Peptides 32 (2011) 2418–2427

Contents lists available at SciVerse ScienceDirect

Peptides

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

A novel immunosuppressory peptide originating from the ubiquitin sequence

Paweł Pasikowski[a], Tomasz Goździewicz[a], Piotr Stefanowicz[a], Jolanta Artym[b], Michał Zimecki[b], Zbigniew Szewczuk[a,*]

[a] Faculty of Chemistry, University of Wrocław, Wrocław, Poland
[b] Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

ARTICLE INFO

Article history:
Received 19 September 2011
Received in revised form 3 October 2011
Accepted 3 October 2011
Available online 8 October 2011

Keywords:
Allograft skin survival
Antibody production
Cryptides
Immunosuppressory peptides
Ubiquitin fragments

ABSTRACT

Ubiquitin is a conservative polypeptide present in every eukaryotic cell. Apart from its involvement in proteasomal degradation and other intracellular signal pathways, it was suggested to play an important role as the extracellular immunomodulator and antimicrobial agent. Moreover, ubiquitin-derived peptides were shown to express significant biological activities. Our previous studies showed a high immunosuppressive potency of the ubiquitin peptic hydrolysate in which we identified over 70 different peptides. The present work focuses on synthesizing the most abundant of these peptides and investigating their immunomodulatory potency. The peptide VKTLTGKTI possessed the highest immunosuppressory activity in AFC experiments, comparable to the previously described LEDGRTLSDY sequence (a previously discovered ubiquitin-derived peptide). Moreover, some of the investigated peptides expressed immunostimulatory effects. These findings support the idea that ubiquitin, together with products of its degradation, could represent a self-regulating immunoregulatory system. Peptide VKTLTGKTI was also tested for its activity to prolong the skin graft survival in mice. The results showed that the investigated peptide significantly extended the skin transplant rejection time, therefore it could be considered as a potential supplementary medicine in the post-transplantation therapy. Moreover, we synthesized two analogs of investigated peptides, first designed to mimic the non-linear epitope consisting of ubiquitin 16–21 and ubiquitin 52–57 fragments, and second designed to mimic the ubiquitin 5–13 hairpin. We also tested their immunosuppressory activity in *in vitro* experiments.

1. Introduction

Ubiquitin is a 76-amino-acid polypeptide expressed in all eukaryotic cells. It is highly conserved through evolution, with only three amino acids difference between the human and the yeast protein, and exhibits a diversity of biological activities. The ubiquitin molecule is structurally well described, with both X-ray [37] and NMR [9] structures reported. It consists of seven β-strands, one α-helix, small 3_{10} helix, and several turns, forming a rigid fold called the β-grasp. The native ubiquitin conformation is extraordinarily resistant to temperature, pH [9], and pressure [28] denaturation and proteolysis [22]. The major role of ubiquitin is covalent modification of other proteins (called ubiquitylation) which directs them to proteasomal degradation [8] or endows them with numerous signal functions [18]. Ubiquitin-dependent pathways regulate a variety of cellular processes, including the immune response [2].

Most of the described processes, involving ubiquitin, occur inside a cell. Nevertheless, the protein was found in body fluids in high concentrations (reaching 50 nM in blood of healthy individuals) [1]. Furthermore, in the case of several diseases elevated ubiquitin concentrations were observed [3,35]. In recent studies ubiquitin was shown to possess a therapeutic potential in *in vivo* experiments. It prolonged the skin allograft survival in fully mismatched mouse strain combinations [7] and alleviated the effects of endotoxic shock induced in pigs [15]. Taken together, these observations suggest that extracellular ubiquitin should be regarded not only as a biomarker of pathological conditions but also as a functional element of the organism defense system [14].

The above cited findings are particularly promising when confronted with strong cytotoxic effects of currently used immunosuppressive drugs. Cyclosporine, tacrolimus, and sirolimus are the most extensively used drugs in humans for treatment of posttransplantational rejection and autoimmune disorders [27]. Unfortunately, their harmful side effects tend to be very devastating for patients and sometimes even exceed the therapeutic benefits, especially when drugs are administered at high doses. Thus, the research leading to discovery of non-toxic immunosuppressants is very extensive. Some researchers suggested synergistic effects of ubiquitin co-administered with tacrolimus and sirolimus [7], which

Abbreviations: AFCs, antibody-forming cells; SRBCs, sheep red blood cells.

* Corresponding author at: Faculty of Chemistry, University of Wrocław, ul. F. Joliot-Curie 14, 50-383 Wrocław, Poland. Tel.: +48 713757212; fax: +48 713282348.
E-mail address: zbigniew.szewczuk@chem.uni.wroc.pl (Z. Szewczuk).

0196-9781/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2011.10.002

can initiate a new therapeutic approach utilizing ubiquitin as an additive to currently used immunosuppressory drugs.

Recently we undertook realization of a concept of the cryptides – functional peptides hidden within the structure of proteins and liberated upon protein degradation [36]. Such bioactive sequences were discovered recently [5]. What is particularly intriguing is that the activities of cryptides can significantly differ from those induced by intact proteins or even oppose them. Our previous study revealed that a nonapeptide fragment (TPQRGDVYT) located in the β164–172 loop of human leukocyte antigen (HLA-DQ) molecules, suppressed the humoral and cellular immune responses and inhibited interaction with certain integrins [29,30]. The corresponding fragments of other MHC class II molecules (HLA-DP and HLA-DR) showed immunological properties similar to those of the HLA-DQ fragment [34]. We also synthesized conformationally restricted analog of β164–172 HLA-DQ fragment which was shown to possess significant immunosuppressive activity [32].

We also found that peptide LEDGRTLSDY corresponding to the ubiquitin 50–59 fragment, containing the retro-RGD sequence, exhibited strong immunosuppressive effects on the cellular and humoral immune responses, comparable to that of cyclosporine [31]. We also pointed out some topological similarities between β164–172 loop of HLA-DQ molecule and mentioned ubiquitin loop [33]. Peptide DGRTL was established as the shortest active sequence originating from LEDGRTLSDY, whereas some of its shorter analogs exhibited rather weak immunostimulatory potency. Also, a cyclic peptide mimicking ubiquitin 50–59 region was synthesized and shown to possess a higher immunosuppressive activity than its linear analog. It has to be also stressed that the investigated peptides were much less toxic than cyclosporine, particularly at higher doses.

Moreover, the peptic digest of ubiquitin showed even a higher immunosuppressive effect than that of intact ubiquitin [11]. The extraordinary activity of this mixture could be explained by the existence of one or more very potent immunosuppressants among the peptides. We established a composition of the digest by identifying over 70 different peptides. The present investigation was focused on synthesizing five of the most abundant components of this mixture and their analogs, in order to investigate their immunosuppressive activity. After performing a series of *in vitro* experiments, aimed to establish their ability to suppress antibody production, we chose the most promising ubiquitin fragment and verified its activity in prolongation of the allograft skin survival in mice. As a result of this study we identified a previously not described ubiquitin-derived peptide possessing significant immunosuppressive effects in the performed experimental models.

2. Materials and methods

2.1. Peptide synthesis

All synthesized ubiquitin-derived sequences are presented in Table 1. The solid phase synthesis of these peptides was carried out manually using standard Fmoc strategy on a Wang resin [38]. The coupling was performed with three equivalents of Nα-Fmoc-protected amino acid, three equivalents of BOP, three equivalents of HOBt, and six equivalents of DIEA in DMF for 2 h. The coupling was monitored using standard Kaiser's test. In the case of a positive result, the resin was washed and the coupling procedure was repeated. The Fmoc group was removed by incubation with 25% piperidine solution in DMF for 20 min.

Peptide IX consists of peptides VI and VIII linked through the lysine residue. To synthesize this compound we performed the synthesis of peptide Boc-Glu(O*t*Bu)-Val-Glu(O*t*Bu)-Pro-Ser(*t*Bu)-Asp(O*t*Bu)-Lys(Mtt) bound to the Wang resin. The Mtt group was selectively removed with the TFA/TIS/DCM mixture (1:5:94), followed by Fmoc synthesis of the segment DGRTLS on the side chain of the lysine residue. For complete scheme of synthesis of compound IX see Fig. 1.

Table 1
The synthesized peptides sharing their sequences with ubiquitin fragments.

Peptide no.	Peptide sequence	Ubiquitin fragment
I	LEDGRTLSDY	50–59
II	AGKQLED	46–52
III	YNIQKESTL	59–67
IV	NVKAKIQDKEGIPPDQ	25–40
V	RLIFAGKQLED	42–52
VI	EVEPSD	16–21
VII	VKTLTGKTITL	5–15
VIIa	KTITL	11–15
VIIb	TGKTITL	9–15
VIIc	TLTGKTITL	7–15
VIId	VKTLT	5–9
VIIe	VKTLTGK	5–11
VIIf	VKTLTGKTI	5–13
VIII	DGRTLS	52–57
IX	DGRTLS ┐ EVEPSDK	16–21 and 52–57
X	┌──────┐ CKTLTGKTC	5–13 analog

The final cleavage of the peptides from the Wang resin (and MBHA resin) was carried out using a mixture of trifluoroacetic acid/water/triisopropylsilane (95:2.5:2.5) or trifluoroacetic acid/water/triisopropylsilane/thioanisol/ethanodithiol (90:2.5:2.5:2.5:2.5) in the case of peptides containing Cys(Trt) for 3 h at room temperature. The peptides were purified by preparative RP-HPLC and transformed into the acetate form. The peptides used in the biological activity tests were at least 98% pure according to the analytical HPLC. The structures of the synthesized peptides were confirmed by a high resolution MS/MS analysis.

Purified linear peptide X was oxidized to its cyclic analog containing the disulfide bridge (the product corresponds to ┌──────┐ CKTLTGKTC). The linear peptide CKTLTGKTC was solubilized in 10 mM PBS buffer (pH 8.0) to the final concentration of 0.1 mg/ml. The solution was stirred in air for 72 h at room temperature and then lyophilized. The cyclic peptide X was purified with RP-HPLC to obtain 98% purity and its structure was confirmed by a high resolution MS/MS analysis.

2.2. CD spectroscopy

CD spectra were measured on a Jasco J-600 spectropolarimeter, at room temperature. Peptide concentrations were 0.07 mg/ml. Path length of 1 mm was used. Each spectrum is the average of at least four scans. The spectra were measured in water, trifluoroethanol (TFE), and mixtures of thereof. The data are presented as residue molar ellipticity (θ).

2.3. Mass spectrometry

High-resolution mass spectrometry was performed on an electrospray ionization (ESI) Bruker ApexUltra spectrometer (Bruker Daltonik, Bremen, Germany), equipped with an Apollo II electrospray ionization source with an ion funnel. The mass spectrometer was operated in the positive ion mode. The instrumental parameters were as follows: scan range m/z 300–2200, capillary voltage 4200 V, end plate offset 3800 V, dry gas – nitrogen 3 L/min, temperature 200 °C, ion energy 2.5 eV. The sample (10 μg/ml) in 1:1 MeCN–H_2O was infused at a flow rate of 3 μL/min. CID-MS/MS experiments were performed using argon as a collision gas. The signals corresponding to investigated peptides were isolated with