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Research Article

STAT1, STAT3 and p38MAPK are involved in the apoptotic effect induced by a chimeric cyclic interferon-α2b peptide

*Viviana C. Blank, Clara Peña, Leonor P. Roguin**

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

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ABSTRACT

In the search of mimetic peptides of the interferon-α2b molecule (IFN-α2b), we have previously designed and synthesized a chimeric cyclic peptide of the IFN-α2b that inhibits WISH cell proliferation by inducing an apoptotic response. Here, we first studied the ability of this peptide to activate intracellular signaling pathways and then evaluated the participation of some signals in the induction of apoptosis. Stimulation of WISH cells with the cyclic peptide showed tyrosine phosphorylation of Jak1 and Tyk2 kinases, tyrosine and serine phosphorylation of STAT1 and STAT3 transcription factors and activation of p38 MAPK pathway, although phosphorylation levels or kinetics were in some conditions different to those obtained under IFN-α2b stimulus. JNK and p44/42 pathways were not activated by the peptide in WISH cells. We also showed that STAT1 and STAT3 downregulation by RNA interference decreased the antiproliferative activity and the amount of apoptotic cells induced by the peptide. Pharmacological inhibition of p38 MAPK also reduced the peptide growth inhibitory activity and the apoptotic effect. Thus, we demonstrated that the cyclic peptide regulates WISH cell proliferation through the activation of Jak/STAT signaling pathway. In addition, our results indicate that p38 MAPK may also be involved in cell growth regulation. This study suggests that STAT1, STAT3 and p38 MAPK would be mediating the antitumor and apoptotic response triggered by the cyclic peptide in WISH cells.

Introduction

Interferons alpha (IFNsα) are a group of related cytokines that exert multiple biological actions, including antiviral, antiproliferative and immunoregulatory responses [1-4]. They are used in the treatment of certain tumors and a variety of viral and autoimmune disorders [5]. Binding of IFNsα (type I IFNs) to specific receptors (Type I receptors) activates different signaling pathways that lead to the generation of IFN-mediated biological activities. Two different subunits of the interferon receptor complex (IFNAR) have been characterized, with IFNAR2 essentially responsible for cytokine binding, and IFNAR1 contributing to the formation of a high affinity receptor complex [6-10]. The classical transduction cascade activated by IFNsα is the Jak/STAT pathway [11-15]. After binding of IFNsα, the activation of the receptor-associated tyrosine kinases Jak1 and Tyk2 induces tyrosine phosphorylation of the receptor subunits and several STAT proteins, which form homodimers and/or heterodimers that translocate to the nucleus and

* *Corresponding author.* IQUIFIB, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956-C1113AAD Buenos Aires, Argentina. Fax: +54 11 4962 5457.
E-mail address: rvroguin@qb.ffyb.uba.ar (L.P. Roguin).

Abbreviations: Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-AMC (AMC: 7-Amino-4-methylcoumarin); IFN-α2b, interferon-α2b; FBS, fetal bovine serum; Jak, Janus-activated kinase; MAPK, mitogen-activated protein kinase; MEM, Minimum Essential Medium; PI, propidium iodide; STAT, signal transducer and activator of transcription; siRNA, small interfering RNA; IFNAR, type I IFN receptor

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regulate the transcription of specific target genes. Besides this classical pathway, other signaling cascades are also required for the generation of IFN-mediated responses, including the involvement of Crk-proteins, phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) pathways [11,13,14,16-19].

In mammalian systems, three main MAPK groups have been identified. These include the extracellular signal-regulated kinases (Erk), the p38 MAP kinases and the Jun amino-terminal kinases (JNK) [11,13,14,16]. Among these MAPK members, the p38 MAPK pathway seems to play an important role in the induction of the antiproliferative activity of IFNsα [11,13,14,16]. In this sense, it has been reported that the activation of p38 is required for the generation of the anti-leukemic effects mediated by IFNα in chronic myelogenous leukemia cells [20] and for the induction of the suppressive effects of IFNα on normal hematopoiesis [21].

We have previously synthesized a chimeric cyclic peptide of the IFN-α2b molecule that inhibits IFN-α2b binding to specific receptors and the proliferation of WISH cells [22]. We further demonstrated that the cyclic peptide induces an apoptotic response by activating both death receptor and mitochondrial pathways [23]. Although we explored the molecular events involved in the induction of apoptosis, the signal transduction pathways activated by the chimeric derivative have not yet been investigated. Therefore, the aim of this study was to determine whether the cyclic peptide is able to stimulate Jak/STAT and MAPK signaling pathways, comparatively studying the intracellular signals induced by the cyclic peptide and IFN-α2b on WISH cells. We then examined to what extent these signaling pathways participate in the regulation of the apoptotic effect induced by the chimeric derivative.

Materials and methods

Chemicals

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 the IFN-α2b chimeric cyclic peptide was previously described [22]. Thus, peptide was always dissolved in a medium containing 0.3 M glycine, 8 M urea, 0.01 M dithiothreitol and 0.014 M 2-mercaptoethanol, pH 8.5. All experimental assays were carried out in the presence of 20 μl of this vehicle per ml of assay medium, including non-treated (control) and IFN-α2b-treated samples. Rabbit polyclonal anti-Jak1, anti-Tyk2, anti-STAT1, anti-STAT3, anti-phospho-STAT1 (Tyr701), anti-phospho-STAT3 (Tyr705), anti-phospho-Tyk2 (Tyr 1054/1055) and mouse monoclonal antibody anti-phosphotyrosine were from Santa Cruz Biotechnology, CA, USA. Rabbit polyclonal antibodies anti-phospho-p38 (Thr180/Tyr182), anti-phospho-p44/42 (Thr202/Tyr204), anti-phospho-JNK (Thr183/Tyr185), the specific anti-p38, p44/42 or JNK proteins, monoclonal antibodies anti-phospho-STAT1 (Ser727) and anti-phospho-STAT3 (Ser727) were purchased from Cell Signaling Technology, Danvers, MA, USA. SB203580, a p38 MAPK inhibitor was from Santa Cruz Biotechnology, CA, 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, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL Gaithersburg, MD, USA), 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. Proliferation assay was performed as described previously [24]. Briefly, cells (25 000 cells/ml) were incubated for 48 or 72 h at 37 °C in 96-well culture microplates with 0.1 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 pre-incubated for 1 h with 10 μM SB203580, a p38-MAPK specific inhibitor. The inhibitor showed no effect on WISH cell proliferation and significantly decreased cytokine-induced p38 MAPK phosphorylation at a 10-μM concentration (data not shown). Cell number was evaluated by colorimetric determination of hexosaminidase levels [25].

Immunoprecipitation of Jak1 protein

WISH cells (1×10^7) were washed and incubated overnight in MEM without FBS. To measure the phosphorylation of Jak1, cells 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. After detaching with a rubber policeman and washing twice with cold PBS, cells were lysed for 30 min at 4 °C in 1 ml of lysis buffer (0.5% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml trysin inhibitor, 1 μg/ml leupeptin, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 10% glycerol, 150 mM NaCl, 50 mM Tris, pH 7.4). Lysates were cleared by centrifugation for 10 min at 12 000 rpm, and the supernatants were incubated in the presence of rabbit polyclonal anti-Jak1 overnight at 4 °C with constant rocking. After incubation, 50 μl of protein A-Sepharose (50% v/v in lysis buffer) (Sigma, St Louis, MO) were added and the preparation was further incubated 2 h at 4 °C and washed twice with lysis buffer. Proteins were dissociated with sample buffer, submitted to 8% SDS–PAGE and then transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NY) for 90 min at 100 V in 25 mM Tris, 195 mM glycine, 20% methanol, pH 8.2. After blocking non-specific antibody binding sites with 10 mM Tris, 130 mM NaCl and 0.05% Tween 20, pH 7.4, (TBS-T), containing 3% bovine seralbumin (BSA), membranes were then incubated overnight at 4 °C with anti-phosphotyrosine mouse monoclonal antibody diluted in TBS-T, containing 1% BSA. After 3–4 washes with TBS-T, membranes were incubated for 1 h at room temperature with anti-mouse IgG (horseradish peroxidase-conjugated goat IgG from Santa Cruz Biotechnology, CA, USA) diluted in TBS-T, 1% BSA. After stripping, membranes were incubated with rabbit polyclonal anti-Jak1 and revealed with anti-rabbit IgG (horseradish peroxidase-conjugated goat IgG from Santa Cruz Biotechnology, CA, USA). The immunoreactive proteins were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NY) according to the manufacturer's instructions. For quantification of band intensity, the western blots were scanned using a densitometer (Gel Pro Analyzer).

Western blot analysis

WISH cells (1×10^6) incubated overnight in MEM 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. After detaching with a rubber policeman and washing twice with cold PBS, cells were treated for 30 min at 4 °C in 10 μl of lysis buffer.

Lysates were cleared by centrifugation for 10 min at 12 000 rpm, and total protein concentration was determined using Bradford reagent. Aliquots containing 100 μg of protein were resuspended in sample buffer, submitted to SDS–PAGE and then transferred onto nitrocellulose membranes as described above. After blocking non-specific antibody binding sites, membranes were incubated overnight at 4 °C with the corresponding primary antibodies diluted in TBS-T, containing 1% BSA. Bound antibodies were revealed with anti-rabbit IgG (horseradish peroxidase-conjugated goat IgG) diluted in TBS-T, 1% BSA. Immunoreactive proteins were visualized as described above. Equal protein loading was confirmed by reprobing membranes with a rabbit anti-actin antibody (Sigma-Aldrich, Inc., Missouri, USA).

RNA interference

STAT3 small interfering RNAs (siRNA), control siRNA, transfection reagent and medium were obtained from Santa Cruz Biotechnology, CA, USA. Cells were transfected according to the manufacturer's instruction. Signal Silence^R STAT1 siRNA kit from Cell Signaling Technology (Danvers, MA, USA) was employed to specifically inhibit STAT1 expression.

Flow cytometry analysis

In order to evaluate the proportion of hypodiploid cells, WISH cells either pre-incubated with 10 μM SB203580 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).

Caspase activity assay

In order to evaluate caspase 3 activity, WISH cells (25,000 cells/ml) were incubated for 72 h with 2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide in the absence or presence of 10 μM SB203580. After treatment, cells were harvested with 0.25% trypsin, 0.03% EDTA, washed twice with cold PBS and centrifuged at 300×*g* for 10 min at 4 °C. Then, 1×10^6 cells were lysed for 30 min at 4 °C in

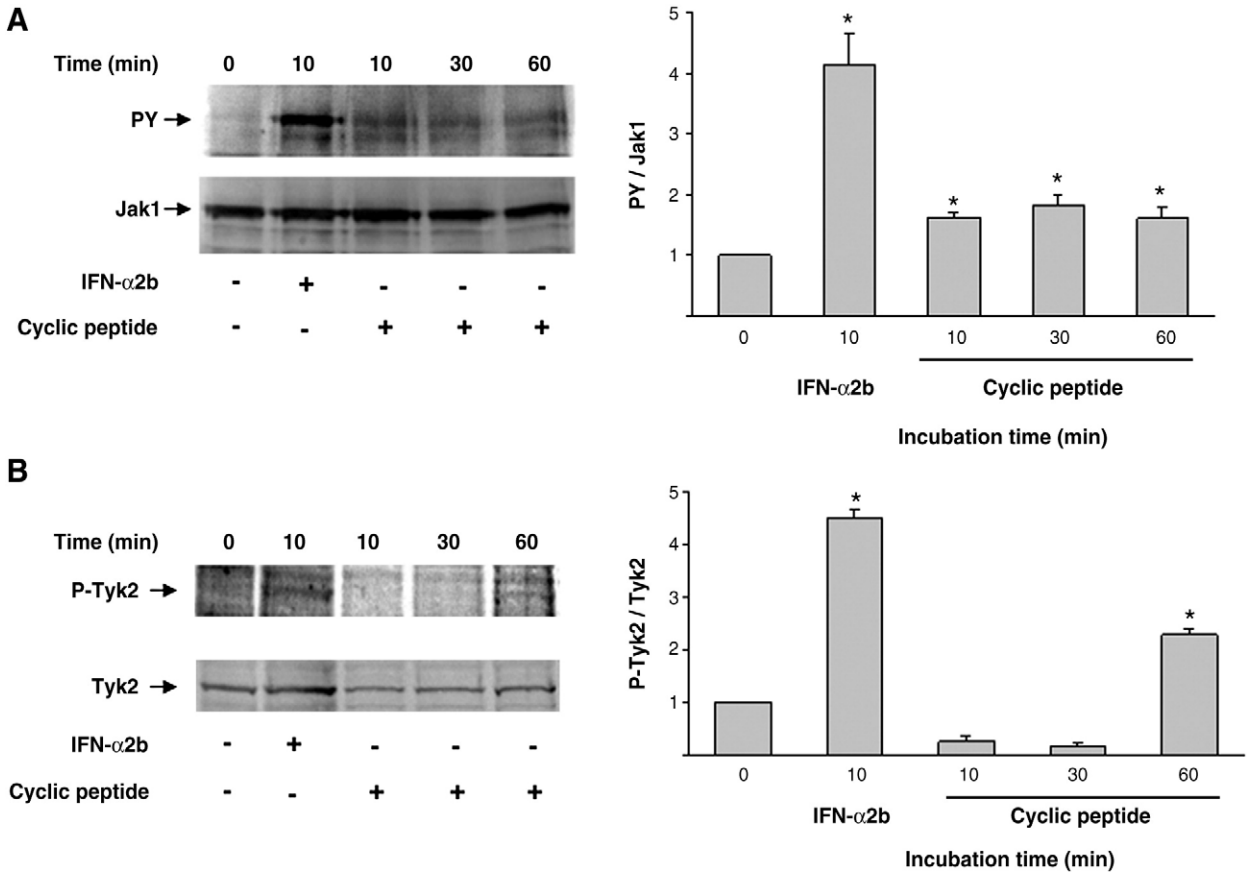

Fig. 1 – Cyclic peptide-induced phosphorylation of Janus kinases. WISH cells were maintained in the absence of FBS for 24 h and then incubated for different times with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. (A) Cells were lysed and incubated with anti-Jak1 and the corresponding immunoprecipitate was analyzed by western blotting with an anti-phosphotyrosine antibody (PY). Membranes were stripped and reprobed with the same anti-kinase antibody employed for immunoprecipitation. (B) Cells lysates were subjected to SDS–PAGE and western blot assays were performed with antibodies against anti-phospho-Tyk2 (P-Tyk2) and anti-Tyk2. Results from one representative experiment are shown (right panel). Quantification of tyrosine phosphorylation was performed by densitometric analysis. Results represent the mean ± SE of three independent experiments (left panel). *$p < 0.005$.

50 μl of lysis buffer (10 mM HEPES, pH 7.4; 50 mM NaCl, 2 mM $MgCl_2$; 5 mM EGTA; 1 mM phenylmethyl sulfonyl fluoride (PMSF); 2 μg/ml leupeptin; 2 μg/ml aprotinin) followed by three cycles of rapid freezing and thawing. Cell lysates were centrifuged at 17,000×*g* for 15 min, and total protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA). Aliquots containing 100 μg of protein were then diluted in assay buffer (20 mM HEPES; 132 mM NaCl; 6 mM KCl; 1 mM $MgSO_4$; 1.2 mM K_2HPO_4, pH 7.4), 20% glycerol, 5 mM DTT and incubated for 2 h at 37 °C with 50 μM of the fluorogenic caspase 3 substrate Ac-DEVD-AMC. Cleavage of substrate was monitored by AMC liberation in an SFM25 Konton Fluorometer using 355 nm excitation and 460 nm emission wavelengths. Results were expressed as a change in fluorescence units (per μg of protein) relative to control.

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

Effect of the chimeric cyclic peptide on the stimulation of the Jak/STAT pathway

It has been reported that Jak1 and Tyk2 tyrosine kinases are activated after binding of IFNsα to IFNα receptor [11-15]. Since we have previously found that the cyclic peptide behaves as a partial agonist of the IFN-α2b [22,23], we decided to examine whether the chimeric peptide-induced tyrosine phosphorylation of these Janus family kinases. Thus, phosphorylation kinetics of Jak1 and Tyk2 was studied in WISH cells incubated in the presence or absence of the cyclic peptide. In parallel, activation of both kinases was also evaluated after IFN-α2b treatment. In order to evaluate Jak1 phosphorylation, cells were lysed, immunoprecipitated with the corresponding anti-Jak1 antibody and then immunoblotted with anti-phosphotyrosine antibody (PY). As shown in Fig. 1A, phosphorylation of Jak1 was observed after 10 min of stimulation with the cyclic peptide and remained elevated for at least 60 min, although activation levels were lower than that obtained after

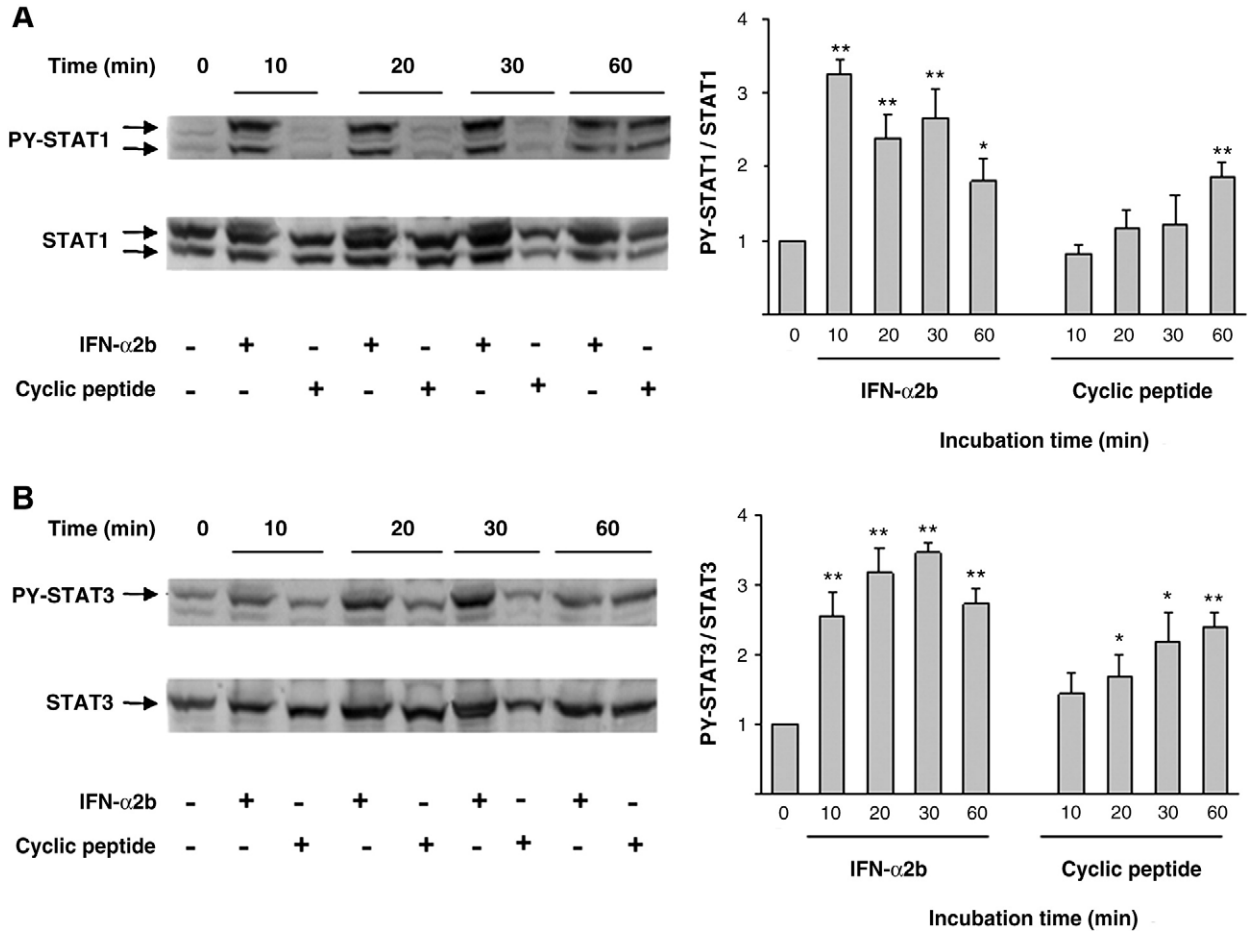

Fig. 2 – Tyrosine phosphorylation of STAT1 and STAT3 by the chimeric cyclic peptide. WISH cells were maintained in the absence of FBS for 24 h and then incubated for different times with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Cells lysates were subjected to SDS–PAGE under the conditions described in Materials and methods. Western blot assays were performed with antibodies against: (A) anti-phosphotyrosine-STAT1 (PY-STAT1) and anti-STAT1, (B) anti-phosphotyrosine-STAT3 (PY-STAT3) and anti-STAT3. Results from one representative experiment are shown (right panel). Data quantification was performed by densitometric analysis. Results represent the mean ± SE of three independent experiments (left panel). *$p<0.05$, **$p<0.005$.

10 min of exposure to IFN-α2b. Tyk2 activation was evaluated by Western blot analyses with anti-phospho-Tyk2 antibody. Tyk2 phosphorylation was evident after 10 min of treatment with IFN-α2b, but 60 min of incubation were required to detect a slight Tyk2 activation in cyclic peptide-treated cells (Fig. 1B).

IFNα activation of Jak kinases leads to tyrosine phosphorylation of several STAT proteins, which form homodimers or heterodimers that translocate to the nucleus and regulate the transcription of IFN-stimulated genes [11-15]. STAT1 and STAT3 phosphorylation kinetics were evaluated by Western blot analyses with phospho-specific antibodies. Tyrosine phosphorylation of STAT1 was detected after 10 min of exposure to IFN-α2b and remained elevated for at least 60 min post stimulation, whereas significant levels of phosphotyrosine-STAT1 were obtained only after 60 min of incubation with the cyclic peptide (Fig. 2A). A more similar pattern of STAT3 activation was obtained in WISH cells treated with IFN-α2b or the cyclic peptide. Thus, STAT3 tyrosine phosphorylation was observed after 10 or 20 min of exposure to IFN-α2b or cyclic peptide, respectively, although minor phosphorylation levels were induced by the peptide (Fig. 2B). Since it has been established that phosphorylation of STAT1 and STAT3 on Ser727 is required for maximal transcriptional activation [11-15,26-28], we decided to explore the ability of the chimeric peptide to induce serine phosphorylation of both STATs. Western blots analyses performed with specific anti-phosphoserine STAT1 and STAT3 antibodies were quite comparable to those obtained with anti-phosphotyrosine antibodies. Thus, serine phosphorylation of STAT1 was observed after 10 min of IFN-α2b treatment or 60 min of peptide incubation (Fig. 3A). STAT3 serine phosphorylation was evident after 10 min of exposure to IFN-α2b or cyclic peptide, and persisted for 60 min (Fig. 3B).

Role of STAT1 and STAT3 in the antiproliferative and apoptotic actions of the chimeric cyclic peptide

The contribution of STAT1 and STAT3 in the growth inhibitory and apoptotic responses induced by the chimeric peptide was further studied by inhibiting STAT protein expression using specific small interfering RNAs (siRNAs). When Western blots assays were performed to determine STAT depletion at the start of experiments, it was found that STAT1 and STAT3 levels diminished

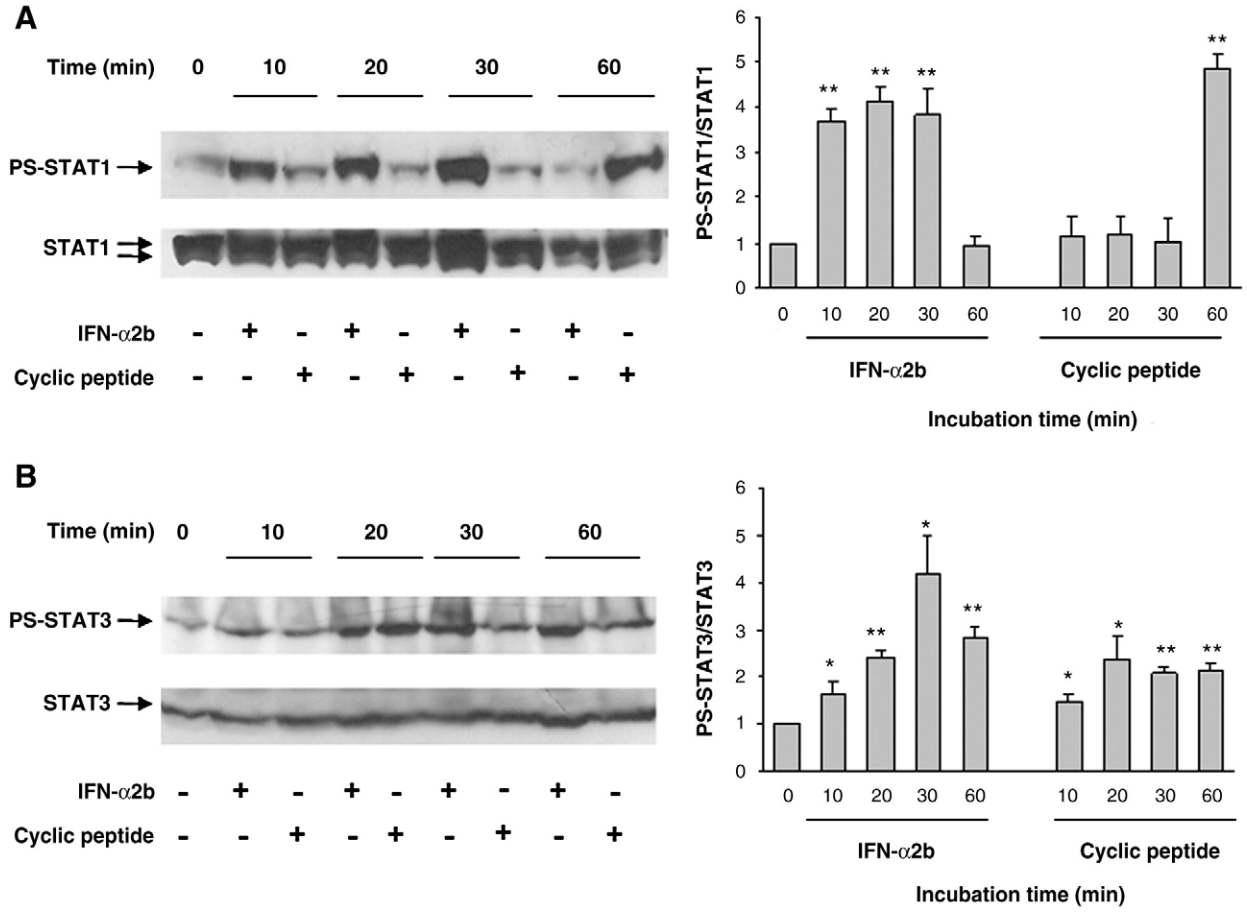

Fig. 3 – Serine phosphorylation of STAT1 and STAT3 by the chimeric cyclic peptide. WISH cells were maintained in the absence of FBS for 24 h and then incubated for different times with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Cells lysates were subjected to SDS–PAGE under the conditions described in Materials and methods. Western blot assays were performed with antibodies against: (A) anti-phosphoserine-STAT1 (PS-STAT1) and anti-STAT1, (B) anti-phosphoserine-STAT3 (PS-STAT3) and anti-STAT3. Results from one representative experiment are shown (right panel). Data quantification was performed by densitometric analysis. Results represent the mean ± SE of three independent experiments (left panel). *$p < 0.05$, **$p < 0.005$.

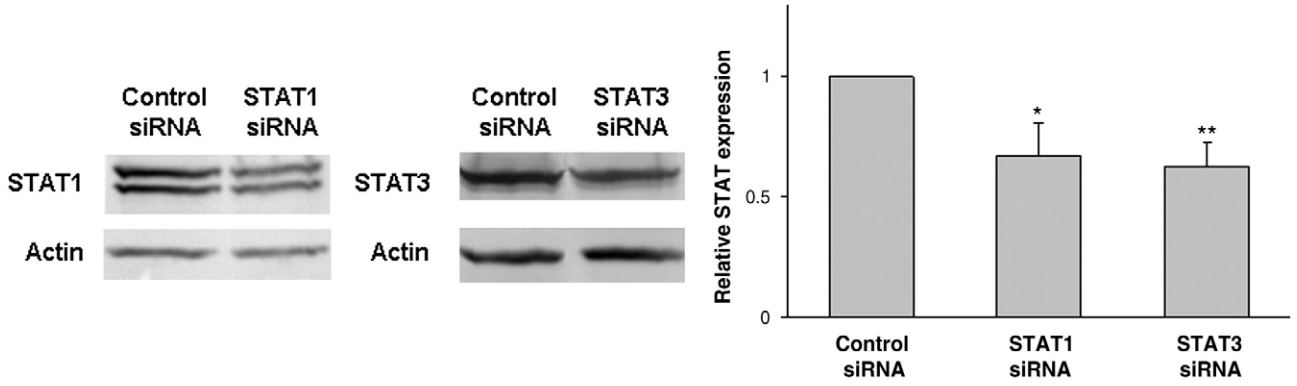

Fig. 4 – STAT1 and STAT3 expression levels after transfection with siRNA. WISH cells were incubated for 24 h in the presence of either specific siRNA or control siRNA according to the manufacturer's instruction. Then, cell lysates were subjected to SDS–PAGE and Western blot with the corresponding anti-STAT antibodies. Equal loading was confirmed by stripping and reprobing each blot for actin. Results from one representative experiment are shown (right panel). Quantification was performed by densitometric analysis (left panel). Results are expressed as mean ± SE of three independent experiments. *$p < 0.01$, **$p < 0.005$.

approximately 40% after 24 h of incubation of WISH cells with the corresponding STATs siRNA (Fig. 4). Tyrosine phosphorylation levels of STAT1 were also analyzed in knocked down cells in comparison with control cells. As shown in Fig. 5, densitometric analyses of Western blots assays revealed that both IFN- and peptide-induced STAT1 tyrosine phosphorylation levels diminished approximately 25% after 10 min or 1 h of incubation with IFN-α2b or peptide, respectively. Thus, a significant inhibition of STAT1 activation was observed. Similar results were obtained for STAT3 knockdown cells (data not shown). Taken together, our results showed that both STATs expression levels as well as activated STATs were affected in knocked down cells. Under these conditions, a significant reduction in the antiproliferative activity induced either by the cyclic peptide or the IFN-α2b was observed (Fig. 6A). Furthermore, a lower percentage of apoptotic cells, represented by the sub-G1 fractions derived from flow cytometry histograms, was obtained for specific siRNA-treated cells with respect to control siRNA cells incubated in the presence of IFN-α2b or cyclic peptide (Fig. 6B). Thus, after transfection with STAT1 siRNA, the sub-G_1 fraction of cells incubated with IFN-α2b decreased from 34 ± 5% to 23 ± 4% and from 28 ± 4% to 20 ± 1% for cyclic peptide-treated cells. In addition, when STAT3 siRNA cells were examined, the percentage of apoptotic cells diminished to 20 ± 2% both under the stimulus of IFN-α2b or the cyclic peptide (Fig. 6B).

Effect of the chimeric cyclic peptide on the stimulation of MAPK pathways

Among the main MAP kinase pathways, the p38 MAPK cascade plays an important role in the induction of IFNα-mediated biological effects [11,13,14,16]. Although the involvement of other members of MAPK, such as p44/42 (Erk) and JNK pathways, in the generation of signals induced by IFNα is still controversial, it

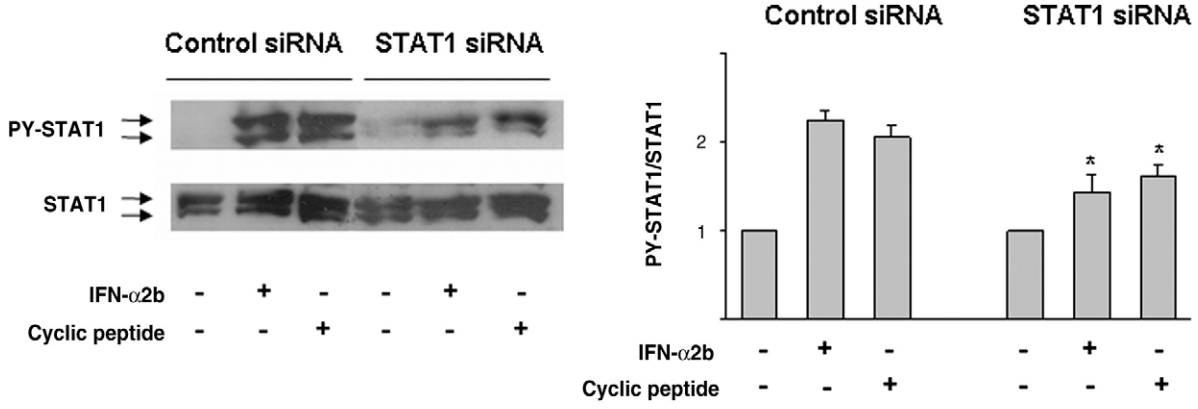

Fig. 5 – Peptide-induced tyrosine phosphorylation levels of STAT1 after transfection with siRNA. WISH cells were incubated for 24 h in the presence of either specific siRNA or control siRNA according to the manufacturer's instruction. Then, cells were maintained in the absence of FBS for 24 h and incubated for 10 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 anti-phosphotyrosine-STAT1 (PY-STAT1) and anti-STAT1. Results from one representative experiment are shown (right panel). Data quantification was performed by densitometric analysis (left panel). Results represent the mean ± SE of three independent experiments. *$p < 0.05$.

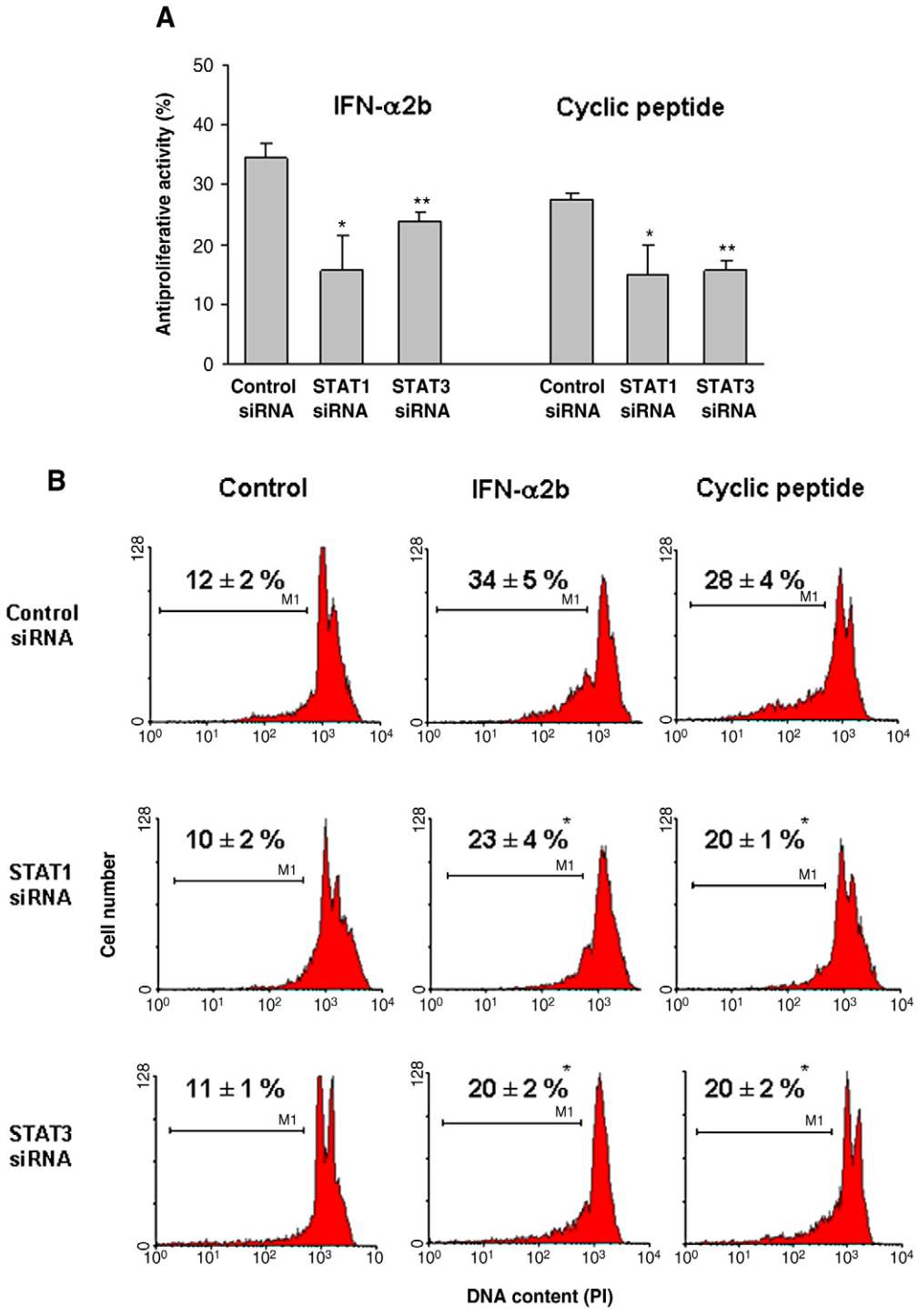

Fig. 6 – Effect of STAT1 and STAT3 depletion on the peptide-induced antiproliferative and apoptotic responses. (A) WISH cells transfected with specific siRNA or control siRNA were incubated for 48 h with 0.1 μg/ml of IFN-α2b or 10 μg/ml of cyclic peptide. The antiproliferative activity, evaluated after determining cell number by colorimetric determination of hexosaminidase levels, was calculated as the percentage of growth inhibition obtained in treated cells with respect to untreated control cells. Results are expressed as mean ± SE of three independent experiments. *$p < 0.05$, **$p < 0.01$. (B) After transfection with specific siRNA or control siRNA, WISH cells were incubated for 48 h in the absence (control) or presence of 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 (IFN-α2b or cyclic peptide-treated cells transfected with control siRNA) is indicated by *$p < 0.05$.

has been recently reported that these kinases are involved in the apoptotic cell death induced by IFNα in different cell lines [29]. To explore if these MAPK pathways are activated in WISH cells stimulated either with the cyclic peptide or IFN-α2b, phosphorylation kinetics of p38, p44/42 and JNK MAPKs were evaluated by Western blot analyses with phospho-specific antibodies. Both cyclic peptide and IFN-α2b rendered a similar phosphorylation pattern of p38 (Fig. 7A). Thus, p38 activation was reached after 15 min of incubation and phosphorylation levels remained elevated for at least 60 min post stimulation (Fig. 7A). No evidence of p44/42 (Fig. 7B) or JNK (Fig. 7C) activation was found after 60 min of exposure to either the cyclic peptide or IFN-α2b.

Since only p38 MAPK activation was observed, we then studied the effect of the specific inhibitor, SB203580 [30], on the antiproliferative activity exhibited by the cyclic peptide and IFN-α2b. When WISH cells were pre-treated with the p38 inhibitor, a significant decrease in the antiproliferative activity induced either by the cyclic peptide or the cytokine was observed (Fig. 8A). In addition, SB203580 treatment also reduced the amount of apoptotic cells obtained after incubation with the peptide or IFN-α2b (Fig. 8B). Thus, when cells incubated in the presence of cyclic peptide were pre-treated with the specific p38 inhibitor, the percentage of hypodiploid cells diminished from 32 ± 4% to 20 ± 5%. A similar behaviour was obtained for cells incubated with IFN-

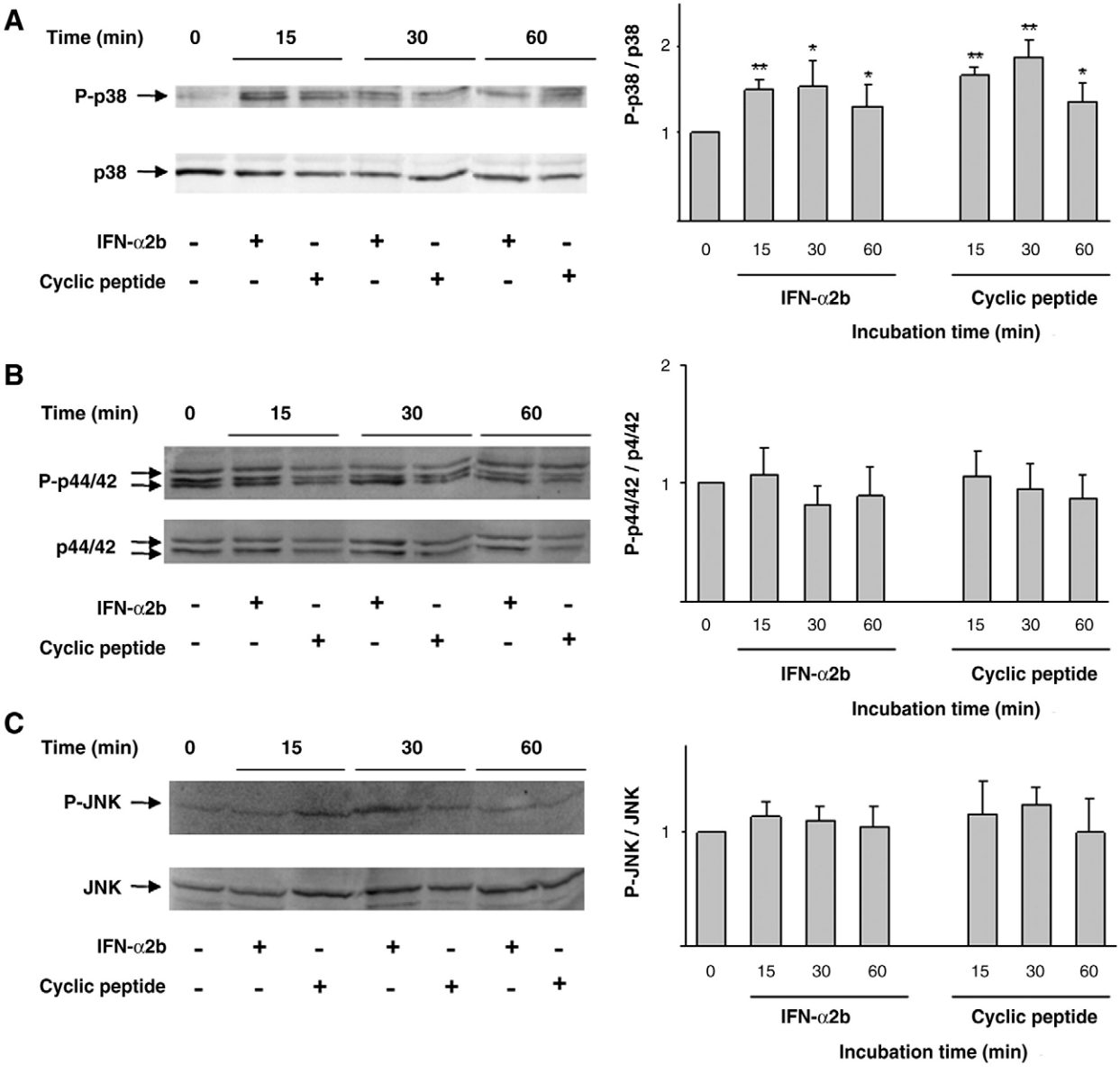

Fig. 7 – Phosphorylation of MAPKs by the cyclic peptide. WISH cells were maintained in the absence of FBS for 24 h and then incubated for different times with 0.2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Cells lysates were subjected to SDS–PAGE under the conditions described in Materials and methods. Western blot assays were performed with antibodies against: (A) anti-phospho-p38 (P-p38) and anti-p38 (p38), (B) anti-phospho-p44/42 (P-p44/42) and anti-p44/42 (p44/42), (C) anti-phospho-JNK (P-JNK) and anti-JNK. Results from one representative experiment are shown (left panels). Quantification of the western blots was performed by densitometric analysis (right panels). Results are expressed as mean ± SE of three independent experiments. *$p < 0.05$, **$p < 0.005$.

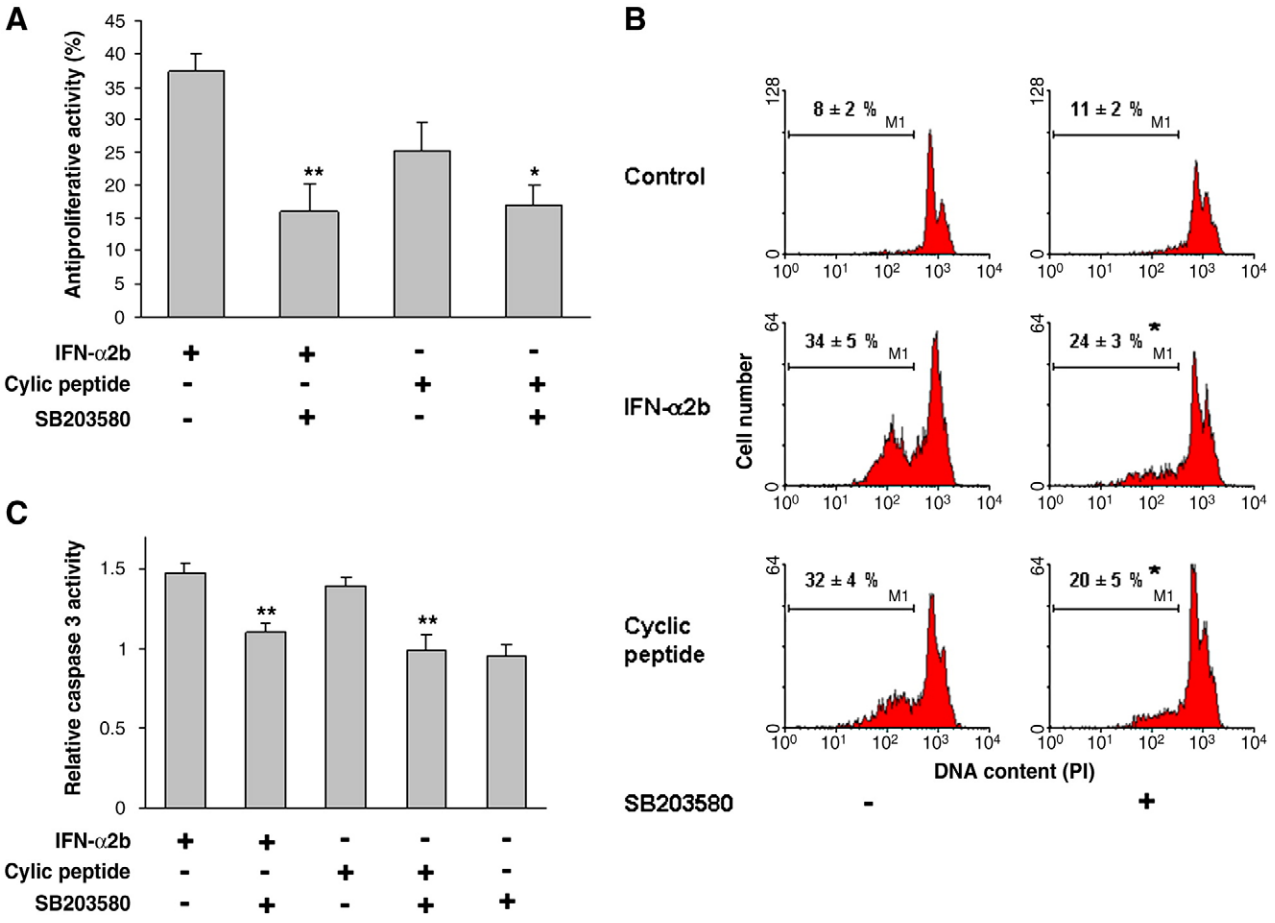

Fig. 8 – Effect of p38 MAPK inhibition on the antiproliferative and apoptotic effect induced by the cyclic peptide. (A) WISH cells were pre-treated for 1 h at 37 °C with or without 10 μM SB203580 (SB) and then incubated for 72 h with 0.1 μg/ml of IFN-α2b or 10 μ g/ml of cyclic peptide. Cell proliferation 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 (IFN-α2b or cyclic peptide-treated cells) is indicated by *$p < 0.02$, **$p < 0.005$. (B) After pre-treatment with or without 10 μM SB as described above, WISH cells were incubated for 48 h in the absence (control) or presence of 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 (IFN-α2b or cyclic peptide-treated cells incubated in the absence of SB) is indicated by *$p < 0.05$. (C) Cells treated in the presence or absence of 10 μM SB were incubated for 72 h with 2 μg/ml of IFN-α2b or 20 μg/ml of cyclic peptide. Caspase 3 activity was determined as indicated in Materials and methods. Enzymatic activities are expressed as percentage of the fluorescence per μg of protein of the treated sample with respect to the fluorescence per μg of protein of the control and represent mean values ± SE of three different experiments ($p < 0.005$).**

α2b since pre-incubation with the inhibitor decreased the percentage of apoptotic cells from 34 ± 5% to 24 ± 3% (Fig. 8B). The effect of SB203580 on the apoptotic response was also measured by determining the proteolytic activity of caspase 3. As shown in Fig. 8C, SB203580 incubation significantly diminished caspase 3 activity in cells stimulated either with the peptide or IFN-α2b.

Discussion

We have previously demonstrated that a chimeric cyclic peptide of the IFN-α2b behaves as an IFN-like molecule, which inhibits WISH cell proliferation by inducing apoptosis [22,23]. In this study, we explored the signaling pathways activated by the cyclic peptide and examined the contribution of some intracellular signals in the peptide-induced apoptotic response.

It is known that IFNα induces the dimerization and activation of the Jak kinases associated to IFNAR1 and IFNAR2 receptor subunits [11-15]. Thus, IFNAR2 interacts with Jak1, whereas IFNAR1 is associated with Tyk2. Activation of Jak1 and Tyk2 kinases was studied at different times in WISH cells stimulated either with the cyclic peptide or the IFN-α2b. As it was expected, cytokine stimulus was enough to phosphorylate both Jak1 and Tyk2 kinases after 10 min of treatment. Although in less extent, Jak1 was also activated by the cyclic peptide after 10 min of

incubation, whereas Tyk2 activation required longer incubation times, since significant levels of phospho-Tyk2 were detected in cyclic peptide-treated cells only after 60 min of exposure. The chimeric peptide herein studied mainly contains 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 [22]. IFN-α2b domains 29–35 and 122–140 have been previously proposed to be involved in the binding to IFNAR2 molecule, whereas the region 78–95 (helix C) would interact with IFNAR1 [31-33]. Other authors have later reported that some residues of helix E and the loop AB constitute the main binding region of IFN-α2b to IFNAR2, being probably minor the contribution of helix D to the interaction [34-37]. This new perspective of the IFN-α2b binding site does not exclude the fact that the sequence of the cyclic peptide herein employed represents a mimotope that would mainly interact with IFNAR2. Thus, Jak1 activation would be mediated by peptide binding to IFNAR2, whereas no Tyk2 phosphorylation would be at first expected. However, as it has been previously suggested that both receptor subunits would be in close proximity in the plasma membrane [38], it is possible to hypothesize that binding of the cyclic peptide to IFNAR2 may lead to a conformational change of this subunit which promotes the dimerization of both receptor chains and the subsequent activation of Tyk2 associated to IFNAR1. Alternatively, since some residues of the helix D have been recently reported to be involved in IFNAR1binding [39], we cannot exclude the possibility that some amino acids corresponding to the helix D of the chimeric peptide could weakly interact with IFNAR1. The late activation levels of Tyk2 could reflect the low-affinity interaction of the complex induced by the peptide with respect to that induced by the IFN-α2b molecule.

After activation of Jak1 and Tyk2 kinases, we studied tyrosine phosphorylation of STAT1 and STAT3 proteins in WISH cells incubated either with the cyclic peptide or the IFN-α2b. Although activation of STAT1 was evident after 10 min of treatment with IFN-α2b, the cyclic peptide showed a delayed response since significant levels of phosphotyrosine-STAT1 were obtained after 60 min of incubation. On the other hand, STAT3 phosphorylation kinetics was not so different in WISH cells treated with IFN-α2b or the cyclic peptide. This result should not be surprising since it has been reported that STAT3 activation is dependent on the intracellular domain of IFNAR2 [40], the receptor subunit almost certainly interacting with the cyclic peptide. Regarding STAT1 activation, we verified that STAT1 signaling was not secondarily activated by the peptide through stimulation of IFN-α2b production since peptide-induced STAT1 phosphorylation remains unchangeable in the presence of a neutralizing antibody that only recognizes the cytokine molecule (data not shown). It is known that STAT2 interacts constitutively with IFNAR2 [41], whereas STAT1 is associated to the IFNAR2–STAT2 complex. After cytokine binding to the receptor complex, STAT2 is recruited by its SH2 domain to a phosphotyrosine site of IFNAR1 [41,42]. This event is followed by the phosphorylation of STAT2 and the recruitment and activation of STAT1 [43,44]. Thus, tyrosine phosphorylation of STAT1 would require the participation of IFNAR1 and the activation of Tyk2 and STAT2. Based on these findings, the late activation of Tyk2 could be responsible of the delay in the formation of phosphotyrosine-STAT1. Thus, it is possible to suggest that STAT1 tyrosine phosphorylation would be dependent on the cyclic peptide-induced dimerization of both receptor chains. The potential interaction of the two receptor subunits after cyclic peptide binding is currently under study.

We also showed that peptide-induced serine phosphorylation of STAT1 and STAT3 was similar to tyrosine phosphorylation. It has been reported that several STAT proteins require serine phosphorylation to regulate a full transcription activity [26-28]. Concerning IFN action, it has been proposed that p38 MAPK could function as a serine kinase for STAT1 in HeLa cells stimulated with IFNα and IFNγ [45], while other reports rendered opposite results in other cell types [46,47]. Later, it was demonstrated that a member of the protein kinase C (PKC) family of proteins, PKCδ, mediates serine phosphorylation of STAT1 and behaves as an upstream regulator of p38 MAPK [48,49]. Although we showed that both peptide and IFN stimuli are able to activate p38 MAPK in WISH cells, the involvement of the p38 pathway in serine phosphorylation of STATs or the possible relationship between p38 and PKCδ remains to be established. Thus, further studies will be required to determine the serine kinase that regulates the phosphorylation of STAT1 and STAT3 induced by the cyclic peptide.

We further studied the participation of STAT1 and STAT3 proteins in the cell growth inhibitory activity and the apoptotic response induced by the cyclic peptide. Comparatively, results obtained in IFN-α2b-treated WISH cells were also evaluated. After blocking STAT1 and STAT3 expression by using specific siRNAs, we demonstrated a significant decrease in the antiproliferative activity induced either by the cyclic peptide or the IFN-α2b. Furthermore, a lower apoptotic effect was observed in cells partially depleted of STAT1 and STAT3 after treatment with the peptide or the cytokine. Thus, our results indicate that both STAT1 and STAT3 transcription factors are involved in the antitumor and apoptotic response mediated by the peptide or the cytokine in WISH cells. Different reports have underlined the role of STATs in cell growth regulation. STAT1 has been generally associated to growth suppressive effects and apoptosis, whereas STAT3 has been related to survival signals [50-52]. In this sense, it was recently found that activation of STAT3 signaling confers protection to IFNα-induced apoptosis in myeloma cell lines [53]. In spite of this finding, other authors have claimed a different role for STAT3. Thus, it was demonstrated that STAT3 expression in an IFN-resistant cell line markedly enhances the sensitivity of the cells to the antiviral and antiproliferative actions of IFNs [54]. In addition, it was also shown that the combination of IL-21 and IFNα triggers STAT3 activation and increases cell-mediated cytotoxicity and the antitumor activity *in vivo* [55].

When activation of p38, p44/42 and JNK MAPK pathways was studied, only p38 MAPK was found to be activated in WISH cells incubated in the presence of the cyclic peptide or the IFN-α2b. Similar p38 phosphorylation kinetics was obtained after treatment with either the peptide or the IFN-α2b. In addition, we showed that pharmacological inhibition of p38 MAPK reduced both the antiproliferative activity and the apoptotic effect induced either by the peptide or the cytokine, suggesting that p38 activation would be involved in the apoptotic response mediated by both stimuli. The role of p38 as a mediator of the antiproliferative activity induced by IFNα in hematopoietic cells has been previously reported [20,21]. It has also been demonstrated that both p38 MAPK and JNK pathways are involved in the apoptotic effect mediated by IFNα on human epidermoid carcinoma KB cells [56]. In addition, although p44/42 (Erk1/2) activation has been related to survival and proliferation, it has recently been shown that,

among other signals, Erk and JNK phosphorylation are involved in the apoptotic response induced by IFNα in a multiple myeloma cell line and a keratinocyte cell line [29]. However, another group has reported that IFNα can inhibit Erk signaling and induce apoptosis in basal cell carcinoma cells [57]. Based on these dissimilar findings, it is reasonable to propose that the role of these cascades on IFN-mediated biological actions depends on the particular cell type studied.

In conclusion, we examined for the first time the signal transduction pathways activated by a chimeric cyclic interferon-alpha2b peptide. Our results showed that the cyclic peptide induces similar signaling pathways to those activated by the cytokine and suggest that this derivative, although represents an epitope that mainly interacts with IFNAR2, would induce the dimerization of both receptor subunits. We also demonstrated that the activation of STAT1, STAT3 and p38 MAPK would be required to generate the antitumor action and the apoptotic effect induced by the cyclic peptide. This mechanistic study, together with the previous characterization of the apoptotic pathways triggered by the peptide [23], supports this chimera as a novel agent potentially useful for cancer therapy.

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