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Analysis of furin (*FURIN*) gene expression in the U-87 MG cell line as a potential target for gene inhibiting therapies in (auto-) immune diseases

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ABSTRACT

Purpose: Furin is a proprotein convertase commonly found in the human body. The enzymatic activity of furin is necessary for the activation of numerous substrates including e.g. hormones and growth factors. Nevertheless, furin is involved in various pathological conditions caused by, among others, chronic inflammation. Therefore furin is considered as a potential target in autoimmune diseases therapy.
We performed an experiment in which the expression of *FURIN* gene in U-87 MG astrocytoma cells was investigated. Additionally, this cell line contains some sequences coding human endogenous retroviruses (HERVs), including *ERVW-1* and its receptor- *SLC1A5*. Deregulation of HERV expression has been observed in some neurodegenerative diseases as well as in inflammatory process.
Material and Methods: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot (WB) techniques were utilized for analysis. Phorbol 12-myristate 13-acetate (PMA) were used for cell stimulation. Short interfering RNAs (siRNA) were used for gene expression inhibition in U-87 MG cells *in vitro*.
Results: Cell stimulation by PMA strongly increased *FURIN* expression, simultaneously downregulating *ERVW-1* ($p<0.01$). Moreover addition of PMA significantly stimulates the autocatalytic action of cellular furin itself resulting in the dissociation of its propeptide that was clearly visible in a time-dependent manner. SiRNA-mediated expression inhibition of *ERVW-1* and *FURIN* influenced the mRNA level for *SLC1A5 (ASCT2)* - primary syncytin-1 receptor, that was significantly lower. *FURIN* inhibition by siRNA caused strong upregulation of *ERVW-1* expression ($p<0.01$).
Conclusion: Our results showed that stimulation by PMA and inhibition expression by siRNA affects the expression of *FURIN* in U-87 MG

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astrocytoma cells. Moreover, furin shows a complex relationship on the expression of *ERVW-1* and *SLC1A5* genes, as well as on the form (precursor or mature) and the amount of the final translation products of the transcripts. The regulation of *FURIN* may pose a potential therapeutic strategy in the treatment of neurodegenerative diseases caused by autoimmunity.

KEYWORDS: neuroinflammation, *FURIN*, HERVs, neurodegenerative diseases, gene therapies

Introduction

Inflammation is a defensive process that is activated by the appearance of a damaging factor(s) in the body. The goal of inflammation is to restore the body's homeostasis. Unfortunately, due to the deregulation of defence mechanisms, an excessive inflammatory reaction is often observed, which causes damage to adjacent tissues. In some cases inflammation process becomes chronic, which is the cause of many life-threatening diseases like atherosclerosis and rheumatoid arthritis (Roe, 2021). In regard to its location, there is a specific type of inflammation namely neuroinflammation. Neuroinflammation involves neuronal tissue and plays a significant role in the development and progression of neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis (MS). Neurodegenerative diseases are a group of diseases in which the degradation and atrophy of cells from the nervous system occur (Oliveira *et al*., 2015). WHO reports from 2007 estimated that approximately 6.8 million people die from neurodegenerative diseases annually. Unfortunately, despite the passage of time, there is still no effective therapy for the treatment of neurodegenerative diseases (Vardi and Merrick, 2007). Hence, there is an urgent need for continuous development in understanding the mechanisms of development of these diseases, which will enable programming of effective treatment. There are numerous indications that two proteins: furin and syncytin-1 play a significant role in the initiation and progression of neurodegenerative diseases mainly due to their pro-inflammatory potential (Dolei, 2018; Valli *et al*., 2019).

Furin is a proprotein convertase that is commonly present within human body. This enzyme is coded by *FURIN* gene that is located on chromosome 15 (15q26.1). The enzymatic activity of furin is necessary for the activation of numerous substrates including e.g. hormones (proinsulin, proparathyroid hormone) and growth factors (transforming growth factor beta 1 precursor, pro-nerve growth factor) (Zhang *et al*., 2022). Despite its crucial role in human physiology furin has been found to be involved in various pathological conditions that are principally caused by chronic inflammatory state. Therefore furin is extensively examined as a potential target in autoimmune diseases therapy both at the level of its enzymatic activity inhibition and, more currently, at the level of gene expression silencing (Valli *et al*., 2019).

The human genome carries approximately 8% of sequences of retroviral origin, that has been incorporated millions of years ago as a result of exogenous retroviral infections. The sequences of human endogenous viruses (HERVs) are mostly unexpressed due to numerous mutations caused them permanently inactive. However, some of HERVs, such as *ERVW-1*, are still transcribed under specific conditions. The

ERVW-1 expression product is a glycoprotein syncytin-1 that plays an important physiological role in the early stages of pregnancy being involved in the formation of the placenta (Noorali *et al*., 2009; Wang *et al*., 2018a). Nevertheless, numerous studies prove that syncytin-1 exhibits some pro-inflammatory properties, mainly observed in brain tissue. The initiation and progression of neuronal inflammation that was related to syncytin-1 activation causes cells damage that has been subsequently demonstrated as a major factor in multiple sclerosis (MS) course (Dolei, 2018). The pathological neuroinflammatory and neurodegenerative potential of syncytin-1 prompt the search for a therapy involving the inhibition of the effective expression of *ERVW-1*. An important fact in the syncytin-1 synthesis is that furin activity is necessary for the transformation of the inactive form of prosyncytin-1 into a functional one. Therefore the regulation of furin gene expression is an important topic to consideration in any pathological conditions that are directly or indirectly responsible for causing autoimmune diseases, including neurodegenerative ones (Dolei *et al*., 2015).

Materials and Methods

In order to investigate the neuronal/glial *FURIN* and *ERVW-1* activity we performed a gene expression analysis using U-87 MG astrocytoma cell line (U-87 MG Cell Line human, No. Cat. 89081402-1VL, Merck, Poznań, Poland). A primary syncytin-1 receptor: the neutral amino acid transporter SLC1A5 coded by *SLC1A5* gene has also been found in this cell line as an expressed mRNA. While *ERVW-1* gene codes for syncytin-1 glycoprotein, furin activity is essential to achieve the syncytin-1 mature form (Machnik *et al*., 2024). Therefore we investigated the *ERVW-1* gene expression behaviour along that for *FURIN* in U-87 MG cells. To date, an altered HERVs expression has been observed in some neurodegenerative diseases as well as in an inflammatory processes (Dolei *et al*., 2019).

RNA extraction

Total RNA was extracted from U-87 MG cells using TRI Reagent (MRC Inc. Cincinnati, OH, USA) according to one-step RNA extraction method described by Chomczynski and Sacchi (1987). Finally RNA was dissolved in nuclease-free water and its concentration was estimated spectrometrically. Next, 500 ng of total RNA was reverse transcribed using GoScript Reverse Transcription System (Promega Corporation, Madison, WI, USA). Reverse transcription (RT) reaction mixture was diluted 1:4 with water before further analysis.

Reverse transcription and real time quantitative polymerase chain reaction

Quantitative analysis of *FURIN*, *ERVW-1* and *SLC1A5* mRNA expression was carried out by real-time quantitative polymerase chain reaction (real time QPCR). An amount of 5 µl of reverse transcription reaction mixture (i.e. an equivalent of 50 ng of total RNA) was used as a template in each reaction. Real-time QPCR was performed using GoTaq qPCR Master Mix (Promega). Reaction mixture (total volume of 25 µl) contained 12.5 µl 2× Master Mix, and 200 nM of each forward and reverse primer. Primer sequences for *ERVW-1 FURIN*, *SLC1A5* as well as for *GAPDH* were listed in our previous paper (Machnik *et al*., 2024). We calculated the relative mRNA quantity of genes of interest based on SYBR-Green I chemistry with a parallel amplification of *GAPDH* mRNA that served as endogenous normalizer. Quantitative PCR was performed on Roche LightCycler 480 Real-Time PCR system (Roche Diagnostics Polska Warsaw,

Poland) as described previously (Schmittgen and Livak, 2008).

Western blotting

After PMA or siRNA treatment, U87-MG cells were lysed directly in wells of 12-well culture plate. To lyse cells, 150 µl of cold RIPA buffer supplemented with 1/100 volume of Halt Protease Inhibitor Cocktail was added (Thermo Fisher Scientific, Warsaw, Poland) and cells were incubated for 10 minutes on ice under shaking. Thereafter protein extracts were centrifuged at 13,000 RPM and total protein concentration was estimated by BCA assay (Thermo Scientific, Warsaw, Poland). Proteins from cell lysates (20 µg) were separated by means of electrophoresis in 10% polyacrylamide gel in the presence of ColorPlusPrestained Protein Marker (New England Biolabs, Lab-Jot, Warsaw, Poland). After separation, proteins were blotted onto PVDF membrane (Merck Millipore, Poznań, Poland).

Immunodetection of specific proteins

Unspecific binding sites were blocked by incubation of membrane in 3% bovine serum albumin (BSA) solution for two hours and then membranes were placed in 3% BSA/1xTTBS containing each of syncytin-1, furin or SLC1A5 -specific antibody at a final dilution of 1:1000. Parallel, an anti-GAPDH antibody (dilution of 1:2000) was used. Incubations were performed for one hour with continuous rocking. Then an Anti-rabbit IgG (whole molecule)-peroxidase antibody (Merck Sigma Aldrich, Poznań, Poland) was added (antibody dilution of 1:10,000) and membranes were incubated for one hour. Finally a specific chemiluminescent signal was developed (Pierce ECL Western Blotting Substrate, Thermo Scientific, Warsaw, Poland) and membranes were scanned using ChemiDoc-It Imaging System (Analytik Jena, Jena, Germany). Measurements of integrated optical density (IOD) representing the amount of the protein of interest in a sample were done using ImageJ software while IOD for GAPDH served as a normalizer (Schneider *et al*., 2012).

Inhibition of gene expression by RNA interference

Small interfering RNA (siRNA) molecules were from Thermo Fisher Scientifics (Warsaw, Poland). For *ERVW-1* and *FURIN* a Silencer Select siRNA was used (siRNA ID: s26921 and s9989, respectively). About 5 nM of each siRNA was delivered to the cells by means of adding of 3 µl of cationic lipid-based cargo (Lipofectamine RNAiMAX Transfection Agent, Thermo Fisher Scientific, Warsaw, Poland) in the 12-well plate wells (Tissue Culture Testplate, SPL Life Sciences, Pocheon-si, Korea). The inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was performed as a control assay (Ambion Silencer GAPDH Positive control siRNA, Thermo Fisher Scientifics, Warsaw, Poland).

Cells stimulation by phorbol myristate acetate (PMA)

U87-MG astrocytoma cell line was stimulated using 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) which is a specific activator of Protein Kinase C (PKC) and hence of NF-kB. PMA has been previously used for induction of ERVW-1 mRNA in monocytoid (MDM) and astrocytic (U373) cell lines (Amos *et al*., 2005). Cells were treated with PMA by adding of the agent directly into cell culture medium at various time intervals ranging from 15 minutes to 5 hours. Thereafter culture medium was replaced with the fresh one and cells incubation was continued for consecutive 24 hours.

Results

We found that cell stimulation by phorbol 12-myristate 13-acetate (PMA) strongly increased *FURIN* expression measured as a relative mRNA expression level (Fig. 1). The addition of PMA stimulated the expression level of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) ($p < 0.01$) as well as influenced the level of mRNA expression for *SLC1A5 (ASCT2)* – a primary syncytin-1 receptor ($p > 0.5$). Interestingly, we observed that the relative mRNA level for *ERVW-1* was significantly reduced after 30 minutes of PMA treatment or in longer time span ($p < 0.01$).

Treatment the astrocytoma cells with of PMA significantly stimulated the autocatalytic activity of cellular furin resulting in the dissociation of its propeptide that was clearly visible in a time-dependent manner (Fig. 2).

FURIN expression inhibition by Small interfering RNA (siRNA) resulted in strong upregulation of *ERVW-1* expression ($p < 0.01$) (Fig. 3A). SiRNA -mediated inhibition of *ERVW-1*

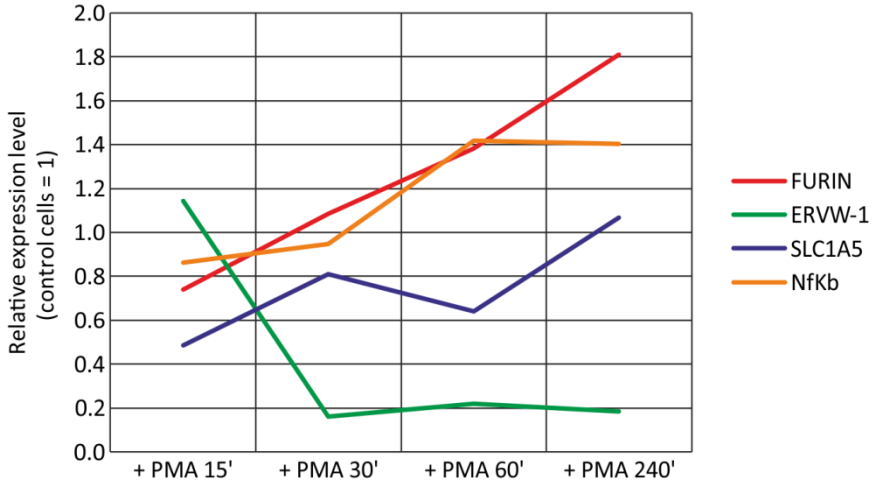

Figure 1. Phorbol 12-myristate 13-acetate (PMA) treatment of U87-MG astrocytoma cells. The relative expression level of *FURIN*, *ERVW-1*, *SLC1A5* as well as *NFKB1* genes is shown at each time point. The expression level that was observed in control (non PMA treated) cells was assumed as a value of 1.

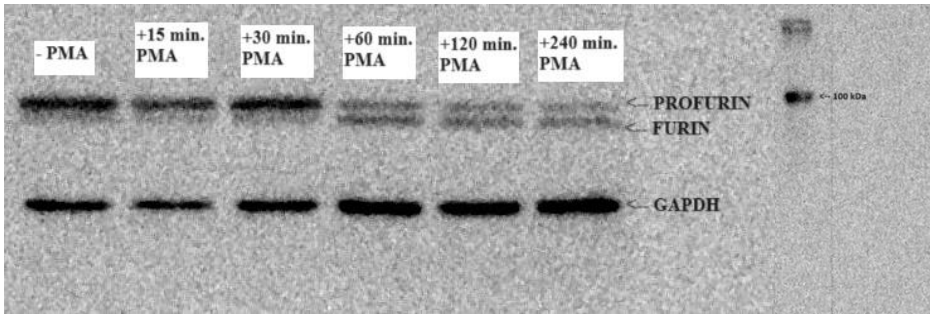

Figure 2. An autocatalytic activity of furin in U87-MG astrocytoma cells after treatment with PMA. The dissociation of profurin to furin was clearly visible in a time-dependent manner. The autocatalytic activity was compared to that observed in control cells (non PMA treated) and normalized with a control protein (GAPDH).

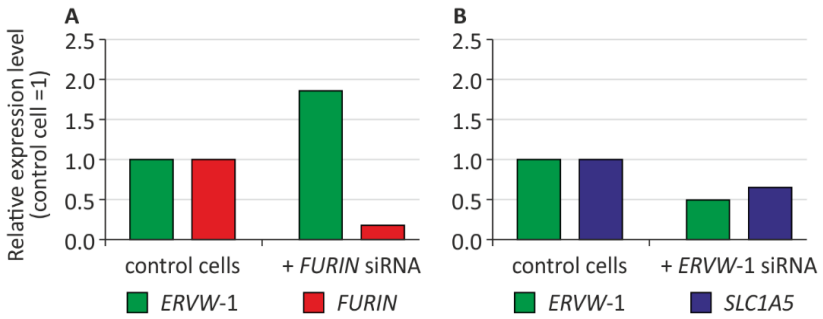

Figure 3. A. Expression level of *ERWV-1* and *FURIN* genes after addition of *FURIN*- specific small interfering RNA (siRNA). B. Expression level of *ERWV-1* and *SLC1A5* genes after addition of *ERVW-1*-specific siRNA. The expression level observed in control, non siRNA-treated cells was assumed as a value of 1.

expression subsequently influenced the mRNA level for *SLC1A5* (*ASCT2*) gene coding for the primary syncytin-1 receptor. It's expression level was lower than that of control cells albeit with no significancy (Fig. 3B).

Discussion

Furin is a proprotein convertase that is responsible for transforming numerous inactive proteins into their active forms thus it is necessary to maintain a proper physiology in vertebrates. Nevertheless in addition to regulating physiological processes, an overexpression of the *FURIN* gene has been observed in many pathological conditions such as atherosclerosis or rheumatoid arthritis (Valli *et al*., 2019).

Among others, furin is responsible for the activation of the protein product of the *ERVW-1* gene coding for syncytin-1 glycoprotein, belonging to the HERV family. HERVs are sequences of human endogenous retroviruses, which constitute approximately 8% of the human genome, but most number of them is transcriptionally inactive. Numerous scientific reports have proven that deregulation of HERV expression occurs in neurodegenerative diseases and also as a result of inflammation (Singh *et al*., 2024).

Antony and colleagues conducted an extensive analysis of the expression of syncytin-1 (*ERVW-1)* gene in multiple sclerosis patients, in which they observed a significant increase in the number of *ERVW-1* mRNA copies in the brain tissue of people with multiple sclerosis compared to healthy controls (Antony *et al*., 2006). In turn, Wang *et al*. observed an excessive expression of the gene encoding syncytin-1 and of CRP (C-reactive protein) in patients suffering from schizophrenia. Scientists confirmed that syncytin-1 activates the CRP protein, which presents a new mechanism for the regulation of inflammation by syncytin-1 in schizophrenia (Wang *et al*., 2018b). In addition to reports indicating the direct involvement of furin- activated syncytin-1 in the course of neurodegenerative diseases associated with inflammation of nervous tissue. There are some indications that furin is involved in the development of these diseases through different mechanisms of action and possibly at another biochemical level. Bennett *et al*. proved that furin activates BACE (beta-site amyloid precursor protein cleaving enzyme) protease, which is a beta-secretase, and that is responsible for the transformation of the precursor form of amyloid into beta-amyloid that forms plaques in Alzheimer's (Bennett *et al*., 2000).

Taking into consideration aspects mentioned above we investigated the connections between the expression of *FURIN*, *ERVW-1*, and *SLC1A5* (syncytin-1 receptor) genes. The understanding of the mechanisms or the relationship between activity of those genes may allow the creation of potential therapy in neurodegenerative diseases that may involves e.g. the regulation of the expression of *FURIN* gene. The use of PMA as a stimulator in cell culture and siRNA as an expression silencer, indicated that *FURIN* gene exhibited an increased or decreased expression level, respectively (Fig. 1, Fig. 3A). That results confirmed the possibility of intentional control of the *FURIN* expression depending on the desirable needs. Moreover, in our study the inhibition of the *ERVW-1* by siRNA resulted in a significant expression reduction of *SLC1A5* gene, which indicates a relationship between mentioned genes (Fig. 3B). In turn, inhibition of the *FURIN* gene by a specific siRNA resulted in strong overexpression of the *ERVW-1* gene, which can be explained as a compensatory mechanism in that a decrease amount of furin protein led to the limited transformation of the precursor form of syncytin-1 protein into its active form, that, in turn initiated cell processes toward the increased transcription of the *ERVW-1* gene that eventually increase the amount of syncytin-1 protein in the cell (Fig. 3A).

After incubating U-87 MG cells with PMA, we also measured the expression levels of *SLC1A5* and *ERVW-1* genes. In the tested time frame, the expression level of the *SLC1A5* gene increased significantly compared to the control sample, but there was a significant decrease in the *ERVW-1* gene expression (Fig. 1). It should be also borne in mind that the protein product of *SLC1A5*, i.e. a neutral amino acid transporter B(0), is not specific only for syncytin-1, but it is the main transporter of amino acids like glutamine, alanine, serine, hence the increase in the expression of *SLC1A5* after using a cellular stimulator should be understood as a result of an activation of cellular metabolism (Ni *et al*., 2023).

To deeper investigate the topic of inflammation we decided to include the NF-κB protein complex in our research. NF-κB is a protein complex that functions as a transcription factor. NF-κB occurs in almost all animal cells and is involved in the cell's response to stimuli such as stress, cytokines, free radicals, or antigens. NF-κB plays a key role in regulating the immune response to infection. Disturbances in the regulation of NF-κB are associated with cancer, inflammation and autoimmune diseases (Poma, 2020). Incubation of U-87 MG cells in culture medium supplemented with PMA resulted in a significant increase in the expression level of the *NFKB1* gene (Fig. 1). An increase in the expression level of *NFKB1* was observed already within the first 15 minutes and was most strongly stimulated for an hour, after which the expression was maintained at the maximum level until the end of the experiment (Fig. 1). This study confirmed the stimulating effect of PMA on the expression of the NF-κB factor gene. It is worth to mention that Abu El-Asrar and colleagues confirmed that furin induces excessive activation of the p65 subunit of NF-κB, which indicates a direct interaction between furin and the NF-κB transcription factor (Abu El-Asrar *et al*., 2022).

In addition we noticed the effect of PMA on the synthesized furin present in the cell. Furin is synthesized in the form of a zymogen, i.e. as profurin which must be activated to fulfill its role in the organism. In the case of profurin, its activation occurs only by means of autocatalysis where the auto-inhibitory

domain is removed (Gawlik *et al*., 2009). It turned out that PMA stimulates the profurin-to-furin autocatalysis and that process was clearly visible in a time-dependent manner (Fig. 2).

Obtained results confirmed findings published previously that syncytin-1 abundance rely on the activity of furin. We showed that either stimulation by PMA or expression inhibition by siRNA enable an intentional regulation of furin in U-87 MG astrocytoma cells. Moreover we noted that furin exhibits a multi-faceted relationship with syncytin-1 (*ERVW-1)* and *SLC1A5* genes, as well as on the form (precursor or mature) and on the amount of the final products.

Due to the fact that there is still ineffective treatment of autoimmune and neurodegenerative diseases, it is important to conduct more investigations in this research topic.

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