ISSN 0233–7657. Biopolymers and Cell. 2012. Vol. 28. N 3. P. 234–238

UDC 577.22

Amphiphysin 1 and 2 interact with latent membrane protein 2A of Epstein-Barr virus and regulate its exosomal secretion

O. V. Dergai, M. V. Dergai, I. Ya. Skrypkina, L. O. Tsyba, A. M. Yaruchik, A. V. Rynditch

State Key Laboratory of Molecular and Cellular Biology
Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03680

o.dergai@gmail.com

Aim. *Latent membrane protein 2A (LMP2A) of Epstein-Barr virus is implicated in the regulation of viral latency. The aim of the current study was to identify proteins interacting with proline-rich motifs of LMP2A.* ***Methods****. In silico prediction with Scansite allowed to recognize amphiphysin 1 (Amph1) as a binding partner of LMP2A. Molecular cloning techniques, site-directed mutagenesis, in vitro binding assay made it possible to study the interaction interface of Amph1/LMP2A complex. Sequential centrifugation steps were used to isolate an exosomal fraction.* ***Results****. LMP2A but not LMP2ΔNT mutant has been found to bind the SH3 domain of Amph1 via three distinct proline-rich motifs located in the N-terminal tail. All three motifs seem to be interchangeable as the presence of at least one of them was sufficient to mediate LMP2A/Amph1 interaction. Furthermore, the binding of LMP2A to Amph1 and related protein amphiphysin 2 was demonstrated by co-immunoprecipitation of endogenous complexes. We have found that inability of LMP2A mutant to bind Amph1 leads to the vanishing of the viral protein from the exosomal fraction.* ***Conclusions****. The latent membrane protein 2A of Epstein-Barr virus forms complexes with endocytic adaptor proteins Amph1 and Amph2. Described interaction might be involved in the regulation of intracellular traffic and secretion of LMP2A.*

Keywords: EBV, LMP2A, Amphiphysin, exosomes.

Introduction. Epstein-Barr virus (EBV) is a member of the herpesvirus family and one of the most common human viruses [1]. EBV is associated with a number of human malignancies, such as Burkitt's lymphoma [2], Hodgkin's lymphoma [3] and nasopharyngeal carcinoma [4]. Only restricted set of viral genes is expressed within the latent phase: LMP1, LMP2A, LMP2B, EBNAs and EBERs. Latent membrane proteins (LMPs) are key players in transformation and survival of infected cells [5]. EBV latency is regulated by LMP2A and LMP2B [5]. LMP2A is a transmembrane protein comprising 12 transmembrane segments and two cytosolic tails. The N-terminal tail is responsible for LMP2A signalosome assembly and signaling, while C-terminal one mediates clusterization of LMP2A molecules [6, 7]. Cytosolic N- and C-terminal tails of LMP2A contain phosphotyrosine-containing (pY) and proline-rich motifs (PRMs). Through the pY-motifs LMP2A interacts with SH2- and PTB-containing proteins [8]. Due to the binding of tyrosine kinases Syk and Lyn, LMP2A mimics activated receptors and establishes its own signaling [9–11].

LMP2A-induced signaling events were uncovered in B-cells, epithelial cells and fibroblasts [5, 10]. LMP 2A activates AKT kinase providing anti-apoptotic and pro-survival signals [11, 12]. Much attention was paid to mitogenic signaling but knowledge about LMP2A internalization and traffic in EBV-positive cells is unclear so far.

Despite the presence of five putative PRMs no interactions with the SH3 domains have been reported. Here

we report the identification of amphiphysins 1 and 2 as protein partners of LMP2A. Inability of LMP2A mutant to interact with Amph1 affects the secretion of LMP2A on exosomes produced by HEK293 cells suggesting a putative role of Amph1 in LMP2A traffic.

Materials and methods. *Antibodies*. A monoclonal antibody against FLAG epitope (clone M2) was purchased from «Sigma» (USA); a monoclonal anti-Omni (D-8) antibody and rabbit polyclonal anti-Omni antibodies (M-21) were from «Santa Cruz» (USA).

DNA constructs. The construct carrying wild type LMP2A CDS [13] was used as a template for PCR amplification of LMP2A of wild type (1–497 aa residues), LMP2AΔNT mutant (105–497 aa residues) and derived P-to-A point mutant constructs with subsequent cloning into *pcDNA4* His/MaxC («Invitrogen», USA). Site-specific mutations were introduced with primer-extension method. LMP2A P2 mutant was generated using the following primers: For 5'-AACACCGCCACCGCAGC GAACGATGAGGAA, Rev 5'-TTCCTCATCGTTCG CTGCGGTGGCGGTGTT, LMP2A P3 mutant: 5'-TG AAGAGGCCGCAGCGGCTTATGAGGACCCA, Rev 5'-TCCTCATAAGCCGCTGCGGCCTCTTCATTAG, LMP2A P4 mutant: 5'-TGACGGGCTCGCTGCCGC TGCCTACTCTCCAC, Rev 5'-GTGGAGAGTAGGC AGCGGCAGCGAGCCCGTCA. The LMP2A P3 + P4 and LMP2A P2 + P3 + P4 were obtained by combining of P-to-A substitution in mentioned motifs (Fig. 1, *A*).

The SH3 domains of Amph 1, PI3Kp85α, Src and endophilin were described previously [14–16]. Full-length CDS of Amph 1 and Amph 2 were amplified by PCR from human embryonal brain cDNA using the High Fidelity PCR enzyme mix. The PCR products were cloned into the pcDNA4 His/MaxC vector to generate Omni-Amph 1 Omni-Amph 2 respectively. All PCR-generated DNA fragments were sequenced to confirm fidelity.

Immunoprecipitation. For immunoprecipitation (IP) the cells were lysed in IP buffer (20 mM Tris-HCl, pH 7.5, 0.5 % NP-40, 150 mM NaCl, 10 % glycerol, 1 mM PMSF and protease inhibitor cocktail («Roche», France)). The HEK293 cell lysate was mixed with antibodies (0.5 μg) and protein A/G Agarose beads («Santa Cruz Biotechnology», USA) prewashed in IP buffer. After overnight incubation at 4 °C the beads were washed three times with IP buffer. The bound proteins were eluted by boiling in 30 μl of Laemmli sample buffer (150 mM Tris-HCl, pH 6.8, 2.5 % glycerol, 10 % SDS, 3 % β-mercaptoethanol and 0.5 % bromophenol blue) and analyzed by SDS-PAGE and Western blotting.

Cell culture and transfection. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum («Sigma»), 50 U/ml penicillin and 100 μg/ml streptomycin. The cells were transiently transfected using polyethyleneimine transfection reagent (JetPEI, Polyplus Transfection) and processed 24 h after transfection.

Protein expression, pull-down assays and Western blot analyses were carried out as described previously [14].

Exosome preparation. Exosomes were isolated as previously described [17] with minor modifications. In brief, HEK293 cells were grown to 60–70 % confluence on 10 cm plates, washed twice with 1 × PBS to remove secreted exosome and metabolites and supplied with fresh complete medium. Then cells were transfected with 15 μg of plasmid DNA. 24 h post-transfection culture medium was collected and cleared by centrifugation at 10,000 *g* for 10 min to remove apoptotic cells and cell fragments. Exosomes were further isolated by centrifugation at 70,000 *g* for 2 h (exosome fraction). Cells were lysed as described above; pelleted exosome fraction was solubilized in Laemmli buffer and analyzed by Western blotting.

Results and discussion. The aim of the current study was to identify proteins that bind directly PRMs located in LMP2A cytosolic tails. The canonical binding site for SH3 domains is PXXP (where X-any amino acid) [18]. Five motifs fitting PXXP consensus were found in the primary structure of LMP2A protein: four motifs (designated P1–P4) in the N-terminal domain and one (P5) in the C-terminal one. *In silico* analysis with Scansite service (www.scansite.mit.edu) evidenced for the presence of at least four motifs (P2, P3, P4 and P5) for binding the SH3 domain of endocytic adaptor protein Amph1. To validate this interaction we performed GST pull-down assay. We have found that the SH3 domain of Amph1 bound Omni-LMP2A, while it was unable to interact with LMP2AΔNT variant that represents the LMP2B isoform, an important negative regulator of LMP2A-dependent signaling (Fig. 1, *B*). Thus, P5 motif is dispensable to mediate interaction

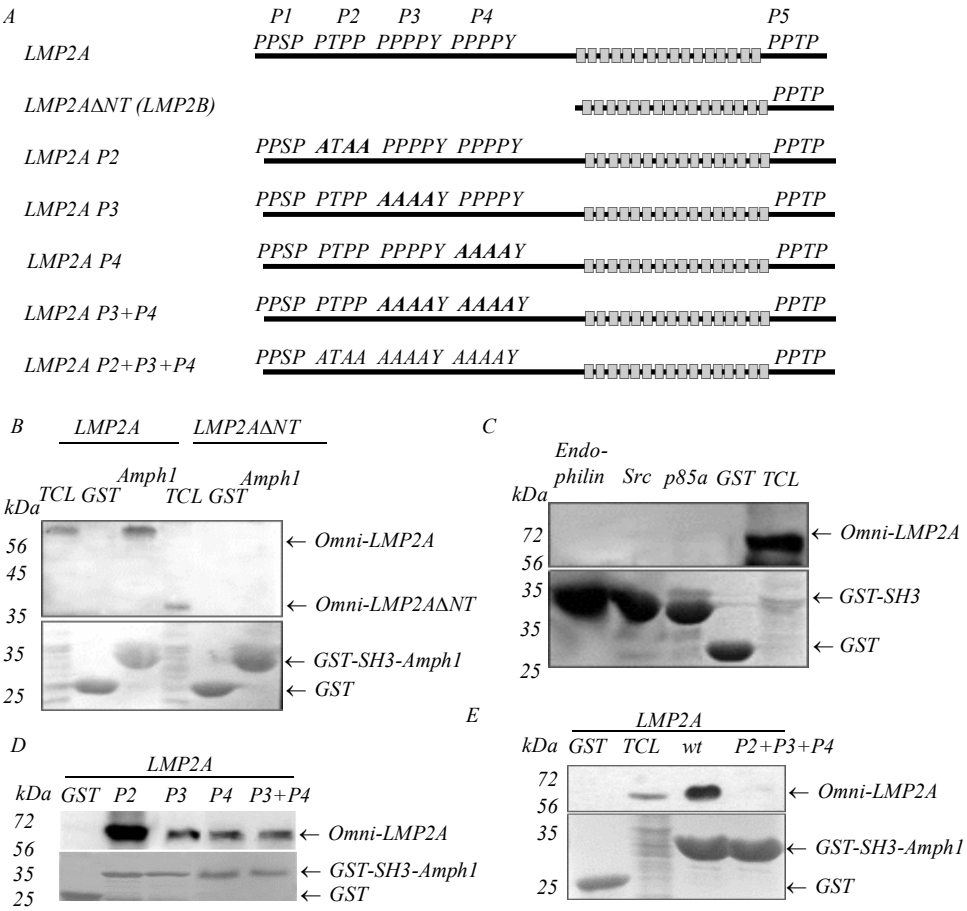

Fig. 1. LMP2A interacts with the SH3 domain of Amph1: *A* – schematic presentation of LMP2A and derived mutants (grey rectangles represent transmembrane segments, P1–P5 designated proline-rich motifs); *B* – lysates of HEK293 cells transiently transfected with Omni-LMP2A or Omni-LMP2AΔNT were incubated with the GST-SH3 domain of Amph1 or GST alone immobilized on glutathione Sepharose beads (protein complexes were eluted and analyzed by Western blotting; proteins were visualized as indicated in the figure; TCL – total cell lysate); *C* – GST-fused SH3 domains of specified proteins were used as a bait to precipitate Omni-LMP2A; *D*, *E* – GST-SH3 domain of Amph1 or GST alone coupled to glutathione-Sepharose beads were incubated with lysates of cells transfected with indicated at the figure LMP2A point mutants; *B*, *D* – WB: anti-Omni, Ponceau S staining; *C*, *E* – WB: nti-Omni, Coomassie staining

between LMP2A and Amph1. We also tested ability of LMP2A to bind the SH3 domains of other proteins: endophilin, Src kinase and p85 regulatory subunit of PI3K. As Fig. 1, *C*, shows, LMP2A failed to interact with the proteins mentioned above. This fact could evidence for the specificity of LMP2A/Amph1 interaction.

Further, we decided to determine which of four potential PRMs found in the N-terminus of LMP2A mediates this interaction. For this aim, prolines in motifs P2, P3 and P4 individually or in combinations P3 + + P4, P2 + P3 + P4 were substituted for alanines. The P1 motif was considered less probable for binding because the sequence of this PRM differs significantly from the consensus. The core motif in P1 is flanked with numerous glycines that is very unusual for classic PRMs, which are typically surrounded with charged amino acid residues [18]. The individual mutations in motifs P2, P3 and P4 had moderate effect on LMP2A binding to the SH3 domain of amphiphysin 1 (Fig. 1, *D*). P3 + P4 LMP2A mutant also bound to the SH3 domain of Amph1 (Fig. 1, *D*, lane *5*) whereas P2 + P3 + P4 mutant of LMP2A was unable to interact with Amph1 (Fig. *1*, *E*). Thus, one can suggest that Amph1 can bind any of three PRMs (P2–P4) in the N-terminal tail of LMP2A. All three motifs seem to be interchangeable as presence of at least one of them was sufficient to mediate LMP2A/Amph1 complex formation.

Moreover, LMP2A was shown to form a complex *in vivo* with Amph1 as well as with a highly related Amph2 (Fig. 2, *A*) in HEK293 cells transiently transfected with corresponding recombinant plasmids. The data obtained may link LMP2A to the endocytic compartment, enabling viral protein to be effectively internalized from the cell surface. Previously, LMP2A was detected in the cytoplasm in the association with different types of vesicles presumably derived from the plasma membrane. LMP2A is secreted on exosome pathway by different cell types [17]. Exosomes are membrane vesicles of endocytic origin after they have passed through multivesicular body [19]. We decided to determine whe-

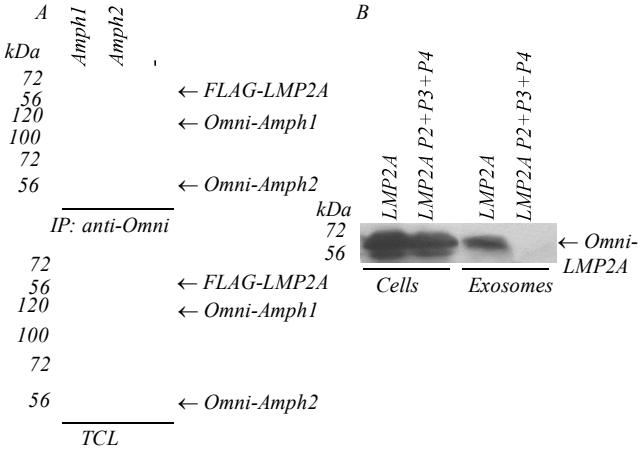

Fig. 2. Amph1 and 2 form complex with LMP2A *in vivo*: *A* – HEK293 cells were co-tranfected with FLAG-LMP2A and Omni-Amph1 or Omni-Amph2 (the lysates of transfected cells were subjected to immunoprecipitation with rabbit polyclonal anti-Omni antibodies; proteins were eluted, resolved by SDS-PAGE and immunoblotted with indicated antibodies: Omni-Amph1 and Omni-Amph2 detected with mouse monoclonal anti-Omni, FLAG-LMP2A with mouse monoclonal anti-FLAG); *B* – immunodetection of Omni-LMP2A and its mutant in lysates of transfected cells and exosomal fraction

ther inability of LMP2A to interact with Amph1 affects secretion of LMP2A on exosomes produced by HEK 293 cells. The wild type LMP2A was found in the exosomal fraction as well as inside of cells, while LMP2A P2 + P3 + P4 had not been detected on exosomes (Fig. 2, *B*). These data imply a role of interaction between LMP2A and Amph1 for intracellular traffic of LMP2A.

The current comprehension of composition of the LMP2A-mediated signalosome and mechanisms of its traffic through the cell remains unclear. Amphiphysins are adaptor proteins that have been implicated in clathrin-mediated endocytosis, regulation of actin cytoskeleton and cellular signaling [20–23]. Amph2 was shown to bind Myc oncoprotein and to function as onco-suppressor [24]. It is possible to speculate that the role of LMP2A in lymphogenesis is not restricted to providing pro-survival stimuli for the cells but also involves deregulation of Amph2/BIN function that leads to the inhibition its tumor-suppressing activity.

Answers for this question might help to improve therapy of EBV-associated lymphomas and make our knowledge deeer in the field of host-pathogen interactions.

Acknowledgements. We are grateful to Dr. O. S'edin for providing expertise and help in ultracentrifugation procedures. We thank Dr. G. Winberg and Dr. L. Matskova for generous gift of pCMV-FLAG-LMP2A plasmid and for stimulating discussions. This work was supported by INTAS Grant (N 05-1000004-7762). OVD is a fellow of Visby Scholarship of Swedish Institute.

О. В. Дергай, М.В. Дергай, І. Я. Скрипкина, Л. О. Циба, А. М. Яручик, А. В. Риндич

Амфіфізин 1 и 2 взаємодіють з латентним мембранним білком 2А (LMP2A) вірусу Епштейна-Барр і регулюють його секрецію

Резюме

Латентний мембранний білок 2А (LMP2A) вірусу Епштейна-Барр є важливим регулятором латентної фази вірусної інфекції. ***Мета****. Ідентифікувати білки, які взаємодіють з пролін-збагаченими мотивами LMP2A.* ***Методи****. Аналіз in silico за допомогою програмного забезпечення Scansite дозволив передбачити можливість взаємодії амфіфізину 1 (Amph1) і LMP2A. Використано загально прийняті техніки молекулярного клонування, сайт-спрямований мутагенез і тест на взаємодію in vitro для подальшого дослідження структурних основ взаємодії комплексу LMP2A/Amph1. Фракцію екзосом отримано за допомогою послідовних центрифугувань.* ***Результати****. Показано, що ізоформа LMP2A, але не LMP2A ΔNT взаємодіє з доменом SH3 амфіфізину 1. Виявлена взаємодія опосередковується трьома різними пролін-збагаченими мотивами, розташованими в N-кінцевій ділянці LMP2A. Всі три мотиви є взаємозамінними, оскільки присутність хоча б одного з них є достатньою для реалізації зв'язування LMP2A з Amph1. Нами продемонстровано взаємодію Amph1 і високоспорідненого з ним Amph2 з LMP2A за допомогою ко-імунопреципітації ендогенних комплексів. Мутант LMP2A за проліновими мотивами не взаємодіяв з Amph1, що спричиняло зникнення його з фракції екзосом.* ***Висновки****. Латентний мембранний білок 2А вірусу Епштейна-Барр утворює комплекси з ендоцитозними адаптерними білками Amph1 і Amph2. Ідентифіковані нові партнери LMP2A можуть впливати на його внутрішньоклітинний трафік та секрецію.*

Ключові слова: вірус Эпштейна-Барра, LMP2A, амфіфізин, екзосоми.

А. В. Дергай, Н. В. Дергай, И. Я. Скрипкина, Л. А. Циба, А. М. Яручик, А. В. Рындич

Амфифизин 1 и 2 взаимодействуют с латентным мембранным белком 2А (LMP2A) вируса Эпштейна-Барр и регулируют его секрецию

Резюме

Латентный мембранный белок 2А вируса Эпштейна-Барр является ключевым регулятором латентной фазы вирусной инфекции. ***Цель****. Идентифицировать белки, способные связываться с пролин-обогащенными мотивами LMP2A.* ***Методы****. Анализ in silico при помощи программного обеспечения Scansite позволил предсказать взаимодействие амфифизина 1 (Amph1) и LMP2A. Использованы стандартные методы молекулярного клонирования, сайт-направленный мутагенез и тест на взаимодействие in vitro для последующего изучения структурных основ взаимодействия комплекса LMP2A/Amph1. Фракцию экзосом получали при помощи последовательных центрифугирований.* ***Результаты****. Показано, что изоформа LMP2A, но не LMP2AΔNT взаимодействует с доменом SH3 Amph1. Выявленное взаимодействие опосредуется тремя разными пролин-обогащенными мотивами, расположенными в N-концевом участке LMP2A. Все три мотива*

являются взаимозаменяемыми, так как присутствия хотя бы одного из них оказывается достаточно для реализации связывания LMP2A с Amph1. Нами продемонстрировано связывание Amph1 и родственного ему Amph2 с LMP2A при помощи ко-иммунопреципитации эндогенных комплексов. Мутант LMP2A по пролиновым мотивам не взаимодействовал с Amph1, что привело к исчезновению его из фракции экзосом. ***Выводы****. Латентный мембранный белок 2А вируса Эпштейна-Барр образует комплексы с эндоцитозными адаптерными белками Amph1 и Amph2. Идентифицированные новые партнеры LMP2A могут влиять на его внутриклеточный трафик и секрецию.*

Ключевые слова: вирус Эпштейна-Барр, LMP2A, амфифизин, экзосомы.

REFERENCES

1. *Kieff E., Liebowitz D.* Epstein-Barr virus and its replication // Fields virology / Eds B. N. Fields, D. M. Knipe.–New York: Raven Press, 1990.–P. 1889–1920.
2. *Callan M. F., Tan L., Annels N., Ogg G. S., Wilson J. D., O'Callaghan C. A., Steven N., McMichael A. J., Rickinson A. B.* Direct visualization of antigen-specific $CD8^+$T cells during the primary immune response to Epstein-Barr virus *in vivo* // J. Exp. Med.–1998.–**187**, N. 9.–P. 1395–1402.
3. *Chapman A. L., Rickinson A. B.* Epstein-Barr virus in Hodgkin's disease // Ann. Oncol.–1998.–**9**, Suppl. 5.–S 5–16.
4. *Chen F., Hu L. F., Ernberg I., Klein G., Winberg G.* Coupled transcription of Epstein-Barr virus latent membrane protein (LMP)-1 and LMP-2B genes in nasopharyngeal carcinomas // J. Gen. Virol.–1995.–**76**, pt 1.–P. 131–138.
5. *Thorley-Lawson D. A.* Epstein-Barr virus: exploiting the immune system // Nat. Rev. Immunol.–2001.–**1**, N 1.–P. 75–82.
6. *Longnecker R., Kieff E.* A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and colocalizes with LMP1 // J. Virol.–1990.–**64**, N 5.–P. 2319–2326.
7. *Matskova L., Ernberg I., Pawson T., Winberg G.* C-terminal domain of the Epstein-Barr virus LMP2A membrane protein contains a clustering signal // J. Virol.–2001.–**75**, N 22.–P. 10941–10949.
8. *Rovedo M., Longnecker R.* Epstein-Barr virus latent membrane protein 2A preferentially signals through the Src family kinase Lyn // J. Virol.–2008.–**82**, N 17.–P. 8520–8528.
9. *Ingham R. J., Raaijmakers J., Lim C. S., Mbamalu G., Gish G., Chen F., Matskova L., Ernberg I., Winberg G., Pawson T.* The Epstein-Barr virus protein, latent membrane protein 2A, co-opts tyrosine kinases used by the T cell receptor // J. Biol. Chem.–2005.–**280**, N 40.–P. 34133–34142.
10. *Merchant M., Longnecker R.* LMP2A survival and developmental signals are transmitted through Btk-dependent and Btk-independent pathways // Virology.–2001.–**291**, N 1.–P. 46–54.
11. *Swart R., Ruf I. K., Sample J., Longnecker R.* Latent membrane protein 2A-mediated effects on the phosphatidylinositol 3-Kinase/ Akt pathway // J. Virol.–2000.–**74**, N 22.–P. 10838–10845.
12. *Matskova L. V., Helmstetter C., Ingham R. J., Gish G., Lindholm C. K., Ernberg I., Pawson T., Winberg G.* The Shb signalling scaffold binds to and regulates constitutive signals from the Epstein-Barr virus LMP2A membrane protein // Oncogene.–2007.–**26**, N 34.–P. 4908–4917.
13. *Winberg G., Matskova L., Chen F., Plant P., Rotin D., Gish G., Ingham R., Ernberg I., Pawson T.* Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases // Mol. Cell. Biol.–2000.–**20**, N 22.–P. 8526–8535.
14. *Dergai M., Skrypkina I., Dergai O., Tsyba L., Novokhatska O., Filonenko V., Drobot L., Rynditch A.* Identification and characterization of a novel mammalian isoform of the endocytic adaptor ITSN1 // Gene.–2011.–**485**, N 2.–P. 120–129.
15. *Dergai M., Tsyba L., Dergai O., Zlatskii I., Skrypkina I., Kovalenko V., Rynditch A.* Microexon-based regulation of ITSN1 and Src SH3 domains specificity relies on introduction of charged amino acids into the interaction interface // Biochem. Biophys. Res. Commun.–2010.–**399**, N 2.–P. 307–312.
16. *Dergai O., Novokhatska O., Dergai M., Skrypkina I., Tsyba L., Moreau J., Rynditch A.* Intersectin 1 forms complexes with SGIP1 and Reps1 in clathrin-coated pits // Biochem. Biophys. Res. Commun.–2010.–**402**, N 2.–P. 408–413.
17. *Ikeda M., Longnecker R.* Cholesterol is critical for Epstein-Barr virus latent membrane protein 2A trafficking and protein stability // Virology.–2007.–**360**, N 2.–P. 461–468.
18. *Zarrinpar A., Bhattacharyya R. P., Lim W. A.* The structure and function of proline recognition domains // Sci. SRKE.–2003.–**2003**, N 179.–re8.
19. *Keller S., Sanderson M. P., Stoeck A., Altevogt P.* Exosomes: from biogenesis and secretion to biological function // Immunol. Lett.–2006.–**107**, N 2.–P. 102–108.
20. *Yamada H., Ohashi E., Abe T., Kusumi N., Li S. A., Yoshida Y., Watanabe M., Tomizawa K., Kashiwakura Y., Kumon H., Matsui H., Takei K.* Amphiphysin 1 is important for actin polymerization during phagocytosis // Mol. Biol. Cell.–2007.–**18**, N 11.–P. 4669–4680.
21. *Wu Y., Liang S., Oda Y., Ohmori I., Nishiki T., Takei K., Matsui H., Tomizawa K.* Truncations of amphiphysin I by calpain inhibit vesicle endocytosis during neural hyperexcitation // EMBO J.–2007.–**26**, N 12.–P. 2981–2990.
22. *Shang W. H., Adachi Y., Nakamura A., Copeland T., Kim S. R., Kamata T.* Regulation of amphiphysin1 by mitogen-activated protein kinase: its significance in nerve growth factor receptor-mediated endocytosis // J. Biol Chem.–2004.–**279**, N 39.–P. 40890–40896.
23. *Peter B. J., Kent H. M., Mills I. G., Vallis Y., Butler P. J., Evans P. R., McMahon H. T.* BAR domains as sensors of membrane curvature: the amphiphysin BAR structure // Science.–2004.–**303**, N 5657.–P. 495–499.
24. *Lundgaard G. L., Daniels N. E., Pyndiah S., Cassimere E. K., Ahmed K. M., Rodrigue A., Kihara D., Post C. B., Sakamuro D.* Identification of a novel effector domain of BIN1 for cancer suppression // J. Cell. Biochem.–2011.–**112**, N 10.–P. 2992–3001.

Received 10.05.11