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Efficient recombinant production of RpII, a Na_V-modulating peptide from the sea anemone *Heteractis magnifica*

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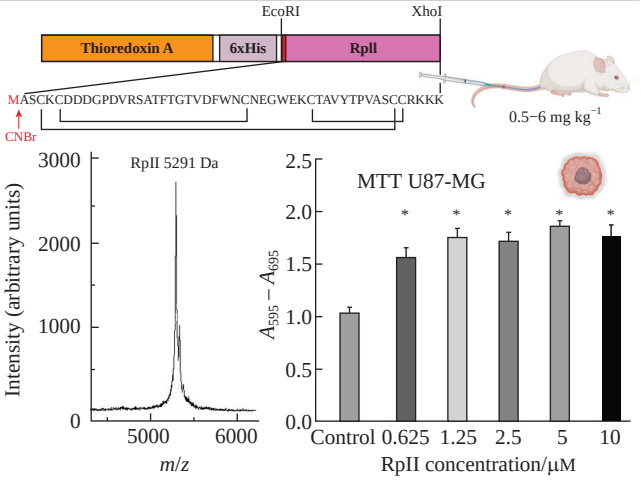
The gene structure and amino acid sequences of the precursor proteins of the sea anemone *Heteractis magnifica* neurotoxin RpII, a modulator of voltage-gated sodium channels, have been determined. A technology has been developed for the recombinant production of RpII in the *Escherichia coli* BL21(DE3) expression system based on the pET32b vector containing the thioredoxin gene, which opens up the possibility of obtaining a pure neurotoxin for further structural and/or pharmacological studies. The neurotoxin exhibits a neurotropic effect when administered intravenously to mice in doses greater than 1 mg kg^{-1}, but is not lethal in doses up to 6 mg kg^{-1} and, in addition, RpII in the nM range does significantly increase the metabolism of human malignant glioblastoma U87-MG.



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Voltage-gated sodium channels (Na_V), responsible for the initiation and propagation of action potentials in excitable cells of the heart, nervous system and skeletal muscle of living organisms,[1] are molecular targets for a fairly large number of compounds, including neurotoxins isolated from animal venom. Abnormal functional activity of Na_V underlies diseases such as epilepsy, neuropathic pain, long QT (the time from the start of the Q wave to the end of the T wave on an electrocardiogram report) syndrome and others.[2,3] To treat these diseases, low molecular weight Na_V blockers are used, the disadvantage of which is side effects that affect the functioning of the cardiovascular, musculoskeletal or nervous systems due to their low selectivity and high toxicity. Peptide neurotoxins produced by venomous animals, including sea anemones, are distinguished by their ability to selectively bind to ion channels and modulate their functional activity.[4] Sea anemone neurotoxins are classified into four structural types based on amino acid sequence identity. The most represented and studied toxins are types I and II, containing 45–54 amino acid residues and presumably having the same ancestor gene.[4] High selectivity, which is achieved through the interaction of neurotoxins of various origins with the variable region of the channel, makes it possible to reduce side effects when used as drugs. In addition, neurotoxins have great potential as molecular tools for studying the structure, mechanism of action, localization and physiological role of ion channels in health and disease.

The sea anemone neurotoxin RpII (δ-SHTX-Rpa1a, UniProt ID: P01534) was first isolated from *Radianthus paumotensis*[5] in 1985 and discovered as a major component of *Heteractis magnifica* venom[6] in 2018, while its electrophysiological profile on Na_V1 expressed in *Xenopus laevis* oocytes was studied[7] in 2020. RpII isolated from *Heteractis crispa* (δ-SHTX-Hcr1f) slows down the inactivation of mammalian Na_V channels expressed in the central nervous system, such as $Na_V1.1$, $Na_V1.2$ and $Na_V1.6$, but not $Na_V1.3$, and does not have the same effect on skeletal muscle $Na_V1.4$ channels, cardiac $Na_V1.5$ channels and PNS $Na_V1.8$ channels.[7] At the same time, RpII activates the insect $BgNa_V1$ and arachnid $VdNa_V1$ channels, being the most potent modulator of Na_V channels among other *H. crispa* neurotoxins: its preferred targets, in descending order, are the channels $Na_V1.2$ > $Na_V1.6$ > > $BgNa_V1$ (EC_{50} 79.5–226.1 nM). According to the results of molecular modeling, the binding site of RpII is apparently localized in the extracellular region of the voltage-sensing VSD-IV domain of the $rNa_V1.2$ channel (Figure 1).[7]

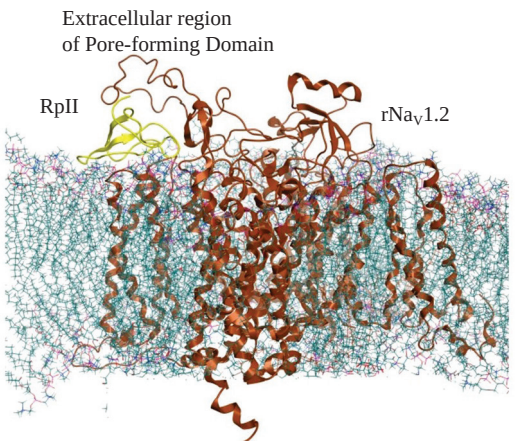

Figure 1 Interaction of RpII with rNa$_V$1.2. Ribbon diagram of the complex of RpII (yellow) with rNa$_V$1.2 (dark orange) embedded in a dipalmitoyl-phosphatidylcholine lipid bilayer (stick representation).

The selectivity for ion channels makes RpII a promising tool to probe Na$_V$, while the low toxicity in *in vivo* models (LD$_{50}$ 4.2 mg kg^{-1} in mice[5]) allows it to be considered for further drug development and, in combination with effects on insect channels, as a potential insecticide. Both potential applications require the development of a recombinant RpII production scheme. Therefore, the task of developing a method for obtaining a peptide with high yield for further study of its biological activity is urgent.

For the first time, the structure of genes encoding Na$_V$ channel modulators of the type II sea anemone toxins has been established. Amplification of cDNA and gDNA using gene-specific primers resulted in fragments of 300 and 750–850 bp in length, respectively. This indicates the presence of several neurotoxin genes, the intron length of which varies within the same organism. It was found that the neurotoxin genes are organized as follows: the intron (470–520 bp) is located in the middle of the propeptide coding sequence.

The precursor protein sequence was amplified from the *H. magnifica* cDNA using gene-specific primers. As a result, two isoforms of RpII precursors were obtained, consisting of a signal peptide, a propeptide ending with a Lys–Arg hydrolysis site and a Gly residue at the C-terminus. The Gly residue at the C-terminus has been shown to undergo hydrolysis, which is widespread among animal toxins.[8–10] They differ by two synonymous substitutions in the signal peptides, with the mature RpII sequence being conserved.

The *Escherichia coli* BL21(DE3) expression system was chosen to produce the recombinant peptide because it is convenient and widespread. However, there is a serious problem with the expression of eukaryotic proteins in *E. coli* cells, especially cysteine-rich peptides. RpII has a β-defensin fold with six cysteine residues, so to ensure its correct folding, the plasmid expression vector pET32b containing the thioredoxin sequence was used (Figure 2). A methionine residue (for cleavage of thioredoxin using cyanogen bromide[11]) introduced before the first amino acid of the peptide, as well as a stop codon introduced before Gly at the C-terminus of the peptide, made it possible to obtain a mature peptide without additional residues.

Expression conditions (temperature, inducer concentration and cell culture time) were selected experimentally. The fusion protein isolated from cell lysate by metal affinity chromatography was hydrolyzed with CNBr and then RpII was purified by RP-HPLC. Its molecular mass according to MS data is 5291 Da, which corresponds to the calculated value. The presence of the fold was confirmed by ^{1}H NMR. The average yield of the target peptide reaches 2.5 mg per 1 liter of cell culture. It is a very significant yield, considering that usually the production of thioredoxin-fused

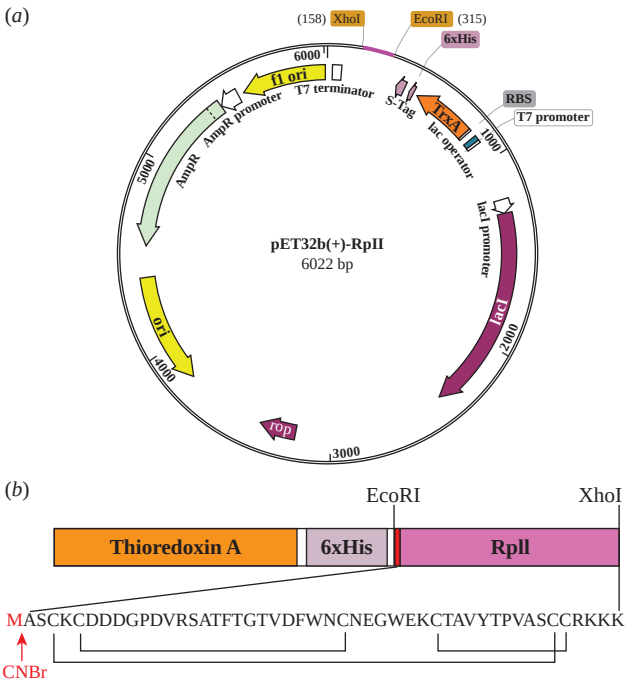

Figure 2 (*a*) Map of the pET32b(+)-RpII expression plasmid. The gene encoding RpII and Met for CNBr hydrolysis was cloned using EcoRI and XhoI restriction sites. (*b*) Schema of the Trx–RpII fusion protein and the amino acid sequence of mature RpII showing disulfide bond topology (UniProt ID: P01534).

cysteine-rich peptides requires refolding steps[12] or purification from inclusion bodies,[13] which increases labor and material costs for obtaining the substance. Thus, a fairly simple and effective method for obtaining pure neurotoxin RpII was developed.

RpII activity was assessed using an *in vivo* acute toxicity assay. At all tested doses from 0.5 to 6 mg kg^{-1}, RpII had no lethal effect. However, after intravenous administration of the toxin in doses of more than 1 mg kg^{-1}, animals showed signs of a neurotropic effect, manifested in the form of shortness of breath, lethargy and an inhibited reaction to external stimuli, which is consistent with previously published data.[5] The normal physical condition of the animals was restored after two hours. When the dose of the toxin was reduced to 0.5 mg kg^{-1}, no obvious manifestations of neurotropic effects were observed.

The pharmacological potential of neurotoxins is due, in particular, to their effect on tumor cells. Roles of Na$_V$ related to cell migration, invasiveness,[14–17] proteolytic degradation of extracellular matrix and cell–cell adhesion[18,19] in cancer cells have been reported. We tested RpII on human malignant glioblastoma U87-MG cells obtained from the American Type Culture Collection (ATCC,

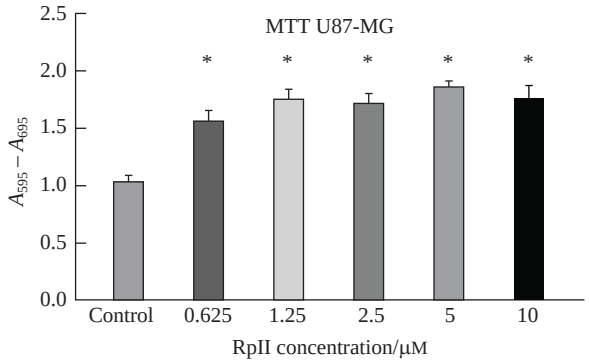

Figure 3 Effect of RpII on the viability of human malignant glioblastoma U87-MG cells. Results of MTT assay of U87-MG cells incubated with increasing concentrations of RpII (0.625–10 μM) for 5 days. All data are presented as mean ±S.E.M. (n = 8). '*' indicates adjusted p-value < 0.0001.

Manassas, VA, USA). Metabolic activity was assessed using the MTT assay [MTT stands for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. It was unexpected that RpII significantly increased the metabolism of U87-MG cells (ANOVA/Dunnett's test, p-value < 0.0001, $n = 8$) at concentrations up to 10 μM (Figure 3), but did not affect the viability of Neuro 2a cells (ATCC, CCL-131, Manassas, VA, USA) or RAW264 cells at the same concentrations (data not shown). Interestingly, the Na_V channel blocker tetrodotoxin (TTX), which inhibits the invasiveness of prostate and breast cancer cell lines,[20–22] was not able to reduce the invasiveness of U87-MG at concentrations 10 and 30 μM; moreover, 30 μM TTX increased the migration of glioblastoma.[23]

As a result, we determined the structure of the RpII precursor and developed a technology for producing the folded and biologically active sea anemone neurotoxin in the prokaryotic expression system. Recombinantly produced RpII may be useful as a tool to study the functions of ion channels, and due to its low toxicity in mammals, it may be considered for detailed study of its pharmaceutical potential.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2024.01.005.

References

1 H. Shen, Q. Zhou, X. Pan, Z. Li, J. Wu and N. Yan, *Science*, 2017, **355**, eaal4326.
2 W. Huang, M. Liu, S. F. Yan and N. Yan, *Protein Cell*, 2017, **8**, 401.
3 D. P. Ryan and L. J. Ptáček, *Neuron*, 2010, **68**, 282.
4 M. M. Monastyrnaya, R. S. Kalina and E. P. Kozlovskaya, *Toxins*, 2023, **15**, 8.
5 H. Schweitz, J. N. Bidard, C. Frelin, D. Pauron, H. P. M. Vijverberg, D. M. Mahasneh, M. Lazdunski, F. Vilbois and A. Tsugita, *Biochemistry*, 1985, **24**, 3554.
6 O. Sintsova, I. Gladkikh, V. Chausova, M. Monastyrnaya, S. Anastyuk, O. Chernikov, E. Yurchenko, D. Aminin, M. Isaeva, E. Leychenko and E. Kozlovskaya, *J. Proteomics*, 2018, **173**, 12.
7 R. S. Kalina, S. Peigneur, E. A. Zelepuga, P. S. Dmitrenok, A. N. Kvetkina, N. Y. Kim, E. V. Leychenko, J. Tytgat, E. P. Kozlovskaya, M. M. Monastyrnaya and I. N. Gladkikh, *Toxins*, 2020, **12**, 44.
8 S. A. Kozlov and E. V. Grishin, *Toxicon*, 2007, **49**, 721.
9 R. M. Metrione, H. Schweitz and K. A. Walsh, *FEBS Lett.*, 1987, **218**, 59.
10 T. Honma, S. Kawahata, M. Ishida, H. Nagai, Y. Nagashima and K. Shiomi, *Peptides*, 2008, **29**, 536.
11 Y. A. Andreev, S. A. Kozlov, A. A. Vassilevski and E. V. Grishin, *Anal. Biochem.*, 2010, **407**, 144.
12 Y. A. Logashina, Y. V. Korolkova, E. E. Maleeva, D. I. Osmakov, S. A. Kozlov and Y. A. Andreev, *Mendeleev Commun.*, 2020, **30**, 214.
13 M. N. Tereshin, A. M. Komyakova, V. N. Stepanenko, I. V. Myagkikh, N. S. Shoshina, Y. V. Korolkova, E. V. Leychenko and S. A. Kozlov, *Mendeleev Commun.*, 2022, **32**, 745.
14 P. Besson, V. Driffort, É. Bon, F. Gradek, S. Chevalier and S. Roger, *Biochim. Biophys. Acta, Biomembr.*, 2015, **1848**, 2493.
15 F. Patel and W. J. Brackenbury, *Int. J. Dev. Biol.*, 2015, **59**, 357.
16 S. Roger, L. Gillet, J.-Y. Le Guennec and P. Besson, *Front. Pharmacol.*, 2015, **6**, 152.
17 R. Stupp, W. P. Mason, M. J. van den Bent, M. Weller, B. Fisher, M. J. B. Taphoorn, K. Belanger, A. A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R. C. Janzer, S. K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J. G. Cairncross, E. Eisenhauer and R. O. Mirimanoff, *N. Engl. J. Med.*, 2005, **352**, 987.
18 G. M. Cooper, *The Cell: A Molecular Approach*, 2nd edn., Sinauer Associates, Sunderland, MA, 2000.
19 X. Sun, D. Zhao, Y.-L. Li, Y. Sun, X.-H. Lei, J.-N. Zhang, M.-M. Wu, R.-Y. Li, Z.-F. Zhao, Z.-R. Zhang and C.-L. Jiang, *Oncol. Rep.*, 2013, **30**, 2852.
20 T. L. Anthony, H. L. Brooks, D. Boassa, S. Leonov, G. M. Yanochko, J. W. Regan and A. J. Yool, *Mol. Pharmacol.*, 2000, **57**, 576.
21 J. B. Arnold, L. Junck and D. A. Rottenberg, *J. Cereb. Blood Flow Metab.*, 1985, **5**, 369.
22 R. McLendon, A. Friedman, D. Bigner, E. G. Van Meir, D. J. Brat, G. M. Mastrogianakis, J. J. Olson, T. Mikkelsen, N. Lehman, K. Aldape, W. K. A. Yung, O. Bogler, J. N. Weinstein, S. VandenBerg, M. Berger, M. Prados, D. Muzny, M. Morgan, S. Scherer, A. Sabo, L. Nazareth, L. Lewis, O. Hall, Y. Zhu, Y. Ren, O. Alvi, J. Yao, A. Hawes, S. Jhangiani, G. Fowler, A. San Lucas, C. Kovar, A. Cree, H. Dinh, J. Santibanez, V. Joshi, M. L. Gonzalez-Garay, C. A. Miller, A. Milosavljevic, L. Donehower, D. A. Wheeler, R. A. Gibbs, K. Cibulskis, C. Sougnez, T. Fennell, S. Mahan, J. Wilkinson, L. Ziaugra, R. Onofrio, T. Bloom, R. Nicol, K. Ardlie, J. Baldwin, S. Gabriel, E. S. Lander, L. Ding, R. S. Fulton, M. D. McLellan, J. Wallis, D. E. Larson, X. Shi, R. Abbott, L. Fulton, K. Chen, D. C. Koboldt, M. C. Wendl, R. Meyer, Y. Tang, L. Lin, J. R. Osborne, B. H. Dunford-Shore, T. L. Miner, K. Delehaunty, C. Markovic, G. Swift, W. Courtney, C. Pohl, S. Abbott, A. Hawkins, S. Leong, C. Haipek, H. Schmidt, M. Wiechert, T. Vickery, S. Scott, D. J. Dooling, A. Chinwalla, G. M. Weinstock, E. R. Mardis, R. K. Wilson, G. Getz, W. Winckler, R. G. W. Verhaak, M. S. Lawrence, M. O'Kelly, J. Robinson, G. Alexe, R. Beroukhim, S. Carter, D. Chiang, J. Gould, S. Gupta, J. Korn, C. Mermel, J. Mesirov, S. Monti, H. Nguyen, M. Parkin, M. Reich, N. Stransky, B. A. Weir, L. Garraway, T. Golub, M. Meyerson, L. Chin, A. Protopopov, J. Zhang, I. Perna, S. Aronson, N. Sathiamoorthy, G. Ren, W. R. Wiedemeyer, H. Kim, S. W. Kong, Y. Xiao, I. S. Kohane, J. Seidman, P. J. Park, R. Kucherlapati, P. W. Laird, L. Cope, J. G. Herman, D. J. Weisenberger, F. Pan, D. Van Den Berg, L. Van Neste, J. M. Yi, K. E. Schuebel, S. B. Baylin, D. M. Absher, J. Z. Li, A. Southwick, S. Brady, A. Aggarwal, T. Chung, G. Sherlock, J. D. Brooks, R. M. Myers, P. T. Spellman, E. Purdom, L. R. Jakkula, A. V. Lapuk, H. Marr, S. Dorton, Y. G. Choi, J. Han, A. Ray, V. Wang, S. Durinck, M. Robinson, N. J. Wang, K. Vranizan, V. Peng, E. Van Name, G. V. Fontenay, J. Ngai, J. G. Conboyu, B. Parvin, H. S. Feiler, T. P. Speed, J. W. Gray, C. Brennan, N. D. Socci, A. Olshen, B. S. Taylor, A. Lash, N. Schultz, B. Reva, Y. Antipin, A. Stukalov, B. Gross, E. Cerami, W. Q. Wang, L.-X. Qin, V. E. Seshan, L. Villafania, M. Cavatore, L. Borsu, A. Viale, W. Gerald, C. Sander, M. Ladanyi, C. M. Perou, D. N. Hayes, M. D. Topal, K. A. Hoadley, Y. Qi, S. Balu, Y. Shi, J. Wu, R. Penny, M. Bittner, T. Shelton, E. Lenkiewicz, S. Morris, D. Beasley, S. Sanders, A. Kahn, R. Sfeir, J. Chen, D. Nassau, L. Feng, E. Hickey, A. Barker, D. S. Gerhard, J. Vockley, C. Compton, J. Vaught, P. Fielding, M. L. Ferguson, C. Schaefer, S. Madhavan, K. H. Buetow, F. Collins, P. Good, M. Guyer, B. Ozenberger, J. Peterson and E. Thomson, *Nature*, 2008, **455**, 1061.
23 A. Malacrida, M. Rivara, A. Di Domizio, G. Cislaghi, M. Miloso, V. Zuliani and G. Nicolini, *Bioorg. Med. Chem.*, 2020, **28**, 115300.

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