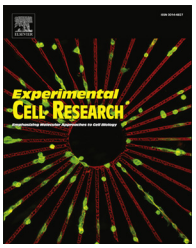
EXPERIMENTAL CELL RESEARCH 319 (2013) 1471–1481

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/yexcr



Research Article

A chimeric cyclic interferon-α2b peptide induces apoptosis by sequential activation of phosphatidylinositol 3-kinase, protein kinase Cδ and p38 MAP kinase

*V.C. Blank, L. Bertucci, V.A. Furmento, C. Peña, V.J. Marino, L.P. Roguin**

Institute of Biochemistry and Biophysics (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956-C1113AAD, Buenos Aires, Argentina

ARTICLE INFORMATION

Article Chronology:
Received 26 October 2012
Received in revised form
13 February 2013
Accepted 22 February 2013
Available online 4 April 2013

Keywords:
IFN-α2b cyclic peptide
PI3K
PKCδ
P38 MAPK
Apoptosis
Phosphoserine-STAT1

ABSTRACT

We have previously demonstrated that tyrosine phosphorylation of STAT1/3 and p38 mitogen-activated protein kinase (p38 MAPK) activation are involved in the apoptotic response triggered by a chimeric cyclic peptide of the interferon-α2b (IFN-α2b) in WISH cells. Since the peptide also induced serine phosphorylation of STAT proteins, in the present study we examined the kinase involved in serine STAT1 phosphorylation and the signaling effectors acting upstream such activation. We first found that p38 MAPK is involved in serine STAT1 phosphorylation, since a reduction of phophoserine-STAT1 levels was evident after incubating WISH cells with cyclic peptide in the presence of a p38 pharmacological inhibitor or a dominant-negative p38 mutant. Next, we demonstrated that the peptide induced activation of protein kinase Cδ (PKCδ). Based on this finding, the role of this kinase was then evaluated. After incubating WISH cells with a PKCδ inhibitor or after decreasing PKCδ expression levels by RNA interference, both peptide-induced serine STAT1 and p38 phosphorylation levels were significantly decreased, indicating that PKCδ functions as an upstream regulator of p38. We also showed that PKCδ and p38 activation stimulated by the peptide was inhibited by a specific pharmacological inhibitor of phosphatidylinositol 3-kinase (PI3K) or by a dominant-negative p85 PI3K-regulatory subunit, suggesting that PI3K is upstream in the signaling cascade. In addition, the role of PI3K and PKCδ in cyclic peptide-induced apoptosis was examined. Both signaling effectors were found to regulate the anti-proliferative activity and the apoptotic response triggered by the cyclic peptide in WISH cells. In conclusion, we herein demonstrated that STAT1 serine phosphorylation is mediated by the sequential activation of PI3K, PKCδ and p38 MAPK. This signaling cascade contributes to the antitumor effect induced by the chimeric IFN-α2b cyclic peptide in WISH cells.

Abbreviations: DNp38, dominant-negative p38 mutant; DNp85, dominant negative mutant of p85 PI3K-regulatory subunit; IFN-α2b, interferon-α2b; FBS, fetal bovine serum; Jak, Janus kinase; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; PI3K, phosphatidylinositol 3-kinase; PKCδ, protein kinase Cδ; STAT, signal transducer and activator of transcription; siRNA, small interfering RNA; IFNAR, type I IFN receptor

*Correspondence to: IQUIFIB, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956-C1113AAD, Buenos Aires, Argentina. Fax: +54 11 4962 5457.

E-mail addresses: rvroguin@qb.ffyb.uba.ar, rvroguin@gmail.com (L.P. Roguin).

0014-4827/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.yexcr.2013.02.024

Introduction

Human alpha interferon (IFNα) consists of a group of structurally related proteins exhibiting pleiotropic biological effects, such as antiviral, antiproliferative and immunoregulatory actions [1–4]. In accordance with their biological activities, IFNα has been employed as a therapeutic agent in the treatment of viral infections, autoimmune disorders and different types of cancers [5–7]. The interaction of IFNα with its receptor leads to the activation of the classical Jak/STAT pathway and other downstream signaling cascades which contribute to elicit the multiplicity of IFN-mediated responses [8–14]. After binding, IFNα induces the dimerization of two different receptor subunits: IFNAR2, which is associated with the tyrosine kinase Jak1, and IFNAR1, which is associated with Tyk2 [8–12]. Activation of Jak1 and Tyk2 leads to tyrosine phosphorylation of both receptor chains and several STAT proteins, which translocate to the nucleus and regulate the transcription of target genes [8–12]. IFNα also regulates a full transcription activity through phosphorylation of the serine residue located in position 727 of STAT1 and STAT3 [15–17]. Besides the Jak/STAT pathway, the activation of several intracellular effectors, including the phosphatidylinositol 3-kinase (PI3K), the mitogen-activated protein kinases (MAPK) ERK, p38 and JNK, and the protein kinase Cδ (PKCδ), is required to generate IFN-mediated responses [10,11,13,14,18].

We have previously synthesized a chimeric cyclic peptide of the IFN-α2b molecule which represents a mimotope mainly interacting with the receptor subunit IFNAR2 [19]. Structurally, it consists of IFN-α2b sequences 30–35 (loop AB) and 122–137 (helix D), with amino acids 30 and 137 linked by two Gly residues and amino acids 122 and 35 connected by a $(Gly)_4$ bridge [19]. The loop AB would constitute the main region involved in IFNAR2 binding, being probably minor the contribution of helix D residues to the interaction [20–23]. This cyclic peptide inhibits WISH cell proliferation and induces an apoptotic response by activating both death receptor and mitochondrial pathways [24]. It was further demonstrated that the peptide induces tyrosine and serine phosphorylation of STAT1 and STAT3, and p38 MAPK activation [25]. Although it has been claimed a role for p38 as a serine kinase for STATs [26], some studies concerning IFN action showed that PKCδ mediates serine phosphorylation of STAT1 [27,28]. It was also demonstrated that even though PKCδ behaves as an upstream regulator of p38 MAPK in IFNα-sensitive leukemia cell lines, PKCδ but not p38 functions as a serine kinase for STAT1 [27]. Based on these facts, we decided to examine whether p38, PKCδ or both kinases regulate the phosphorylation of STAT1 induced by the cyclic peptide. The involvement of PI3K as a regulator of this activation pathway was also evaluated. In addition, we investigated the contribution of these serine kinases in the peptide-induced cell growth inhibitory activity and apoptotic response.

Materials and methods

Reagents

Recombinant human IFN-α2b with a specific activity of 2×10^8 U/mg protein was supplied by Bio Sidus S.A., Buenos Aires, Argentina. Synthesis and purification of IFN-α2b chimeric cyclic peptide was performed as described previously [19]. The peptide was dissolved in a medium containing 0.3 M glycine, 8 M urea, pH 8.5. Experimental assays including control samples (without peptide) were performed in the presence of 20 μl of this vehicule per ml of assay medium. Rabbit polyclonal anti-STAT1, anti-PKCδ, anti-p38, anti-phospho-STAT1 (Ser727), anti-phospho-PKCδ (Thr 507), anti-phospho-p38 (Thr180/Tyr182), p38-MAP kinase inhibitor SB203580, PKCδ inhibitor rottlerin and PI3K inhibitor Ly294002 were from Santa Cruz Biotechnology, CA, USA. Rabbit polyclonal anti-actin antibody was from Sigma-Aldrich, Inc., MO, USA.

Cell culture and proliferation assay

WISH (ATCC CCL-25) cells were grown at 37 °C under 5% CO_2 atmosphere in Minimum Essential Medium (MEM, Gibco BRL, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, MD, USA), 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. Proliferation assay was performed as described previously [29]. Briefly, cells (25,000 cells/ml) were incubated for 48 or 72 h at 37 °C in 96-well culture microplates with 0.2 μg/ml IFN-α2b or 10 μg/ml of cyclic derivative in a total volume of 0.2 ml of the corresponding culture medium. In some experiments, cells were seeded 24 h before and pre-incubated for 1 h with 10 μM SB203580, 1 μM rottlerin or 2 μM Ly294002. Cell number was evaluated by colorimetric determination of hexosaminidase levels [30]. None of the inhibitors diminished WISH cell proliferation in the concentrations specified above (data not shown). Rottlerin and Ly294002 significantly decreased IFN-induced PKCδ and Akt phosphorylation at 1 μM and 2 μM concentrations, respectively (data not shown). SB concentration and effectiveness were assessed previously [25].

Western blot analysis

Western blot assays were performed as previously described [25]. Briefly, WISH cells incubated overnight in culture medium without FBS were treated in the presence or absence of 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide at 37 °C for different times. Cells were then solubilized in lysis buffer and 100 μg of protein were submitted to SDS–PAGE, followed by transfer to a nitrocellulose membrane. After blocking non-specific antibody binding sites, membranes were incubated overnight at 4 °C with the corresponding primary antibodies. Bound antibodies were then revealed with anti-rabbit IgG (horseradish peroxidase-conjugated goat IgG). Immunoreactive proteins were visualized using the ECL detection system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Quantification of the intensity of each band was performed with a densitometer (Gel Pro Analyzer). In some experiments, cells were pre-incubated for 1 h with 10 μM of SB203580, 1 μM rottlerin or 2 μM Ly294002.

Transfection and RNA interference

PKCδ small interfering RNA (siRNA), control siRNA, transfection reagent and medium were obtained from Santa Cruz Biotechnology, CA, USA. Cells were transfected with 10^{-4} nM siRNA according to the manufacturer's instruction. Dominant negative mutant of p38MAPK (DNp38) was a gift form Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA, USA). Dominant negative mutant of p85 PI3K-regulatory subunit (DNp85) was

provided by William Hahn (Dana Farber Cancer Institute, Boston, MA, USA). Cells were transfected with DNp38 or DNp85 plasmids (Addgene plasmids 20352 and 10888, respectively), or with the corresponding empty vectors using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA), according to the manufacturer's protocol.

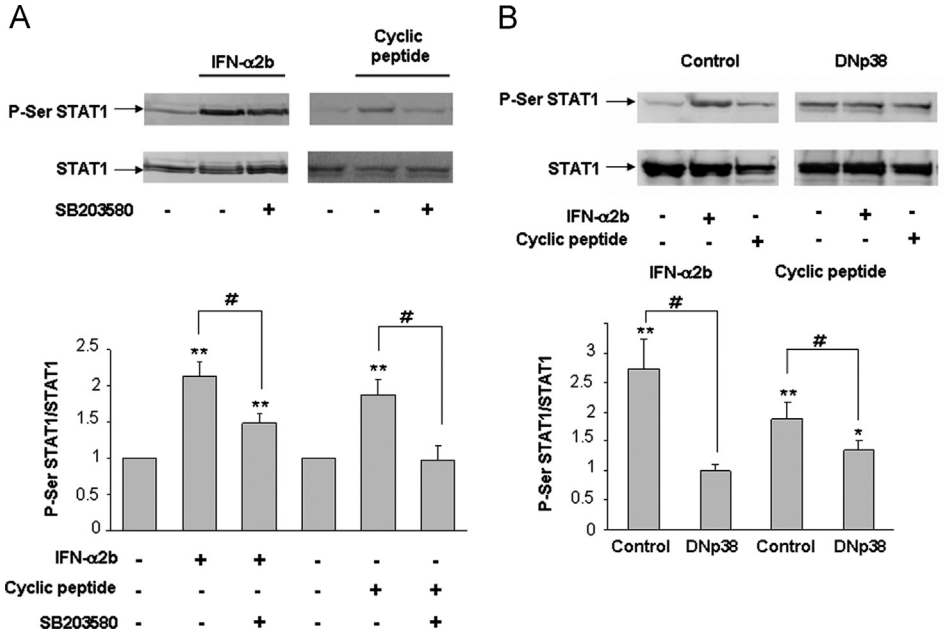


Fig. 1 – Effect of p38 MAPK inhibition on serine phosphorylation of STAT1 induced by the cyclic peptide. (A) WISH cells maintained in the absence of FBS for 24 h were pre-treated for 1 h at 37 °C with or without 10 μM SB203580 and then incubated for 30 min with 0.2 μg/ml of IFN-α2b or 1 h with 20 μg/ml of cyclic peptide. Cells lysates were subjected to SDS-PAGE under the conditions described in Materials and methods. Western blot assays were performed with antibodies against phosphoserine-STAT1 (P-Ser STAT1) and STAT1. (B) Cells were transfected with the empty vector (control) or the DNp38 mutant construct for 5 h. After removing the transfection medium, cells were incubated overnight in growth medium, starved for 24 h and then treated for 30 min with 0.2 μg/ml of IFN-α2b or 1 h with 20 μg/ml of cyclic peptide. Western blot assays were performed as described above. Results from one representative experiment are shown (top panel). Data quantification was performed by densitometric analysis (mean ± SE of three independent experiments, bottom panel). $^{*}p<0.01$, $^{**}p<0.005$, significantly different from non-stimulated cells; $^{\#}p<0.05$, significantly different from stimulated-cells incubated in the absence of inhibitor or DNp38.

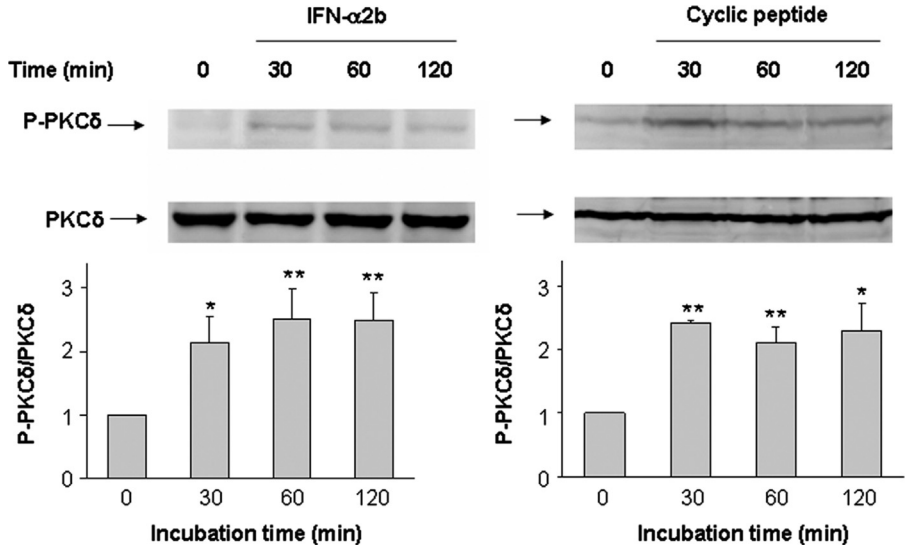


Fig. 2 – Cyclic-peptide induced PKCδ phosphorylation. WISH cells were maintained in the absence of FBS for 24 h and then incubated for different times with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Cells lysates were subjected to SDS-PAGE and Western blot assays were performed with antibodies against phospho-PKCδ (P-PKCδ) and PKCδ. Results from one representative experiment are shown (top panel). Data quantification was performed by densitometric analysis (mean ± SE of three independent experiments, bottom panel). $^{*}p<0.05$, $^{**}p<0.005$.

Flow cytometry analysis

In order to evaluate the proportion of hypodiploid cells, WISH cells pre-incubated with 10 μM SB203580, 1 μM rottlerin, 2 μM Ly294002, or transfected with the corresponding siRNA were incubated for 48 h at 37 °C in the presence or absence of 2 μg/ml IFN-α2b or 10 μg/ml of cyclic derivative in culture medium containing 1% FBS. After harvesting and washing with cold PBS, cells were fixed overnight with 1 ml of 70% ethanol and kept at 4 °C. Then, cells were washed twice with PBS and resuspended in 500 μl of 0.1% sodium citrate buffer, pH 8.4, 0.1% Triton X-100 and 50 μg/ml propidium iodide (PI), overnight at 4 °C. Stained cells were analyzed for DNA content by using a FACScan flow cytometer (Becton Dickinson, CA, USA).

Statistical analysis

The values are expressed as mean ± SE. Statistical analysis of the data was performed by using the Student's *t*-test. *P* values <0.05 were considered statistically significant.

Results

p38 MAPK and PKCδ are required for peptide-induced STAT1 Ser727 phosphorylation

We have previously demonstrated that the IFN-α2b cyclic peptide regulates WISH cell proliferation by inducing tyrosine phosphorylation of Jak1 and Tyk2 kinases, tyrosine and serine phosphorylation of STAT1 and STAT3, and activation of p38 MAPK pathway [25]. Although it has been proposed that p38 MAPK could act as a kinase for serine STAT1 phosphorylation in HeLa cells stimulated with IFNα [26], an IFNα induced serine phosphorylation of STAT1 not related to p38 activation was found in different leukemia cell lines [31] and dendritic cell precursors [32]. In order to evaluate if peptide-induced phosphorylation of STAT1 on Ser727 is mediated by p38 MAPK, WISH cells were pre-treated with SB203580, a specific p38 MAPK inhibitor, and then incubated in the presence or absence of the cyclic peptide. In parallel, the effect of SB203580 on IFN-α2b-induced phosphorylation of Ser727 STAT1 was also examined. Consistent with our previous results, serine phosphorylation of STAT1 was determined after 60 min and 30 min of exposure to cyclic peptide or IFN-α2b, respectively [25]. As shown in Fig. 1A, SB203580 significantly diminished phophoserine-STAT1 levels induced after treatment with either cyclic peptide or IFN-α2b, suggesting that p38 would be required for STAT1 serine phosphorylation. To confirm these results, the effect of blocking p38 activation with a DNp38 mutant expression construct was then determined. Cells were transfected with either a control empty vector or the mutant p38 construct, and phospho-Ser727 STAT1 levels were examined. A significant reduction of STAT1 serine phosphorylation was evident in DNp38-treated cells after incubating either with the cyclic peptide or IFN-α2b (Fig. 1B), indicating a role for p38 in serine STAT1 phosphorylation.

It has also been demonstrated that PKCδ, but not p38 kinase, behaves as a serine kinase for STAT1 in various leukemia cell lines

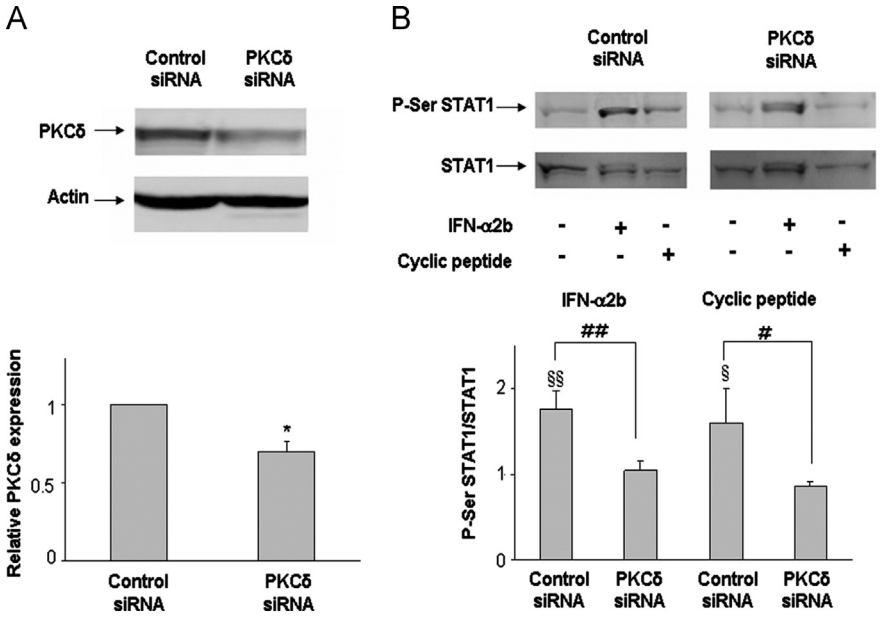


Fig. 3 – Serine phosphorylation levels of STAT1 after transfection with PKCδ siRNA. WISH cells were incubated for 24 h in the presence of either PKCδ siRNA or control siRNA according to the manufacturer's instruction. (A) Cell lysates were subjected to SDS-PAGE and Western blot with anti-PKCδ antibody. Equal loading was confirmed by stripping and reprobing each blot for actin. (B) Cells seeded in 24 well plates were maintained in the absence of FBS for 24 h and incubated for 30 min or 1 h with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide, respectively. Cells lysates were subjected to SDS-PAGE under the conditions described in Material and methods. Western blot assays were performed with antibodies against P-Ser STAT1 and STAT1. Results from one representative experiment are shown (top panel). Quantification was performed by densitometric analysis (mean ± SE of three independent experiments, bottom panel). $^{*}p<0.001$; $^{\S\S}p<0.005$, $^{\S}p<0.05$, **significantly different from non-stimulated cells;** $^{\#\#}p<0.005$, $^{\#}p<0.05$, **significantly different from cells transfected with control siRNA.**

sensitive to type I IFNs [27,28,33,34]. We first examined whether the cyclic peptide induced PKCδ activation. To this end, WISH cells were incubated for different times in the presence or absence of cyclic peptide, and Western blots analyses of cell lysates were performed with anti-phospho-PKCδ antibody. Phosphorylation of PKCδ was detected after 30 min of exposure to cyclic peptide and remained elevated for at least 120 min post stimulation (Fig. 2). A similar pattern of PKCδ phosphorylation was obtained after cell treatment with IFN-α2b. Based on these results, we decided to determine whether PKCδ was also mediating the Ser727 phosphorylation of STAT1. To this end, when cells pre-treated with rottlerin, a PKCδ inhibitor, were incubated in the presence or

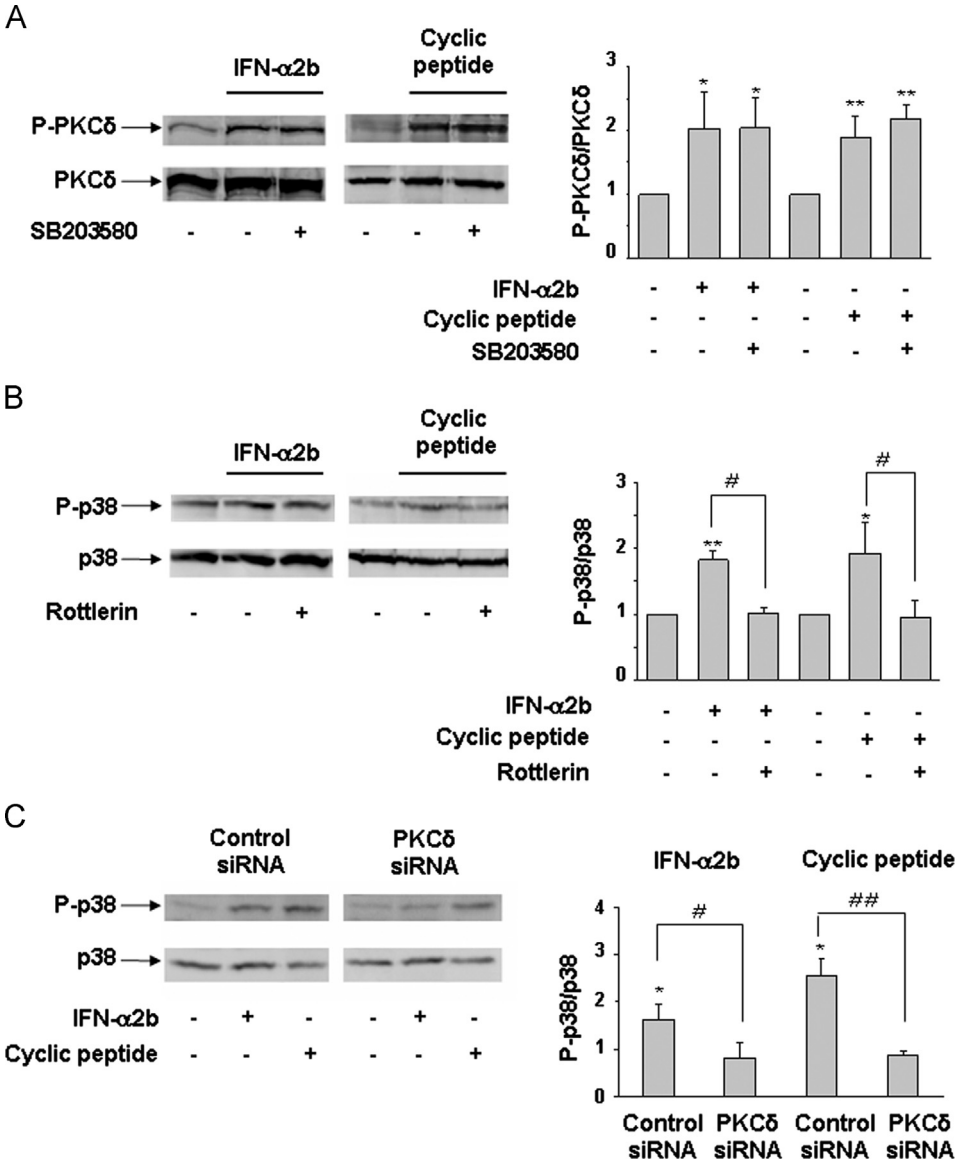


Fig. 4 – Cyclic peptide-induced PKCδ-dependent activation of p38. WISH cells maintained in the absence of FBS for 24 h were pre-treated for 1 h at 37 °C with or without 10 μM SB203580 (A) or 1 μM rottlerin (B) and then incubated for 30 min with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Cells lysates were subjected to SDS-PAGE under the conditions described in Materials and methods. Western blot assays were performed with antibodies anti-P-PKCδ and anti-PKCδ (A) or anti-phospho p38 (P-p38) and anti-p38 (B). Results from one representative experiment are shown (left panel). Data quantification was performed by densitometric analysis (mean ± SE of three independent experiments, right panel). $^{*}p<0.05$, $^{**}p<0.01$, significantly different from non-stimulated cells; $^{\#}p<0.005$, significantly different from stimulated-cells incubated in the absence of rottlerin (C) WISH cells were incubated for 24 h in the presence of either PKCδ siRNA or control siRNA according to the manufacturer's instruction. Then, cells were maintained in the absence of FBS for 24 h and incubated for 30 min with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Cells lysates were subjected to SDS-PAGE under the conditions described in Material and methods. Western blot assays were performed with antibodies anti-P-p38 and anti-p38. Results from one representative experiment are shown (left panel). Data quantification was performed by densitometric analysis (mean ± SE of three independent experiments, right panel).$^{*}p<0.05$, significantly different from non-stimulated cells; $^{\#\#}p<0.005$, $^{\#}p<0.05$, significantly different from cells transfected with control siRNA.

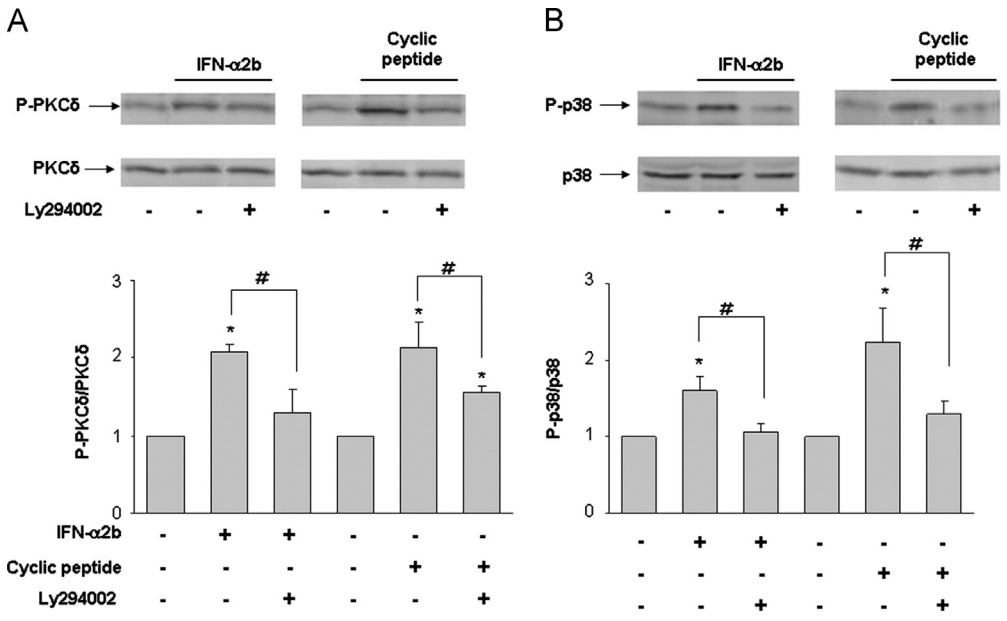


Fig. 5 – Effect of pharmacological inhibition of PI3K on PKCδ and p38 phosphorylation. WISH cells maintained in the absence of FBS for 24 h were pre-treated for 1 h at 37 °C with or without 2 μM Ly294002 and then incubated for 30 min with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Cells lysates were subjected to SDS-PAGE under the conditions described in Materials and methods. Western blot assays were performed with antibodies (A) anti-P-PKCδ, anti-PKCδ; (B) anti-P-p38, anti-p38. Results from one representative experiment are shown (top panel). Data quantification was performed by densitometric analysis (mean ± SE of three independent experiments, bottom panel). *$p < 0.005$, significantly different from non-stimulated cells; #$p < 0.005$, significantly different from stimulated-cells incubated in the absence of inhibitor.

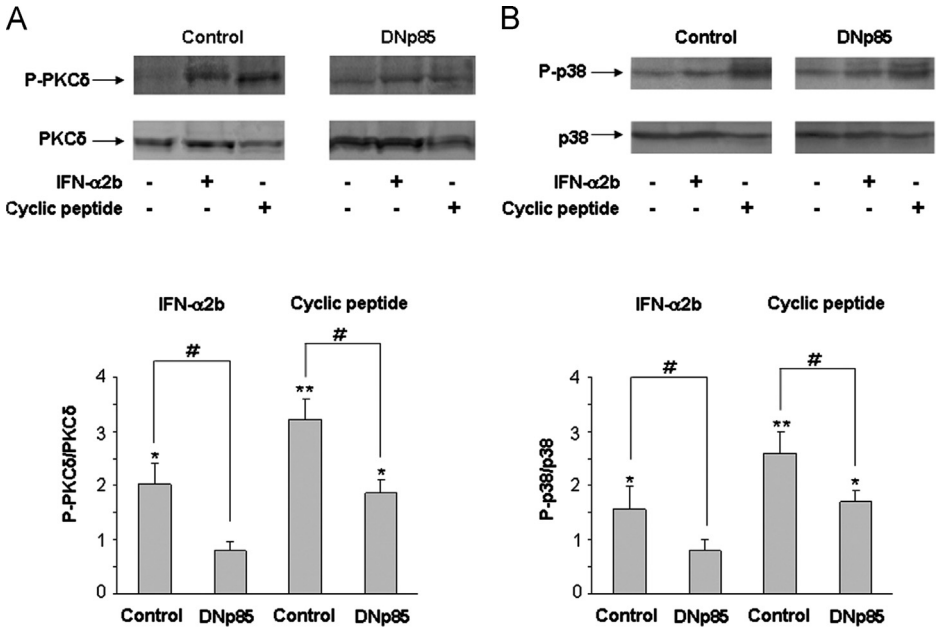


Fig. 6 – Phosphorylation levels of PKCδ and p38 after transfection with a DNp85 mutant. WISH cells were transfected with the empty vector (control) or the DNp85 mutant construct for 5 h. After removing the transfection medium, cells were incubated overnight in growth medium, starved for 3 h and then treated for 30 min with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Western blot assays were performed with antibodies (A) anti-P-PKCδ, anti-PKCδ; (B) anti-P-p38, anti-p38. Results from one representative experiment are shown (top panel). Data quantification was performed by densitometric analysis (mean ± SE of three independent experiments, bottom panel). *$p < 0.05$, **$p < 0.01$, significantly different from non-stimulated cells; #$p < 0.05$, significantly different from stimulated-cells incubated in the absence of DNp85.

absence of cyclic peptide or IFN-α2b, Ser727 STAT1 phosphorylation levels were significantly reduced after stimulation with either cyclic peptide (~30%) or IFN-α2b (~38%), suggesting that PKCδ activation would be involved in STAT1 serine phosphorylation (data not shown). In order to confirm these results, PKCδ protein expression was blocked by using specific small interfering RNA (siRNA). Western blots assays were performed to determine PKCδ depletion after incubating WISH cells with either siRNA control or an specific siRNA for PKCδ. As shown in Fig. 3A, PKCδ levels diminished approximately 30% after 24 h of incubation with the specific siRNA. Serine phosphorylation levels of STAT1 induced either by the cyclic peptide or IFN-α2b were next analyzed in knocked down cells in comparison with control cells. Densitometric analyses of Western blots assays revealed a significant decrease in STAT1 serine phosphorylation levels induced by any stimulus (Fig. 3B).

PI3K is required for peptide-induced PKCδ and p38 activation

Since we found that both PKCδ and p38 MAPK are involved in cyclic peptide- and IFN-α2b-induced serine STAT1 phosphorylation in WISH cells, we decided to examine if these serine kinases are part of a common pathway or if they are mediating separate independent pathways. PKCδ activation induced either by the cyclic peptide or IFN-α2b after 30 min of incubation of WISH cells was measured in the presence or absence of SB203580 or rottlerin (Fig. 4A and B). Densitometric analyses of Western blots showed that SB203580 was not able to inhibit PKCδ phosphorylation, but rottlerin significantly blocked p38 activation, suggesting that PKCδ regulates p38 phosphorylation. The PKCδ-dependent activation of p38 was also confirmed by using PKCδ knocked down cells obtained after transfection with specific siRNA. Thus, when PKCδ-depleted WISH cells were stimulated by the cyclic peptide or IFN-α2b, a significant reduction of phospho-p38 levels was observed (Fig. 4C).

It has been shown that IFNα-dependent PKCδ phosphorylation occurs downstream of PI3K activation [35]. On the other hand, it has been determined that p38 stimulation induced by type I IFNs in leukemia cells is independent of PI3K activation [31]. Thus, based on these findings, we further inquired whether PI3K, a pathway commonly activated by IFNα, is required for cyclic peptide-induced PKCδ and p38 activation. WISH cells, pre-treated with the PI3K inhibitor Ly294002, were incubated with cyclic peptide or IFN-α2b for 30 min, and PKCδ and p38 phosphorylation were then examined by Western blot. As shown in Fig. 5A and B, phospho-PKCδ and phospho-p38 expression levels induced either by the cyclic peptide or the IFN-α2b molecule were significantly reduced in the presence of Ly294002. In addition, a dominant-negative mutant of p85 (DNp85) was employed to confirm these results. Inhibition of PI3K pathway also diminished the amount of phospho-PKCδ and phospho-p38 induced by the peptide or the IFN-α2b, suggesting that PI3K would be acting as an upstream regulator of PKCδ and p38 kinases (Fig. 6A and B).

Role of PI3K and PKCδ in the antiproliferative and apoptotic actions of the chimeric cyclic peptide

We have previously demonstrated that p38 MAPK would be mediating the antiproliferative activity and the apoptotic response triggered by the cyclic peptide in WISH cells [25]. Based on the results herein obtained, we decided to examine whether PI3K and PKCδ were also regulating these effects. Cell growth assays were performed with WISH cells pre-treated with rottlerin or Ly294002 and then stimulated with the cyclic peptide or IFN-α2b. As shown in Fig. 7A, cell treatment with either rottlerin or Ly294002 significantly diminished the antiproliferative activity exhibited by the cyclic peptide and IFN-α2b. A similar behavior was observed for both stimuli in cells depleted of PKCδ after siRNA transfection (Fig. 7B). We next evaluated the effect of both inhibitors on the peptide- and IFN-α2b-induced apoptotic action. Thus, the percentage of hypodiploid cells obtained after incubating WISH cells in the presence of the cyclic peptide diminished from 23±3% to 8±2% when cells were pre-incubated with rottlerin, or 12±1% after Ly294002 treatment (Fig. 8A). In a similar way, when cells were incubated with I

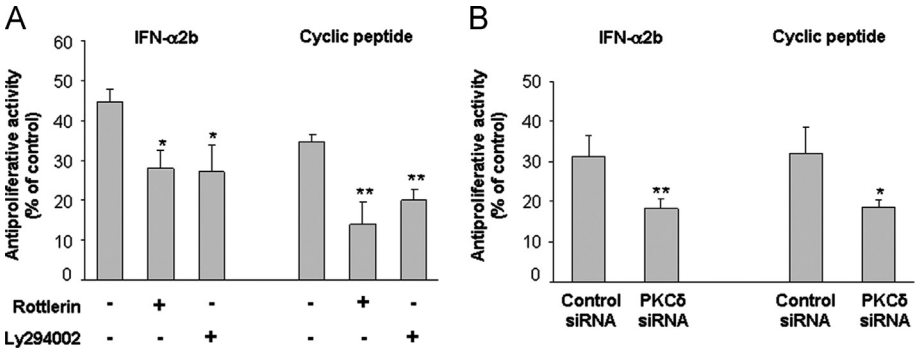


Fig. 7 – Effect of PKCδ and PI3K inhibition on the antiproliferative effect induced by the cyclic peptide. (A) WISH cells were pre-treated for 1 h at 37 °C with or without 1 μM rottlerin or 2 μM Ly294002 and then incubated for 72 h with 0.2 μg/ml of IFN-α2b or 10 μg/ml of cyclic peptide. (B) WISH cells transfected with PKCδ siRNA or control siRNA were incubated for 48 h with 0.1 μg/ml of IFN-α2b or 10 μg/ml of cyclic peptide. Cell proliferation was evaluated by colorimetric determination of hexosaminidase levels. The antiproliferative activity was calculated as the percentage of growth inhibition obtained in treated cells with respect to untreated control cells. Results represent the mean±SE of three different experiments. Statistical significance in comparison with the corresponding control values (cells either incubated in the absence of inhibitor or transfected with control siRNA) is indicated by $^{*}p < 0.05$, $^{**}p < 0.01$.

FN-α2b, pre-incubation with rottlerin or Ly294002 decreased the percentage of apoptotic cells from 29±5% to 11±2% or 10±1%, respectively (Fig. 8A). Results obtained with rottlerin were further confirmed with cells treated with siRNA. Consequently, after transfection with PKCδ siRNA, the sub-G_1 fraction of cells incubated with cyclic peptide diminished from 24±4% to 12±2%, and from 29±4% to 14±4% for IFN-α2b-treated cells (Fig. 8B).

Discussion

We have recently demonstrated that STAT1, STAT3 and p38 MAPK were involved in the apoptotic response triggered by a chimeric cyclic peptide of the IFN-α2b in WISH cells [25]. We found that the peptide stimulated p38 activation and both tyrosine and

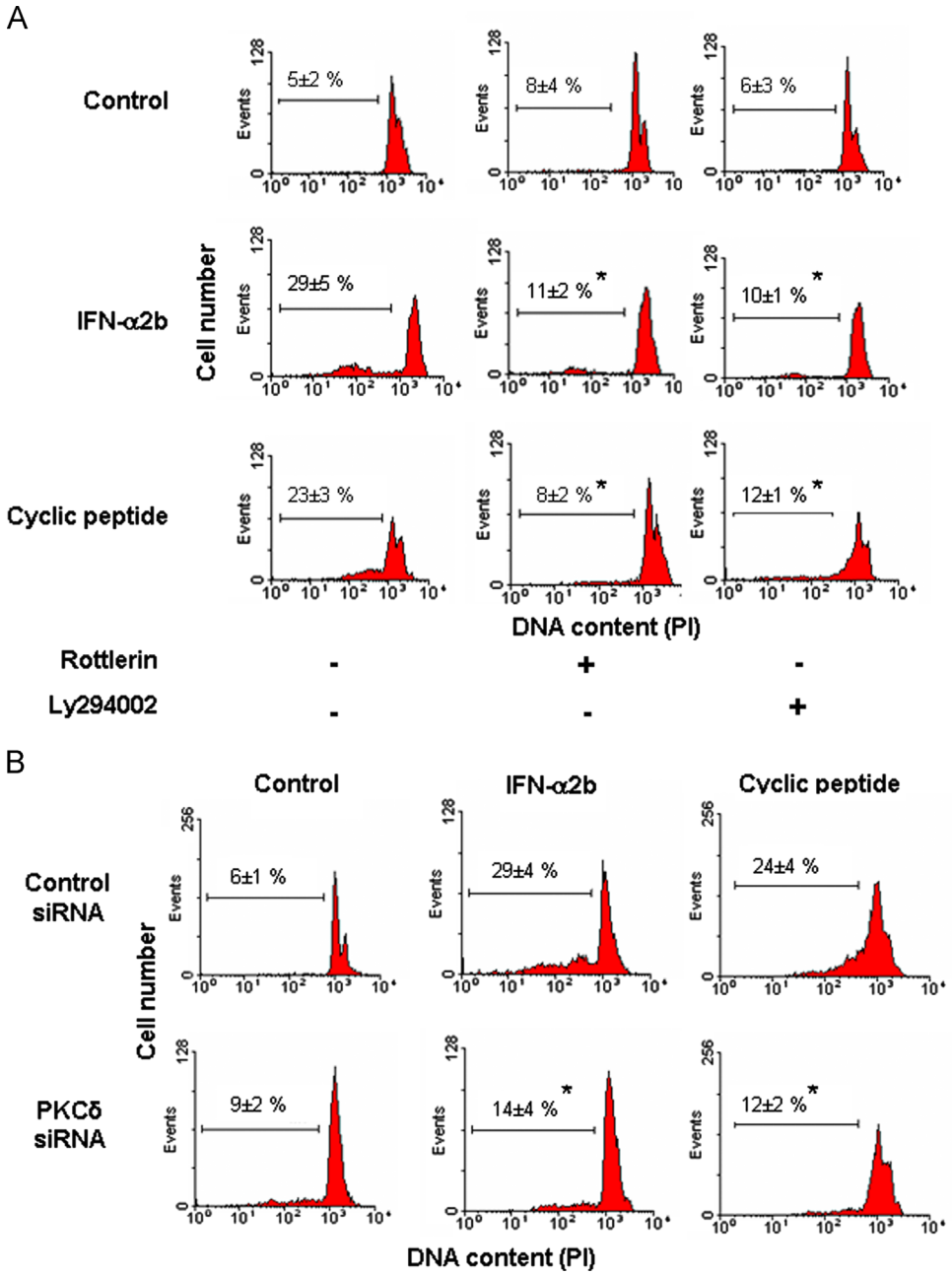


Fig. 8 – Effect of PKCδ and PI3K inhibition on the apoptotic effect induced by the cyclic peptide. (A) WISH cells were pre-treated for 1 h at 37 °C with or without 1 μM rottlerin or 2 μM Ly294002 and then incubated for 48 h with 2 μg/ml of IFN-α2b or 10 μg/ml of cyclic peptide. Hypodiploid DNA content was evaluated by flow cytometry after propidium iodide staining. The percentage of apoptotic cells±SE of three different experiments is shown in each histogram. Statistical significance in comparison with the corresponding control values is indicated by *$p<0.005$. (B) WISH cells transfected with PKCδ siRNA or control siRNA were incubated for 48 h with 2 μg/ml of IFN-α2b or 10 μg/ml of cyclic peptide. Results represent the mean±SE of three different experiments. Statistical significance in comparison with the corresponding control values is indicated by *$p<0.01$.

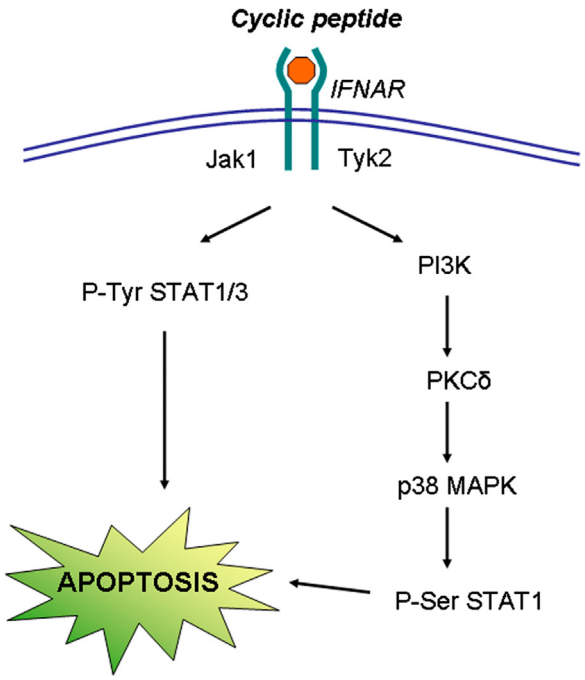


Fig. 9 – Schematic representation of the signaling pathways involved in the apoptotic cell death induced by the cyclic peptide.

serine phosphorylation of STAT1 and STAT3 proteins [25]. In the present study, we decided to examine whether p38 is involved in STAT1 phosphorylation on Ser727 and the upstream regulators of p38 activation Similarly to the results obtained for IFN-α2b-stimulated WISH cells, a significant reduction of phophoserine-STAT1 levels induced by the cyclic peptide was observed after treating cells either with an specific pharmacological inhibitor of p38 MAPK or with a dominant-negative p38 mutant. Thus, our results showed that p38 MAPK plays a role as a mediator of serine phosphorylation of STAT1, although it remains to be established whether the p38 kinase itself or other possible downstream kinases contribute to the observed effect. In accordance to our data, a role for p38 MAPK in STAT1 serine phosphorylation has also been reported in HeLa cells exposed to IFNα or IFNγ [26]. In spite of these findings, some studies performed with diverse leukemia cell lines [27,31] and dendritic cell precursors [32] have established that IFNα-induced Ser727 STAT1 phosphorylation would be independent of p38 activation. These controversial results may reflect a difference in the cell type studied. In this respect, taking into account that WISH cells were established via HeLa contamination [36], a similar behavior could be expected for both related cell lines. Thus, depending on the cellular background, different serine kinases could be acting as regulators for serine STAT1 phosphorylation.

Since various studies performed in leukemia cell lines have provided evidence about the involvement of PKCδ, but not p38, in the IFNα-stimulated serine phosphorylation of STATs [11,27,28,33,34], we inquired whether this kinase could someway contribute to the peptide-stimulated signaling. We first demonstrated that the cyclic peptide effectively induced PKCδ activation in a similar manner to that showed by IFN-α2b. Next, we found that after treatment of cells with a PKCδ pharmacological inhibitor or with a specific PKCδ siRNA, cyclic peptide- or IFN-α2b-induced Ser727 STAT1 phosphorylation levels were significantly decreased, suggesting that PKCδ also contributes to serine STAT1 phosphorylation. However, unlike results obtained in leukemia cells [27,28], our data showed that PKCδ and p38 are part of a common pathway that finally lead to serine STAT1 phosphorylation, since blocking of PKCδ, either with a pharmacologic inhibitor or with specific siRNA, significantly reduced phospho-p38 levels obtained after incubating WISH cells with either cyclic peptide or IFN-α2b.

In the search of an upstream regulator of PKCδ and p38, we sought to examine the possible participation of PI3K. In this sense, there is multiple evidence involving the PI3K pathway in IFNα-mediated signaling [11,14,35,37,38]. In addition, it has also been reported that IFNγ induces Ser727 STAT1 phosphorylation through activation of the PI3K pathway, which in turn functions as an upstream effector of PKCδ [39,40]. Results herein obtained showed that cyclic peptide- or IFN-α2b-induced phosphorylation levels of PKCδ and p38 were significantly inhibited by a specific PI3K pharmacological inhibitor or a dominant-negative p85 mutant, indicating that PI3K would be upstream in the signaling cascade.

As it was established in a previous work [25], we found that the cyclic peptide activated similar signaling components to those stimulated by the IFN-α2b, suggesting that this derivative, although represents an epitope that mainly recognizes IFNAR2 [19], would induce the dimerization of both receptor subunits. We also demonstrated that the signaling effectors studied are certainly mediating the growth inhibitory activity and the apoptotic response triggered by the cyclic peptide in WISH cells. In this respect, we and others have previously shown a role of p38 in the apoptotic effect mediated by IFNα [11,13,25,41,42]. Different reports have also revealed that PI3K and PKCδ contribute to the IFNα-induced apoptosis [33,36,39,43]. In the present study, we found that PI3K functions as an upstream regulator of PKCδ and p38 MAPK in WISH cells. As it was mentioned before, other signaling pathways could be activated in a different cellular context. Thus, some studies performed with leukemia cells have shown that PKCδ, instead of being related to p38, mediates the IFNα-stimulated apoptosis through JNK activation [33,35].

In conclusion, the whole results lead us to propose the signaling cascade that would be responsible for the apoptotic cell death induced by the cyclic peptide (Fig. 9). Briefly, at least both tyrosine STAT1/3 phosphorylation previously studied [25] and serine STAT1 phosphorylation are related to the apoptotic response triggered by the chimeric peptide. Herein, we demonstrated that STAT1 serine phosphorylation is mediated by activation of PI3K, PKCδ and p38 signaling effectors. These findings provide the molecular mechanism of action of a chimeric IFN-α2b cyclic peptide putative valuable for the treatment of cancer patients.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by grants from CONICET (PIP 1261) and the Universidad de Buenos Aires (UBACYT 20020100100081), Argentina.

REFERENCES

[1] S. Pestka, J.A Langer, K.C. Zoon, C.E. Samuel, et al., (1987)., Interferons and their actions, Annu. Rev. Biochem. 56 (1987) 727–777.

[2] G.C. Viscomi, M. Grimaldi, E. Palazzini, S. Silvestri, et al., (1995)., Human leukocyte interferon alpha: structure, pharmacology, and therapeutic applications, Med. Res. Rev. 15 (1995) 445–478.

[3] G.R. Stark, I.M. Kerr, B.R. Williams, R.H. Silverman, R.D. Schreiber, et al., (1998)., How cells respond to interferons, Annu. Rev. Biochem. 67 (1998) 227–264.

[4] M.M. Brierley, E.N. Fish, et al., (2002)., Review: IFN-alpha/beta receptor interactions to biologic outcomes: understanding the circuitry, J. Interferon Cytokine Res. 22 (2002) 835–845.

[5] S. Parmar, L.C. Platanias, et al., (2003)., Interferons: mechanisms of action and clinical applications, Curr. Opin. Oncol. 15 (2003) 431–439.

[6] G. Antonelli, et al., (2008)., Biological basis for a proper clinical application of alpha interferons, New Microbiol. 31 (2008) 305–318.

[7] J. Kirkwood, et al., (2002)., Cancer immunotherapy: the interferon-alpha experience, Semin. Oncol. 29 (3 Suppl. 7) (2002) 18–26.

[8] S. Uddin, L.C. Platanias, et al., (2004)., Mechanisms of type-I interferon signal transduction, J. Biochem. Mol. Biol. 37 (2004) 635–641.

[9] M.M. Brierley, E.N. Fish, et al., (2005)., Stats: multifaceted regulators of transcription, J. Interferon Cytokine Res. 25 (2005) 733–744.

[10] M. Caraglia, M. Marra, G. Pelaia, R. Maselli, M. Caputi, S.A. Marisco, A. Abbruzzese, et al., (2005)., Alpha-interferon and its effects on signal transduction pathways, J. Cell Physiol. 202 (2005) 323–335.

[11] L.C. Platanias, et al., (2005)., Mechanisms of type-I- and type-II-interferon-mediated signalling, Nat. Rev. Immunol. 5 (2005) 375–386.

[12] C. Schindler, D.E. Levy, T. Decker, et al., (2007)., JAK-STAT signaling: from interferons to cytokines, J. Biol. Chem. 282 (2007) 20059–20063.

[13] L.C. Platanias, et al., (2003)., The p38 mitogen-activated protein kinase pathway and its role in interferon signalling, Pharmacol. Ther. 98 (2003) 129–142.

[14] S. Kaur, S. Uddin, L.C. Platanias, et al., (2005)., The PI3' kinase pathway in interferon signalling, J. Interferon Cytokine Res. 25 (2005) 780–787.

[15] Z. Wen, Z. Zhong, J.E. Darnell Jr., et al., (1995)., Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation, Cell 82 (1995) 241–250.

[16] Z. Wen, J.E. Darnell Jr., et al., (1997)., Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3, Nucleic Acids Res. 25 (1997) 2062–2067.

[17] C.M. Horvath, et al., (2000)., STAT proteins and transcriptional responses to extracellular signals, Trends Biochem. Sci. 25 (2000) 496–502.

[18] L. Thyrell, L. Hjortsberg, V. Arulampalam, T. Panaretakis, S. Uhles, M. Dagnell, B. Zhivotovsky, I. Leibiger, D. Grandér, K. Pokrovskaja, et al., (2004)., Interferon alpha-induced apoptosis in tumor cells is mediated through the phosphoinositide 3-kinase/mammalian target of rapamycin signaling pathway, J. Biol. Chem. 279 (2004) 24152–24162.

[19] C. Peña, V.C. Blank, V.J. Marino, L.P. Roguin, et al., (2005)., Synthesis and biological properties of chimeric interferon-alpha 2b peptides, Peptides 26 (2005) 1144–1149.

[20] J. Piehler, L.C. Roisman, G. Schreiber, et al., (2000)., New structural and functional aspects of the type I interferon–receptor interaction revealed by comprehensive mutational analysis of the binding interface, J. Biol. Chem. 275 (2000) 40425–40433.

[21] J.H. Chill, S.R. Quadt, R. Levy, G. Schreiber, J. Anglister, et al., (2003)., The human type I interferon receptor: NMR structure reveals the molecular basis of ligand binding, Structure 11 (2003) 791–802.

[22] S.R. Quadt-Akabayov, J.H. Chill, R. Levy, N. Kessler, J. Anglister, et al., (2006)., Determination of the human type I interferon receptor binding site on human interferon-alpha2 by cross saturation and an NMR-based model of the complex, Protein Sci. 15 (2006) 2656–2668.

[23] J. Kumaran, L. Wei, L.P. Kotra, E.N. Fish, et al., (2007)., A structural basis for interferon-alpha–receptor interactions, FASEB J. 21 (2007) 3288–3296.

[24] V.C. Blank, C. Peña, L.P. Roguin, et al., (2007)., A cyclic chimeric interferon-α2b peptide induces apoptosis in tumor cells, Cancer Biol. Ther. 6 (2007) 1787–1793.

[25] V.C. Blank, C. Peña, L.P. Roguin, et al., (2010)., STAT1, STAT3 and p38MAPK are involved in the apoptotic effect induced by a chimeric cyclic interferon-α2b peptide, Exp. Cell Res. 316 (2010) 603–614.

[26] K.C. Goh, S.J. Haque, B.R. Williams, et al., (1999)., p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons, EMBO J. 18 (1999) 5601–5608.

[27] S. Uddin, A. Sassano, D.K. Deb, A. Verma, B. Majchrzak, A. Rahman, A.B. Malik, E.N. Fish, L.C. Platanias, et al., (2002)., Protein kinase C-delta (PKC-delta) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727, J. Biol. Chem. 277 (2002) 14408–14416.

[28] S. Kaur, S. Parmar, J. Smith, E. Katsoulidis, Y. Li, A. Sassano, B. Majchrzak, S. Uddin, M.S. Tallman, E.N. Fish, L.C. Platanias, et al., (2005)., Role of protein kinase C-delta (PKC-delta) in the generations of the effects of IFN-alpha in chronic myelogenous leukemia cells, Exp. Hematol. 33 (2005) 550–557.

[29] V.C. Blank, C. Peña, L.P. Roguin, et al., (2000)., Suitable experimental conditions are required to characterize IFN-α2b synthetic peptides, Eur. J. Biochem. 267 (2000) 5711–5716.

[30] U. Landegren, et al., (1984)., Measurement of cell numbers by means of the endogenous enzyme hexosaminidase, applications to detection of lymphokines and cell surface antigens, J. Immunol. Methods 67 (1984) 379–388.

[31] S. Uddin, F. Lekmine, N. Sharma, B. Majchrzak, I. Mayer, P.R. Young, G.M. Bokoch, E.N. Fish, L.C. Platanias, et al., (2000)., The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon alpha-dependent transcriptional activation but not serine phosphorylation of Stat proteins, J. Biol. Chem. 275 (2000) 27634–27640.

[32] R. Takauji, S. Iho, H. Takatsuka, S. Yamamoto, T. Takahashi, H. Kitagawa, H. Iwasaki, R. Iida, T. Yokochi, T. Matsuki, et al., (2002)., CpG-DNA-induced IFN-alpha production involves p38 MAPK-dependent STAT1 phosphorylation in human plasmacytoid dendritic cell precursors, J. Leukoc. Biol. 72 (2002) 1011–1019.

[33] N. Yanase, M. Hayashida, Y. Kanetaka-Naka, A. Hoshika, J. Mizuguchi, et al., (2012)., PKC-δ mediates interferon-α-induced apoptosis through c-Jun NH2-terminal kinase activation, BMC Cell Biol. 13 (2012) 7.

[34] K.W. Zhao, D. Li, Q. Zhao, Y. Huang, R.H. Silverman, P.J. Sims, G.Q. Chen, et al., (2005)., Interferon-alpha-induced expression of phospholipid scramblase 1 through STAT1 requires the sequential activation of protein kinase Cdelta and JNK, J. Biol. Chem. 280 (2005) 42707–42714.

[35] T. Panaretakis, L. Hjortsberg, K.P. Tamm, A.C. Björklund, B. Joseph, D. Grandér, et al., (2008)., Interferon alpha induces nucleus-independent apoptosis by activating extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase downstream of phosphatidylinositol 3-kinase and mammalian target of rapamycin, Mol. Biol. Cell 19 (2008) 41–50.

[36] S.M. Gartler, et al., (1968)., Apparent HeLa cell contamination of human heteroploid cell lines, Nature 217 (1968) 750–751.

[37] S. Kaur, A. Sassano, A.M. Joseph, B. Majchrzak-Kita, E.A. Eklund, A. Verma, S.M. Brachmann, E.N. Fish, L.C. Platanias, et al., (2008)., Dual regulatory roles of phosphatidylinositol 3-kinase in IFN signaling, J. Immunol. 181 (2008) 7316–7323.

[38] L. Thyrell, L. Hjortsberg, V. Arulampalam, T. Panaretakis, S. Uhles, M. Dagnell, B. Zhivotovsky, I. Leibiger, D. Grandér, K. Pokrovskaja, et al., (2004)., Interferon alpha-induced apoptosis in tumor cells is mediated through the phosphoinositide 3-kinase/mammalian target of rapamycin signaling pathway, J. Biol. Chem. 279 (2004) 24152–24162.

[39] H. Nguyen, C.V. Ramana, J. Bayes, G.R. Stark, et al., (2001)., Roles of phosphatidylinositol 3-kinase in interferon-γ-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression, J. Biol. Chem. 276 (2001) 33361–33368.

[40] D.K. Deb, A. Sassano, F. Lekmine, B. Majchrzak, A. Verma, S. Kambhampati, S. Uddin, A. Rahman, E.N. Fish, L.C. Platanias, Activation of protein kinase C δ by IFN-γ, J. Immunol. 171 (2003) 267–273 (2003).

[41] I.A. Mayer, A. Verma, I.M Grumbach, S. Uddin, F. Lekmine, F. Ravandi, B. Majchrzak, S. Fujita, E.N. Fish, L.C. Platanias, et al., (2001)., The p38 MAPK pathway mediates the growth inhibitory effects of interferon-alpha in BCR-ABL-expressing cells, J. Biol. Chem. 276 (2001) 28570–28577.

[42] A. Verma, D.K. Deb, A. Sassano, S. Uddin, J. Varga, A. Wickrema, L. C. Platanias, et al., (2002)., Activation of the p38 mitogen-activated protein kinase mediates the suppressive effects of type I interferons and transforming growth factor-beta on normal hematopoiesis, J. Biol. Chem. 277 (2002) 7726–7735.

[43] L. Hjortsberg, C. Lindvall, M. Corcoran, V. Arulampalam, D. Chan, L. Thyrell, M. Nordenskjold, D. Grandér, K. Pokrovskaja, et al., (2007)., Phosphoinositide 3-kinase regulates a subset of interferon-alpha-stimulated genes, Exp. Cell Res. 313 (2007) 404–414.