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Suppression of the α-isoform of class II phosphoinositide 3-kinase gene expression leads to apoptotic cell death☆

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Abstract

Phosphoinositide 3-kinases (PI3Ks) have known to be key enzymes activating intracellular signaling molecules when a number of growth factors bind to their cell surface receptors. PI3Ks are divided into three classes (I, II, and III) and enzymes of each class have different tissue-specificities and physiological functions. Class II PI3Ks consist of three isoforms (α, β, γ). Although the α-isoform (PI3K-C2α) is considered ubiquitous and preferentially activated by insulin rather than the β-isoform, the physiological significance of PI3K-C2α is poorly understood. The present study aimed to determine whether PI3K-C2α is associated with the suppression of apoptotic cell death. Different sense- and antisense oligonucleotides (ODNs) were synthesized based on the sequence of C2 domain of PI3K-C2α gene. Transfection of CHO-IR cells with two different antisense ODNs clearly reduced the protein content as well as mRNA levels of PI3K-C2α whereas neither the nonspecific mock- nor sense ODNs affected. The decrease of PI3K-C2α gene expression was paralleled by cellular changes indicating apoptotic cell death such as nuclear condensation, formation of apoptotic bodies, and DNA fragmentation. PI3K-C2α mRNA levels were also reduced when cells were incubated in growth factor-deficient medium. Supplementing growth factors (serum or insulin) into medium lead to an increase of PI3K-C2α mRNA levels. This finding strongly suggests that PI3K-C2α is a crucial survival factor.

Keywords: PI3K-C2α; α-isoform of class II phosphoinositide 3-kinase; C2 domain; CHO-IR cells; Chinese hamster ovary cells expressing human insulin receptors; Apoptotic cell death; DNA fragmentation; Nuclear condensation; Apoptotic body; Survival factor

Phosphoinositide 3-kinases (PI3Ks) have been characterized as enzymes phosphorylating the D-3 position of the inositol ring of phosphoinositides and involved in diverse cellular events, such as cell migration, cell proliferation, oncogenic transformation, cell survival, and intracellular trafficking of proteins [1,2]. Mammalian PI3Ks are divided into three classes based on their structure and substrate specificity [3]. Class I PI3Ks are divided into two types, class IA and class IB, having different regulatory and catalytic subunits. Class IA PI3K consists of a p110 catalytic subunit that associates with an SH2 domain-containing subunit p85, and is activated by the majority of tyrosine kinase-coupled transmembrane receptors whereas class IB PI3K consists of a p101 regulatory subunit that associates with p110γ catalytic subunit, and is activated by heterotrimeric G proteins [1]. Class III PI3K is homologous to the *Saccharomyces cerevisiae* Vps34p and plays an essential role in protein trafficking through the lysosome [4]. In contrast to class I PI3Ks, which are mainly cytosolic,

☆ *Abbreviations:* PI3K-C2α, α-isoform of class II phosphoinositide 3-kinase; CHO-IR, Chinese hamster ovary cells expressing human insulin receptors; ODNs, oligonucleotides.

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class II PI3K enzymes are predominantly associated with membrane fractions of cells [5]. Class II PI3Ks consists of three isoforms, PI3K-C2α, PI3K-C2β, and PI3K-C2γ [6]. The expression of PI3K-C2α and PI3K-C2β was considered ubiquitous [7,8] whereas PI3K-C2γ was mainly found in the liver [9,10]. Moreover, PI3K-C2α is preferentially activated by insulin rather than PI3K-C2β in a number of cell lines [11]. Most of biological activities of PI3Ks have been described exclusively in class I PI3Ks, however, little is known about the nature of biological activities of class II PI3Ks.

In this study, we found that the suppression of PI3K-C2α gene expression induced a variety of cellular changes representing apoptotic death. In addition, growth factors such as serum or insulin stimulated PI3K-C2α mRNA levels. This finding strongly suggests that PI3K-C2α is a crucial survival factor.

Materials and methods

Materials. CHO-IR cells (generous gift from Dr. M. Bernier, NIA/NIH, Baltimore, USA) used in this study have been previously described [12]. Phosphorothioate oligonucleotides (ODNs) and PCR primers were synthesized by Genotech (Korea). FuGENE reagents (Roche) were used to deliver ODNs into the cells. Electrophoresis reagents, such as 8% polyacrylamide gels, Tris–borate–EDTA running buffer, were from Koma Biotech (Korea). Monoclonal antibody against PI3K-C2α (PI3-Kinase p170) was from Beckon–Dickinson Biosciences (USA) and monoclonal antibodies against Bcl-2 (C-2) and ERK2 (D-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Ham's F-12 medium, D-PBS, trypsin–EDTA, and H33342 were from Sigma and fetal bovine serum (FBS) was from US Bio-Tech. Genomic DNA was extracted with Puregene kit (Gentra, Minneapolis, MN, USA). Trizol reagent and Superscript One-step RT-PCR system were from Life Technologies (Rockville, MD, USA).

Cell culture and transfection. CHO-IR cells were grown in Ham's F-12 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS, and maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. Fully grown cells were plated at a density of 5×10^5 cells in a 35 mm culture dish. Delivery of PI3K-C2α ODNs into the cells was performed with FuGENE reagent as per a manufacturer's protocol. The sequences of ODNs were as follows: sense-1 (S1) 5′-GGAGGAGCTG TGAAATTA-3′, antisense-1 (As1) 5′-TAATTTCACAGCTCCTCC-3′, sense-2 (S2) 5′-GTTACTGAAGATGGAGCT-3′, antisense-2 (As2) 5′-CAGCTCCATCTTCAGCAAC-3′, and mock 5′-TTCACTGACT TCCAACCT-3′. After 48–72 h of delivery of ODNs, total RNA or genomic DNA was extracted for the measurement of PI3K-C2α mRNA levels or the degree of DNA fragmentation, respectively.

PI3K-C2α mRNA expression. Expression of PI3K-C2α mRNA was observed by RT-PCR or Northern blot analysis. Total cellular RNA was extracted by homogenizing cells in Trizol reagent by a manufacturer's protocol. The pelleted RNA was dissolved in 50 μl of diethyl pyrocarbonate (DEPC)-treated Rnase-free water and quantitated using a UV spectrophotometer. At first, cDNA was synthesized by a reverse transcription reaction of equal amount (2 μg) of total RNA with oligo(dT) primers (Life Technologies). Next, 2 μl cDNA was amplified by *Taq* DNA polymerase and the PCR profile consisted of initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 30 s, extension at 70 °C for 30 s, and the final extension at 70 °C for 7 min. Human primers, designed from the C2 domain of PI3K-C2α sequence, were used and the expected length of its PCR product is 242 base pairs (bp). The nucleotide sequences of primers were as follows: sense 5′-AGATG TAGCAGCCAAAAG-3′ and antisense 5′-CCATGATGAAAAGAG TAC-3′, respectively. RT-PCR was also performed using primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA as an internal control. Primers for the G3PDH gene were 5′-TCCACCACC CTGTTGCTGTAG-3′ (sense) and 5′-GACCACAGTCCATGACAT CACT-3′ (antisense). The products were resolved on a 2% agarose gel. For Northern blot analysis, the RT-PCR product (242 bp) of PI3K-C2α mRNA was used as a template to prepare radiolabeled cDNA probe. Total RNA from each sample was heated in the presence of 17% formaldehyde (v/v) and 50% formamide (v/v) at 65 °C for 15 min and then applied to a 1.2% (w/v) agarose gel containing 17% formaldehyde. After electrophoresis, RNA was transferred from the gel to nylon membrane (Nytran, Schleicher & Schuell, Keene, NH, USA) by capillary action. The blots were fixed by UV cross-linking, and then hybridized with a 242 bp human PI3K-C2α cDNA probe which was labeled with [α-^{32}P]dCTP (Amersham) by the random-priming procedure with a Megaprime labeling kit (Amersham). Hybridization was carried out as described before [13].

Western blot analysis. Unless otherwise indicated, cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin A). The same amount of proteins was separated by SDS–PAGE on 4–20% polyacrylamide gel and electrotransferred onto PVDF membrane. The membrane was incubated in blocking buffer [5% nonfat dried milk in Tris-buffered saline (TBS)–0.1% Tween 20 (TBS-T)] for 1 h at room temperature and then probed with different primary antibodies (1:1000–1:5000). After a series of washes, the membrane was further incubated with different horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000–1:10,000). The signal was detected with enhanced chemiluminescence (ECL) detection system (Intron, Korea).

DNA fragmentation. Internucleosomal DNA fragmentation analysis was performed as described previously [14]. Cells were lysed in lysis solution (Puregene) and incubated overnight at room temperature prior to the addition of 20 μg/ml Rnase A for 1 h at 37 °C. The samples were deproteinized, followed by DNA precipitation with 2-propanol. Equal amounts of DNA from each sample (1 μg) were 3′-OH-labeled with 5 U Klenow fragment of DNA polymerase I (New England Biolabs) and 0.5 μCi [α-^{32}P]dCTP (Amersham) in the presence of 10 mM Tris–HCl, pH 7.5, and 5 mM $MgCl_2$. The samples were electrophoresed on a 6% polyacrylamide gel. Following electrophoresis, the gel was dried and analyzed by autoradiography using Kodak BioMax film and intensifying screens.

Observation of apoptotic body. The degree of apoptosis was also determined by staining cultured cells directly with H33342 (10 μg/ml), a cell membrane-permeable DNA-specific fluorescent dye. The fluorescent apoptotic bodies among cell populations were observed under a fluorescent microscope equipped with a CoolSNAP-Pro color digital camera (Media Cybernetics, Silver Spring, MD, USA).

Results and discussion

Although both PI3K-C2α and PI3K-C2β are implicated in signaling downstream of epidermal growth factor and platelet-derived growth factor receptors [15], their specific roles as well as their intracellular targets have not been characterized. The present study aimed to determine whether PI3K-C2α is involved in maintaining cellular viability. To do this, we made an attempt to suppress the gene expression of PI3K-C2α in CHO-IR cells by a liposome-mediated delivery of the antisense

ODNs designed from sequences within the C2 domain of human PI3K-C2α gene in CHO-IR cells. Following the delivery of antisense ODNs (0–250 nM), total RNA was extracted daily and subjected to RT-PCR or Northern blot analysis to measure PI3K-C2α mRNA levels. The reduction of PI3K-C2α mRNA levels was initially observed after 48 h of delivery (Figs. 1A and B). The degree of reduction was dependent on the dose of antisense ODNs, clearly at higher doses (>125 nM) of ODNs. Upon decreases of PI3K-C2α mRNA levels and its protein content, intracellular genomic DNA was fragmented, showing the laddering pattern after acrylamide gel electrophoresis (Fig. 1C). However, these results might have come from the nonspecific toxicity of antisense ODN itself. In order to make sure the gene-specific suppression of PI3K-C2α, we developed another sequence-unrelated antisense-, sense-, and nonspecific mock ODNs, which are also designed from sequences within the C2 domain of PI3K-C2α gene. After delivery of these ODNs into cells, experiments to determine the degree of apoptosis were performed. In addition, the intracellular PI3K-C2α protein content was also determined with the immunoblot experiment using anti-PI3K-C2α p170 antibody (BD). The decrease of the PI3K-C2α protein was clearly observed only in cells

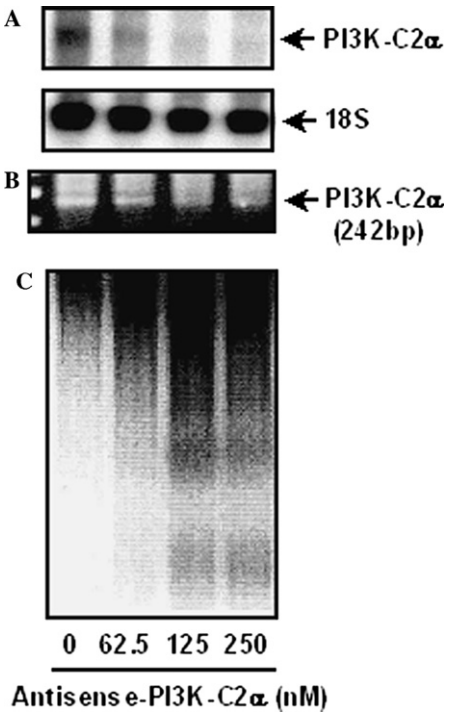

Fig. 1. Suppression of PI3K-C2α mRNA expression by transfection with an antisense oligonucleotide-1 (As1) in CHO-IR cells. Cells were transfected with different concentrations of As1 oligonucleotides (0–250 nM) and further incubated for an additional 48 h. Total RNA was extracted for Northern blot analysis (A) or RT-PCR analysis (B) to measure the steady state PI3K-C2α mRNA levels. Otherwise, genomic DNA was extracted for DNA fragmentation analysis (C).

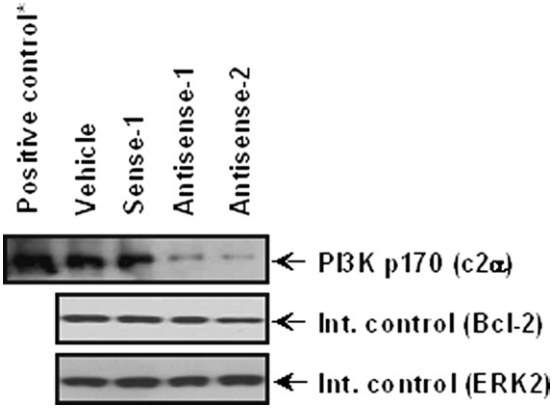

Fig. 2. Decrease of intracellular PI3K-C2α protein content by transfection of CHO-IR cells with different antisense oligonucleotides against PI3K-C2α gene sequences. Cells were transfected with different oligonucleotides (250 nM) (sense-1, antisense-1, and antisense-2) and further incubated for an additional 48 h. Cells were lysed in ice-cold lysis buffer (200 μl). Same amounts of proteins were separated by SDS–PAGE on 4–20% polyacrylamide gel and subjected to immunoblot analysis using an antibody to PI3K-C2α (1:1000) as described in Materials and methods. Blots were repeatedly stripped and reprobed with different antibodies to Bcl-2 and ERK as internal standards. *Positive control, prepared from rat cerebrum lysate, was provided together with anti PI3-kinase p170 antibody by Beckon–Dickinson Biosciences.

receiving the two different antisense (As1 and As2) ODNs but not in cells receiving sense ONDs (Fig. 2). Apoptotic DNA fragmentation was not observed in cells receiving the nonspecific mock- or sense (S1, S2) ODNs, however, clear DNA fragmentation patterns were observed in cells receiving the two different antisense ODNs (As1 and As2) (Fig. 3A). When stained with H33342, apoptotic bodies were also observed exclusively in cells receiving the two different antisense ODNs (As1 and As2) (Fig. 3B). From the cell cycle analysis using flow cytometer, apoptotic subG1 peaks were prominent in cells receiving the two different antisense ODNs (As1 and As2) (data not shown). These results imply that the suppression of PI3K-C2α gene expression by antisense ODNs should be responsible for the occurrence of cellular changes representing apoptotic cell death. It also suggests that PI3K-C2α is a possible key player to maintain cellular viability by inhibiting apoptotic cell death or by promoting cell proliferation. We further examined the effect of growth factors (serum and insulin) on PI3K-C2α mRNA levels. CHO-IR cells were incubated in the presence or absence of insulin (10 nM) or serum (10% FBS) for 18 h. When incubated in serum-free medium, cells were readily apoptotic from the DNA fragmentation pattern, and the message of PI3K-C2α gene disappeared (Fig. 4). However, the addition of serum or insulin to the serum-free medium recovered the levels of PI3K-C2α mRNA and suppressed the occurrence of DNA fragmentation induced by growth factor deprivation (Fig. 4). These results strongly suggest that PI3K-

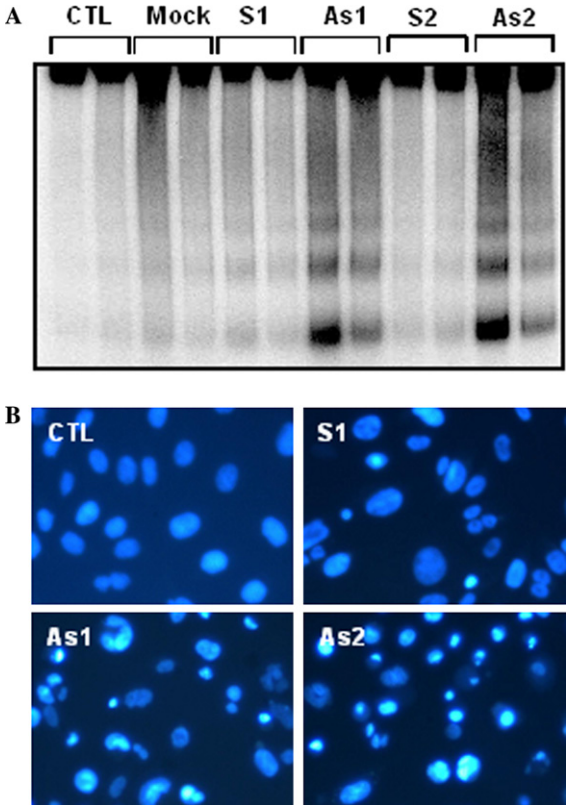

Fig. 3. Blockade of PI3K-C2α gene expression leads to apoptotic cell death. CHO-IR cells were transfected with different oligonucleotides derived from sequences within C2 domain of PI3K-C2α gene. Transfected cells were further incubated in normal culture medium containing oligonucleotides (200 nM) as indicated. Genomic DNA was isolated after 72 h, used for analysis of DNA fragmentation (A). Otherwise, cells were stained with H33342, a cell membrane-permeable DNA-specific fluorescent dye to observe apoptotic bodies with condensed chromatin (B). S1, sense oligonucleotide-1; As1, antisense oligonucleotide-1; S2, sense oligonucleotide-2; and As2, antisense oligonucleotide-2.

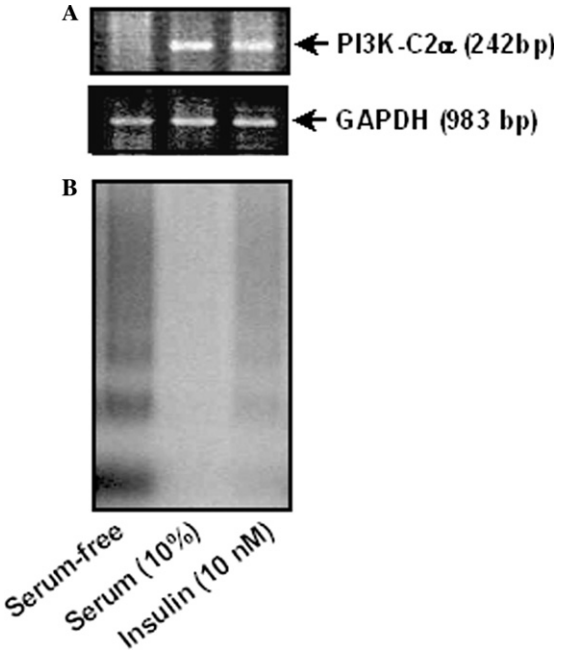

Fig. 4. Stimulation of PI3K-C2α gene expression by growth factors. Fully grown CHO-IR cells were serum-starved for 4 h and further incubated with growth factors (serum, insulin) for 18 h. Total RNA was extracted for the RT-PCR analysis (A) to measure the steady state PI3K-C2α mRNA levels. Otherwise, genomic DNA was extracted for DNA fragmentation analysis (B).

C2α is an important survival factor to prevent cells from apoptotic death.

Little is known about how PI3K-C2α is activated or the biological processes that it controls. Recently, a few studies have reported that PI3K-C2α lies downstream of the monocyte chemotactic peptide-1 chemokine receptor [16], the insulin receptor [11], platelet-derived growth factor (PDGF), and the epidermal growth factor receptor (EGFR) [15]. More recently, it was reported that PI3K-C2α is phosphorylated in proliferating cells at the G2/M transition of the cell cycle and the protein level of PI3K-C2α is regulated by proteolysis in a cell cycle-dependent manner [17]. These evidences strongly suggest that PI3K-C2α is one of key players modulating the cell cycle progression. Thus, a cell that is deprived of PI3K-C2α protein or the enzyme's functional integrity might stop cell cycle progression, or otherwise, it might get into apoptotic processes. In the present study, suppression of PI3K-C2α gene expression by delivery of its C2 domain-specific antisense ODNs resulted in a marked induction of apoptotic events. While it is necessary to be cautious to define the exact role of PI3K-C2α, the present study provides a strong evidence supporting the notion that PI3K-C2α is a potent survival factor where cells were stimulated by growth factors such as insulin, PDGF, or EGF which are known to promote cell proliferation. Based on this finding, studies to elucidate the nature of the upstream regulatory factors or the downstream targets of PI3K-C2α are necessary to understand the biological significance of this signaling protein.

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References

[1] R. Katso, K. Okkenhaug, K. Ahmadi, S. White, J. Timms, M.D. Waterfield, Cellular function of phosphoinositide 3-kinases: Implications for development, homeostasis, and cancer, Annu. Rev. Cell Dev. Biol. 17 (2001) 615–675.
[2] D.A. Cantrell, Phosphoinositide 3-kinase signalling pathways, J. Cell Sci. 114 (2001) 1439–1445.

[3] J. Domin, M.D. Waterfield, Using structure to define the function of phosphoinositide 3-kinase family members, FEBS Lett. 410 (1997) 91–95.
[4] S. Volinia, R. Dhand, B. Vanhaesebroeck, L.K. MacDougall, R. Stein, M.J. Zvelebil, J. Domin, C. Panaretou, M.D. Waterfield, A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p–Vps15p protein sorting system, EMBO J. 14 (1995) 3339–3348.
[5] B. Vanhaesebroeck, M.D. Waterfield, Signaling by distinct classes of phosphoinositide 3-kinases, Exp. Cell Res. 253 (1999) 239–254.
[6] S.S. El Sheikh, J. Domin, P. Tomtitchong, P. Abel, G. Stamp, E.N. Lalani, Topographical expression of class IA and class II phosphoinositide 3-kinase enzymes in normal human tissues is consistent with a role in differentiation, BMC Clin. Pathol. 3 (2003) 1–14.
[7] A. Arcaro, S. Volinia, M.J. Zvelebil, R. Stein, S.J. Watton, M.J. Layton, I. Gout, K. Ahmadi, J. Downward, M.D. Waterfield, Human phosphoinositide 3-kinase C2beta, the role of calcium and the C2 domain in enzyme activity, J. Biol. Chem. 273 (1998) 33082–33090.
[8] J. Domin, F. Pages, S. Volinia, S.E. Rittenhouse, M.J. Zvelebil, R.C. Stein, M.D. Waterfield, Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin, Biochem. J. 326 (1997) 139–147.
[9] M. Rozycka, Y.J. Lu, R.A. Brown, M.R. Lau, J.M. Shipley, M.J. Fry, cDNA cloning of a third human C2-domain-containing class II phosphoinositide 3-kinase, PI3K-C2gamma, and chromosomal assignment of this gene (PIK3C2G) to 12p12, Genomics 54 (1998) 569–574.
[10] L.K. Ho, D. Liu, M. Rozycka, R.A. Brown, M.J. Fry, Identification of four novel human phosphoinositide 3-kinases defines a multi-isoform subfamily, Biochem. Biophys. Res. Commun. 235 (1997) 130–137.
[11] R.A. Brown, J. Domin, A. Arcaro, M.D. Waterfield, P.R. Shepherd, Insulin activates the alpha isoform of class II phosphoinositide 3-kinase, J. Biol. Chem. 274 (1999) 14529–14532.
[12] D. Park D, S.K. Pandey, E. Maksimova, S. Kole, M. Bernier, Akt-dependent antiapoptotic action of insulin is sensitive to farnesyltransferase inhibitor, Biochemistry 39 (2000) 12513–12521.
[13] W. Lee-Kwon, D. Park, M. Bernier, Involvement of the Ras/extracellular signal-regulated kinase signalling pathway in the regulation of ERCC-1 mRNA levels by insulin, Biochem. J. 331 (1998) 591–597.
[14] W. Lee-Kwon, D. Park, P.V. Baskar, S. Kole, M. Bernier, Antiapoptotic signaling by the insulin receptor in Chinese hamster ovary cells, Biochemistry 37 (1998) 15747–15757.
[15] A. Arcaro, M.J. Zvelebil, C. Wallasch, A. Ullrich, M.D. Waterfield, J. Domin, Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors, Mol. Cell. Biol. 20 (2000) 3817–3830.
[16] S.J. Turner, J. Domin, M.D. Waterfield, S.G. Ward, J. Westwick, The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K-C2alpha, J. Biol. Chem. 273 (1998) 25987–25995.
[17] S.A. Didichenko, C.M. Fragoso, M. Thelen, Mitotic and stress-induced phosphorylation of HsPI3K-C2alpha targets the protein for degradation, J. Biol. Chem. 278 (2003) 26055–26064.