Carbohydrate Polymers 98 (2013) 224–232

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Fucan effect on CHO cell proliferation and migration

Leonardo Thiago Duarte Barreto Nobre[a,b], Arthur Anthunes Jacome Vidal[a], Jailma Almeida-Lima[a], Ruth Medeiros Oliveira[a], Edgar Jean Paredes-Gamero[b], Valquiria Pereira Medeiros[b], Edvaldo Silva Trindade[c], Celia Regina Cavichiolo Franco[c], Helena Bonciani Nader[b], Hugo Alexandre Oliveira Rocha[a,*]

[a] Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte – UFRN, Av. Senador Salgado Filho, 3000, Lagoa Nova, 59072-970 Natal, RN, Brazil
[b] Disciplina de Biologia Molecular, Depto. de Bioquímica, Universidade Federal de São Paulo – UNIFESP, Rua Tres de maio, 100, 4° andar, Vila Clementino, São Paulo, SP, Brazil
[c] Departamento de Biologia Celular, Universidade Federal do Paraná – UFPR, Curitiba, PR, Brazil

ARTICLE INFO

Article history:
Received 20 March 2013
Received in revised form 17 May 2013
Accepted 19 May 2013
Available online 25 May 2013

Keywords:
Fucoidan
Antiproliferative
Cancer
MAPK pathway

ABSTRACT

Fucan is a term used to denominate sulfated L-fucose rich polysaccharides. Here, a heterofucan, named fucan B, was extracted from the *Spatoglossum schröederi* seaweed. This 21.5 kDa galactofucan inhibited CHO-K1 proliferation and migration when fibronectin was the substrate. Fucan B derivatives revealed that such effects depend on their degree of sulfation. Fucan B did not induce cell death, but promoted G_1 cell cycle arrest. Western blotting and flow cytometry analysis suggest that fucan B binds to fibronectin and activates integrin, mainly integrin $\alpha5\beta1$, which induces FAK/RAS/MEK/ERK activation. FAK activation inhibits CHO-K1 migration on fibronectin and ERK blocks cell cycle progression. This study indicates that fucan B could be applied in developing new antitumor drugs.

1. Introduction

Sulfated fucans comprise a family of polydisperse polysaccharides composed of sulfated L-fucose and heterofucans, also known as fucoidans. They are not widespread in nature, occurring only in brown seaweed and tunicates (Pomin, 2010), therefore, seaweeds are its most important source. Each seaweed synthesizes its own fucan possessing unique structural characteristics, which reflect in their biological, pharmacological and biotechnological proprieties. Furthermore, these structural characteristics can be altered by biotic and abiotic factors to which the seaweed is exposed, as well as extraction and purification methods used in the attempt of their obtention. Recent reviews regarding seaweed fucans have been restricted to their structural characteristics and primary biological/pharmacological activities (Jiao, Yu, Zhang, & Ewart, 2011; Li, Lu, Wei, & Zhao, 2008).

Several fucans showed antiproliferative activity against different types of cells by blocking their passage in the G_1 phase of the cell cycle (Moreau et al., 2006). However, in most cases, the antiproliferative activity of seaweed fucans is associated with their ability to induce apoptosis in several cell lines. Apoptosis induction is often related to caspase activation (Aisa et al., 2005; Athukorala et al., 2009; Yamasaki-Miyamoto, Yamasaki, Tachibana, & Yamada, 2009); however, other proteins involved in cell survival pathways may be affected by the presence of sulfated polysaccharides (SP) in the culture medium. A recently published study reported a heterofucan, extracted from the seaweed *Sargassum filipendula*, with pro-apoptotic activity in which such compound inhibited HeLa (adenocarcinoma cervical cell) cell proliferation by inducing apoptosis through a caspase-independent mechanism where apoptosis is promoted mainly by mitochondrial release of an apoptosis-inducing factor (AIF) into cytosol (Costa et al., 2011).

The brown seaweed, *Spatoglossum schröederi*, synthesizes three heterofucans known as fucan A (Almeida-Lima et al., 2011), fucan B and fucan C (Rocha, Bezerra, et al., 2005; Rocha, Moraes, et al., 2005). The 21.5 kDa fucan B was purified and its structure was suggested to contain a central core composed of β-(1-4) linked galactose, partially sulfated at C3. 25% of galactose residues are linked in C2 to a tetrasaccharide formed of 3-sulfated α-(1-4) linked fucose, and one or two nonsulfated β-(1-4) linked xylose, at the reducing and nonreducing end, respectively (Rocha, Moraes, et al., 2005). Using a biotinylated fucan B as a probe, it was reported that it binds specifically to fibronectin, thereby hindering CHO cells adhesion to this matrix molecule (Rocha, Franco, et al., 2005), yet, its antiproliferative activity was not evaluated. Therefore, the aim of the present study was to evaluate its on CHO cell proliferation, cell cycle and

* Corresponding author.
E-mail addresses: hugo@cb.ufrn.br, hugo-alexandre@uol.com.br (H.A.O. Rocha).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.05.040

cell migration using several suitable methods. This investigation may provide evidence for the development of a possible alternative chemotherapeutic treatment for cancer.

2. Materials and methods

2.1. Fucan B extraction

The marine algae *S. schroederi* was collected on the seashore of Nisia Floresta, RN, Brazil. Immediately after collection, the algae was dried at 50 °C under ventilation and grounded in a blender. The seaweed was then treated with acetone to eliminate lipids and pigments. 100 g of defatted, dried, and powdered algae were suspended in 500 mL of 0.25 M NaCl, and the pH was adjusted to 8.0 with NaOH. Prolav 750 (3 mg/mL) (Prozyn Biosolutions, São Paulo, SP, Brazil), a mixture of alkaline proteases, was added to the mixture for proteolytic digestion. After 18 h of incubation at 60 °C with constant agitation, the mixture was filtered and the filtrate fractionated by acetone precipitation as follows: 0.5 volume of ice-cold acetone was added to the solution under gentle agitation and maintained at 4 °C for 24 h. The precipitate formed was collected by centrifugation (10,000 × *g*, 20 min), dried under vacuum, dissolved in distilled water, and analyzed. The operation was repeated by adding 0.6, 0.7, 0.9, 1.1, 1.3, and 2.0 volumes of acetone to the supernatant. The fraction precipitated with 0.9 volume of acetone contains the sulfated fucan B used in the present work. This polysaccharide was further purified by ion exchange chromatography (Lewatite from Bayer, São Paulo, Brazil) with increasing concentrations of NaCl (0.25–3.0 M). The eluates were precipitated with 2 volumes of methanol (18 h, 4 °C) being the precipitates collected by centrifugation (10,000 × *g*, 15 min), dried, and dissolved in distilled water for further analysis.

2.2. Agarose gel electrophoresis

Agarose gel electrophoresis of the acidic polysaccharides was performed in 0.6% agarose gel (7.5 cm × 10 cm × 0.2 cm thick) prepared in 0.05 M 1,3-diaminopropane acetate buffer pH 9.0, as previously described (Dietrich & Dietrich, 1976). Aliquots of the polysaccharides (about 50 μg) were applied to the gel and subjected to electrophoresis. The gel was fixed with 0.1% cetyltrimethylammonium bromide solution for 2 h, dried, and stained for 15 min with 0.1% toluidine blue in 1% acetic acid in 50% ethanol. The gel was, then, destained with the same solution without the dye.

2.3. Chemical analysis and monosaccharide composition

Total sugars, sulfate, protein and glucuronic acid were determined as previously described (Rocha, Moraes, et al., 2005).

The polysaccharides were hydrolyzed with 2 M HCl for 2 h at 100 °C and sugar composition was determined using a LichroCART® 250-4 column (250 mm × 40 mm) packed with Lichrospher® 100 NH2 (5 μm) coupled to a LaChrom Elite® HPLC system from VWR-Hitachi equipped with a refractive index detector (RI detector model L-2490). The sample mass used was 0.2 mg and analysis time was 25 min. The chromatogram was compared to those of sugar references: arabinose, fructose, fucose, galactose, glucose, glucosamine, mannose, and xylose.

The molecular weight of the fucans was determined by HPLC in 0.2 M NaCl, 0.5% ethanol, using a GF-250 column (Asahipak GF series, Asahi Chemical Industry Co., Yakoo, Japan). The column was calibrated with standard glycosaminoglycans, as described earlier (Rocha, Moraes, et al., 2005).

2.4. Fourier transformed infrared spectroscopy (FT-IR)

Sulfated polysaccharides (10 mg) were mixed thoroughly with dry potassium bromide. A pellet was prepared and the infrared spectrum between 500 and 4000 cm^{-1} was measured on a Thermo-Nicolet Nexus 470 ESP FT-IR spectrometer. Thirty-two scans with a resolution of 4 cm^{-1} were averaged and referenced against air.

2.5. Unspecific fucan desulfation

Polysaccharide desulfation was performed by solvolysis in dimethyl sulfoxide as used previously for desulfation of a sulfated fucans (Rocha, Moraes, et al., 2005). Using different reaction times, 1 h and 5 h, two desulfated fucan B; Fuc B 2.0 M 1 h and Fuc B 2.0 M 5 h, containing 15.0% and 7.6% of sulfate groups respectively, were obtained.

2.6. Cell culture

Chinese hamster ovary (CHO) cells (ATCC CCL-61) were donated by Prof. Jeffrey D. Esko (Glycobiology Research and training Center, UCSD, San Diego, California, USA). The cells were grown in F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum – FBS (Cutilab, Campinas, SP, Brazil) and penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (Sigma-Aldrich, St Louis, MO, USA), and maintained at 37 °C in 2.5% CO_2.

2.7. MTT assay

For MTT assay, cells were seeded into 96-well plates at a density of 5×10^3 cells/well and allowed to attach overnight in a 300 μL of fresh medium, incubated at 37 °C, 2.5% CO_2. Cells were, then, incubated for 24 h at 37 °C and 2.5% CO_2 in the presence of different concentrations (0.1–0.5 mg/mL) of Fuc B. After incubation, traces of sulfated polysaccharide fractions were removed by washing the cells twice with 200 μL PBS and applying 100 μL of fresh medium, as well as 10 μL of 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in PBS, to determine the effect of algal sulfated polysaccharides on cell viability. Cells were, then, incubated for 4 h at 37 °C, 2.5% CO_2. In order to solubilize the product of MTT cleavage, 100 μL of isopropanol containing 0.04 M HCl was added to each well and thoroughly mixed using a multichannel pipette. Within 1 h of HCl–isopropanol addition, absorbance at 570 nm was measured using a Multiskan Ascent Microplate Reader (Thermo Labsystems, Franklin, MA).

2.8. BrdU incorporation

Cells were seeded into 96-well plates at a density of 5×10^3 cells/well and allowed to attach overnight in 300 μL of fresh medium and incubated at 37 °C, 2.5% CO_2. In some cases the cells were added to fibronectin or vitronectin coated wells. After 12 h, the medium was removed and the fucan B in F12 medium was added to a final concentration of 0.1–0.5 mg/mL and incubated for 24 h, at 37 °C and 2.5% CO_2. After incubation, traces of fucan B were removed by washing the cells twice with 200 μL PBS and the BrdU incorporation was determined according to the manufacturer's instruction (BrdU cell proliferation assay kit – Cell Signaling, Danvers, MA, USA).

2.9. Annexin V-FITC/pi double staining and analysis by flow cytometry

In order to evaluate the effects of fucan B on cell death, a FITC/Annexin V Apoptosis Kit was used, with Dead Cell Annexin FITC and PI, for flow cytometry (Invitrogen, Catalog n° V13242).

Cells were grown in 6-well plates until they reached confluence of 2×10^5 cells/mL when they were stimulated to enter G_0 in a medium without serum for 48 h. Next, cells were to exit G_0 by adding F-12 medium supplemented 10% FBS, in the presence of fucan B (0.5 mg/mL). A negative control was prepared without the presence of fucan B. After 24 h, the cells were harvested using 2 mM EDTA PBS, collected and washed with cold PBS. The supernatant was discarded and cells were resuspended in 200 μL of 1× binding buffer. 5 μL of Annexin V-FITC and 1 μL of PI solution 100 μg/mL was added in 100 μL of cell suspension. Cells were incubated for 15 min at room temperature and kept under light protection. After the incubation time, 400 μL of binding buffer for annexin V (1×) was added and cells which were, then, analyzed by flow cytometry (flow cytometer FACSCANTO II, BD Biosciences), measuring fluorescence emission at 530–575 nm for annexin V and 630/22 nm for PI. FlowJo software v. 7.6.3 (Tree Star, Inc., CA, USA) was used for data analysis.

2.10. Expression of α5 integrin subunit

CHO-K1 cells were grown in the presence of fucan B (0.5 mg/mL) for 24 h, as described above. Cells were removed from the bottles with 2 mM EDTA PBS for 10 min, and 10^6 cells were suspended and washed 3 times with PBS containing 1% BSA. The cell suspensions were, then, incubated with MAB anti-α5 antibody (2 μg/mL) in PBS + 1% BSA for 1 h at 4 °C, washed with PBS and incubated with the secondary antibody (fluorescein isothiocyanate-conjugate goat antimouse IgG) for 30 min at 4 °C. Cells were washed 3 times with the FACS solution, as recommended by the manufacturer. Analyses of 40,000 events were performed using a FACSCANTO flow cytometer (Becton Dickinson Immunocytometry System, CA, USA).

2.11. Effect of fucan B on the CHO cells sulfated glycosaminoglycans synthesis

Fucan B effect on the synthesis of heparan sulfate and chondroitin sulfate by CHO-K1 cells was performed essentially as previously described (Rocha, Bezerra, et al., 2005). Cell protein was estimated by a Coomassie Blue method. All the experiments were performed in triplicate for each data point. The bars of the figures indicate the ±SD.

2.12. Western blotting

CHO cells at 80% confluence were incubated with fucan B and washed after 12, 24 and 48 h in ice-cold PBS. They were then scraped into 200 μL lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF], and protease inhibitors (1 mg/mL aprotinin, 10 mg/mL leupeptin and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride) and, then, kept on ice for 2 h. Protein extracts were cleared by centrifugation and protein concentrations were determined using a BCA protein assay kit (Pierce, USA), with bovine serum albumin as standard. An equal volume of sodium dodecyl sulfate (SDS) gel loading buffer [100 mM Tris–HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 0.1% bromophenol blue and 20% glycerol] was added to samples, which were subsequently boiled for 10 min. From each sample, 50 μg of protein was loaded onto SDS-PAGE and blotted onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 1% fat-free dried milk or 2% bovine serum albumin in Tris–buffered saline (TBS), with 0.05% Tween 20 (TBST), and incubated overnight at 4 °C with the appropriate primary antibody at 1:1000 dilution. After washing in TBST, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilution, in blocking buffer for 1 h. Intensity of the specific immunoreactive bands was detected by enhanced chemiluminescence (ECL), according to the manufacturer's protocol (Kirkegaard and Perry Laboratories), quantified by densitometry and expressed as a ratio of actin.

2.13. Motility assay

The motility assay was conducted as described by (Franco et al., 2009). Briefly, haptotactic motility experiments were performed using transwell motility chambers (8 μm pore size) (Costar Corp.). The undersurface of the polycarbonate membrane was coated with fibronectin (10 μg/mL in PBS). An insoluble solid-phase gradient was established by floating the filters (40 min at 37 °C), which were then blocked with 1% BSA (1 h, 37 °C). Cells (5×10^5) were suspended in 100 μL of F-12 medium and added to the upper chamber in the presence or absence (control) of different concentrations of fucan B, incubated at 37 °C in a humid atmosphere of 2.5% CO_2 for 5 h. Following incubation, cells on the upper surface were removed using a cotton-tipped applicator. Membranes were fixed with 2% formaldehyde for 10 min and stained with 1% toluidine blue in Borax for 10 min. The cells which migrated were visualized using an inverted microscope (Nikon, TE-300) and counted.

2.14. Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was done by one-way ANOVA using the Prisma 5.01 software. Tukey post-tests were performed for multiple group comparison. In all cases, statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Purification and characterization of fucan B

Fucans from *S. schröederi* were extracted using proteolysis and submitted to precipitation with different concentrations of acetone. The fractions underwent electrophoresis on agarose gel (Fig. 1A), indicating that the fraction obtained with 90% of acetone (v/v), denominated F0.9, was a fucan B-rich fraction, as previously reported (Rocha, Moraes, et al., 2005). The fucan B from fraction F0.9 was further purified using ion exchange chromatography on a Lewatite resin. Sulfated polysaccharides were separated into four new fractions eluted with 1.0, 1.5, 2.0, and 3.0 M NaCl. These were named F1.0 M, F1.5 M, F2.0 M, and F3.0 M, respectively. The compounds were submitted to electrophoresis on agarose gel and, as shown in Fig. 1B, only a single band was obtained in F1.0 M, F1.5 M, and F2.0 M. Electrophoretic mobilities of these bands correspond to fucan B electrophoretic mobility, as previously reported (Rocha, Moraes, et al., 2005). This indicates that we obtained three fractions of fucan B. Fraction F3.0 M was rich in fucan C (data not shown).

The chemical composition of the different fucan B fractions, F1.0 M, F1.5 M and F2.0 M, is shown in Table 1. All of them are composed of xylose, fucose, galactose, sulfate and a trace of glucuronic acid. The monosaccharide molar ratios of F1.0 M, F1.5 M, F2.0 M are very similar. The main difference in their chemical composition is the proportion of sulfate, which ranged from 14.1% (F1.0 M) to 19.0 (F2.0 M).

FT-IR spectroscopy is an easy-to-use technique and provides fast results. It is also a powerful tool for showing similarities between molecules. Table 2 illustrates the main signals observed in the FT-IR spectra of fucan B fractions from *S. schröederi*.

Characteristic sulfate absorptions were identified in the FT-IR spectra of fucan B fractions: signals around 1257 cm^{-1} are caused primarily by asymmetric S=O stretching vibration (Pielesz & Binias, 2010), while those at approximately 1043–1046 cm^{-1} are attributed mainly to symmetric C—O vibration associated with a C—O—SO_3 group (Zhang et al., 2010). These signals are strong,

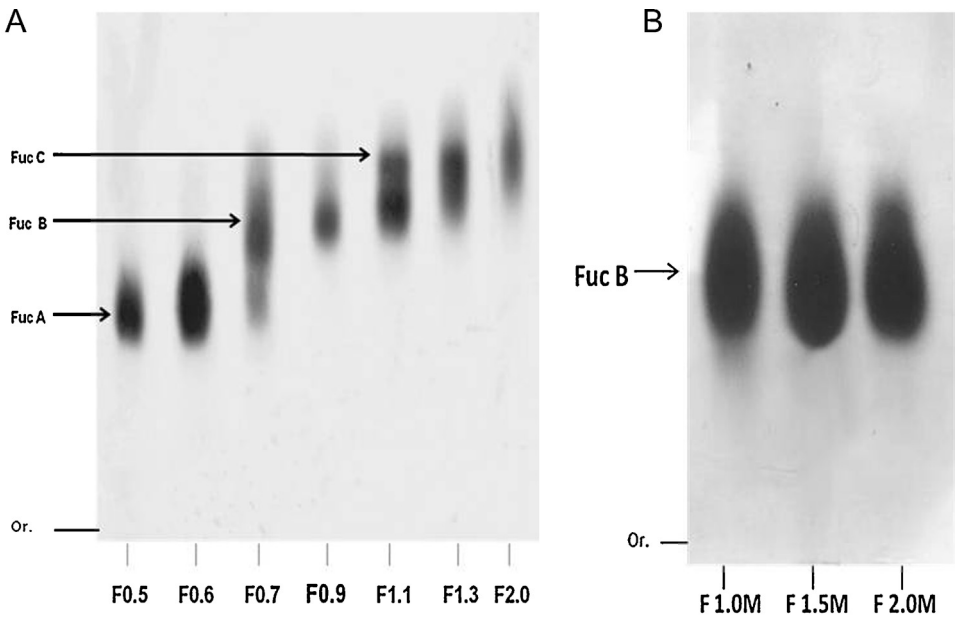

Fig. 1. (A) Electrophoresis in 0.05 M diaminopropane acetate buffer, pH 9.0, of fractions obtained by acetone precipitation. (B) Fucans B obtained through ion exchange chromatography. About 5 μL (50 μg) of each polysaccharide was applied in agarose gel prepared in diaminopropane acetate buffer and subjected to electrophoresis, as described in Section 2. F0.5; F0.6; F0.7; F0.9; F1.1; F1.3; F2.0 – fractions obtained by acetone precipitation. F1.0 M; F1.5 M; F2.0 M – fucan B fractions obtained from ion exchange chromatography. Or. – origin.

Table 1
Chemical composition of fucans from brown seaweed *S. schröederi*.

Fucans	Yield (%)	Sulfate (%)	Protein (%)	Molar ratio				
				Fucose	Galactose	Mannose	Xylose	Glucuronic acid
F1.0 M	32.9	14.1	nd	1.0	0.5	0.1	0.3	>0.1
F1.5 M	18.1	17.8	nd	1.0	1.9	0.0	0.5	>0.1
F2.0 M	38.8	19.0	nd	1.0	2.0	0.0	0.5	>0.1
Fuc B 2.0 M 1 h	–	15.0	nd	1.0	2.0	0.0	0.5	>0.1
Fuc B 2.0 M 5 h	–	7.5	nd	1.0	1.8	0.0	0.5	>0.1

nd – not detected; Fuc B 2.0 M 1 h and Fuc B 2.0 M 5 h – desulfated fucans B (see Section 2)

confirming the presence of a significant amount of sulfate in the polysaccharides. The band at 848 cm^{-1} is caused by the bending vibration of C—O—S of sulfate groups at the axial C-4 position (Pielesz & Binias, 2010). The IR features at 583 cm^{-1} are the result of symmetric O=S=O deformation of sulfates (Synytsya et al., 2010). In addition, all fractions showed signals at 3446–3447 cm^{-1} from the stretching vibration of O—H (Camara et al., 2011). The band around 1632–1645 cm^{-1} was caused by the carboxyl group of uronic acid, which overlaps with signals of H_2O deformation (Camara et al., 2011; Pielesz & Binias, 2010). The band at about 1420 cm^{-1} is caused by symmetric CH_2 deformation of galactose and asymmetric deformation of CH_3 from fucose (Pielesz & Binias, 2010).

The molecular weight of fucan B was calculated as 21.0 kDa (F1.0 M), 21.5 kDa (F1.5 M) and 21.5 (F2.0 M) using HPLC analysis.

Overall, the chemistry, FT-IR spectrum, and molecular weight of the fucan B F2.0 M isolated here were the same as those already reported for fucan B (Rocha, Moraes, et al., 2005), suggesting that we obtained the same fucan B as previously described. In addition, data showed that F1.0 M and F1.5 M are B fucans with less sulfate content.

Table 2
IR spectrum data of fucan B fractions from the brown seaweed *S. schröederi*.

Sulfated polysaccharides	IR signals (cm^{-1})
F1.0 M	3446; 2034; 1632; 1420; 1257; 1043; 848; 582
F1.5 M	3445; 2031; 1645; 1421; 1257; 1045; 848; 583
F2.0 M	3447; 2031; 1645; 1420; 1257; 1045; 848; 583

3.2. Antiproliferative activity of fucans B against CHO cells

In order to evaluate the antiproliferative effect of fucan B, we selected wild-type CHO-K1 and mutant CHO-745 cells, since the glycosaminoglycans (GAG) produced by mutant cells were about 5% of those synthesized by CHO-K1 (Franco et al., 2009). However, this decline in GAG did not affect CHO-745 proliferation, which is similar to that of the CHO-K1 cells (Franco et al., 2001). Therefore, since the effect of fucan B was similar in both cell lines, we can rule out the possibility that this occurs due to repulsion between fucans and GAG content of the cell surface. Our data showed that fucans B 1.0 M, 1.5 M and 2.0 M are the same compound, with different degrees of sulfation (Tables 1 and 2). As showed in Fig. 2, fucan B 2.0 M displayed a dose-dependent effect, reaching saturation at around 0.4 mg/mL for both cell lines. Thus, using a concentration of 0.4 mg/mL, we compared the antiproliferative effect of three fucans B. Fig. 2 also demonstrates that, regardless of the degree of sulfation, all fucans B exhibit antiproliferative activity at the same extension. During the treatment period, no cell morphology alterations were observed. Since fucan B 2.0 M showed the highest yield (Table 1), it was selected for subsequent testing and from here after it is referred to as fucan B or Fuc B.

It has been reported that fucan bioactivities are closely related to several structural parameters, such as degree of sulfation, sulfation

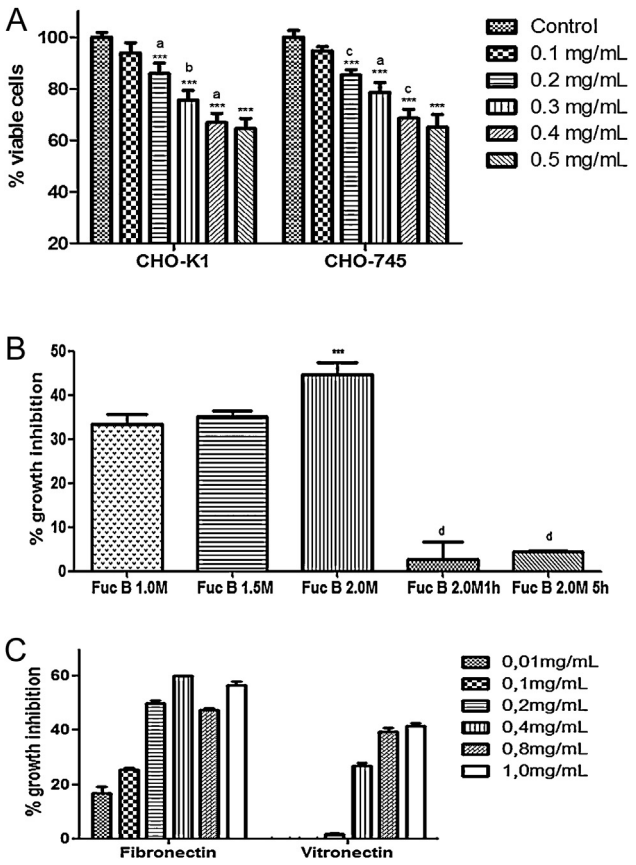

Fig. 2. (A) Effect of fucan B on CHO cell proliferation. The results are presented as percentage of control cells normalized to 100% in the absence of fucan B (mean ± SD of four determinations). (B) Effect of fucan B fractions and desulfated fucans B (Fuc B 2.0 M 1 h e Fuc B 2.0 M 5 h) on CHO-K1 proliferation. (C) Effect of fucan B on CHO-K1 growing on coated surface with extracellular matrix proteins. The rate of cell proliferation inhibition was determined using BrdU. Results are expressed as percentage of control cells normalized to 100% in the absence of polysaccharide (mean ± SD of four determinations). ***$P < 0.001$ compared to control. [a]$P < 0.05$ compared to other concentrations. [b]$P < 0.01$ compared to other concentrations. [c]$P < 0.001$ compared to other concentrations. [d]$P < 0.001$ compared to other fucans.

position, type of sugar and glycosidic branching. Structural analyses of oligofucans obtained from fucoidan of *Ascophyllum nodosum* showed that anticoagulant activity requires sulfation at all O-2 and some O-3 positions of fucose residues, whereas sulfation at O-4 seems unnecessary. In addition, 3- and/or 4-linked fucose residues are always present (Chevolot et al., 1999). However, there are no data regarding sulfation position for antiproliferative fucans. When fucans from *A. nodosum* were desulfated, they lost anticoagulant activity, although their ability to inhibit tumor cell proliferation was not affected (Haroun-Bouhedja, Ellouali, Sinquin, & Boisson-Vidal, 2000). In other words, the degree of sulfation is not as important to *Ascophyllum* fucan antiproliferative activity as sulfation position.

In order to determine the role of sulfate groups in the antiproliferative effect of fucan B, we used desulfated fucan B 2.0 M 1 h and Fuc B 2.0 M 5 h (see Section 2), containing 15% and 7.5% of sulfate groups, respectively. Data indicated (Fig. 2B) that none of the desulfated polysaccharides displayed antiproliferative activity. This is an interesting result because Fuc B 1.0 M contains 14.1% sulfate groups, less than fucan B 2.0 M 1 h. Nevertheless, the smaller amount of sulfate did not alter the antiproliferative activity of Fuc B 1.0 M in comparison to other fucans B. Since the desulfation method applied in this study removes the sulfate groups nonspecifically, specific relationship between sulfate position and bioactivity cannot be made. In addition, when we compared the monosaccharide composition of desulfated fucans with the native fucan we did not find any difference between them (Table 1). The data indicate that the sulfation position, as observed for *Ascophyllum* fucans, is more important than the degree of sulfation for fucan B antiproliferative activity. Further investigation using more specific desulfation methods will help to determine the role of sulfation position on fucan B antiproliferative activity.

Fucans can bind several growth factors and also affect cell metabolism (Irhimeh, Fitton, Ko, Lowenthal, & Nordon, 2011). Thus, fucan B was incubated with CHO-K1 cells and different FBS concentrations to identify whether the effect of fucans depends on the interaction of fucan and FBS molecules. We found that the amount of FBS did not affect fucan B antiproliferative activity (data not shown).

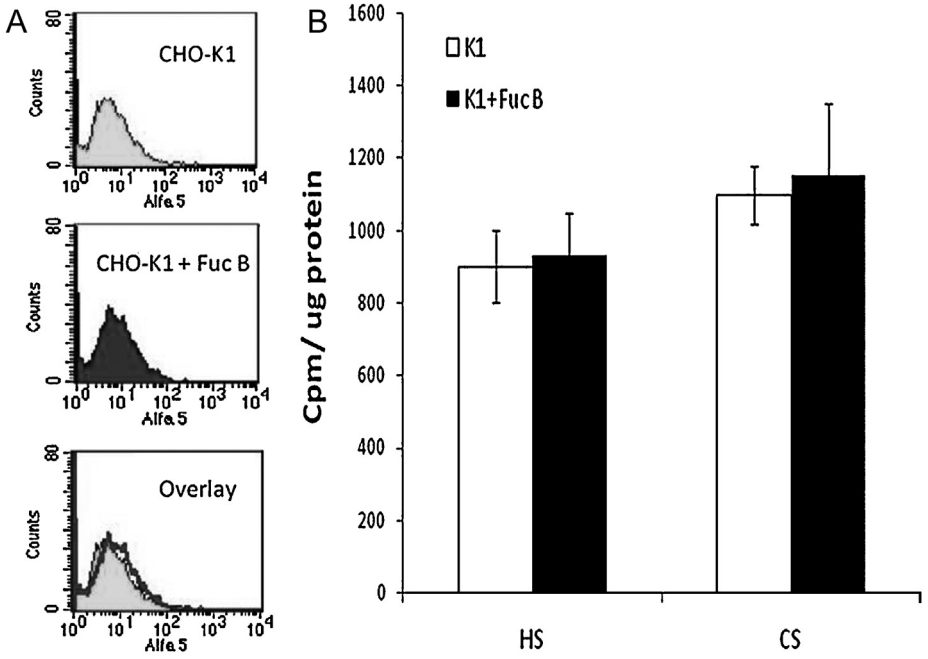

Fig. 3. (A) α5 expression of CHO-K1 in the presence of fucan B. (B) Stimulation of glycosaminoglycan synthesis by CHO-K1 exposed to fucan B. HS, heparan sulfate; CS, chondroitin sulfate; Fuc B – fucan B.

3.3. Fucan B binds fibronectin and inhibits CHO cell proliferation

Previously, confocal microscopy and flow cytometry showed that Fuc B binds the extracellular matrix of CHO cells, but not the CHO cell surface (Rocha, Bezerra, et al., 2005). In addition, fucans from *A. nodosum* are able to bind fibronectin and affect the cytoskeleton of MDA-MB-231 cells. In fact, there are, at least, four binding sites along the fibronectin chain for fucans, two of which are also heparin-binding sites (Liu et al., 2005). Thus, in order to identify whether the Fuc B molecular target is found in the extracellular matrix, CHO-K1 cells were seeded into wells coated with fibronectin (FN), collagen I, collagen IV, laminin (LN) or vitronectin (VN). Fucan B (from 0.01 mg/mL to 1 mg/mL) was then added and cells were stimulated to grow.

When cells were coated onto LN or collagens, Fuc B did not showed antiproliferative activity (data not shown), although it acted as an antiproliferative molecule when VN and particularly FN were used as substrate for cell proliferation (Fig. 2C). Indeed, Fuc B inhibits around 60% of CHO-K1 cell proliferation, which is greater than the effect observed when using plastic as substrate. Data indicate that Fuc B binds extracellular matrix proteins, mainly FN, and acts as an antiproliferative compound.

FN contains a binding site for proteoglycan-mediated cell adhesion (Dreyfuss et al., 2009), as well as an Arg-Gly-Asp (RGD) motif, which efficiently acts as the binding site for integrin-mediated cell adhesion (Kim, Turnbull, & Guimond, 2011). The α5β1 integrin and cell surface proteoglycans, such as syndecan IV, are the major FN adhesion receptors. These cooperate in signaling events, including cell proliferation and cell death. Moreover, we previously demonstrated that the α5β1 integrin in CHO cells is a hybrid proteoglycan, containing both heparan and chondroitin sulfate chains (Franco et al., 2009).

The antiproliferative effect of fucan B may be related to reduce FN cell surface receptors. To rule out this possibility, CHO-K1 cells were grown for 24 h in the presence or absence (control) of Fuc B. Next, we applied flow cytometry and found no difference between the expression of α5 integrin subunit on CHO cells grown in the presence or absence of Fuc B for 24 h (Fig. 3A). Another set of experiments showed that levels of heparan and chondroitin sulfate of

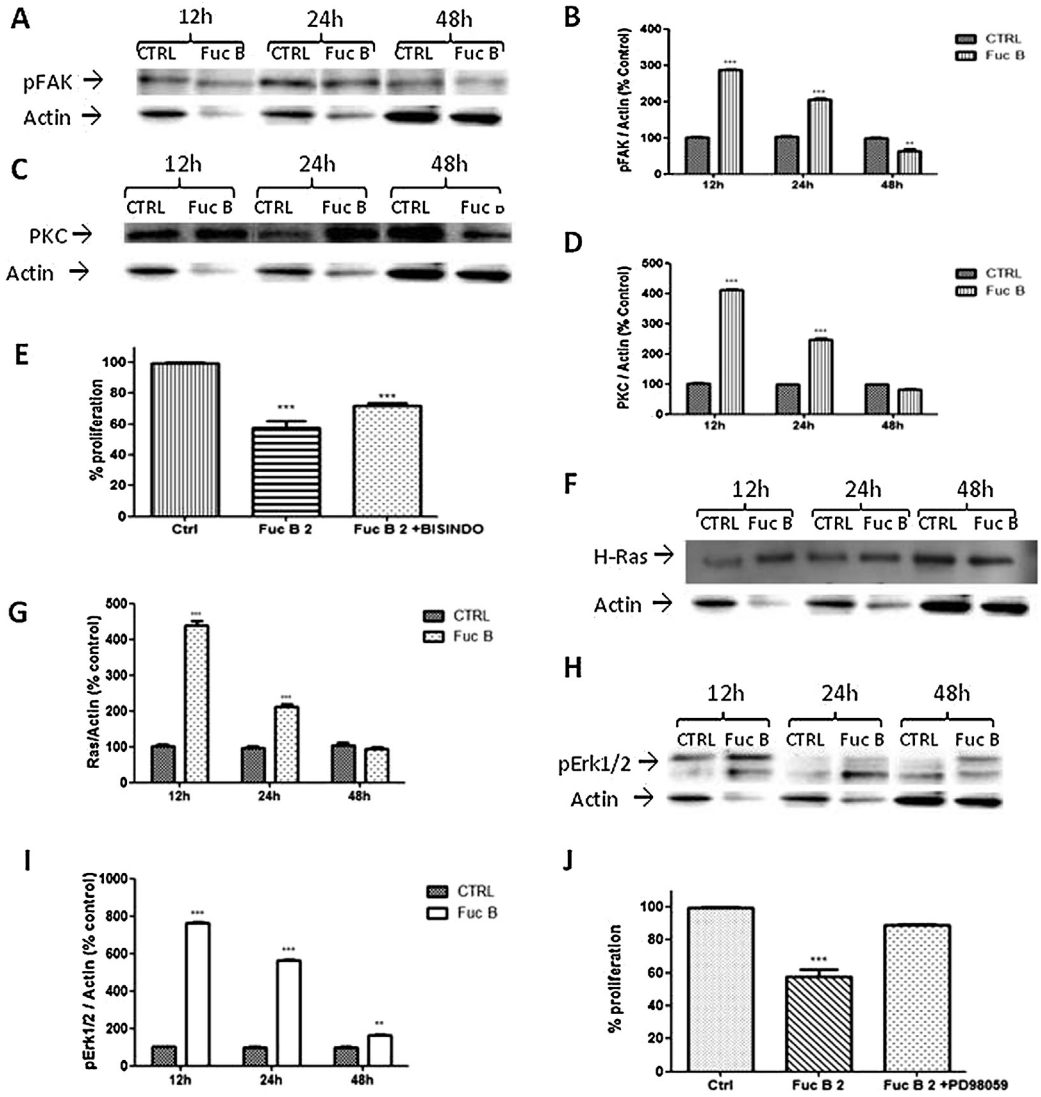

Fig. 4. Regulation of several intracellular proteins by fucan B. Western blot analysis was performed several times on CHO-K1 cells exposed to fucan B (0.5 mg/mL). 50 μg of protein from cell lysates was loaded per line, and expression of pFAK, PKC, RAS, and pERK1/2 (Figs. 5A, C, F and H) was analyzed by blotting with the corresponding specific antibody. One representative blot of three independent experiments is presented. Densitometry was carried out and data were expressed as the ratio between pFAK, PKC, RAS, and pERK1/2 and β-actin levels (Fig. 5B, D, G and I). CHO-K1 were stimulated to grow in the presence or absence of fucan B (0.5 mg/mL), PKC inhibitor Bisindolylmaleimide (Fig. 5E) or MEK inhibitor (PD98059) (Fig. 5J) for 24 h, after determining the rate of cell proliferation using BrdU.

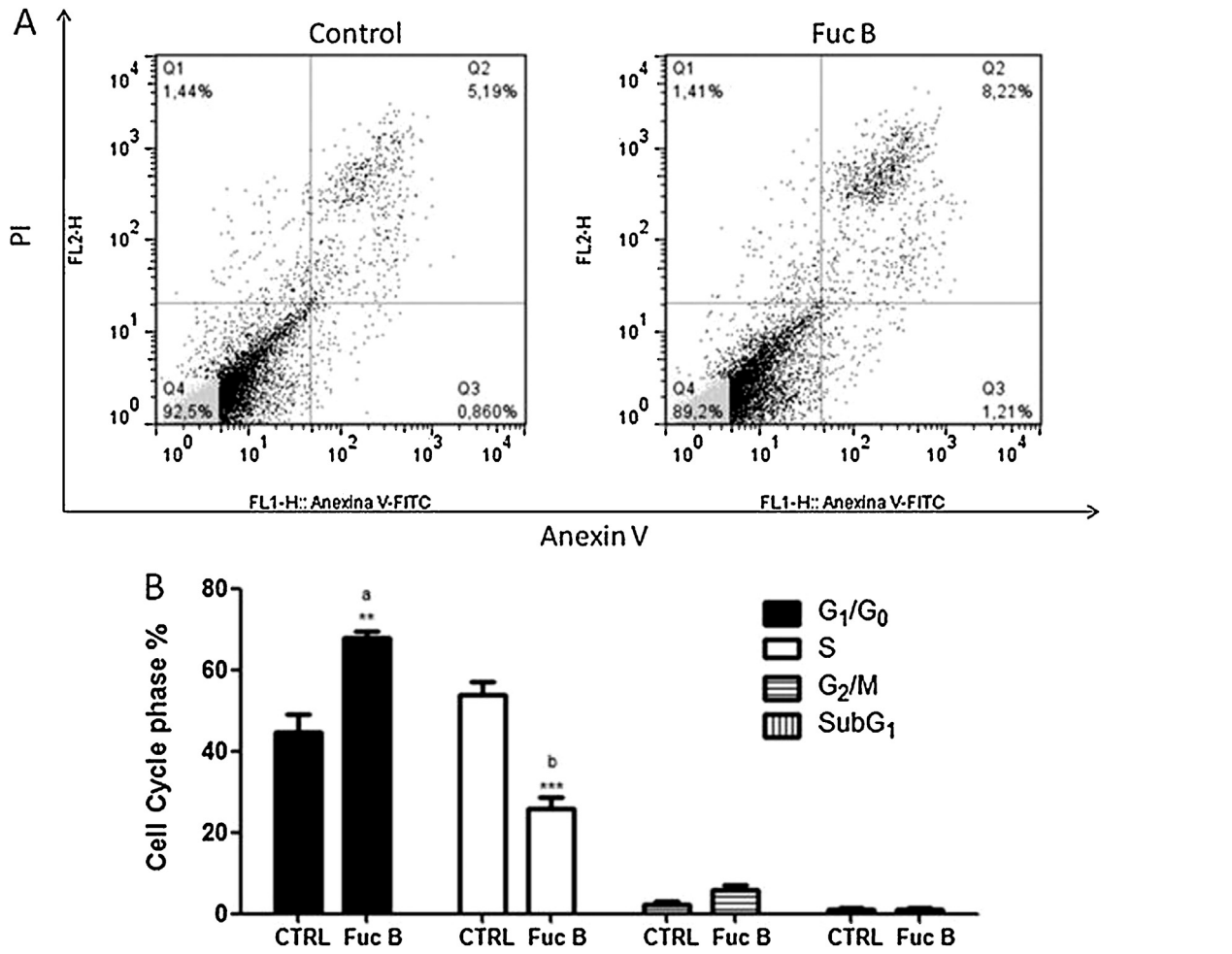

Fig. 5. (A) Flow cytometry analysis of apoptotic death of CHO-K1 cells by fucan B. One representative FACS assay of three independent experiments is presented. Annexin−/PI− (Q4), viable cells; Annexin+/PI− (Q3), cells undergoing apoptosis; Annexin+/PI+ (Q2), cells in end-stage apoptosis or already dead; Anexin−/PI+ (Q1), cells in necrosis. (B) Effect of fucan B on CHO-K1 cell cycle. CHO-K1 cells were grown to 50% confluence, treated for 24 h with fucan B, harvested and fixed with paraformaldehyde, then stained with PI and analyzed using flow cytometry.

CHO-K1 cells did not change when these cells were incubated with Fuc B (Fig. 3B).

3.4. Fucan B binds FN and active MAPKs

Cell-matrix communication occurs mainly through the binding of matrix proteins to receptors present on the cell surface, and in the case of the fibronectin, it occurs with membrane integrins. Thus, the next step was to investigate which proteins responsible for intracellular signaling could be affected by Fuc B. The first to be investigated was focal adhesion kinase (FAK), since it is closely associated with integrins (Telci et al., 2008). Western blot analysis demonstrated that treating CHO cells with Fuc B induced superior expression of focal adhesion kinase (FAK) (Fig. 4A and B). The highest level of expression of FAK occurred with 12 h of exposure to Fuc B, which was decreased to match the control at 48 h.

Activating FAK leads to activation of several intracellular proteins (Telci et al., 2008), among them, protein kinase C (PKC). After exposure to Fuc B, cells exhibited activation of PKC (Fig. 4C and D). However, when the cells were incubated with Fuc B and a specific PKC inhibitor, the antiproliferative effect of Fuc B was not significantly affected, despite showing a tendency to decrease (Fig. 4E).

FAK can promote activation of the Ras protein, leading to activation of the MAPK/ERK pathway, an important cell survival pathway. Thus, using Western blot analysis, we found that treating CHO cells with Fuc B induces activation of Ras (Fig. 4F and G). As observed with FAK, the highest levels of RAS activation were observed after 12 h of exposure to fucan. Following 48 h of treatment with Fuc B, RAS levels became similar to those recorded in cells not exposed to Fuc B.

The next protein analyzed was ERK. Fig. 4H and I shows that cells exposed to Fuc B exhibited the same pattern of ERK expression observed for FAK and RAS. A high amount of ERK was recorded after 12 h of incubation with Fuc B, which decreased time-dependently.

RAS occasionally regulates the activity of downstream effectors such as MEK and ERK. Therefore, a specific MEK inhibitor was used to assess whether the antiproliferative effect of Fuc B was dependent on this protein. CHO cells were exposed to Fuc B and the MEK inhibitor (PD98059) for 24 h. Next, cell proliferation was analyzed using BrdU incorporation. Data demonstrated that the MEK inhibitor abolished the Fuc B effect (Fig. 4J).

3.5. Effect of fucan B on CHO cell cycle

Data showed that the antiproliferative effect of fucan B is dependent on activation of the FAK/RAS/MEK/ERK cellular signaling route. Several studies show that fucans can induce cell death by stimulating this pathway (Kim et al., 2011; Kim, Lee, Choi, & Moon, 2010; Li et al., 2008; Liu et al., 2005; Nabeyrat, Jones, Fenwick, Barnes, & Donnelly, 2003). Thus, in order to obtain further

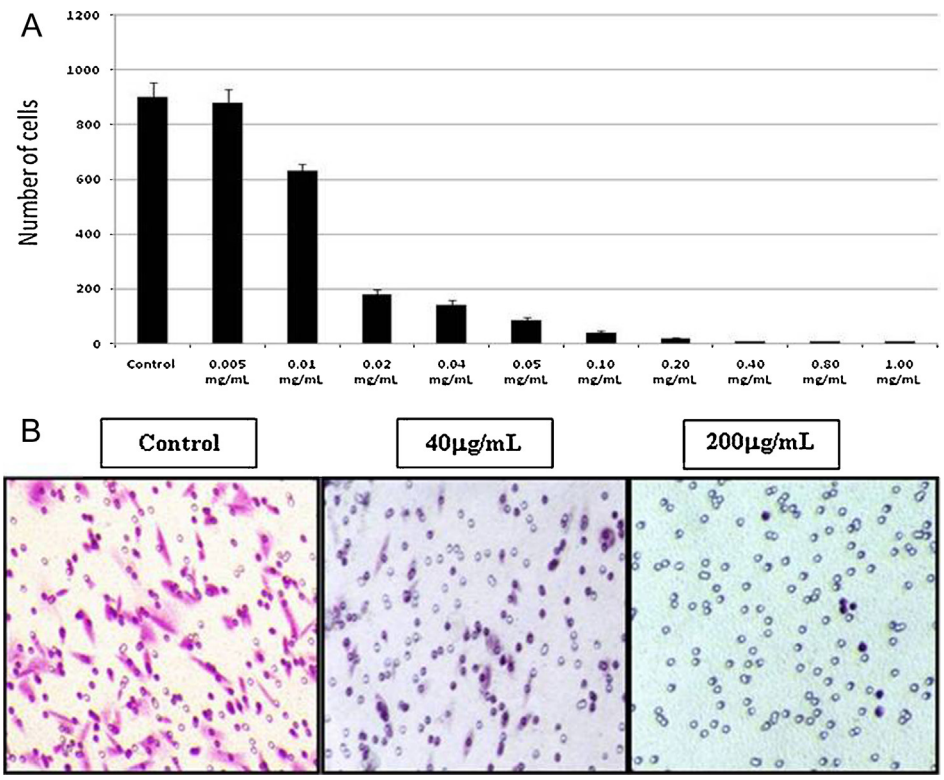

Fig. 6. (A) Motility on fibronectin of CHO-K1 cells exposed to fucan B. Cells on the other side of the membrane were stained with 1% toluidine blue and counted. Results are expressed as mean ± SD of three determinations. (B) CHO-K1 transwell migration at 40 µg/mL and 200 µg/mL of fucan B. Cells show purple or dark blue color.

insight on the mechanism of Fuc B induced cell death, cells were incubated with Fuc B, stained with annexin-V (apoptosis indicator) and iodide propidium (necrosis indicator) and submitted to flow cytometry analysis. As seen in Fig. 5A, Fuc B did not increase the percentage of iodide propidium or annexin V-positive cells in comparison to the control. This data suggest that Fuc B does not induce cell death.

In order to establish the effect of Fuc B on CHO cell cycle progression, flow cytometry analysis was performed on cells treated with Fuc B. Administration of fucan promoted a 20% increase in the proportion of cells in the G_1 phase and a corresponding decrease in the proportion of cells in the G_2/M phase (Fig. 5B). Other fucans inhibit cell proliferation by blocking the cell cycle. For instance, fucans from the seaweeds *Turbinaria ornata* (Deslandes et al., 2000) and *Bifurcaria bifurcata* inhibit lung carcinoma (NSCLC-N6) proliferation by blocking its passage in the G_1 phase of the cell cycle (Moreau et al., 2006). This effect was also observed with sulfated fucans from the seaweed *A. nodosum*, which inhibit proliferation of NSCLC-N6 cell lines by blocking cell growth in the G_1 phase (Riou et al., 1996). However, the molecular mechanisms of these fucans as blocking cell cycle compounds are yet to be determined.

3.6. Effect of fucan B on CHO cell migration

Integrins interact with the extracellular matrix, initiating several intracellular pathways that play important roles during many processes, including cell proliferation, differentiation and migration (Schaller, 2010). The FAK pathway is involved in the regulation of cell motility. Published data regarding the role of FAK in cell migration are intriguing, given that activation of this molecule is typically associated with increased migration (Schaller, 2010). However, several studies show that phosphorylation of FAK also inhibits migration (Borensztajn, Peppelenbosch, & Spek, 2010; Schaller, 2010). Since Fuc B increases FAK phosphorylation, we investigated whether Fuc B could regulate the CHO-K1 cell migration on fibronectin. Fig. 6 indicates that Fuc B inhibits cell migration dose-dependently, reaching saturation at around 0.1 mg/mL. However, when laminin was the substrate, no effect was observed until 1 mg/mL of Fuc B (data not shown).

A number of studies have shown that fucans inhibit endothelial cell migration by binding to several growth factors (Giraux et al., 1998; Lake et al., 2006). However, since we did not use fetal bovine serum in the cell migratory test, this possibility was ruled out. Thus, we hypothesized that Fuc B binds fibronectin and activates integrin, leading to decreased cell migration. This effect is related to the ability of the polysaccharide to promote phosphorylation of FAK.

4. Conclusion

This study evaluated the fucan B effect on CHO-K1 cell proliferation and migration. Fucan B exhibited a dose-dependent effect, which depended on its degree of sulfation. Data suggest that fucan B binds fibronectin and activates integrin, which induces FAK/RAS/MEK/ERK activation. ERK blocks cell cycle progression. FAK activation also inhibits CHO-K1 migration on fibronectin. This information could help the development of new antitumor agents. Furthermore, our data indicate that further studies are needed to evaluate the *in vivo* effect of fucan B.

Acknowledgments

This study was funded by grants from CNPq, MCT, FAPESP, FAPERN and CAPES. J. Almeida-Lima, L. Nobre, A. Vidal and R. Oliveira are recipients of fellowships from the CNPq or CAPES. We would like to thank Dr. Marcelo Lima for helpful suggestions.

References

Aisa, Y., Miyakawa, Y., Nakazato, T., Shibata, H., Saito, K., Ikeda, Y., et al. (2005). Fucoidan induces apoptosis of human HS-sultan cells accompanied by activation of caspase-3 and down-regulation of ERK pathways. *American Journal of Hematology*, *78*(1), 7–14.

Almeida-Lima, J., Dantas-Santos, N., Gomes, D. L., Cordeiro, S. L., Sabry, D. A., Costa, L. S., et al. (2011). Evaluation of acute and subchronic toxicity of a non-anticoagulant, but antithrombotic algal heterofucan from the Spatoglossum schroederi in Wistar rats. *Revista Brasileira De Farmacognosia*, *21*(4), 674–679.

Athukorala, Y. A., Jee, G. N., Kim, Y., Kim, G., Ha, S., Kang, J., et al. (2009). Antiproliferative activity of sulfated polysaccharide isolated from an enzymatic digest of Ecklonia cava on the U-937 cell line. *Journal of Applied Phycology*, *21*(5), 307–314.

Borensztajn, K., Peppelenbosch, M. P., & Spek, C. A. (2010). Coagulation Factor Xa inhibits cancer cell migration via LIMK1-mediated cofilin inactivation. *Thrombosis Research*, *125*(6), e323–e328.

Camara, R. B., Costa, L. S., Fidelis, G. P., Nobre, L. T., Dantas-Santos, N., Cordeiro, S. L., et al. (2011). Heterofucans from the brown seaweed Canistrocarpus cervicornis with anticoagulant and antioxidant activities. *Marine Drugs*, *9*(1), 124–138.

Chevolot, L., Foucault, A., Chaubet, F., Kervarec, N., Sinquin, C., Fisher, A. M., et al. (1999). Further data on the structure of brown seaweed fucans: Relationships with anticoagulant activity. *Carbohydrate Research*, *319*(1–4), 154–165.

Costa, L. S., Telles, C. B., Oliveira, R. M., Nobre, L. T., Dantas-Santos, N., Camara, R. B., et al. (2011). Heterofucan from Sargassum filipendula induces apoptosis in HeLa cells. *Marine Drugs*, *9*(4), 603–614.

Deslandes, E., Pondaven, P., Auperin, T., Roussakis, C., Guézennec, C., Stiger, J., et al. (2000). Preliminary study of the in vitro antiproliferative effect of a hydroethanolic extract from the subtropical seaweed *Turbinaria ornata* (Turner) J. Agardh on a human non-small-cell bronchopulmonary carcinoma line (NSCLC-N6). *Journal of Applied Phycology*, *12*(3), 257–262.

Dietrich, C. P., & Dietrich, S. M. (1976). Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. *Analytical Biochemistry*, *70*(2), 645–647.

Dreyfuss, J. L., Regatieri, C. V., Jarrouge, T. R., Cavalheiro, R. P., Sampaio, L. O., & Nader, H. B. (2009). Heparan sulfate proteoglycans: Structure, protein interactions and cell signaling. *Anais da Academia Brasileira de Ciencias*, *81*(3), 409–429.

Franco, C. R., Rocha, H. A., Trindade, E. S., Santos, I. A., Leite, E. L., Veiga, S. S., et al. (2001). Heparan sulfate and control of cell division: Adhesion and proliferation of mutant CHO-745 cells lacking xylosyl transferase. *Brazilian Journal of Medical and Biological Research*, *34*(8), 971–975.

Franco, C. R., Trindade, E. S., Rocha, H. A., da Silveira, R. B., Paludo, K. S., Chammas, R., et al. (2009). Glycosaminoglycan chains from alpha5beta1 integrin are involved in fibronectin-dependent cell migration. *Biochemistry and Cell Biology-Biochimie et Biologie Cellulaire*, *87*(4), 677–686.

Giraux, J. L., Matou, S., Bros, A., Tapon-Bretaudiere, J., Letourneur, D., & Fischer, A. M. (1998). Modulation of human endothelial cell proliferation and migration by fucoidan and heparin. *European Journal of Cell Biology*, *77*(4), 352–359.

Haroun-Bouhedja, F., Ellouali, M., Sinquin, C., & Boisson-Vidal, C. (2000). Relationship between sulfate groups and biological activities of fucans. *Thrombosis Research*, *100*(5), 453–459.

Irhimeh, M. R., Fitton, J. H., Ko, K. H., Lowenthal, R. M., & Nordon, R. E. (2011). Formation of an adherent hematopoietic expansion culture using fucoidan. *Annals of Hematology*, *90*(9), 1005–1015.

Jiao, G. L., Yu, G. L., Zhang, J. Z., & Ewart, H. S. (2011). Chemical Structures and Bioactivities of Sulfated Polysaccharides from Marine Algae. *Marine Drugs*, *9*(2), 196–223.

Kim, S. H., Turnbull, J., & Guimond, S. (2011). Extracellular matrix and cell signalling: The dynamic cooperation of integrin, proteoglycan and growth factor receptor. *Journal of Endocrinology*, *209*(2), 139–151.

Kim, W. J., Lee, S. J., Choi, Y. D., & Moon, S. K. (2010). Decursin inhibits growth of human bladder and colon cancer cells via apoptosis, G1-phase cell cycle arrest and extracellular signal-regulated kinase activation. *International Journal of Molecular Medicine*, *25*(4), 635–641.

Lake, A. C., Vassy, R., Di Benedetto, M., Lavigne, D., Le Visage, C., Perret, G. Y., et al. (2006). Low molecular weight fucoidan increases VEGF165-induced endothelial cell migration by enhancing VEGF165 binding to VEGFR-2 and NRP1. *Journal of Biological Chemistry*, *281*(49), 37844–37852.

Li, B., Lu, F., Wei, X. J., & Zhao, R. X. (2008). Fucoidan: Structure and bioactivity. *Molecules*, *13*(8), 1671–1695.

Liu, J. M., Bignon, J., Haroun-Bouhedja, F., Bittoun, P., Vassy, J., Fermandjian, S., et al. (2005). Inhibitory effect of fucoidan on the adhesion of adenocarcinoma cells to fibronectin. *Anticancer Research*, *25*(3B), 2129–2133.

Moreau, D., Thomas-Guyon, H., Jacquot, C., Jugé, M., Culioli, G., Ortalo-Magné, A., et al. (2006). An extract from the brown alga Bifurcaria bifurcata induces irreversible arrest of cell proliferation in a non-small-cell bronchopulmonary carcinoma line. *Journal of Applied Phycology*, *18*(1), 87–93.

Nabeyrat, E., Jones, G. E., Fenwick, P. S., Barnes, P. J., & Donnelly, L. E. (2003). Mitogen-activated protein kinases mediate peroxynitrite-induced cell death in human bronchial epithelial cells. *American Journal of Physiology – Lung Cellular and Molecular Physiology*, *284*(6), L1112–L1120.

Pielesz, A., & Binias, W. (2010). Cellulose acetate membrane electrophoresis and FTIR spectroscopy as methods of identifying a fucoidan in Fucusvesiculosus Linnaeus. *Carbohydrate Research*, *345*(18), 2676–2682.

Pomin, V. H. (2010). Structural and functional insights into sulfated galactans: A systematic review. *Glycoconjugate Journal*, *27*(1), 1–12.

Riou, D., Colliec-Jouault, S., Pinczon du Sel, D., Bosch, S., Siavoshian, S., Le Bert, V., et al. (1996). Antitumor and antiproliferative effects of a fucan extracted from ascophyllum nodosum against a non-small-cell bronchopulmonary carcinoma line. *Anticancer Research*, *16*(3A), 1213–1218.

Rocha, H. A., Bezerra, L. C., de Albuquerque, I. R., Costa, L. S., Guerra, C. M., de Abreu, L. D., et al. (2005). A xylogalactofucan from the brown seaweed Spatoglossum schroederi stimulates the synthesis of an antithrombotic heparan sulfate from endothelial cells. *Planta Medica*, *71*(4), 379–381.

Rocha, H. A., Franco, C. R., Trindade, E. S., Veiga, S. S., Leite, E. L., Nader, H. B., et al. (2005). Fucan inhibits Chinese hamster ovary cell (CHO) adhesion to fibronectin by binding to the extracellular matrix. *Planta Medica*, *71*(7), 628–633.

Rocha, H. A., Moraes, F. A., Trindade, E. S., Franco, C. R., Torquato, R. J., Veiga, S. S., et al. (2005). Structural and hemostatic activities of a sulfated galactofucan from the brown alga Spatoglossum schroederi. An ideal antithrombotic agent? *Journal of Biological Chemistry*, *280*(50), 41278–41288.

Schaller, M. D. (2010). Cellular functions of FAK kinases: Insight into molecular mechanisms and novel functions. *Journal of Cell Science*, *123*(Pt 7), 1007–1013.

Synytsya, A., Kim, W., Kim, S., Pohl, R., Synytsya, A., Kvasnička, F., et al. (2010). Structure and antitumour activity of fucoidan isolated from sporophyll of Korean brown seaweed Undaria pinnatifida. *Carbohydrate Polymers*, *81*, 41–48.

Telci, D., Wang, Z., Li, X., Verderio, E. A., Humphries, M. J., Baccarini, M., et al. (2008). Fibronectin-tissue transglutaminase matrix rescues RGD-impaired cell adhesion through syndecan-4 and beta1 integrin co-signaling. *Journal of Biological Chemistry*, *283*(30), 20937–20947.

Yamasaki-Miyamoto, Y., Yamasaki, M., Tachibana, H., & Yamada, K. (2009). Fucoidan induces apoptosis through activation of caspase-8 on human breast cancer MCF-7 cells. *Journal of Agricultural and Food Chemistry*, *57*(18), 8677–8682.

Zhang, Z., Wang, F., Wang, X., Liu, X., Hou, Y., & Zhang, Q. (2010). Extraction of the polysaccharides from five algae and their potential antioxidant activity in vitro. *Carbohydrate Polymers*, *82*(1), 118–121.