# EXPERIMENTAL ARTICLES

UDC 577.112:576.385.5 doi: 10.15407/biotech8.05.019

# RECOMBINANT FLUORESCENT SENSOR OF HYDROGEN PEROXIDE HYPER FUSED WITH ADAPTOR PROTEIN RUK/CIN85: DESIGNING OF EXPRESSION VECTOR AND ITS FUNCTIONAL CHARACTERIZATION

*A. V. Bazalii*[1]
*A. V. Vorotnikov*[2]
*P. A. Tiurin-Kuzmin*[2]
*V. A. Tkachuk*[2]
*S. V. Komisarenko*[1]
*L. B. Drobot*[1]

[1]Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv

[2]Faculty of Fundamental Medicine, Lomonosov Moscow State University, Russian Federation

*E-mail: bazalii@biochem.kiev.ua*

Received 30.09.2015

The aim of this study was to design the expression vector encoding fluorescent sensor of hydrogen peroxide HyPer fused with adaptor protein Ruk/CIN85 as well as to check its subcellular distribution and ability to sense hydrogen peroxide. It was demonstrated that in transiently transfected HEK293 and MCF-7 cells Ruk/CIN85-HyPer is concentrated in dot-like vesicular structures of different size while HyPer is diffusely distributed throughout the cell. Using live cell fluorescence microscopy we observed gradual increase in hydrogen peroxide concentration in representative vesicular structures during the time of experiment. Thus, the developed genetic construction encoding the chimeric Ruk/CIN85-HyPer fluorescent protein represents a new tool to study localized $H_2O_2$ production in living cells.

***Key words:*** recombinant proteins, adaptor protein Ruk/CIN85, hydrogen peroxide, sensor of hydrogen peroxide HyPer, Ruk/CIN85-HyPer.

Hydrogen peroxide ($H_2O_2$), depending on the intracellular concentration and localization, is not only the cytotoxic factor but also the important regulator of redox-dependent signaling networks. As new second messenger, $H_2O_2$ is involved into the control of plethora fundamental biological processes such as activation of immune cells, cell proliferation, differentiation, migration, invasion, apoptosis, and gene expression [1–3]. The data are accumulated regarding participation of $H_2O_2$ in the development of oncological, neurodegenerative and inflammatory diseases, diabetes [4].

Despite the important physiological role of $H_2O_2$ in cell, today, there are few experimental techniques to detect $H_2O_2$ *in cellulo*. With the most methods, virtually all reactive oxygen species (ROS) are detected that is their biggest drawback. To detect the levels of intracellular ROS, dichlorofluorescein (DCF) derivatives are the most frequently used because they fluoresce after oxidation by ROS. At the same time, these compounds are characterized by a number of disadvantages. They are sensitive to multiple types of ROS as well to nitrogen species and cannot be targeted to specific intracellular compartments [5]. In addition, DCF derivatives can produce ROS upon exposure to light [6, 7]. It would be cautioned, that visualization of ROS with DCF can result in artifactual ROS generation and signal amplification. A genetically encoded, highly specific fluorescent probe for hydrogen peroxide detection inside living cells (HyPer) was developed in 2006 by Belousov et al. [8]. HyPer consists of circularly permuted yellow fluorescent protein (cpYFP) inserted into the regulatory domain of the prokaryotic $H_2O_2$-sensing protein, OxyR. Fluorescent

properties of cpYFP are dependent on protein conformation. Wild type OxyR contains two domains: $H_2O_2$-sensitive regulatory domain (amino acids 80–310) and DNA-binding domain (amino acids 1–79). In the presence of $H_2O_2$ the reduced form of OxyR converts into its oxidized, DNA-binding form. The key residues that undergo the oxidative modification are Cys199 and Cys208. Cys199 is located in a hydrophobic pocket. Exposure of OxyR to $H_2O_2$ converts Cys199 to a sulfenic acid derivative that is released from the hydrophobic pocket and forms a disulfide bond with Cys208. This modification leads to a dramatic conformational change in the flexible region (amino acids 205–222) of regulatory domain of OxyR and, hence, to an YFP spectral change. It was demonstrated that HyPer has submicromolar affinity to $H_2O_2$ and, at the same time, it is insensitive to other oxidants [8].

Important sources of ROS (superoxide radical and its dismutation product $H_2O_2$) in cell are NADPH oxidases. According to our previous results, the adaptor protein Ruk/CIN85 can form the intracellular complex with protein Tks4 [9, 10], which functions as organizer subunit of NADPH-oxidase complex mediated by Nox1 [11, 12].

To elucidate the possible colocalization between Ruk/CIN85 and $H_2O_2$ production inside living cells it was aimed to develop the recombinant plasmid vector encoding chimeric protein Ruk/CIN85-HyPer as well as to check the peculiarities of intracellular $H_2O_2$ production in transiently transfected human breast adenocarcinoma MCF-7 cells using time-lapse imaging.

## Materials and Methods

*Plasmids and reagents.* The following plasmids were used in the work: linearized positive selection cloning vector pJET1.2/blunt with a lethal insert that allows for efficient recovery of blunt-ended polymerase chain reaction (PCR) products (Thermo Fisher Scientific); pEGFP-N1 (BD Biosciences Clontech, GenBank Accession #U55762); PH-Btk-HyPer-MigRI encoding the PH domain of tyrosine kinase Btk fused with Hyper (provided by A. Vorotnikov, Lomonosov Moscow State University, RF); pRc-$Ruk_1$ (provided by V. Buchman, Cardiff University, Great Britain). Restriction endonucleases BamHI, EcoRI, XhoI, NotI, Pfu DNA Polymerase, T4 DNA Ligase, Silica Bead DNA Gel Extraction Kit, agarose, LB medium and IPTG were purchased from Fermentas. GeneJET Plasmid Miniprep Kit was from Thermo Fisher Scientific. Cell culture reagents were from HyClone. FuGene 6 Transfection Reagent was from Roche Molecular Biochemicals, Germany. Monoclonal antibody that recognizes SH3A domain of adaptor protein Ruk/CIN85 was described earlier [13].

*Plasmid construction.* The sequence encoding Hyper was created by PCR using as a template the plasmid PH-Btk-Hyper-MigR1. The primers used were as follows: forward primer, 5′-TCCGGATCCAATGGAGATGGCAAG-3′ and reverse primer, 5′-TTAAGCGGCCGC-TATTAAACCGCCTGTTTT-3′. The sequence encoding Rukl was created by PCR using as a template the plasmid pRc-$Ruk_1$ and following primers: forward primer, 5′-GAGCTCGAGA-TGGTGGAGGCCATA-3′ and reverse primer, 5′-TTCGAATTCGTTTTGATTGGAGAGC-3′. PCR amplification was performed under the following conditions: incubation at 94 °C for 5 min; 28 cycles at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 3 min; and extension at 72 °C for 5 min. PCR amplification products were extracted and purified using Silica Bead DNA Gel Extraction Kit. At first, Hyper cDNA was subcloned into pJET1.2/blunt vector using CloneJET™ PCR Cloning Kit. Then, reaction mixture was transformed into competent *E. coli* DH10B cells. Three positive clones verified with PCR were picked. To confirm the correct orientation of insert, the purified plasmid DNA was validated using digestion with Not1. At next stage, the Hyper cDNA was cut out and inserted into eukaryotic vector pEGFP-N1, instead of EGFP cDNA, at recognition sequences for restriction endonucleases BamH1 and Not1. The plasmid carrying the Hyper nucleotide sequence was designated Hyper-N1. Similarly, Rukl cDNA was obtained followed by insertion into Hyper-N1 at recognition sequences for restriction endonucleases Xho1 and EcoR1. The resulting plasmid was termed Ruk/CIN85-Hyper-N1. The obtained constructs were verified by DNA sequencing.

*Cell culture.* Human embryonic kidney HEK293 cells and human breast adenocarcinoma MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% $CO_2$ at 37 °C. Cells were passaged (1:3 split) every 3 to 4 days by trypsinization in solution containing 0.25% trypsin and 0.02% EDTA.

*Cell transfection, protein preparation and Western blot analysis.* HEK293 and MCF-7 cells have almost undetectable levels of Ruk/CIN85.

HEK293 cells were transiently transfected either with pRuk$_1$-HyPer-N1 or pHyPer-N1 by calcium phosphate precipitation essentially as described in [14]. MCF-7 cells were transfected according to the manufacturer's instructions using FuGene 6 Transfection Reagent. Cell imaging was performed 24–36 h after transfection. Transfected HEK-293 cells were scraped in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X100, 2 mM EDTA, 1 mM PMSF, complete protease inhibitor cocktail tablet — Roche), mechanically triturated through a 1-ml syringe and centrifuged at 12 000 g for 20 min at 4 °C. Protein content was determined using bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (30 µg per sample) were separated by electrophoresis on 10%-th polyacrylamide gel and transferred to nitrocellulose membranes. Membrane was incubated with monoclonal anti-Ruk/CIN85 antibody, which recognizes an epitope within the first SH3A domain present only in the full-length form of Ruk/CIN85 protein [13]. Secondary anti-mouse peroxidase-conjugated IgG (Sigma-Aldrich) were used in 1:5 000 dilution. Enhanced chemiluminescence kit (Amersham Pharmacia Biotech, USA) was used for detection of immunoreactive bands. To investigate the subcellular localization of both Hyper and Ruk/CIN85-Hyper, HEK293 cells were cultured on glass cover slips, fixed with 3% paraformaldehyde and mounted on glass slides with Vectashield mounting medium (Vector Laboratories). Fluorescence was visualized using confocal laser scanning microscopy (LSM 510 META, Zeiss).

*Time-lapse imaging of $H_2O_2$ in living cells* was performed essentially as described in [15]. Cultured MCF-7 cells on glass bottom dishes (Willco-Dish, the Netherlands) were analyzed in a live cell chamber with 5% $CO_2$ at 37 °C 24–36 h following transfection using laser scanning confocal inverted microscope Leica TCS SP5 (Leica Microsystems GmbH, Germany) equipped with 63x oil immersion objective, on-stage cell culture box, temperature controller set to 37 °C and humidifier unit. To excite HyPer fluorescence, the 405 nm diode laser (Coherent, USA) and 488 nm argon laser (Ar-Ion laser LASOS LGK 7872 ML, LASOS Lasertechnik GmbH, Germany) were used. The probe emission was detected at indicated time points in a 500–600 nm interval. The filming was carried out at the succession of channels one after the other at intervals between adjacent images indicated at picture. The relation between fluorescence intensity in second excitation channel (488 nm) to fluorescence intensity in first excitation channel (405 nm) was calculated for each image and depending on the result the colour was attributed to point (white colour–high relation, much $H_2O_2$, black colour–low relation, less $H_2O_2$).

## Results and Discussion

It is now well documented that ROS, especially $H_2O_2$, generated by NADPH oxidases participate in signal transduction, so-called "redox signaling", that involves the selective reversible modification of thiol groups in regulatory proteins [1]. The specificity, efficiency and spatiotemporal characteristics of redox-signalling are dependent on the formation of multimolecular signalling complexes containing NADPH oxidases [16–18]. In its turn, the localized production of $H_2O_2$ in vicinity of specific substrates leads to their oxidized modification and hence the modulation of signalling responses. It can be suggested that adaptor/scaffold proteins consisting of domains and motifs involved in intermolecular interactions play key roles in compartmentalization of ROS-mediated signal transduction.

Our previous studies revealed that adaptor protein Ruk/CIN85, which contains multiple $SH_3$ domains and Pro-rich motifs in its structure, can form the intracellular complex with adaptor protein Tks4 [9, 10] functioning as organizer subunit of NADPH-oxidase complex mediated by Nox1 [11, 12]. In addition, it was shown by us that ROS production by human colorectal adenocarcinoma HT-29 cells is positively correlated with Ruk/CIN85 expression [19] while increased levels of Ruk/CIN85 in weakly invasive human breast adenocarcinoma MCF-7 cells contribute to their malignant phenotype [20]. Taking into account these data and important roles of ROS in carcinogenesis, we hypothesized the potential regulatory interrelation between Ruk/CIN85 and $H_2O_2$ production in tumour cells. To check the possible co-localization of these signalling molecules in living cells we decided to fuse the cDNA coding for Hyper with Ruk/CIN85 cDNA.

To construct a plasmid encoding the chimeric fluorescent sensor of $H_2O_2$ Ruk/CIN85-HyPer, the following strategy was used. At first stage, the Hyper cDNA was inserted into eukaryotic vector pEGFP-N1, instead of EGFP cDNA, at recognition sequences for

restriction endonucleases BamH1 and Not1. At next stage, the $Ruk_1$ cDNA was subcloned into obtained genetic construction at recognition sequences for restriction endonucleases Xho1 and EcoR1 (vector maps at Fig. 1, *A*, *C*). The size and correct orientation of inserts were validated by restriction analysis (Fig. 1, *B*, *D*). The experimental procedure of cloning is described in details in "Materials and methods".

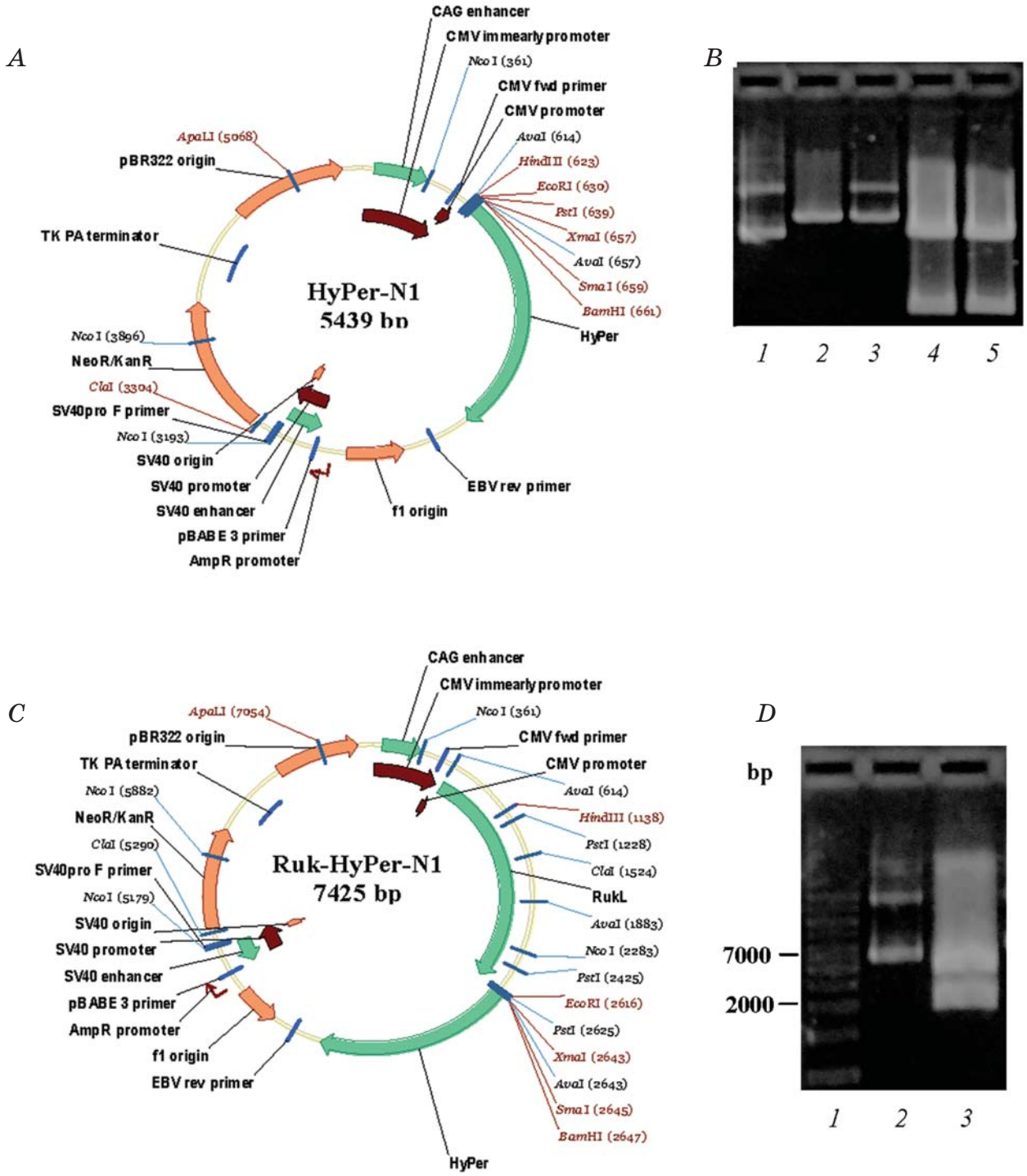


*Fig. 1.* **Cloning of Hyper vectors:**
*A* — HyPer-N1 vector map; *B* — Restriction analysis of HyPer-N1 (*1* — HyPer-N1 plasmid; *2* — HyPer-N1 digested with EcoRI; *3* — HyPer-N1 digested with XhoI; *4* — HyPer-N1 digested with BamHI and NotI; *5* — HyPer-N1 digested with EcoRI and NotI); *C* — Ruk/CIN85-HyPer-N1 plasmid map; *D* — Restriction analysis of Ruk/CIN85-HyPer-N1 (*1* — GeneRulerTM 1 kb DNA Ladder (#SM0311); *2* — Ruk/CIN85-HyPer-N1 plasmid; *3* — Ruk/CIN85-HyPer-N1 digested with EcoRI and XhoI)

The expression efficiency of Hyper and Ruk/CIN85-Hyper as well as the peculiarities of their intracellular localization were tested in HEK293 cells transiently transfected with obtained plasmids using $Ca^{2+}$-phosphate precipitation. Western blotting of Ruk/CIN85-Hyper in lysates of transfected HEK293 cells with monoclonal antibody recognizing N-terminal SH3A domain of Ruk/CIN85 confirmed the assumed molecular weight of recombinant protein about 120 kDa (Fig. 2, *A*). As can be seen from Fig. 2, *B*, the fluorescence (405 nm excitation and 520 nm emission) of the Hyper protein in control HEK293 cells transfected with Hyper-N1 is characterized by diffuse pattern throughout the cell. On the contrary, the fluorescence of Ruk/CIN85-Hyper in HEK293 cells transfected with Ruk/CIN85-Hyper-N1was clearly detectable as green spots of different size after fixation with paraformaldehyde. In a number of previous publications we and others have been demonstrated punctate localization pattern of both endogenous and exogenous Ruk/CIN85 characteristic for proteins involved in membrane trafficking. Specifically, Ruk/CIN85 was found associated with a subset of COPI-coated vesicles of the Golgi complex [21]. In turn, we revealed that in MCF-7 cells GFP-Ruk/CIN85 localized and concentrated in the perinuclear region and in round juxtamembrane structures, which were also clathrin-positive [22]. It was also shown that in COS-7 cells Ruk/CIN85 was located on the edges of some dark circular areas, part of which were filled with endocytic EGF [23]. Authors demonstrated that although some cells expressing exogenous Ruk/CIN85 showed very large dot-like structures, these dots are not aggresomes, as they failed to co-localize with Hsp70, the chaperone involved in the unfolded protein response. It was suggested that these dots could be aggregates of Ruk/CIN85 and its binding partners.

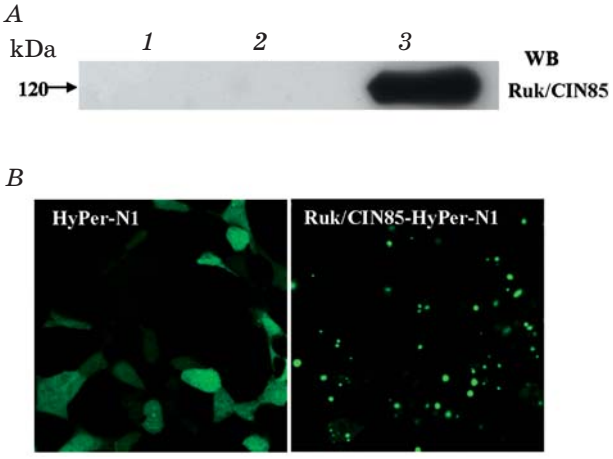


*Fig. 2.* **Recombinant proteins Hyper and Ruk/CIN85 Hyper are expressed effectively in transiently transfected HEK293 cells:** *A* —Western blotting of Ruk/CIN85-Hyper in Triton X-100-soluble cellular lysates using monoclonal antibody to SH3A domain of adaptor protein (*1* — Non-transfected HEK293 cells; *2* — HEK293 cells transfected with Hyper-N1; *3* —HEK293 cells transfected with Ruk/CIN85-Hyper-N1); *B* —Immunofluorescence microscopy of Hyper and Ruk/CIN85-Hyper in transiently transfected HEK293 cells

To verify if the novel constructed Hyper protein fused with Ruk/CIN85 is able to detect hydrogen peroxide, the changes of the fluorescence ratio were analyzed via live cell fluorescence microscopy as described in "Materials and Methods". As could be seen from Fig. 3, during the time of experiment, increase in hydrogen peroxide concentration in representative vesicular structures was observed going along with a pseudocolour change from green via yellow towards red. It is important to emphasize that co-localization of Ruk/CIN85 and $H_2O_2$ generation actually takes place at the edges of detected vesicular structures. Although the nature and biological significance of these vesicular structures are currently unknown, the data obtained provide novel insights into the potential role of adaptor protein Ruk/CIN85 in localized $H_2O_2$ production.

In a number of recent publications it was shown that endosomal NADPH oxidases are critically involved in the control of redox-dependent signalling [16]. Internalization of activated receptors to a compartment enriched with NADPH oxidases and associated signalling molecules is expected to facilitate regulation of redox-mediated signal transduction. In particular, treatment of SMC with TNF-α induced a dynamin-dependent endosomal generation of ROS that was inhibited by shRNA to Nox1 [24]. Using the Hyper probes immobilized within intracellular membrane subcompartments, it was demonstrated that ER-associated $N_2O_2$ production becomes activated faster

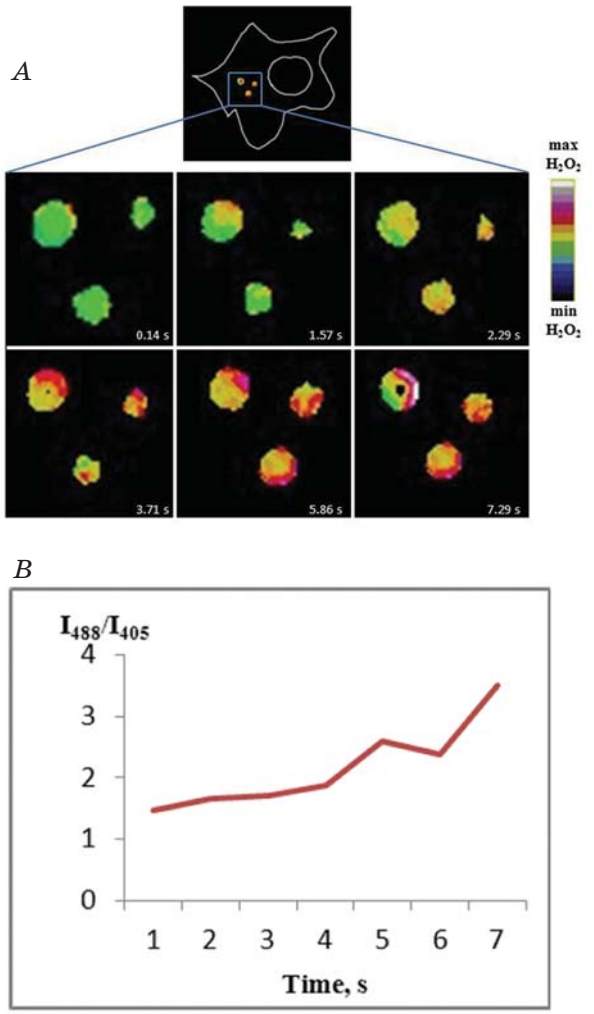


*Fig. 3.* **Hydrogen peroxide detection via live cell fluorescence microscopy in MCF-7 cells expressing the chimeric protein Ruk/CIN85-Hyper:**
*A* — Individual fragments of imaging in representative region of cell that corresponds to denoted time;
*B* — Time course of $H_2O_2$ generation associated with representative region of cell presented at *A*

than endosomal $H_2O_2$ appears in growth factors-treated cells [25]. Endosomal Nox1 and Nox2 were shown to regulate c-Src activation following hypoxia/reoxygenation injury [26]. There are also data that NADPH oxidase is internalized by clathrin-coated pits and localizes to a Rab27A/B GTPase regulated secretory compartment in activated macrophages [27]. All these findings are important in light of regulatory role of Ruk/CIN85 in ligand-induced endocytosis of different types of membrane receptors as well as in intracellular membrane trafficking [21, 28].

The exact nature of Ruk/CIN85-containing vesicular structures associated with hydrogen peroxide generation has to be established to improve our understanding the mechanisms of redox-dependent signalling in living cells. So, with the chimeric Ruk/CIN85-Hyper fluorescent protein presented herein we offer a new tool for studying hydrogen peroxide generation within intracellular membrane structures.

This work was partially supported by bilateral grant between National Academy of Sciences (NAS) of Ukraine and Russian Fund for Basic Research (RFBR)–2012.

## REFERENCES

1. *Finkel T.* Reactive oxygen species and signal transduction. *IUBMB Life*. 2001, 52 (1–2), 3–6.
2. *Lambeth J. D.* Nox enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol*. 2004, 64 (10), 3580–3585.
3. *Drobot L. B., Samoylenko A. A., Vorotnikov A. V., Tyurin-Kuzmin P. A., Bazalii A. V., Kietzmann T., Tkachuk V. A., Komisarenko S. V.* Reactive oxygen species in signal transduction. *Ukr Biokhim Zh*. 2013, 85 (6), 208–216.
4. *Griendling K. K., Sorescu D., Ushio-Fukai M.* NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*. 2000, 86 (5), 494–501.
5. *Crow J. P.* Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species. *Nitric Oxide*. 1997, 1 (2), 145–157.
6. *Marchesi E., Rota C., Fann Y. C., Chignell C. F., Mason R. P.* Photoreduction of the fluorescent dye 2′-7′-dichlorofluorescein: a spin trapping and direct electron spin resonance study with implications for oxidative stress measurements. *Free Radic. Biol. Med*. 1999, 26 (1–2), 148–161.
7. *Rota C., Fann Y. C., Mason R. P.* Phenoxyl free radical formation during the oxidation of the

fluorescent dye 2′,7′-dichlorofluorescein by horseradish peroxidase. Possible consequences for oxidative stress measurements. *J. Biol. Chem.* 1999, 274 (40), 28161–28168.
8. *Belousov V. V., Fradkov A. F., Lukyanov K. A., Staroverov D. B., Shakhbazov K. S., Terskikh A. V., Lukyanov S.* Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat. Meth.* 2006, 3 (4), 281–286.
9. *Havrylov S., Rzhepetskyy Y., Malinowska A., Drobot L., Redowicz M. J.* Proteins recruited by SH3 domains of Ruk/CIN85 adaptor identified by LC-MS/MS. *Proteome Sci.* 2009, 16 (7). doi: 10.1186/1477-5956-7-21.
10. *Bazalii A. V., Samoylenko A. A., Petukhov D. M., Rynditch A. V., Redowicz M.-J., Drobot L. B.* Interaction between adaptor proteins Ruk/CIN85 and Tks4 in normal in tumor cells of different tissue origins. *Biopolimers and Cell.* 2014, 30 (1), 33–37.
11. *Gianni D., Diaz B., Taulet N., Fowler B., Courtneidge S. A., Bokoch G. M.* Novel p47(phox)-related organizers regulate localized NADPH oxidase 1 (Nox1) activity. *Sci Signal.* 2009, 2 (88), ra54.
12. *Gianni D., DerMardirossian C., Bokoch G. M.* Direct interaction between Tks proteins and the N-terminal proline-rich region (PRR) of NoxA1 mediates Nox1-dependent ROS generation. *Eur. J. Cell. Biol.* 2011, 90 (2–3), 164–171.
13. *Mayevska O., Shuvayeva H., Igumentseva N., Havrylov S., Barska M., Bobak Ya., Volodko N., Baranska J., Buchman V., Drobot L.* Expression of adaptor protein Ruk/CIN85 isoforms in cell lines of various tissue origins and human melanoma. *Exp. Oncol.* 2006, 28 (4), 275–281.
14. *Webster G. A., Perkins N. D.* Transcriptional cross talk between NF-kappaB and p53. *Mol. Cell. Biol.* 1999, 19 (5), 3485–3495.
15. *Tiurin-Kuz'min P. A., Agaronian K. M., Morozov Ya. I., Mishina N. M., Belousov V. V., Vorotnikov A. V.* NADPH oxidase controls EGF-induced proliferation via the ERK1/2-independent mechanism. *Biofizika.* 2010, 55 (6), 1048–1056.
16. *Ushio-Fukai M.* Compartmentalization of redox signaling through NADPH oxidase-derived ROS. *Antioxid. Redox. Signal.* 2009, 11 (6), 1289–1299.
17. *Mishina N. M., Tyurin-Kuzmin P. A., Markvicheva K. N., Vorotnikov A. V., Tkachuk V. A., Laketa V., Schultz C., Lukyanov S., Belousov V. V.* Does cellular hydrogen peroxide diffuse or act locally? *Antiox. Redox Signal.* 2011, 14 (1), 1–7.
18. *Malinouski M., Zhou Y., Belousov V. V., Hatfield D. L., Gladyshev V. N.* Hydrogen Peroxide Probes Directed to Different Cellular Compartments. *PLoS ONE.* 2011, 6 (1), e14564.
19. *Samoylenko A. A., Byts N. V., Pasichnyk G. V., Kozlova N. V., Bazalii A. V., Gerashchenko D. S., Shandrenko S. G., Vorotnikov A. V., Kietzmann T., Komisarenko S. V., Drobot L. B.* Recombinant lentivirus-mediated silencing of adaptor protein Ruk/CIN85 expression influences biological responses of tumor cells. *Biotechnol. acta.* 2013, 6 (4), 182–189. (In Ukrainian).
20. *Samoylenko A., Vynnytska-Myronovska B., Byts N., Kozlova N., Basaraba O., Pasichnyk G., Palyvoda K., Bobak Y., Barska M., Mayevska O., Rzhepetsky Y., Shuvayeva H., Lyzogubov V., Usenko V., Savran V., Volodko N., Buchman V., Kietzmann T., Drobot L.* Increased levels of the HER1 adaptor protein Rukl/CIN85 contribute to breast cancer malignancy. *Carcinogenesis.* 2012, 33 (10), 1976–1984.
21. *Havrylov S., Ichioka F., Powell K., Borthwick E. B., Baranska J., Maki M., Buchman V. L.* Adaptor Protein Ruk/CIN85 is Associated with a Subset of COPI-Coated Membranes of the Golgi Complex. *Traffic.* 2008, 9 (5), 798–812.
22. *Nikolaienko O. V., Skrypkina I. Ya., Tsyba L. O., Drobot L. B., Rynditch A. V.* ITSN1 and Ruk/CIN85 colocalized to clathrin-coated pits in MCF-7 cells. *Biopolymers and Cell.* 2009, 25 (5), 424–427.
23. *Zhang J., Zheng X., Yang X., Liao K.* CIN85 associate with endosomal membrane and binds phosphatidic acid. *Cell Res.* 2009, 19 (6), 733–746.
24. *Miller F. J., Chu X., Stanic B., Tian X., Sharma R. V., Davisson R. L., Lamb F. S.* A differential role for endocytosis in receptor-mediated activation of Nox1. *Antioxid. Redox. Signal.* 2009, 12 (5), 583–593.
25. *Lukyanov K. A., Belousov V. V.* Genetically encoded fluorescent redox sensors. *Biochim. Biophys. Acta.* 2014, 1840 (2), 745–756.
26. *Li Q., Zhang Y., Marden J. J., Banfi B., Engelhardt J. F.* Endosomal NADPH oxidase regulates c-Src activation following hypoxia/reoxygenation injury. *Biochem. J.* 2008, 411 (3), 531–541.
27. *Ejlerskov P., Christensen D. P., Beyaie D., Burritt J. B., Paclet M. H., Gorlach A., van Deurs B., Vilhardt F.* NADPH oxidase is internalized by clathrin-coated pits and localizes to a Rab27A/B GTPase-regulated secretory compartment in activated macrophages. *J. Biol. Chem.* 2012, 287 (7), 4835–4852.
28. *Dikic I., Giordano S.* Negative receptor signalling. *Curr. Opin. Cell Biol.* 2003, 15 (2), 128–135.

# РЕКОМБІНАНТНИЙ ФЛУОРЕСЦЕНТНИЙ СЕНСОР ПЕРОКСИДУ ГІДРОГЕНУ HYPER, ЗЛИТИЙ ІЗ АДАПТЕРНИМ ПРОТЕЇНОМ RUK/CIN85: КОНСТРУЮВАННЯ ЕКСПРЕСІЙНОГО ВЕКТОРА ТА ФУНКЦІОНАЛЬНА ХАРАКТЕРИСТИКА

*А. В. Базалій*[1]
*А. В. Воротніков*[2]
*П. О. Тюрін-Кузьмін*[2]
*В. А. Ткачук*[2]
*С. В. Комісаренко*[1]
*Л. Б. Дробот*[1]

[1]Інститут біохімії ім. О. В. Палладіна НАН України, Київ
[2]Факультет фундаментальної медицини, Московський державний університет ім. М. В. Ломоносова, РФ

*E-mail: bazalii@biochem.kiev.ua*

Метою дослідження було конструювання експресійного вектора, що кодує флуоресцентний сенсор пероксиду гідрогену, злитого з адаптерним протеїном Ruk/CIN85, з'ясування його субклітинної локалізації та здатності детектувати $H_2O_2$. Встановлено, що в тимчасово трансфікованих клітинах HEK293 та MCF-7 Ruk/CIN85-HyPer концентрується в «дот»-подібних везикулярних структурах різних розмірів, тоді як HyPer розподілений у клітині дифузно. Із використанням прижиттєвої флуоресцентної мікроскопії продемонстровано зростання концентрації $H_2O_2$ у репрезентативних везикулярних структурах упродовж зазначеного часу експерименту. Одержана генетична конструкція, що кодує химерний протеїн Ruk/CIN85-HyPer, є новим інструментом для дослідження локалізованого продукування $H_2O_2$ у живих клітинах.

***Ключові слова:*** рекомбінантні протеїни, адаптерний протеїн Ruk/CIN85, пероксид гідрогену, сенсор пероксиду гідрогену HyPer, Ruk/CIN85-HyPer.

# РЕКОМБИНАНТНЫЙ ФЛУОРЕСЦЕНТНЫЙ СЕНСОР ПЕРОКСИДА ГИДРОГЕНА HYPER, СЛИТЫЙ С АДАПТЕРНЫМ ПРОТЕИНОМ RUK/CIN85: КОНСТРУИРОВАНИЕ ЭКСПРЕССИОННОГО ВЕКТОРА И ФУНКЦИОНАЛЬНАЯ ХАРАКТЕРИСТИКА

*А. В. Базалий*[1]
*А. В. Воротников*[2]
*П. А. Тюрин-Кузьмин*[2]
*В. А. Ткачук*[2]
*С. В. Комисаренко*[1]
*Л. Б. Дробот*[1]

[1]Институт биохимии им. А. В. Палладина НАН Украины, Киев
[2]Факультет фундаментальной медицины, Московский государственный университет им. М. В. Ломоносова, РФ

*E-mail: bazalii@biochem.kiev.ua*

Целью работы было конструирование экспрессионного вектора, кодирующего флуоресцентный сенсор пероксида гидрогена, слитого с адаптерным протеином Ruk/CIN85, выяснение его субклеточной локализации и способности детектировать $H_2O_2$. Установлено, что во временно трансфицированных клетках HEK293 и MCF-7 Ruk/CIN85-HyPer концентрируется в «дот»-подобных везикулярных структурах разных размеров, в то время как HyPer распределен в клетке диффузно. С использованием прижизненной флуоресцентной микроскопии было продемонстрировано возрастание концентрации $H_2O_2$ в репрезентативных везикулярных структурах в течение указанного времени эксперимента. Полученная генетическая конструкция, кодирующая химерный протеин Ruk/CIN85-HyPer, является новым инструментом для исследования локализованной продукции $H_2O_2$ в живых клетках.

***Ключевые слова:*** рекомбинантные протеины, адаптерный протеин Ruk/CIN85, пероксид гидрогена, сенсор пероксида гидрогена HyPer, Ruk/CIN85-HyPer.