Journal of Proteomics xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

A Cyclin D2-derived peptide acts on specific cell cycle phases by activating ERK1/2 to cause the death of breast cancer cells

Lilian C. Russo [a], Christiane B. Araujo [b,c], Leo K. Iwai [c], Emer S. Ferro [b], Fabio L. Forti [a,*]

[a] Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP 05508-000, Brazil
[b] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-000, Brazil
[c] Special Laboratory of Applied Toxinology (LETA), Center of Toxins, Immune Response, and Cell Signaling (CETICS), Butantan Institute, São Paulo, SP 05503-000, Brazil

ARTICLE INFO

Article history:
Received 26 November 2015
Received in revised form 9 June 2016
Accepted 21 June 2016
Available online xxxx

Keywords:
Pep5
Cell death
Breast cancer cells
ERK1/2 activation
Cell cycle
Cyclin D2

ABSTRACT

Protein degradation by the proteasome generates functional intracellular peptides. Pep5, a peptide derived from Cyclin D2, induces cell death in tumor cell lines and reduces the volume of rat C6 glioblastoma tumors *in vivo*. Here, we chose the human MDA-MB-231 breast cancer cells to evaluate the mechanism of cell death induced by pep5 in different phases of the cell cycle. Fluorescently labeled pep5, monitored by real time confocal microscopy, entered the MDA-MB-231 cells 3 min after application and localized to the nucleus and cytoplasm. Pep5-induced cell death was increased when the MDA-MB-231 cell population was arrested at the G1/S transition or in S phase compared to asynchronous cells. Pep5 induced permanent extracellular signal-regulated kinase (ERK1/2) phosphorylation in MDA-MB-231 cells synchronized in G1/S or S phase. Affinity chromatography followed by mass spectrometry identified CLIC1 and Plectin as the only two proteins that interacted with pep5 in both asynchronous and synchronized MDA-MB-231 cells. These interactions could explain the long-lasting ERK1/2 phosphorylation and the cytoskeleton perturbations in the MDA-MB-231 cells, in which the stress fibers' integrity is affected by pep5 treatments. These data suggest that pep5 has potential therapeutic properties for treating specific types of cancers, such as breast cancer cells.

Biological significance: Pep5, a natural intracellular peptide formed by the degradation of Cyclin D2 through the ubiquitin–proteasome system, induces cell death when reintroduced into MDA-MB-231 breast cancer cells, which express low levels of Cyclin D2, specifically in G1/S arrested cells or in cells that are passing through S phase. Under these conditions, pep5 is able to interact with different intracellular proteins, primarily cytoskeleton and proteasome components, which can lead to cellular apoptosis. Together, our data suggest that pep5 is an intracellular peptide with therapeutic potential for treating specific types of tumors with low expression of Cyclin D2 by inhibiting cell proliferation.

1. Introduction

In eukaryotes, approximately 90% of the malformed or functionally dead proteins undergo intracellular proteolysis through the ubiquitin–proteasome system (UPS) [1]. Thus, the UPS contributes to different cellular functions, such as cell cycle progression, antigen presentation, proliferation, differentiation and gene expression [2]. The proteasome is responsible for the extra-lysosomal cleavage of antigenic proteins and the generation of peptides that are presented by the class I major histocompatibility complex. In cancer biology, the UPS is important for cell cycle progression and can lead to cyclin degradation [3]. Proteasome inhibition has been targeted in the clinic to treat patients with recurrent multiple myeloma [4], and it has also been studied in hepatocellular carcinoma [5], bladder [6], and breast cancer [7].

Hundreds of the non-antigenic peptides that are generated by the UPS remain inside cells before they are completely degraded by peptidases, producing what is known as *intracellular peptides* [8]. Many of these intracellular peptides are common in various tissues and cells, whereas many others are specific to different cells lines and tissues [9]. It was shown that intracellular peptides perform different functions [8], including modulating signal transduction in response to G-protein coupled receptor agonists [10,11], facilitating insulin-induced glucose uptake in 3T3-L1 adipocytes [12] and modulating protein–protein interactions *in vitro* [13]. Moreover, a proteasome peptide product that is capable of modulating specific protein–protein interactions increases the cytosolic Ca^{2+} concentration in a dose-dependent manner [13]. Taken together, it has been suggested that natural intracellular peptides may exert pleiotropic biological functions [14].

Previous peptidomic studies performed in HeLa cells demonstrated a variation in the intracellular peptide profile at different phases of the cell cycle [15]. Among the identified peptides, a peptide named pep5, which originated from Cyclin D2, is increased in S phase of the cell

* Corresponding author at: Av. Prof. Lineu Prestes, 748, Bl.09i - Sl. 922, CEP 05508-900, Cidade Universitária, São Paulo, SP, Brazil.
E-mail address: flforti@iq.usp.br (F.L. Forti).

http://dx.doi.org/10.1016/j.jprot.2016.06.028
1874-3919/

cycle [15]. Cyclin D2 is degraded by the UPS during the G1/S transition of the cell cycle [16], suggesting that pep5 can be generated endogenously at this transition state of the cell cycle. A synthetic pep5 fused to a cell penetrating peptide (cpp), or pep5-cpp, induced cell death in different cell lines *in vitro* and reduced the rat C6 glioblastoma volume by almost 50% *in vivo* [15].

Cyclin D2 can be either overexpressed or underexpressed, depending on the type of cancer. Cyclin D2 is overexpressed in colon cancer and lymphoid leukemia, which led to the hypothesis that it exhibits proto-oncogene activity [17–20]. Cyclin D2 gene is epigenetically silenced in prostate cancer [21,22], and its expression is gradually suppressed throughout the development and progression of prostate cancer [23]. The restoration of the Cyclin D2 levels may prevent prostate cancer carcinogenesis [24]. Similarly, most of the studied breast cancer cell lines [25,26], as well as lung, pancreatic and gastric cancers [26–28], express low levels of Cyclin D2.

Thus, it is suggested that in some cancers, Cyclin D2 may act as a tumor suppressor, which makes it a candidate for cancer treatment and prevention [29]. In fact, some cancer treatments already target Cyclin D2, such as valproic acid, which reduces the prostate cancer carcinogenesis by restoring Cyclin D2 expression [24,30]. Therefore, pep5 could present a relevant therapeutic potential by preventing the carcinogenesis of cancers with low Cyclin D2 expression.

Here, we aim to further investigate the molecular mechanism underlying pep5-induced cell death. We demonstrated that fluorescent pep5-cpp effectively penetrates into the cytoplasm and nucleus of the MDA-MB-231 breast cancer cell line, which expresses low levels of Cyclin D2, resulting in cell death. The cell death effects of pep5 are more evident in cells synchronized at the G1/S transition or S phase of the cell cycle than in asynchronous cells, and are accompanied by specific and permanent ERK1/2 phosphorylation. Additionally, we verified that pep5 can interact with proteins linked to different cellular functions, including Chloride intracellular channel protein 1 and Plectin. These proteins are known to regulate ERK1/2 activation and are linked to different cytoskeletal filaments. Pep5-cpp causes a significant perturbation in the cytoskeleton by affecting the integrity of stress fibers. Therefore, the cell death-inducing activity of pep5 was further characterized, and the putative cellular targets of pep5 in the MDA-MB-231 breast cancer cell line were identified.

2. Materials and methods

2.1. Cells

The MDA-MB-231 breast cancer cell line was cultured in DMEM (Dulbecco's Modified Eagle Medium; Life Technologies, Carlsbad, CA, USA), containing 10% fetal bovine serum (FBS, Cultilab, Campinas, SP, BR), 0.025 g/L penicillin (Sigma-Aldrich, Saint Louis, MO, USA) and 0.1 g/L streptomycin (Life Technologies) at 37 °C and 5% CO_2.

2.2. Peptides

Peptide 5 (pep5; WELVVLGKL) and the control scrambled peptide (SCB; VNMVPVGWASR) were synthesized with or without the cell penetrating peptide (cpp YGRKKRRQRRR) covalently bound to their C-terminus [15,31–33] at a purity >95% (Proteimax Biotechnology Inc., São Paulo, SP, BR). In all assays, the cells were maintained in serum-free DMEM during the peptide treatment to minimize the possible interactions between the peptide and the serum proteins.

2.3. Pep5-cpp distribution within cells, as evaluated by confocal microscopy

We investigated the entry and intracellular localization of pep5-cpp in living MDA-MB-231 cells using pep5-cpp labeled with a 490 dye molecule at its C-terminus (Proteimax Biotechnology). The cells were analyzed with a LSM 510 confocal microscope (Zeiss, Oberkochen, DEU). Fluorescent pep5-cpp (10 or 50 μM) was added to the cells in serum-free DMEM, and the fluorescence of the cells was monitored (Ex/Em: 488/505–530 nm) in real time for approximately 12 min after treatment.

2.4. Cell cycle synchronization and flow cytometry

The MDA-MB-231 cells were synchronized using the double-thymidine block procedure. Briefly, the cells were treated with 2 mM thymidine (Sigma-Aldrich) for 18 h at 37 °C and 5% CO_2, washed twice in phosphate-buffered saline (PBS), and the medium was replaced with DMEM containing 10% FBS for 9 h. The cells were treated with 2 mM thymidine a second time for 17 h, washed twice in PBS, and the medium was replaced with DMEM containing 10% FBS (called the releasing step). The cells were harvested at different times (0 h, 4 h and 10 h, in which at least 70% of the cells were in G_1/S, S and G_2/M phases) and analyzed by flow cytometry or submitted to additional assays. The control cells were always asynchronous. The cell cycle was analyzed by flow cytometry using a FACS-Verse (BD Biosciences, San Jose, CA, USA) instrument. The cells were fixed in 70% ethanol, washed in PBS and incubated with propidium iodide (10 μg/mL), RNAse (10 μg/mL), Triton X-100 (0.1%) and sodium citrate (0.1%). A total of 30,000 events were acquired for each sample, and the data were analyzed using Kaluza software (v.1.2, Beckman Coulter, Brea, CA, USA).

2.5. Effects of pep5-cpp addition at different phases of the cell cycle

To analyze the effect of pep5-cpp on G1/S phase at the end of the double-thymidine block procedure (0 h), the cell population was arrested at the G1/S transition of the cell cycle (0 h). At this moment, the cells were washed with PBS and treated with 25 μM peptide (pep5, cpp, SCB-cpp or pep5-cpp) in serum-free DMEM for 15 min. After the peptide treatments, the cells were washed with PBS and maintained in DMEM plus 10% FBS (release step) to continue their progression through the cell cycle until they were collected at 4 h (t4), 8 h (t8) and 22 h (t22) after peptide treatment. Alternatively, at the end of the double-thymidine block procedure (0 h), the cells were released by FBS treatment for 4 h to allow the population to arrive in S phase and analyze the effect of pep5-cpp on S phase. The cells were washed twice with PBS and treated with 25 μM peptide in serum-free DMEM for 15 min. At the end of the peptide treatment, the medium was replaced with DMEM containing 10% FBS, and the cells were collected at 4 h (t4), 8 h (t8) and 22 h (t22) after peptide treatment (Supplementary Fig. S1).

2.6. Immunoblotting

To map the cellular signaling networks involved in cell cycle progression and genomic stability that may be affected by pep5, immunoblotting assays were performed on asynchronous MDA-MB-231 cells or cells synchronized in G1/S or S phases. The cells were treated with 25 μM SCB-cpp or pep5-cpp peptides for 15 min, 30 min, 45 min, 1 h, 2 h and 4 h and lysed with RIPA buffer (50 mM Tris pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, and 10 mM $MgCl_2$) containing protease and phosphatase inhibitors (1 mM DTT, 1 mM PMSF, 2 μg/mL leupeptin, 1 mM NaF, 1 mM Na_3VO_4, 2 μg/mL aprotinin and 2 μg/mL pepstatin). Fifty micrograms of each lysate were loaded onto a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk diluted in T-TBS (Tris-buffered saline buffer containing 0.1% Tween 20) for 1 h at RT and then incubated with specific antibodies. To analyze the canonical MAPK pathway, we verified the phosphorylation of the ERK1/2 (anti-ERK1/2 Thr202/Tyr204, 1:1000; Cell Signaling Technology, Danvers, MA, USA), p38 (anti-p38 Thr180/Tyr182, 1:1000; Santa Cruz Biotechnology, Dallas, TX, USA) and JNK (anti-JNK Thr183/Tyr185, 1:1000; Santa Cruz Biotechnology) proteins, while we verified AKT phosphorylation (anti-AKT Ser473, 1:1000; Cell

Please cite this article as: L.C. Russo, et al., A Cyclin D2-derived peptide acts on specific cell cycle phases by activating ERK1/2 to cause the death of breast cancer cells, J Prot (2016), http://dx.doi.org/10.1016/j.jprot.2016.06.028