Biochimie 107 (2014) 350–357

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Proliferation and migration activities of fibroblast growth factor-2 in endothelial cells are modulated by its direct interaction with heparin affin regulatory peptide

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ARTICLE INFO

Article history:
Received 3 April 2014
Accepted 2 October 2014
Available online 12 October 2014

Keywords:
Growth factors
Interaction
Proliferation
Migration
Protect and label

ABSTRACT

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. In normal or pathological angiogenesis, angiogenic growth factors activate cognate receptors on endothelial cells. Fibroblast growth factor-2 (FGF-2) and heparin affin regulatory peptide (HARP) are two heparin-binding growth factors and were described for their pro-angiogenic properties on human umbilical endothelial cells (HUVEC). We now show that HARP acts as a mediator of FGF-2's stimulatory effects, since it is able to inhibit the proliferation and migration of HUVEC induced by FGF-2. We demonstrate by ELISA and optical biosensor binding assay that HARP and FGF-2 interact through direct binding. We have adapted a previously developed structural proteomics method for the identification of residues involved in protein–protein interactions. Application of this method showed that two sequences in HARP were involved in binding FGF-2. One was in the C-thrombospondin type 1 repeat (C-TSR-1) domain and the other in the C-terminal domain of HARP. The identification of these regions as mediating the binding of FGF-2 was confirmed by ELISA using synthetic peptides, which are as well mediators of FGF-2-induced proliferation, migration and tubes formation on HUVEC *in vitro*. These results imply that besides a regulation of the proliferation, migration and angiogenesis of HUVEC by direct interaction of FGF-2 with its receptors, an alternative pathway exists involving its binding to growth factors such as HARP.

1. Introduction

Angiogenesis plays a key role in physiological and physiopathological processes. The formation of new capillaries from the endothelium of existing vasculature involves different steps, including degradation of the basement membrane, migration and proliferation of endothelial cells and stabilization of the new vascular tubes [1]. This process is tightly regulated by the balance between different pro and anti-angiogenic molecules, including growth factors. Among the pro-angiogenic growth factors, vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) represent the most important and extensively studied.

Abbreviations: a.a., amino acid; ALK, anaplastic lymphoma kinase; ASP, aspartic acid; CTGF, connective tissue growth factor; FGF-2, fibroblast growth factor-2; GLU, glutamic acid; HARP, heparin affin regulatory peptide; HBGF, heparin binding growth factors; HS, heparan sulfates; HUVEC, human umbilical vein endothelial cells; MK, midkine; RPTP β/ζ, receptor-type protein tyrosine phosphatase beta/zeta; $VEGF_{165}$, vascular endothelial growth factor 165; TSR-1, thrombospondin type 1 repeat.

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[1] Célia Dos Santos was funded by a Marie Curie Intra-European fellowship. This work was supported in part by a grant from ANR (ANR-06-RIB 016-02), a grant from INCA (PL06-093) and by the Centre National de la Recherche Scientifique.

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http://dx.doi.org/10.1016/j.biochi.2014.10.002
0300-9084/

These two polypeptides are particularly implicated in normal and tumor angiogenesis [2,3].

Heparin affin regulatory peptide (HARP), also called pleiotrophin, is a 136 amino acid secreted polypeptide that forms with midkine (MK) a two-member family of heparin-binding growth factors (HBGF). Initially reported as a neurite outgrowth promoting molecule, intensive research on its ability to stimulate cell growth showed controversial results. Indeed, it appeared that HARP produced in mammalian system is mitogenic whereas HARP produced in bacteria is not [4]. In support of its role in angiogenesis, HARP has also been found to induce the migration of endothelial cells *in vitro* and *in vivo* [5,6]. HARP is a developmentally regulated cytokine that is highly expressed in the embryonic nervous system, but its expression drops dramatically after the perinatal phase and during adulthood. However, HARP has been shown to be overexpressed in various tumor cell lines and primary human tumors [7–9]. Two transmembrane proteins with intracellular catalytic domains have been described as HARP receptors: the receptor-type protein tyrosine phosphatase beta/zeta (RPTP β/ζ) and the anaplastic lymphoma kinase (ALK) receptor. The mitogenic and anti-apoptotic activities of HARP were initially linked to the high-affinity tyrosine kinase receptor ALK [10,11], whereas the neurite outgrowth, cell migration and adhesion activities of HARP were associated with the chondroitin sulfate proteoglycan RPTP β/ζ [12,13]. Structure-function studies have revealed that different regions of HARP are implicated in its biological activities. Thus, the C-terminal tail of HARP was reported to be implicated in the mitogenic, angiogenic and transforming activity of the protein [14,15]. The central region of HARP is composed of two β-sheet domains containing one thrombospondin type 1 repeat (TSR-1) motif each. These domains are linked by a flexible linker and maintained by disulfide bonds. The β-sheet domains were reported to be responsible for the binding of HARP to heparin and to its dimerization [16]. Interestingly, the β-sheet domains of HARP were also demonstrated to be responsible for the direct binding of HARP to $VEGF_{165}$. This direct binding inhibited the interaction of $VEGF_{165}$ to its receptors on endothelial cells causing a down-regulation of its angiogenic activity [17]. This inhibitory effect of HARP on $VEGF_{165}$, observed both *in vitro* and *in vivo*, suggested a dual regulatory function of HARP as both an angiogenic and angiostatic molecule.

In view of the foregoing, we hypothesized that HARP might also bind directly to FGF-2, which also plays an important role during angiogenesis, and interfere with its biological activity. The present study determined that HARP inhibited proliferation, migration and tubes formation of human umbilical vein endothelial cells (HUVEC) induced by FGF-2. Interaction between the two growth factors was highlighted by ELISA and optical biosensor binding assays. Moreover, we adapted a new approach to identify two main sequences of HARP interacting with FGF-2. So it appeared that the activities of FGF-2 can be modulated by its binding to HARP.

2. Materials and methods

2.1. Materials

Recombinant fibroblast growth factor-2 (FGF-2) was purified in the laboratory by sequential heparin Sepharose and Mono-S chromatography from bacteria [18] or by sequential heparin Sepharose and heparin HPLC affinity chromatography [19]. Recombinant Heparin Affin Regulatory Peptide (HARP) of bacterial origin was produced and purified in the laboratory as previously described [20]. Peptides P13–39 (SDCGEWQWSVCVPTSGDCGLGTREGTR) derived from the N-TSR-1 domain, P65–97 (AECKYQFQAWGECDLNTALKTRTGSLKRALHNA) derived from the C-TSR-1 domain and P111–136 (KLTKPKPQAESKKKKKEGKKQEKMLD) from the C-terminal tail of HARP were purchased from Altergen (Schiltigheim, France). Human umbilical vein endothelial cells (HUVEC), EGM-2 BulletKit medium were purchased from Lonza (Emerainville, France). Polyclonal antibodies against HARP and FGF-2 were from R&D Systems (France).

2.2. Proliferation and migration assays

HUVEC were routinely cultured in EBM-2 medium supplemented with 2% (v/v) fetal bovine serum (FBS) and BulletKit. Cultures were grown at 37 °C in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2. All experiments were carried out between passages 2 and 5. For cell proliferation assays, HUVEC were seeded into twelve-well plates (10 000 cells/well) in EBM-2 supplemented with 2% (v/v) FBS in the presence or absence of FGF-2 and HARP, P65–97, or P111–136. The cells were counted on day 5. For cell migration assays, HUVEC were seeded into a 24-well chemotaxis chamber (Transwell, Corning Costar, France). Polycarbonate filters of 8 μm pore size were coated with 10 μg/ml type I collagen R (Serva, Heidelberg, Germany) for 1 h and dried under sterile air. The EBM-2 medium supplemented with 1% (v/v) FBS was then placed in the lower chamber, with or without FGF-2, HARP, and different concentrations of P65–97 or P111–136 which served as chemoattractant. Cells (100 000/well) suspended in EBM-2/1% (v/v) FBS were seeded in the upper compartment. Transwells were incubated for 5 h at 37 °C, and treated as described previously [5].

2.3. ELISA binding assay

HARP or FGF-2 was coated at 1 μg/ml in 10 mM CAPS buffer and incubated overnight at 4 °C. For P111–136, P65–97 and P13–39 peptides, a range of concentrations was chosen for coating. After washing the wells with PBS containing 0.05% (v/v) Tween 20, non-specific binding sites were blocked by adding PBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma–Aldrich), for 1 h at 37 °C. After a rinse step, binding of FGF-2 or HARP was achieved in PBS containing 1% (w/v) BSA for 2 h at room temperature. The bound protein was characterized using 0.25 μg/ml of polyclonal antibody targeting HARP or FGF-2 and incubated for 1 h at room temperature. A peroxidase-labelled polyclonal anti-IgG was used at a concentration of 80 ng/ml for 30 min at 37 °C. Peroxidase activity was detected using 3,3,5,5-tetramethylbenzidine dihydrochloride substrate according to the supplier's instructions. Absorbance was measured at 450 nm.

2.4. Optical biosensor binding assays

HARP was immobilized on aminosilane surfaces using bissulfosuccinimidyl suberate (BS^3) as the cross linker following the manufacturer's recommendations (NeoSensors, Sedgefield, UK). No more than 700 arc *s* HARP was immobilized on the surface (1 arc $s = 1/3600°$, 600 arc $s = 1$ ng protein bound per mm^2). Binding assays were carried out in PBS supplemented with 0.02% (v/v) Tween 20 (PBST) at 20 °C following previously described methods [21–23].

2.5. Data analysis

To avoid artifactual second phase binding sites [24], assays were designed as described previously [25].

2.6. Protect and Label procedure

The method was adapted from the one described by Ori et al. [26], which was developed to identify binding sites in proteins for heparin. FGF-2 was biotinylated on its thiol residues using maleimide-PEG2-Biotin, No-Weigh™ Format (Pierce), as indicated by

the manufacturer. A Strep-Tactin™ Sepharose resin (200 µl) from IBA (Goettingen, Germany) was packed in a mini-column. The column was equilibrated with 4 × 250 µl PBS and loaded with 80 µg of biotinylated FGF-2 in a final volume of 250 µl. Loading was repeated three times to ensure complete binding. The mini-column was then washed with 4 × 250 µl PBS and HARP (40 µg in a final volume of 250 µl PBS) was applied to the column. Loading was repeated three times to ensure complete binding. The Strep-Tactin™ Sepharose column with bound HARP was washed with 100 µl PBS containing 50 mM sulfo–NHS–acetate (Pierce) and then incubated with 100 µl PBS containing 50 mM sulfo–NHS–acetate for 5 min at room temperature to ensure complete blocking (acetylation) of exposed lysines. To stop the reaction, the column was extensively washed with 4 × 250 µl PBS. Acetylated protein was eluted from Strep-Tactin™ Sepharose beads with 4 × 50 µl 8 M urea buffered with 400 mM NH_4HCO_3 pH 7.8. After elution, the acetylated protein was biotinylated on the free amino groups, protected by the interaction with HARP, with EZ-Link® NHS-biotin (Pierce) and then digested overnight with 1.5 µg of chymotrypsin (Sigma–Aldrich) at 37 °C. After digestion, biotinylated peptides were purified on Strep-Tactin Sepharose beads. The eluate was concentrated by rotary evaporation, desalted using a C18 ZipTip™ (Millipore Ltd, Watford, UK) according to manufacturer's instruction and analyzed by mass spectrometry.

2.7. ESI-Q-TOF mass spectrometry

After dehydration, sample was diluted in 20 µl 0.1% (v/v) formic acid/HPLC grade water. Data dependent analysis (DDA) was performed on the top 10 most intense peptides in the parent spectrum. Depending on the *m*/*z* of the peptide selected for MS/MS, the corresponding energy ramp was applied.

MS/MS spectra were processed using MassLynx v4.0 software (Waters Corporation). Peak lists were generated using the MaxEnt3 algorithm applying a 5% base peak intensity cut-off. Data analysis was performed using the MS-tag tool of the Protein Prospector package v.5.2.2 (http://prospector2.ucsf.edu) with the following parameters: digest = chymotrypsin; max missed cleavages = 5; possible modifications = acetyl (K), biotin (K), carbamidomethyl (C), carboxymethyl (C); parental ion tolerance = 0.2 Da; fragment ion tolerance = 0.8–1 Da; non-specific cleavage = at 1 termini. The UniProt ID of the protein analyzed was used as a pre-search parameter. All other settings were used as default values.

2.8. In vitro angiogenesis assay on Matrigel

Tubes formation capacity of the HUVEC induced by FGF-2 was tested on Matrigel™ Basement Membrane Matrix (BD Biosciences, Bedford, UK). Briefly, 1.5 × 10^4 HUVEC cells per well of a 96-well plate were seeded onto a Matrigel™ layer in EBM-2 medium supplemented with 2% (v/v) FBS. Immediately after plating, the cells were treated with 0.6 nM FGF-2, 3 nM of HARP, and different concentrations of the peptides P65–97 or P111–136. Eight hours after cell plating, tubular network structures were observed and quantified, using an Axiovert 10 photozoom inverted microscope connected to a digital camera (Axiocam MRm Zeiss, Germany). Quantification of pseudo-capillaries was determined by analyzing triplicates for each condition of treatment. The results are expressed as the number of tubes per field.

2.9. Statistical analysis

The statistical analyses were performed using the GraphPad PrismTM version 5.00 software from GraphPad Software Inc. (San Diego, CA, USA). The results were expressed as means ± standard deviation (SD) or standard error mean (SEM) of at least three determinations for each test from three independent experiments. Statistical analyses were carried out using the unpaired two-tailed *t*-test. The statistical significance of the differences is given as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$; ns: non significant.

3. Results

3.1. HARP inhibited proliferation and migration of HUVEC induced by FGF-2

Despite HARP being described as an angiogenic factor, it has also been reported to bind $VEGF_{165}$ and to inhibit the angiogenic activities of the latter on HUVEC. As the proliferation of these cells can also be stimulated by FGF-2, we questioned first if HARP could inhibit their stimulation by FGF-2. To avoid any confused proliferating effect of the eukaryotic recombinant HARP, we have used the prokaryotic recombinant protein devoid of proliferating activity on endothelial cells as previously described [27]. When HUVEC were cultured *in vitro* in presence of 0.6 nM FGF-2, a 5-fold increase in cell number was observed (Fig. 1A). When increased concentrations

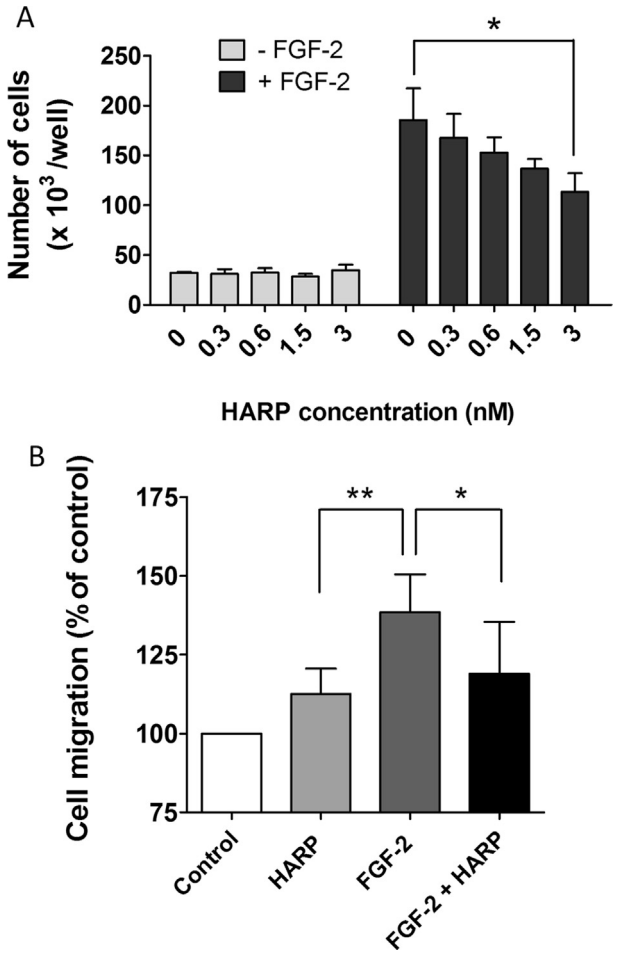

Fig. 1. Effect of HARP on the proliferation and migration of HUVEC induced by FGF-2. A. HUVEC were seeded with increasing concentrations of HARP (0, 0.3, 0.6, 1.5, 3 nM) in the presence or absence of FGF-2 (0.6 nM). After 5 days of culture, cells were trypsinized and counted. $^{*}p < 0.05$. B. Migration was measured using the transfilter assay. 10^5 cells per well were seeded in the upper compartment while FGF-2 (1.2 nM), HARP (6 nM) or combination of both proteins were placed in the lower chamber to serve as chemo-attractant. Coloration and quantification of migrated cells were performed as described in "Material and Methods". $^{*}p < 0.05$ $^{**}p < 0.01$. Data are the means ± SD of three experiments, each carried out in triplicate.

of HARP were added, a dose-dependent inhibition of the proliferation of the cells occurred, reaching 38% for 3 nM HARP. In the absence of FGF-2, HARP had no effect on HUVEC proliferation as previously reported [17]. In addition, the migration activity of FGF-2 was explored in presence or absence of HARP (Fig. 1B). When 1.2 nM FGF-2 was added to Boyden chambers, migration of endothelial cells was stimulated by 38% compared to incubation in a medium lacking the growth factor. Migration was slightly increased by 6 nM of HARP. When the two proteins were added together at these concentrations, a significant inhibition of 15% occurred in cell migration when compared with FGF-2 alone. All together, these results demonstrated that HARP could interfere with the effects of FGF-2 on endothelial cells.

3.2. Interaction of FGF-2 with HARP

In a previous study, we showed that HARP could bind directly to $VEGF_{165}$ and inhibited its biological activities on HUVEC. Thus the inhibitory effect of HARP on the growth- and migratory stimulatory activities of FGF-2 could likewise be due to a direct interaction of HARP and FGF-2. The possibility of an interaction between HARP and FGF-2 was first tested using an ELISA. FGF-2 or HARP were first coated with 100 ng of protein and then incubated with HARP or FGF-2 at various concentrations ranging from 0 to 60 nM. In each condition, a dose-dependent and specific binding was observed with a maximum at 30 nM of HARP or FGF-2 added (Figs. 2A and B). The EC_{50}'s of both

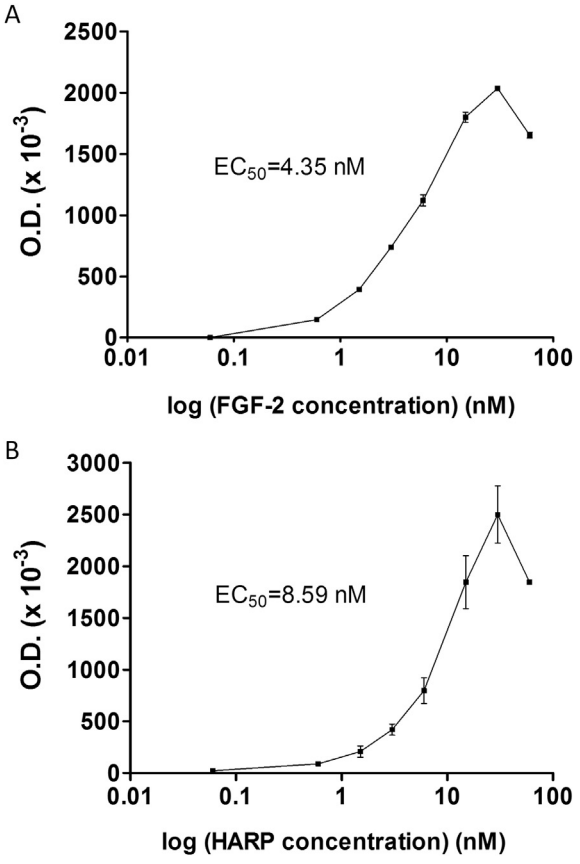

Fig. 2. Interaction between HARP and FGF-2 determined by ELISA assay. A. HARP (100 ng) was coated on 96-well plate, and incubated in the presence or absence of different concentrations of FGF-2 ranging from 0.6 to 60 nM. Bound FGF-2 was detected with FGF-2 antibodies. B. FGF-2 (100 ng) was coated on 96-well plate, and incubated in the presence or absence of different concentrations of HARP ranging from 0.6 to 60 nM. Bound HARP was detected with HARP antibodies. Data are the mean ± SD of three experiments, each carried out in triplicate.

proteins are very similar. Indeed, the concentration of FGF-2 needed to bind 50% of HARP was 4.35 nM, while the concentration of HARP needed to bind 50% of FGF-2 was 8.59 nM. As we deliberately used a prokaryotic HARP to evaluate the biological effects of its interaction with FGF-2, we tested if the eukaryotic recombinant protein was also able to bind FGF-2. When using in a similar ELISA assay, the eukaryotic HARP interacts with FGF-2 in a dose-dependent manner with an EC_{50} of 7.70 nM (data not shown). This concentration being very comparable to the one determined with the use of prokaryotic HARP, this result confirm that the source of production of the growth factor is not impacting its ability to bind FGF-2. To characterize the interaction of FGF-2 with HARP, the kinetics of the binding reaction were determined. As shown in Fig. 3A, when increasing concentrations of FGF-2 were added to a cuvette with a HARP-derivatized surface, the level of binding increased dose dependently; in the absence of HARP, there was no detectable binding of FGF-2 to the aminosilane surface (data not shown and [21]). Analysis of the binding curves with a one site model resulted in a random distribution of the data about the model (Fig. 3D–I). A two-site model did not improve this measure of goodness of fit (data not shown). A plot of the slope of initial rate against FGF-2 concentration was fitted by a straight line (Fig. 3B), as was a plot of the observed on-rate k_{on} against the concentration of FGF-2 (Fig. 3C). Taken together, these analyses indicate that, at the concentrations used for the experiment, the interaction of FGF-2 with HARP was monophasic. The association reaction was characterized by a fast k_a equal to 390 000 M^{-1} s^{-1}. The k_d was measured at 0.023 s^{-1}, consequently FGF-2/HARP binding is defined by a high affinity of 58 nM (Table 1). The values of K_d calculated from the kinetic parameters are not so different to those calculated from the extent of binding when this had reached a maximum (Table 1), indicating that these are the appropriate binding parameters for this interaction. Interestingly, during the regeneration step in these experiments, it was observed that 95% of the FGF-2 bound to HARP could be dissociated by adding 2 M NaCl. This observation suggested that driving forces for the binding of HARP and FGF-2 are ionic interactions.

3.3. Identification of the FGF-2 binding sites on HARP

HARP and FGF-2 are both heparin binding growth factors. During the optical Biosensor binding assays we showed that heparin acted as a competitor between HARP and FGF-2 interaction by reducing their binding (data not shown). Thus, to identify the HARP binding sites on FGF-2, we adapted a method developed by Ori et al. [26] to investigate the interaction between proteins and glycosaminoglycans. This method, called “Protect and Label”, is based on the protection by the polysaccharide of the residues located in the heparin binding sites against chemical modification. FGF-2 was biotinylated on its exposed thiol, which is on a surface of the protein that is not involved in ionic interactions, so that it could be bound to Strep-Tactin™ Sepharose beads. After HARP binding FGF-2, exposed amines were protected by acetylation. Acetylated protein was eluted from Strep-Tactin™ Sepharose beads with urea and any protected amines, which must be implicated in the interaction, were labeled with NHS-biotin (see subsection 2.6 in “Material and Methods” section). Labeled peptide sequences were identified by MALDI-Q-TOF mass spectrometry. The ten most intense ions were automatically selected in the quadrupole, and MS/MS data were acquired to assign chemical modifications introduced during the “Protect and Label” procedure (Table 2). A modification-specific marker ion at m/z = 310.2, assigned as Lys(biotin)-NH_3, was in fact observed in all the fragmentation spectra analyzed. We point out one exception for ion m/z = 954.7 (Supplementary spectrum S10) characterized by a specific ion marker at m/z = 227.1 corresponding to biotin.

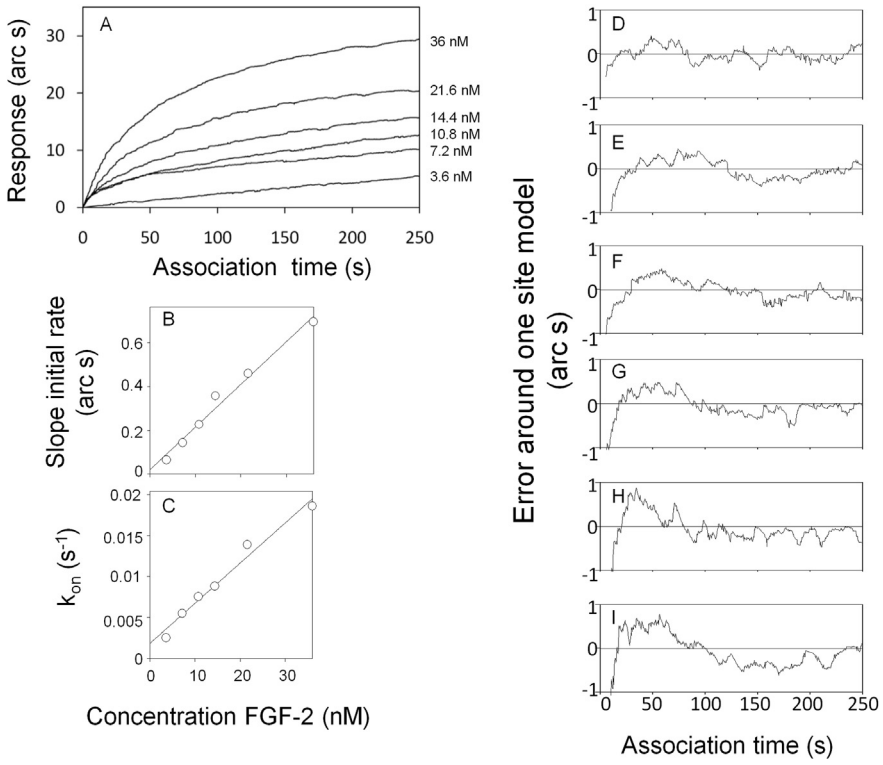

Fig. 3. Binding of FGF-2 to immobilized HARP. The binding kinetics of FGF-2 to immobilized HARP were measured, as described under subsection 2.4 of "Material and Methods". Data shown are the result of one representative experiment of three. A. FGF-2 at different concentrations was added to a HARP-derivatized cuvette containing PBST, and the association reaction was followed for 250 s, by which time at least 90% of the fitted one site binding curve was covered. The concentration of FGF-2 in nanomolar is indicated. B. Linear relationship between the slope of initial rate of association and concentration of FGF-2. C. Linear relationship between k_{on}, determined from a one-site model, and concentration of FGF-2. D–I, the distribution of the data points (jagged line) around one-site binding model (horizontal line at 0 arc s) is shown for each of the concentrations of HARP used in the binding assay in panel A. Instrument noise is ±1 arc *s*. Concentrations of FGF-2 are 3.6 nM (D), 7.2 nM (E), 10.8 nM (F), 14.4 nM (G), 21.6 nM (H), 36 nM (I).

The "Protect and Label" procedure allowed us to identify two main regions of HARP involved in binding FGF-2, the HARP C-terminus between amino acids 107 to 125 and a small part of the C-TSR-1 domain including amino acids 58 to 71. As a consequence, we used the synthetic peptides P111–136 and P65–97, already studied for their anti-proliferative and anti-tumor activities on cancer cells [28,29], to test if they bind directly to FGF-2. As shown in Fig. 4A, the binding of FGF-2 on P111–136 or P65–97 is dose dependent and saturable. The EC_{50}'s of both peptides showed that P111–136 bound FGF-2 with higher affinity than P65–97. Indeed, the concentration of P111–136 needed to obtain a 50% reduction of FGF-2 maximum binding is almost thirty times lower (EC_{50} = 0.15 μM) than that observed with P65–97 (EC_{50} = 3.95 μM). As control, a peptide P13–39, derived from the N-TSR-1 domain of HARP was found to be unable to bind FGF-2. These results confirm the importance of the C-TSR-1 and the C-terminus domains of HARP in the interaction with FGF-2. To determine if these regions of HARP could have differential activities with regard to either cell proliferation or migration, we studied the effects of P65–97 and P111–136 on the activities of HUVEC induced by FGF-2. Interestingly, both peptides were able to inhibit FGF-2-dependent proliferation of HUVEC (Fig. 4B). At 5 μM, P65–97 reduced, significantly and up to 26%, endothelial cells growth. Similar effects were observed using P111–136, with a significant inhibition of proliferation when adding 1 μM of the peptide (22% of inhibition). With regard to HUVEC migration, P65–97 was able to reduce FGF-2 activity by approximately 20% using 1, 5 or 10 μM of the peptide (Fig. 4C). On the contrary, P111–136 inhibited HUVEC migration dependent to FGF-2 only at the higher concentration of 10 μM, implying that the C-terminal region has less inhibitory activity on cell migration than the C-TSR-1 domain. In order to investigate if angiogenic activity of FGF-2 on HUVEC is modified in presence of the peptides, we realized a Matrigel™ assay. The formation of pseudo-capillaries was induced by FGF-2 with an increase of 35% compared to control (Fig. 4D). When HARP was added, the tube formation induced by FGF-2 was inhibited by 16% ($p < 0.07$). The implication of the C-TSR-1 and C-terminal domains of HARP was confirmed as the addition of the peptides P65–97 and P111–136 inhibited significantly the *in vitro* angiogenic activity of FGF-2. At 1 and 10 μM of P65–97 added, the inhibition reached 37% and 44% respectively. When treated with the same concentrations of P111–136, HUVEC's tube formation was inhibited by 25 and 35% respectively.

4. Discussion

HARP is known as a pro-angiogenic growth factor capable of promoting the growth, migration and formation of capillary networks of endothelial cells [5,30]. In a previous study, the migration of human glioma cells was demonstrated to be stimulated by the interaction of 18 kDa HARP with the receptor protein tyrosine phosphatase β/ζ (RPTP β/ζ). In contrast, a naturally C-terminally truncated 15 kDa form of HARP was reported to interact with the anaplastic lymphoma kinase (ALK) receptor and further stimulate the proliferation of these cells [31]. Our results are in agreement with these findings. The recombinant 18 kDa HARP used in our study was unable to stimulate the proliferation of HUVEC, but slightly increased their migration, and HUVEC were found to express RPTP β/ζ but not ALK (data not shown). In addition, a former

Table 1
Kinetics of FGF-2 binding to immobilized HARP. Binding parameters were determined in an optical biosensor (see subsection 2.4 and 2.5 of "Material and Methods").

Immobilized ligand	Ligate	k_a[a] (M^{-1} s^{-1})	r[b]	k_d[c] (s^{-1})	K_d (kinetic)[d] (nM)	K_d (equilibrium)[e] (nM)
HARP	FGF-2	390,000 ± 83383	0.983	0.023 ± 0.005	58 ± 17	32 ± 10

[a] S.E. of the k_a was derived from the deviation of the data from a one-site binding model, calculated by matrix inversion using the FASTFit software provided with the instrument. No evidence was found for a two-site model of association. At least four independent sets of k_{on} were measured and the four resulting values for k_a and their errors were combined.
[b] The correlation coefficient of the linear regression through the k_{on} values is represented by r.
[c] The k_d is the mean ± S.E. of at least five values, obtained at high concentrations of FGF-2 in the presence of competing heparin.
[d] The K_d (kinetic) was calculated from the ratio of k_d/k_a and its S.E. is the combined S.E. of the two kinetic parameters.
[e] The K_d (equilibrium) was calculated from the extent of binding at equilibrium and its S.E. is the combined S.E. of three independent determinations of K_d.

study demonstrated the ability of HARP to stimulate serum-starved HUVEC [5]. However, the role of HARP in angiogenesis is more complex, since subsequently it has been shown that HARP binds to $VEGF_{165}$ and inhibits its angiogenic activity [17]. In this study, we demonstrated that HARP can also inhibit FGF-2-induced proliferation and migration of HUVEC.

The inhibitory activity of HARP was linked to a direct interaction between HARP and FGF-2. The binding of FGF-2 to HARP was characterized by a high affinity ($K_d = 58$ nM) and a fast association rate constant ($k_a = 390\,000\ M^{-1}\ s^{-1}$). The latter is characteristic of interactions with an electrostatic component. Indeed, binding of FGF-2 and HARP was abrogated by 2 M NaCl and by heparin. This suggested that the heparin-binding sequence of one or both of the proteins could be implicated in their interaction. It is established that proteins that interact through heparin binding sites can have activities on cells. For example, neuropilin, which binds FGF-2 with a K_d similar to that of HARP for FGF-2 also enhances the activity of FGF-2 in HUVEC [32]. Therefore, proteins that interact through heparin binding sites can clearly have activities in the presence of cellular heparan sulfates (HS). The affinities of HARP and FGF-2 for heparin ($K_d = 13$ nM [16] and $K_d = 39$ nM [33], respectively) are similar to the affinity of the two growth factors for themselves. Studies have also demonstrated that the affinity of HARP for FGF-2 is higher or equivalent to that of FGF-2 for HS and for heparin-derived oligosaccharides [21,23]. In our study, the presence of endogenous polysaccharides in the extracellular microenvironment of HUVEC did not prevent HARP to inhibit the growth and migratory stimulatory activities of FGF-2. Therefore, the balance between the availability of each growth factor and their binding structures in HS may ultimately determine whether HARP inhibits that activity of FGF-2 or not.

The binding of FGF-2 to HARP depended on ionic interactions. Consequently, we were able to adapt a technique, originally described to identify the amino acid sequences proteins which interact with heparin [26], to identify regions in HARP that bind to FGF-2. The present adaptation of this strategy to protein–protein interactions involving electrostatic bonding highlights its versatility, particularly since a number of such interactions between extracellular proteins have been documented in recent years [17,32,34]. We demonstrated that the C-TSR-1 and the C-terminal domain of HARP were involved in the binding of this growth factor to FGF-2. The first sequence (a.a.60 to a.a.71) corresponds to a domain already described as involved in heparin binding, the C-TSR-1 composed with three β-sheets [35]. Though the N-TSR-1 domain of HARP has been reported previously as necessary for the binding of HARP to heparin [35], subsequent studies have indicated that this is not the case [26,29]. Thus, the interaction of C-TSR-1 with FGF-2 is entirely consistent with the observed ability of heparin to abrogate the binding of the two proteins *via* an interaction with the heparin-binding site of HARP. Interestingly, FGF-2 and $VEGF_{165}$ shared the particularity to bind HARP through the β-sheet domain C-TSR-1. This domain is characterized by a strong sequence homology with TSP-1 (Thrombospondin-1) which was described as an inhibitor of the angiogenic activity of FGF-2 through a direct interaction of the proteins [36]. FGF-2, *via* its binding to TSP-1, is sequestrated in the extracellular matrix inhibiting its specific interaction through receptors at the cell surface. It represses as a consequence its mitogenic and migration activities. The TSR domain is characterized by the motif WXXWXXC within W residues are important in the angiostatic properties of the TSR-1 domain [17,37]. The TSR-1 domain is found in a variety of molecules involved in protein–protein interactions. As an example, the connective tissue growth factor (CTGF) contained a TSR-1 domain which was demonstrated to interact directly with $VEGF_{165}$ inhibiting its *in vitro* and *in vivo* angiogenic activity [34]. The second sequence identified (a.a.107 to a.a.125) is the beginning of the 26 amino acid C-terminal tail of HARP. It was previously demonstrated that the HARP C-terminal-end, containing amino acid residues 111–136, was implicated in the binding of HARP to its ALK receptor [14,15], and was necessary for the downstream biological activities of the growth factor. More recently this C-terminal-end region was also demonstrated to interact with the RPTPβ/ζ [38]. This result is

Table 2
Protect and Label strategy labelled peptides. For each sample, peptides are sorted in a descending order according to the intensity of their parental ion. Labelled peptides were identified using the tools MS-tag and MS-product of the package Protein-Prospector v5.2.2 (http://prospector2.ucsf.edu). Assigned MS/MS spectra are available as supplementary data.

Peptide	m/z Observed	m/z Theoritical	Error (Da)	Sequence	Residues	Supplementary spectrum
1	1744.4	1744.8	−0.419	K(Biotin)K(Acetyl)QFGAECKYQF	60–71	S1
2	1929.2	1928.88	0.114	KK(Biotin)QFGAECK(Biotin)YQF	60–71	S2
3	1992.8	1992.07	0.769	TK(Acetyl)PKPQAESKK(Biotin)K(Biotin)K	113–125	S3
4	1782.4	1781.81	0.583	K(Biotin)K(Biotin)QFGAECKYQ	60–70	S4
5	1870	1869.83	0.170	NWK(Acetyl)K(Biotin)QFGAEC (Carboxymethyl)K(Acetyl)Y	58–69	S5
6	946.1	946.48	−0.381	K(Biotin)K(Acetyl)QFGA	60–65	S6
7	929.9	929.46	0.442	K(Biotin)PCGKL	107–112	S7
8	903.6	904.47	−0.871	K(Biotin)KQFGA	60–65	S8
9	1766.2	1765.99	0.201	TK(Biotin)PK(Acetyl)PQAESKKKK	113–125	S9
10	954.7	954.37	0.330	GAEC(Carboxymethyl)K(Biotin)Y	64–69	S10

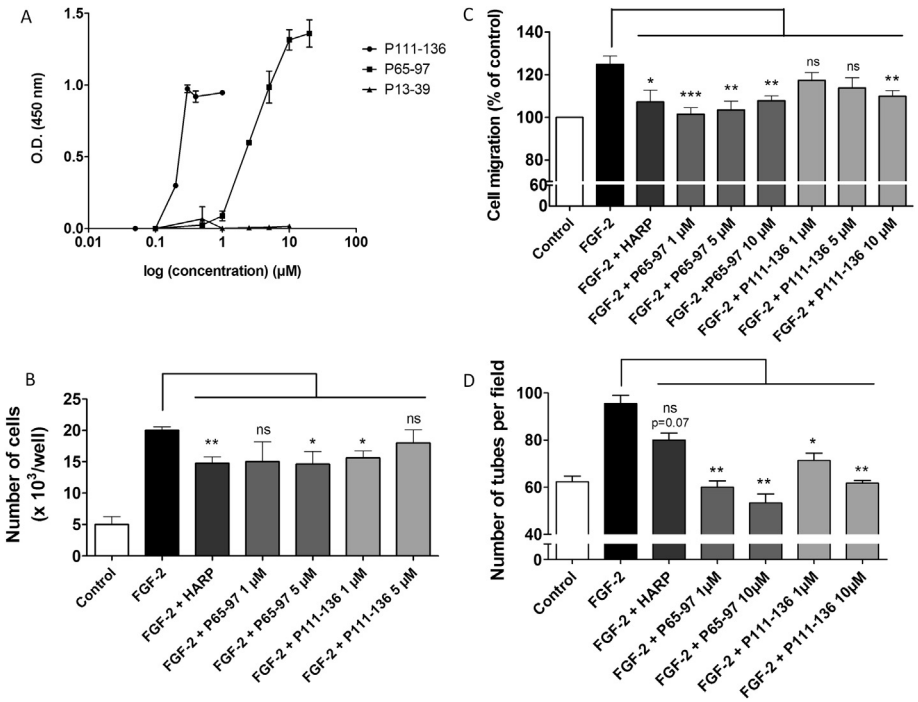

Fig. 4. Interaction of FGF-2 with peptides derived from HARP and their effects on HUVEC's proliferation, migration and tubes formation induced by FGF-2. A. Peptides containing the sequences of HARP identified by "Protect and Label" were tested in ELISA assay for their binding to FGF-2. Different concentrations of N-TSR-1, C-TSR-1 ranging from 0.05 to 1 μM and P111–136 ranging from 0.1 to 20 μM were coated on 96 wells-plates and incubated in the presence of 30 nM of FGF-2. Bound FGF-2 was detected with FGF-2 antibodies. B. HUVEC were seeded with increasing concentrations of P65–97 or P111–136 (1 and 5 μM) in the presence of FGF-2 (0.6 nM). After 5 days of culture, cells were trypsinized and counted. $^{*}p < 0.05$, $^{**}p < 0.01$, ns = non significant. C. Migration was measured using the transfilter assay. 100 000 cells per well were seeded in the upper compartment while FGF-2 (0.6 nM), and P65–97 or P111–136 (1, 5 and 10 μM) were placed in the lower chamber to serve as chemo-attractant. Coloration and quantification of migrated cells were performed as described in subsection 2.2 of "Material and Methods". $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, ns = non significant. D. HUVEC's tubes formation was measured using the Matrigel™ assay. 1.5×10^4 cells per well were seeded on a Matrigel™ layer while FGF-2 (0.6 nM), HARP (3 nM) and P65–97 or P111–136 (1 and 10 μM) were added in the corresponding wells. $^{*}p < 0.05$, $^{**}p < 0.01$, ns = non significant. Data are the means ± SD of three experiments, each carried out in triplicate.

consistent with the observation that not only does HARP inhibit HUVEC migration induced by FGF-2, but by binding to FGF-2, it also loses its own ability to stimulate efficiently cell migration. In our study, we showed that peptides corresponding to the C-TSR-1 domain and C-terminal region of HARP were able to inhibit FGF-2-dependent proliferation of HUVEC. It is to mention that the peptides concentrations used in our study affecting FGF-2 biological activities are in the same range as the ones previously described for interfering with the biological activities of HARP [29,38]. The use of distinct range of concentrations between the growth factors and the peptides (nanomolar vs micromolar) could be due to the presence of proteases in the conditioned medium. These enzymes would target preferentially the peptides for being smaller a.a. sequences and less stable, as devoid of clear secondary structure. With regard to migration and tubes formation, the inhibitory effect of C-TSR-1 domain seemed more effective than the activity of the C-terminal region. If HARP C-terminal-end binds with higher affinity the receptor, it could explain why only a high concentration of the peptide, P111–136, is needed to induce inhibition of FGF-2-induced migration and tubes formation. Although the peptides we used were synthetic, we showed in a precedent study that natural fragments of HARP could be generated by MMP-2 proteolysis [39]. This cleavage was found to inhibit HARP-induced proliferation and migration of murine fibroblasts NIH 3T3, supporting the idea that peptides can derive from endogenous growth factors in cells, and modulate their biological activities.

The protection of lysine residues in HARP, by FGF-2 binding, may be due to their direct involvement in the protein–protein interaction, or to their protection through a conformational change in HARP elicited by FGF-2 binding. The demonstration that two peptides corresponding to the same regions of HARP, P111–136 and P65–97, bind FGF-2 in an ELISA indicates that the identified lysines are more likely to be directly involved in mediating the interaction. The domains of HARP found to interact with FGF-2 are particularly rich in basic residues. Since the protein–protein interaction is strongly dependent on electrostatic interactions, we speculate that HARP binds an acidic patch of amino-acids on FGF-2. Although, FGF-2 is very basic (its original name was basic FGF and its pI is 9.6), it possesses small patches of negative charge exposed to solvent. In particular, GLU 67, GLU 68, GLU 87, ASP 87, ASP 99, GLU 100, are more or less aligned across the surface of FGF-2, forming a weakly acidic patch (weak due to neighboring basic residues), that is distinct from the heparin-binding sites on FGF-2 [26] and its receptor-binding sites [40,41]. We speculate that this or an analogous acidic patch is the site of interaction of HARP in FGF-2.

The inhibition of the growth and migration stimulatory activity of FGF-2 on HUVEC by HARP is not unique, in that HARP similarly inhibits the activity of $VEGF_{165}$ and the same was described for CTGF and $VEGF_{165}$ [17,34]. Moreover, there is a further regulatory relationship between HARP and FGF-2, at least in prostate epithelial cells [42]. Thus, HARP expression is required for FGF-2-induced LNCaP cells proliferation and migration and FGF-2 is able to induce the expression and secretion of HARP by these cells.

The results of the present study suggest that the regulation of the activities of growth factors such as HARP, FGF-2 and $VEGF_{165}$ is extremely complex, involving interaction with cognate receptors, HS co-receptors and direct interactions between the growth factors. All of which can be followed by a change in the levels of specific growth factors in the regulatory network due to the activation of specific transcriptional events downstream of activated

receptors. This highlights the importance of readouts of activity, rather than simply expression of growth factors and their receptors, in measurements of functional changes, between different states of cells and or tissues in development and in disease.

Conflict of interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.10.002.

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