic conditions triggered by HDAC inhibition a cleaved band at ~24 kDa could be detected by anti-PU.1 antibody (detects C-terminal part of PU.1) while full-length PU.1 disappears under these conditions (Fig. 1b). Interestingly, Zhao and coworkers could demonstrate before that PU.1 is cleaved into two C-terminal fragments of ~24 and ~16 kDa under apoptotic conditions in leukemia cell lines [16]. The cleavage sites were mapped at HVLD₉₇↓T and GEAD₁₅₁↓G and this cleavage process is specifically catalyzed by caspase-3 (Fig. 1a) [16].

To validate and confirm the specificity of caspase-3 for PU.1 in vitro-translated PU.1 was subjected to a caspase cleavage assay

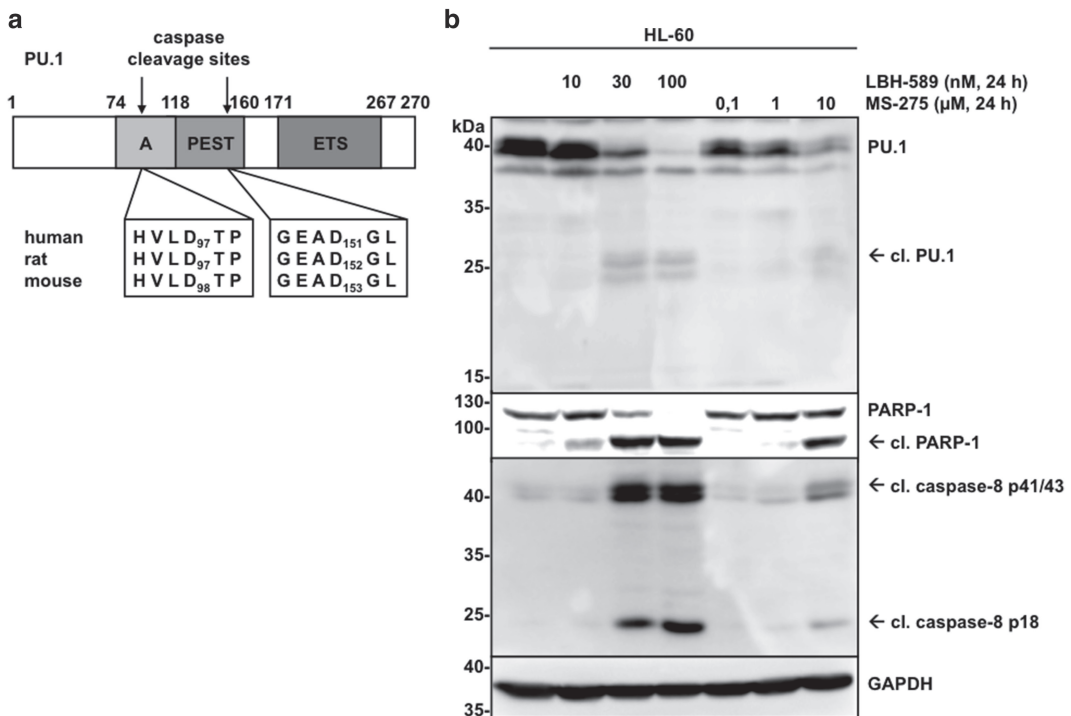


Fig. 1 PU.1 is cleaved upon HDACi treatment in HL-60 cells. **(a)** Schematic overview of human PU.1 protein: The caspase cleavage sites at positions D97 and D151; activation domain (amino acids 74–118); PEST domain (domain enriched in proline (P), glutamic acid (E), serine (S), and threonine (T), amino acids 118–160); ETS-domain (amino acids 171–267). **(b)** HL-60 cells were incubated with 10, 30, and 100 nM LBH589, or with 0.1, 1, and 10 μM MS-275 for 24 h. Cell lysates were analyzed by immunoblotting using antibodies against PU.1, PARP-1 as a marker for apoptosis, cleaved-caspase-8 and GAPDH as loading control

with recombinant caspases 1–10 (Fig. 2). Alternatively, the caspase assay can be performed with purified recombinant proteins or with immunoprecipitated proteins [17, 18]. Figure 2 demonstrates that only caspase-1 and caspase-8 were able to hydrolyze full-length PU.1, whereas caspase-3 failed to do so. Caspase-8 processing of PU.1 resulted in the appearance of a single ~16 kDa fragment, whereas caspase-1-mediated PU.1 cleavage resulted in the generation of an ~24 kDa and a main ~16 kDa fragment detected with the C-terminal PU.1 antibody (Fig. 2). These data are in agreement with the two reported caspase cleavage sites at HVLD₉₇↓T and GEAD₁₅₁↓G [16]. Caspase-1 and -8 were able to cleave PU.1 to various degrees in vitro with caspase-8 being the most efficient to hydrolyze PU.1 as judged by the complete disappearance of full-length PU.1 and the absence of the ~24 kDa cleavage fragment. The use of antibodies has got its limitations especially when more than one cleavage event occurs. In this case the synthesis of an in vitro [³⁵S] methionine-labeled target protein provides a useful alternative to analyze cleavage events if no suitable antibody is available [19].

Our in vitro data suggest that caspase-8 might cleave PU.1 in HL-60 cells that are exposed to apoptosis-inducing concentrations of HDACi. To confirm caspase-8 activation under conditions where the HDACi treatment results in PU.1 cleavage, we made use of an antibody that specifically recognizes the activated and cleaved form of caspase-8. Figure 1b demonstrates that caspase-8 activation correlates with PU.1 cleavage, suggesting that HDACi-induced PU.1 cleavage is caspase-8 dependent. While caspase-8-mediated PU.1 cleavage in vitro results in the generation of a C-terminal ~16 kDa PU.1 fragment, HDACi-induced apoptosis in vivo mainly generates a C-terminal ~24 kDa PU.1 fragment, indicating that under these conditions only one PU.1 cleavage site is targeted by caspase-8.

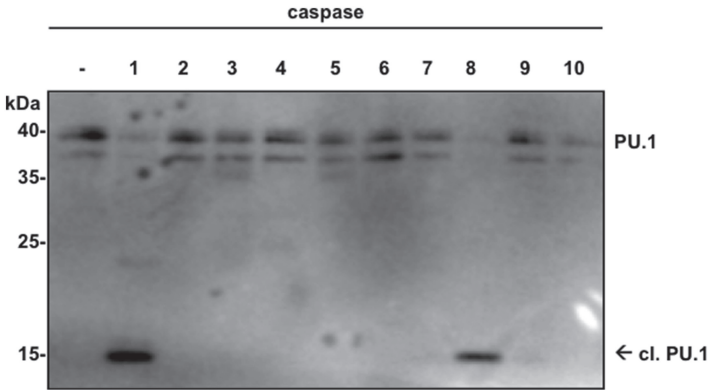


Fig. 2 PU.1 is a substrate of caspase-1 and caspase-8. Caspase assay using in vitro-translated PU.1 and recombinant human caspases 1–10. The reaction was stopped after 30 min by adding sample buffer and proteins were analyzed by immunoblotting using PU.1 antibodies

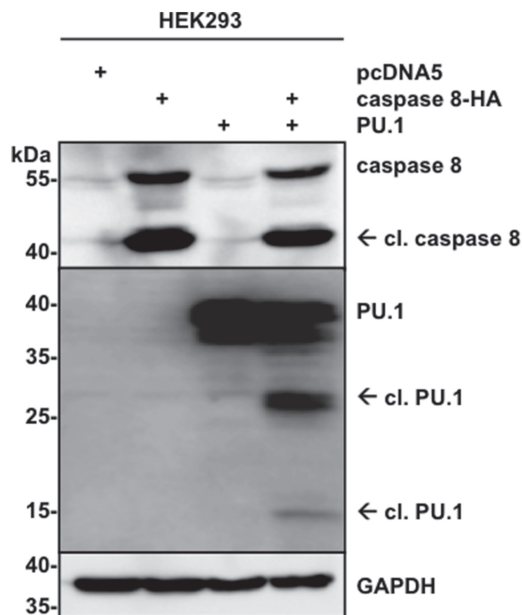


Fig. 3 PU.1 is cleaved by caspase-8 in cells. HEK293 cells were transfected with 1 μ g of the indicated plasmids for 16 h using the calcium phosphate transfection method. Cells were lysed and subjected to immunoblotting using antibodies recognizing caspase-8, PU.1, and GAPDH

On the other hand, coexpression of PU.1 and caspase-8 in HEK293 cells results in the generation of both PU.1 cleavage products, indicating that caspase-8 has access to PU.1 in the cell and is capable of inducing PU.1 cleavage at both sites (Fig. 3).

Caspase-mediated cleavage of a substrate protein can lead to a gain, loss, or change of a protein's functions [7]. To analyze the contribution of PU.1 hydrolysis towards HDACi-induced apoptosis in HL-60 cells the creation of a cleavage-resistant PU.1 mutant should help to investigate the importance of caspase-8-mediated PU.1 cleavage. In addition, the overexpression of PU.1 cleavage fragments should complement these studies to address the biological effect of the HDACi-mediated PU.1 cleavage.

2 Materials

Materials and antibodies listed below are routinely used in our laboratory. However, equipment and reagents from other providers should be equally suitable.

2.1 Preparation of Whole-Cell Extracts

1. TLB lysis buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (v/v), 1 mM DTT. Freshly add 0.5 mM NaF, 1 mM NaVO₃, and a protease inhibitor cocktail.

2. Phosphate-buffered saline (PBS).
3. SDS-PAGE loading buffer (2×): 20 mM Tris-HCl pH 6.8, 20 % glycerol (v/v), 4 % SDS (w/v), spatula tip bromophenol blue. Freshly add 200 mM DTT.
4. Bio-Rad Protein Assay Dye Reagent Concentrate (Bio Rad).

2.2 SDS-Polyacrylamide Gel Electrophoresis

1. Separating gel buffer: 1.5 M Tris-HCl pH 8.8.
2. Stacking gel buffer: 2 M Tris-HCl pH 6.8.
3. 20 % SDS: 20 % (w/v) aqueous solution.
4. 20 % Ammonium persulfate (APS): 20 % (w/v) aqueous solution.
5. Tetramethylethylenediamine (TEMED).
6. 30 % Acrylamide/bisacrylamide 29/1.
7. Methanol.
8. Mini PROTEAN 3 system-casting stand with corresponding casting frames, combs, and glass plates (Bio-Rad).
9. SDS-running buffer: 25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS (*see Note 1*).
10. Protein ladder.

2.3 Protein Transfer: Western Blot

1. Polyvinylidene difluoride (PVDF) membrane.
2. Whatman paper (3 M).
3. Methanol.
4. Transfer buffer: 25 mM Tris, 250 mM glycine, 10 % methanol.
5. Semidry transfer blotter.

2.4 Antibody Detection

1. Antibodies, *see* Subheading 2.8.
2. Nonfat dry milk.
3. BSA.
4. PBS-T: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.05 % Tween 20.
5. Enhanced chemiluminescence (ECL): 100 mM Tris-HCl pH 8.8, 2.5 mM Luminol, 0.2 mM poly-coumaric acid.
6. 30 % H₂O₂ (v/v).

2.5 In Vitro Translation

1. TNT® Quick Coupled Transcription/Translation Systems (Promega; # L4610).
2. PEQ176P2-PU.1 was subcloned into the EcoRI/HindIII sites of pCDNA3.1 (+).

2.6 Caspase Assay

1. Recombinant human caspases (1–10) (PromoKine; PK-RP577-K233).
2. Caspase assay buffer: 50 mM Hepes pH 7.2, 50 mM NaCl, 0.1 % Chaps (v/v), 10 mM EDTA, 5 % Glycerol (v/v). Freshly add 10 mM DTT.

**2.7 Calcium
Phosphate
Transfection**

1. Hepes-buffered saline (HBS): 21 mM Hepes pH 6.95, 136.9 mM NaCl, 5 mM KCl, 0.89 mM Na₂HPO₄.
2. 2.5 M CaCl₂.

**2.8 Antibodies
and HDAC Inhibitors**

1. PU.1 (Santa Cruz; sc-352 [rabbit]).
2. GAPDH (Santa Cruz; sc-32233 [mouse]).
3. Caspase-8 (Cell Signaling; #9746 [mouse]).
4. PARP-1 (Cell Signaling; #9542 [rabbit]).
5. Cleaved caspase-8 (Asp 391) (Cell Signaling; #9496 [rabbit]).
6. LBH589 (Selleckchem).
7. MS-275 (Selleckchem).

3 Methods

**3.1 Preparation
of Whole-Cell Extracts**

1. Human HL-60 cells are cultured in 60 mm dishes and stimulated with increasing concentrations of the HDACis LBH589 (10 nM, 30 nM, 100 nM) or MS-275 (0.1 μM, 1 μM, 10 μM) for 24 h.
2. The following steps are performed on ice. Cells are centrifuged at 200×*g* for 5 min at 4 °C and the supernatant is carefully aspirated. Before cell lysis, cells are washed with ice-cold PBS and centrifuged at 200×*g* for 5 min at 4 °C and the supernatant is aspirated.
3. Cells are lysed immediately using 200 μl ice-cold complete TLB lysis buffer. Vortex and keep on ice for 20 min.
4. A centrifugation step at 20,000×*g* for 20 min at 4 °C is performed to remove cell debris. 3 μl of the supernatant is used for protein concentration.
5. Protein concentration is measured using Bio-Rad Protein Assay Dye Reagent Concentrate at OD 595.
6. Mix 150 μl protein lysate with 150 μl 2× SDS-PAGE loading buffer and heat at 95 °C for 5 min. Spin down the protein samples and perform SDS-PAGE with equal protein amounts.

**3.2 SDS-
Polyacrylamide Gel
Electrophoresis**

1. Cast the 12.5% acrylamide (v/v) separating gel using 4.9 ml H₂O, 6.0 ml acrylamide, 3.8 ml 1.5 M Tris-HCl (pH: 8.8), and 75 μl 20% SDS (w/v). 15 μl TEMED (catalyst) and 40 μl 20% APS (w/v) (radical former) should be added last. After vortexing the mixture is immediately poured into the assembled gel plate to ¾ of the volume. Cover the surface of the gel with 500 μl isopropanol to ensure proper polymerization of the gel.
2. After the separating gel is polymerized (approx. 10 min) the isopropanol is carefully removed using filter paper. Cast the stacking gel using 4 ml H₂O, 635 μl acrylamide, 313 μl 2 M

Tris (pH: 6.8), 25 μ l 20 % SDS (w/v), 5 μ l TEMED, and 40 μ l 20 % APS (w/v) and quickly insert the comb.

3. After polymerization of the stacking gel (approx. 10 min) the comb should be removed carefully and the slots carefully rinsed with ddH₂O.
4. Assemble the gel in an electrophoresis chamber (Bio-Rad) and fill it up with SDS-running buffer (*see* **Note 2**).
5. Use a Hamilton syringe to load equal amounts of protein samples (*see* Subheading 3.1), and load a protein ladder as size standard.

3.3 Protein Transfer: Western Blot

Western blotting involves the transfer of proteins separated on SDS-polyacrylamide gel electrophoresis to a solid matrix such as a PVDF membrane. Western blotting can be performed by either complete immersion of a gel-membrane sandwich in a transfer buffer (wet transfer) or placing the gel-membrane sandwich between absorbent Whatman paper soaked in transfer buffer (semidry transfer). Semidry transfer is generally more rapid and efficient than wet transfer and is in general better suited to low-molecular-weight protein (smaller 150 kDa).

1. A PVDF membrane (usually 6 \times 9 cm) is equilibrated in methanol for 20 s. Afterwards the PVDF membrane is washed in ddH₂O for 2 min and incubated in transfer buffer for 5 min for equilibration (*see* **Note 3**).
2. Take the polyacrylamide gel and remove the stacking gel. The protein gel is also incubated in transfer buffer for 5 min for equilibration (*see* **Note 3**).
3. In the meantime soak four Whatman papers (same size as PVDF membrane) in transfer buffer.
4. Carefully arrange Whatman papers, polyacrylamide gel, and PVDF membrane on the semidry blotter in the following order: (anode +), 2 \times Whatman paper, PVDF membrane, polyacrylamide gel, 2 \times Whatman paper (cathode -). Avoid air bubbles between layers as they result in insufficient protein transfer.
5. The transfer should run for 1 h at 50 mA per polyacrylamide gel.
6. Disassemble the semidry blotter, transfer the PVDF membrane to a tray with PBS-T, and incubate the membrane on a rocking platform for 5 min.

3.4 Antibody Detection

1. Incubate the PVDF membrane in PBS-T with 5 % (w/v) non-fat dry milk for 1 h on a rocking platform to block unspecific binding sites on the membrane.
2. Wash the PVDF membrane three times for 5 min in PBS-T. Add the membrane to a tray with PBS-T 5 % (w/v) BSA and PARP-1 antibody (e.g., 9542) diluted 1:2000 (*see* **Note 4**). Incubate overnight at 4 °C on a rocking platform.

3. Wash the PVDF membrane three times for 5 min in PBS-T on a rocking platform to wash away unbound antibodies.
4. Incubate the PVDF membrane for 1 h at room temperature with the secondary antibody diluted 1:8000 in PBS-T with 5 % nonfat dry milk.
5. Afterwards the membrane is washed three times for 5 min with PBS-T to wash away unbound secondary antibodies.
6. The signal is detected by autoradiography using ECL. In brief, 10 ml ECL buffer is mixed with 10 μ l 30% H₂O₂ (v/v). Incubate the PVDF membrane for 1 min in ECL solution, wipe off excessive solution, and develop the PVDF membrane.

3.5 *In Vitro* Translation

For the in vitro synthesis of proteins cell-free systems derived from cells engaged in a high rate of protein synthesis have been developed. The most frequently used cell-free translation systems consist of extracts from rabbit reticulocytes, wheat germ, or *Escherichia coli*. They contain all macromolecular components (tRNAs, aminoacyl-tRNA synthetases, ribosomes, initiation, elongation and termination factors, etc.) for efficient translation of exogenous RNA. Whereas standard translation systems (reticulocyte lysates or wheat germ extracts) use RNA as template, coupled systems start with DNA templates, which are transcribed into RNA and subsequently translated. Such systems typically combine a prokaryotic phage RNA polymerase and promoter (T7, T3, or SP6) with eukaryotic or prokaryotic extracts to synthesize proteins from DNA templates. The TNT[®] Quick Coupled Transcription/Translation System (Promega) is a coupled transcription/translation kit for eukaryotic in vitro translation purposes and is available for transcription/translation of genes cloned downstream of the T7 or SP6 RNA polymerase promoters. It simplifies in vitro reactions by combining RNA polymerase, nucleotides, salts, and recombinant RNasin ribonuclease inhibitor with the reticulocyte lysate in a single master mix (*see Note 5*). Starting with circular plasmid DNA in vitro transcription/translation results may be generated in 3–4 h and can be used to analyze posttranslational modifications subsequently (*see Subheading 3.6*).

1. Rapidly thaw the TNT[®] Quick Master Mix by hand-warming and store it on ice.
2. Pipet 40 μ l TNT[®] Quick Master Mix, 1 μ l 1 mM methionine (*see Note 6*), and 2 μ l pCDNA3-PU.1 (0.5 μ g/ μ l) and fill up to a final volume of 50 μ l with nuclease-free H₂O.
3. Incubate the reaction at 30 °C for 60–90 min.
4. Analyze the results by Western blotting (*see Subheadings 3.2–3.4*).

3.6 Caspase Assay

Before performing the caspase assay the efficacy of the in vitro translation should be tested and analyzed by Western blotting.

1. 2 μ l in vitro-translated PU.1, 0.1 unit/enzyme recombinant human caspases 1–10 (PromoKine), and caspase assay buffer to a final volume of 20 μ l are incubated at 37 °C for 30 min (*see* **Note 7**).
2. The caspase reaction is stopped, by adding equal amounts of 2 \times SDS loading dye.
3. The caspase assay is analyzed by Western blotting (*see* Subheadings 3.2–3.4).

3.7 Calcium Phosphate Transfection

Transient transfection of plasmid DNA into cells is an indispensable tool in molecular biology. Though plenty of lipid-based transfection reagents are commercially available, a quick, efficient, and inexpensive method is to transfect eukaryotic cells via the calcium phosphate co-precipitation method. Calcium phosphate transfection typically results in transient expression of the delivered plasmid DNA and the efficacy can be close to 100 % depending on the cell lines used.

1. For transient transfections HEK293 cells are seeded in 6-well plates 1 day prior to transfections.
2. 1 μ g of plasmid DNA is diluted in 160 μ l HBS buffer and afterwards 8.5 μ l 2.5 mM CaCl_2 is added drop-wise.
3. The transfection mixture is carefully vortexed and incubated for 25 min at room temperature.
4. Finally the transfection mixture is added drop-wise to the cells (50% confluency is desired) and the cells are incubated for 16–24 h.
5. The transfected cells are lysed and analyzed by Western blotting (*see* Subheadings 3.1–3.4).

4 Notes

1. A solution of a tenfold concentrated SDS running buffer can be used as a stock.
2. SDS-PAGE polyacrylamide gels can be stored up to 2 weeks wrapped in wet paper and sealed in a plastic bag to prevent drying out at 4 °C. Longer storage is not recommended as it results in structurally unstable gels.
3. The equilibration step in Western blotting refers to the soaking of your PVDF membrane and your polyacrylamide gel in transfer buffer for at least 5 min. The equilibration step ensures that any contaminating electrophoresis buffer salts are removed, the protein gel is allowed to shrink to its final size before the

transfer, and it prevents the protein gel or membrane to dry out if you cannot do the transfer immediately.

4. After the protein transfer from the gel it is important to block the PVDF membrane to prevent nonspecific binding of the antibodies during subsequent steps. The suitable blocking buffer (nonfat dry milk, BSA, etc.) should improve the sensitivity of the assay by reducing background. For most antibodies a recommendation for the blocking buffer is provided. In other cases this has to be tested empirically.
5. To reduce RNase contamination Recombinant RNasin® Ribonuclease inhibitor is included in the TNT® Quick Master Mix (Promega).
6. If no suitable antibody is available coupled in vitro reactions from plasmid DNA can be used to synthesize [³⁵S] methionine-labeled proteins by incorporating 2 µl [³⁵S] methionine (1000 Ci/mmol at 10 mCi/ml) in the transcription/translation reaction.
7. The amount of time for the caspase/substrate assay can vary considerably and should be titrated/adjusted.

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