Heparin affin regulatory peptide (HARP) is an heparin-binding growth factor, highly expressed in several primary human tumors and considered as a rate-limiting angiogenic factor in tumor growth, invasion, and metastasis. Implication of this protein in carcinogenesis is linked to its mitogenic, angiogenic, and transforming activities. Recently, we have demonstrated that the C-terminal residues 111-136 of HARP are required for its mitogenic and transforming activities (Bernard-Pierrot, I., Delbe, J., Caruelle, D., Barritault, D., Courtj, Y., and Milhiet, P. E. (2001) J. Biol. Chem. 276, 12228-12234). In this paper, HARP deleted of its last 26 amino acids was shown to act as a dominant negative effector for its mitogenic, angiogenic, transforming, and tumor-formation activities by heterodimerizing with the wild type protein. Similarly, the synthetic corresponding peptide P111-136 displayed in vitro inhibition of wild type HARP activities, but in this case, the inhibition was mainly explained by the competition of the peptide with HARP for the binding to the extracellular domain of the high affinity ALK receptor.

The expression of polypeptide growth factors is tightly regulated and contributes to the timely development of tissues during embryonic and neonatal growth. As depicted in several studies, gene expression of these growth factors is deregulated in solid tumors, and these polypeptides contribute to autocrine and paracrine stimuli leading to the development of the tumor tissues. Among these growth factors, angiogenic polypeptides appear to be very interesting therapeutic targets because angiogenesis plays a key role in cancer development, and negative regulators of angiogenesis are under clinical evaluation (reviewed in Ref. 1).

Heparin affin regulatory peptide (HARP) (2), also known as pleiotrophin (3) or heparin-binding growth-associated molecule (4), is an 18-kDa-secreted polypeptide that constitutes with Midkine (MK), a two-member family among the heparin-binding growth factors. The pattern of expression of HARP suggests that this molecule has functions in neuronal migration and in epithelium-mesenchyme interactions during the embryonic development (5). However, although HARP is mainly down-regulated after birth, it seems to play a key role in cell growth and differentiation during adulthood. It seems particularly involved in tumor growth and angiogenesis (6). HARP mRNA and/or protein are highly expressed in neuroblastoma, glioblastoma, and gastrointestinal, prostate, and primary breast cancers (7, 8). They are also retrieved in tumor cell lines of different origins including pancreas (9), lung (10), and ovary (11). In pancreatic cancers, HARP expression by tumors is correlated with its elevated level in patient sera (8).

HARP displays several in vitro biological activities. It induces neurite-outgrowth in embryonic neural cells through its binding to the heparan sulfate proteoglycan N-syndecan or to the chondroitin sulfate proteoglycan RPTPβ (12, 13). In addition, HARP stimulates cellular proliferation of a wide range of cell types including fibroblast, endothelial, and epithelial cells (2, 7, 14). HARP also displays transforming activity because transfection of NIH-3T3 or normal rat kidney (NRK) fibroblasts with the HARP cDNA led to morphological transformations, anchorage independent growth, and tumor formation in nude mice (15). In agreement with its role in angiogenesis, HARP induces migration of aortic endothelial cells in collagen (16) and enhances plasminogen activator activity in the same cells (17). Recently, the mitogenic and transforming activities of HARP have been linked to its binding to the tyrosine kinase receptor anaplastic lymphoma kinase (ALK) inducing its phosphorylation and recruitment of downstream effector molecules such as IRS-1, Shc, phospholipase C-γ, and phosphatidylinositol 3-kinase (18).

NMR experiments have clearly demonstrated that the HARP molecule is organized into two β-sheet domains linked by a flexible linker, and this structure is maintained through five intrachain disulfide bonds (19). Each β-sheet domain contains a heparin-binding site, which plays a role in the modulation of HARP mitogenic activity (20). In addition, we have shown that at least one heparin-binding domain is involved in the dimerization of HARP (21). Two clusters of basic residues are located
at the N-terminal and C-terminal regions, and we have recently shown that the lysine-rich C-terminal tail of HARP is needed for its mitogenic and tumor-formation activities but not for the neurite outgrowth-promoting activity (22). Indeed the deletion mutant HΔ111–136, deleted of its last 25 amino acids, was shown to display no mitogenic activity on BEL cells or NIH-3T3 cells and shown to be unable to induce tumor formation in nude mice but to stimulate the neurite outgrowth of rat embryonic neurons.

In this paper, we demonstrate for the first time that HΔ111–136 and the corresponding synthetic polypeptide P₁₁₁–₁₃₆, lacking mitogenic, angiogenic, and tumor formation activities, could act as potent inhibitors of these HARP biological activities.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture medium, fetal calf serum (FCS), and G418 were supplied by Invitrogen. Superblocker® solution was purchased from Pierce, horseradish peroxidase-conjugated rabbit anti-goat, goat anti-rabbit immunoglobulins G was purchased from Jackson, and 3,3′,5′-trime-thylbenzene dihydrochloride substrate led disuccinimidyl suberate were purchased from Interchim (Montluçon, France). Goat anti-human HARP antibodies were from R&D (Oxon, United Kingdom). Heparin-Sepharose gel and Mono-S column were from Amersham Biosciences, Immobilon-P from Millipore Corp. (Saint-Quentin en Yvelines, France), BM chemiluminescence® and Fu-GENE® were from Roche Mannheim (Meylan, France), and mouse anti-phospho-p44/p42 MAP kinase was from New England BioLabs (Saint-Quentin en Yvelines, France). [Methyl-3H]thymidine was provided by ICN (Orsay, France) and Matrigel was from BD PharMingen. Peptides P₁₁₁–₁₃₆ (KLTKPKFQAESSKKKEEGKKQEKMLD) and P₁–₂₁ (AEEAGKKEKPEKVKKSDCGEW) were synthesized by the System laboratoire (Nîmes, France). Recombinant fibroblast growth factor-2 (FGF-2), MK, and mutated and wild type (WT) HARP were purified in the laboratory by sequential heparin-Sepharose and Mono-S chromatographies from bacteria and conditioned media of eukaryotic cells, respectively (22). Bovine angiogenin was a generous gift from Dr. G. Spik (UMR8576 CNRS, Villeneuve d’Ascq, France).

**Thymidine Incorporation Assay and Phosphorylation of MAP Kinase**—The incorporation of [methyl-3H]thymidine by serum-starved NIH-3T3 cells was performed as previously described (22). 2.5 × 10⁴ NIH-3T3 cells were seeded in 35-mm culture dishes for 24 h, serum-starved for 24 h, and stimulated 5 min at 37 °C with samples. Cells were lysed with electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.02% bromphenol blue, 2% SDS, and 5% 2-mercaptoethanol), and the presence of p42/p44 or phospho-p42/p44 MAP kinase was determined using an immunometric assay (25), and clones secreting the largest amount of HARP were selected. For these clones, the presence of both HARP and mutant proteins in the culture medium was further investigated by Western blotting after heparin-Sepharose purification.

**Soft Agar Assay**—2 × 10⁴ cells of the clonally selected MDA-MB-231 cell line were seeded in triplicate into 6-well plates containing agar and DMEM supplemented with 10% FCS. After 14 days, colonies with diameters greater than 50 μm were scored as positive using a phase contrast microscope equipped with a measuring grid.

**Tumor Formation in Nude Mice**—Tumor formation in 5-week-old male athymic nude mice (Nu/Nu; IFFA CREDO Laboratories) was tested by subcutaneous injection of 4 × 10⁶ cells suspended in 100 μl of DMEM in each flank. Tumor size was measured twice a week, starting from the second week following injection. Mice were sacrificed 6 weeks after injection.

**Chemical Cross-linking Experiment**—Purified recombinant HARP or HΔ111–136 (15 pmol) were pre-incubated for 60 min at 25 °C in the presence of 10 μg/ml heparin and treated with 0.5 μM disuccinimidyl suberate according to the manufacturer’s protocol, and products were analyzed using a 15% acrylamide SDS-PAGE and anti-HARP Western blotting assay using HARP as a probe or using an antiphospho p42/p44 MAP kinase antibody (B) and an anti-p42/p44 MAP kinase antibody (C), respectively.
hours after transfection, medium was changed and hygromycin (Sigma) was added to the medium at 0.5 mg/ml. After 10 days of selection, the medium was changed to the serum-free AIM-V synthetic medium (In-vitrogen). The AIM-V production media were collected every 2–3 days.

The secreted RECA-His protein was purified from the AIM-V production media over a nickel-nitrilotriacetic acid superflow column (Qiagen, Courtaboeuf, France) following the manufacturer’s protocol. Rabbits were immunized with the purified RECA-His protein. IgG fractions of the immune sera were immunopurified using DEAE chromatography.

Cell-free RECA-HARP Binding Studies—Purified recombinant HARP, HΔ111–136, FGF-2, MK, angiogenin, peptides P111–136 and P1–21 were coated on a 96-well EIA plate (costar). Nonspecific binding sites were blocked with PBS, 0.05% Tween 20 (v/v) (PBS-T) containing 3% BSA (w/v). Wells were then incubated with RECA diluted in PBS-T containing 1% BSA for 1 h at 37 °C and exposed for 1 h at 37 °C to rabbit anti-RECA antibodies diluted at 1 µg/ml in PBS-T containing 1% BSA. Bound antibodies were visualized using a peroxidase-labeled goat anti-rabbit antibodies at a 1:5000 dilution in PBS-T containing 1% BSA, and the peroxidase activity was detected with 3,3′,5,5′-tetramethyl benzidine dihydrochloride substrate according to the supplier.

RESULTS

Inhibition of HARP Mitogenic Activity by HΔ111–136—The mitogenic activity of HARP in the presence of different concentrations of the HΔ111–136 mutant was investigated on serum-starved NIH-3T3 cells. A 2-fold increase of [³H]thymidine incorporation was observed for 2 nM HARP and inhibited in a dose-dependant manner by addition of HΔ111–136 (Fig. 1A). A maximum 70% inhibitory effect was observed for 7.5 nM HΔ111–136 corresponding to a molecular ratio of 3:1 between mutant and HARP protein. As expected from previous results (22), no mitogenic activity of HΔ111–136 alone was observed. In a control experiment, stimulation of [³H]thymidine incorporation induced by 0.2 nM FGF-2 was not affected by HΔ111–136 (Fig. 1A). The specificity of the HΔ111–136 inhibitory effect was also tested with the structurally related molecule MK, which displayed no
effects upon 2 nM HARP stimulation (data not shown). Because the HARP mitogenic signal, at least in BEL and NIH-3T3 cells, is transduced through the MAP kinase signaling pathway leading to the phosphorylation of ERK1 and ERK2 (22, 27), the ability of HARP to induce MAP kinase phosphorylation in the presence of HΔ111–136 was evaluated (Fig. 1B). As expected from cell proliferation experiments ERK1 and ERK2 phosphorylation induced by HARP was inhibited in a dose-depended manner by the addition of HΔ111–136.

**Inhibition of in Vitro and in Vivo HARP Angiogenic Activity by HΔ111–136**—As the HΔ111–136 mutant protein specifically inhibited the HARP-induced cell proliferation, its effect on the angiogenic activity of HARP was investigated *in vitro* and *in vivo* using aortic bovine endothelial cells cultured on a three-dimensional collagen gel (23) and a mouse Matrigel plug assay (24), respectively. *In vitro*, 20 nM HARP induced a tubular network structure formation within 72 h (Fig. 2B). As compared with the HARP-treated cells (Fig. 2A), 60 nM HΔ111–136 inhibited the HARP-induced angiogenic effect by more than 66% (Fig. 2E), whereas no significant effect was observed either in cells treated with 60 nM HΔ111–136 alone (Fig. 2C) or in control cells (Fig. 2A). A tubular network comparable with that formed using HARP was also obtained with 3 nM FGF-2, and no significant effect was observed with HΔ111–136 (data not shown). In the *in vivo* angiogenesis assay, a 3-fold increase in the infiltration of endothelial cells was observed when 3 nM HARP was added to Matrigel as compared with control (Matrigel alone) (Fig. 3, A, C, and G). Addition of 10 nM HΔ111–136 with 3 nM HARP in Matrigel, corresponding to a 3:1 ratio, resulted in a 50% inhibition of the angiogenic activity of HARP, whereas no Matrigel infiltration was observed with HΔ111–136 alone (Fig. 3, B, D, and G). As a control, 10 nM FGF-2 was tested and a more pronounced increase, as compared with HARP, of the infiltration of Matrigel by endothelial cells was obtained. As expected from the *in vitro* assay, no significant inhibition of FGF-2 by HΔ111–136 was observed (Fig. 3, E, F, and G).

**In Vitro and in Vivo Inhibition of HARP-transforming Activity by HΔ111–136**—The human breast cancer cell line MDA-MB 231 has been previously demonstrated to produce endogenous HARP, which acts in an autocrine manner. These cells were therefore used to test the inhibitory effect of the HΔ111–136 mutant protein on HARP-transforming activity. MDA-MB 231 cells were transfected with pCDNA3 alone (control) or pCDNA3-HΔ111–136, and clones were selected for their ability to secrete HARP. The presence of both HARP and HΔ111–136 proteins in the culture media was checked by Western blotting experiments, and proteins were similarly secreted (data not shown). Two representative clones (control and HΔ111–136-expressing cells) were further studied for their ability to grow in soft agar. Under our experimental conditions, HΔ111–136-expressing clones formed 2-fold fewer colonies than control cells (Table I). These results suggested that HΔ111–136 had inhibited the anchorage-independent growth of MDA-MB 231 cells. To further investigate the inhibitory effect of HΔ111–136 protein on HARP-transforming activity, one HΔ111–136 clone and one control clone were tested for their ability to form tumors in nude mice. Six weeks after the injection, ten of twelve mice injected with MDA-MB 231 control cells had developed tumors, whereas only one mouse injected with MDA-MB 231 cells expressing HΔ111–136 had developed a tiny tumor (Table I). A difference of weight between the two pools of mice could also be noticed. To ensure that tumors derived from G418-resistant-injected cells, tumor fractions were dispersed and cultured in medium containing 600 µg/ml of the antibiotic. Under these conditions, most cells from the tumors appeared to be G418-resistant (data not shown). These results indicate that the ability of MDA-MB 231 cells to form tumors in nude mice was strongly inhibited by a dominant negative effect of HΔ111–136 mutant protein and that the transforming activity of these cells is really dependent on their HARP expression.

**Molecular Mechanisms Involved in the Dominant Negative Effect of HΔ111–136**—Two hypothesis could be raised to explain the dominant negative effect: i) because HARP had been demonstrated to homodimerize (21), HΔ111–136 could form a non-functional heterodimer with HARP and ii) the mutant protein could compete against HARP for binding to the high affinity receptor ALK and consequently block signal transduction. To determine whether HΔ111–136 was capable of heterodimerizing with HARP, we performed chemical cross-linking and Western blotting experiments. As expected, HARP could form homodimers in the presence of heparin (Fig. 4, lane 2) (21), and similarly, HΔ111–136 could also homodimerize (Fig. 4, lane 1). However, in the presence of WT and mutant proteins, both homo- and heterodimers were observed (Fig. 4, lane 3).

The ability of HΔ111–136 to bind to the high affinity ALK
receptor was investigated by enzyme-linked immunosorbent assay using the recombinant extracellular domain of the ALK receptor (RECA) produced in high eukaryotic cells. Recombinant purified HARP (500 ng/well) was coated and incubated with increasing concentrations of RECA ranging from 0 to 600 ng/well (Fig. 5A). Anti-RECA immunodetection revealed that RECA binds HARP in a dose-dependent manner reaching a plateau at a RECA concentration of 300 ng/well. Then, various quantities of either HARP, HΔ111–136, or control basic proteins including MK, FGF-2, and angiogenin were coated and incubated with 300 ng of RECA per well (Fig. 5B). RECA displayed a specific and saturable binding to the precoated HARP and MK proteins, whereas only background signals were detected with the HΔ111–136 mutant protein, FGF-2, or angiogenin. These results strongly suggest the absence of HΔ111–136 binding to its high affinity receptor and could explain the absence of signal transduction using this mutant (Fig. 1B, lane 4) (22).

Inhibition of WT HARP in Vitro Biological Activities by the P111–136 Peptide—Taken together, these results and our previous report seem to implicate the lysine rich C-terminal domain of HARP in the binding to its high affinity receptor ALK. To confirm this hypothesis, the ability of the peptide P111–136, corresponding to the last 25 amino acids of HARP, to interact with RECA was tested using the enzyme-linked immunosorbent assay described above. The P1–21 peptide was used as a control because it also contained a lot of lysines and has a similar backbone size. Various amounts of P111–136 and of the P1–21 N-terminal peptide of HARP (28) were coated and incubated with 300 ng of RECA per well (Fig. 5C). A specific and saturable signal was observed when P111–136 was immobilized, whereas only a background signal was detected using P1–21. Our results strongly strengthened the fact that HARP binds to the ALK receptor via its C-terminal domain and suggested that P111–136 could act as a dominant negative effector to inhibit WT HARP biological activities. This assumption was validated because P111–136 inhibited, in a dose-dependent manner, the [3H]thymidine incorporation induced by HARP (Fig. 6A). About 75% inhibition was observed on serum-starved NIH 3T3 cells with 10 nM P111–136, although P1–21 was unable to inhibit HARP stimulation. Inhibition of the in vitro transforming activity of HARP by P111–136 was also evaluated in soft agar assays using MDA-MB 231 cells. Cells were seeded in soft agar and various amounts of P1–21 and P111–136 were added in culture medium. As shown in Fig. 6B, the transforming activity of WT HARP was prevented in a dose-dependent manner.
manner by addition of P111–136, and no inhibition was observed with P1–21. The number of colonies was 50% decreased by 10 μM P111–136 peptide.

**DISCUSSION**

HARP is expressed in many human tumors and tumoral cell lines including neuroblastoma, glioblastoma, melanoma, pancreatic, and breast cancers (6–10) and can be an in vitro rate-limiting angiogenic factor in tumor growth and metastasis (29, 30). According to these studies, this molecule is now considered as an interesting target in cancer therapy (9, 29). Recently, we have demonstrated the involvement of the C-terminal 111–136 amino acid of HARP in the mitogenic and in the transforming activities of this growth factor. These results prompted us to investigate the potential dominant negative effect of the C-terminal-truncated protein for the different characterized biological activities of HARP.

**Dominant Negative Effect of HΔ111–136—Inhibition of HARP mitogenic, transforming, and angiogenic activities**

was observed in the presence of HΔ111–136 suggesting that similar structural determinants of the HARP molecule were implicated in these three activities. The dominant negative effect of HΔ111–136 on HARP-transforming activity was investigated using MDA-MB 231 cells. These cells were previously shown to express HARP and its high affinity receptor ALK, which is essential for the malignant phenotype (31, 32). In vitro, stable overexpression of the HΔ111–136 mutant protein by the MDA-MB 231 cell line resulted in a 50% decrease of colony formation in soft agar assay suggesting that the anchorage-independent growth was only partially due to HARP and that other growth factors are implicated. Similar partial inhibition had already been observed with other tumoral cell lines such as colorectal Colo 357 cells and melanoma WI 582 or 1205 Lu cells using HARP ribozyme targeting or HARP antisense strategies (9, 30, 33). However, in vivo, tumor formation into nude mice, usually obtained with MDA-MB 231 cells, was completely abolished when HΔ111–136-expressing MDA-MB 231 cells were injected suggesting that both the growth advantage and the angiogenic activity were inhibited. The dominant negative effect of HΔ111–136 on angiogenic activity was also supported by its ability to in vitro inhibit the formation of capillary network in a three-dimensional collagen gel and to strongly decrease in vivo the HARP-induced infiltration of Matrigel plugs by endothelial cells.

**Molecular Mechanism Involved in the Dominant Negative Effect**—The dimerization of growth factors such as FGF-1 and FGF-2 is responsible for receptor dimerization, cellular activation, and cell proliferation (34), and such a mechanism has been proposed for HARP (21). In regards to the results presented here, it seems that heterodimerization of the C-terminal mutant with HARP mainly explains its dominant negative effect. This heterodimerization is favored for a 3:1 molecular ratio between HΔ111–136 and HARP suggesting that under these conditions an excess of the mutant protein can dissociate HARP homodimers, inducing the formation of non-functional heterodimers. A similar mechanism had been proposed for HARP-transforming activities (35). These results strengthen the role of HARP dimers as the active forms of HARP for its mitogenic, angiogenic, and transforming activities.

During this study, we also investigated the binding of HΔ111–136 and HARP to the extracellular domain (RECA) of the tyrosine kinase receptor ALK, recently identified as a part of the HARP signaling pathway involved in mitogenic and transforming activities. The involvement of ALK in the HARP biological activities that we evaluated was first attested by the ability of RECA to bind HARP using the mitogenic activity assay with NIH-3T3 cells. RECA therefore induced a dose-depandent inhibition of the [3H]thymidine incorporation due to HARP and transfection of non-responsive Chinese hamster ovary cells with the ALK cDNA-induced [3H]thymidine incorporation and cellular proliferation of the selected clones by HARP (data not shown). The absence of interaction between HΔ111–136 and RECA that we have observed strongly suggests that HARP binds to ALK via its C-terminal domain. This assumption was reinforced by the binding of RECA to the synthetic peptide corresponding to the truncated mutant HΔ111–136 and the dominant negative effects of this peptide for HARP in vitro biological activities. The specificity of the interaction between the C-terminal part of HARP and RECA was mainly validated by the fact that P1–21, the HARP N-terminal peptide with basic properties close to P111–136 (pI of 9.37 versus 10.08), was not able to interact with RECA. Correlatively, a mutant deleted of the basic N-terminal cluster of lysines (HΔ1–12) kept mitogenic, angiogenic, and transforming activities and a binding capacity to RECA (data not shown). During these experiments, specific binding of MK to RECA was also observed, suggesting that ALK could be a common receptor for MK and HARP as observed with PTPζ (36). This hypothesis is currently under study.

In conclusion, we have demonstrated the dominant negative effects on HARP biological activities of the truncated mutant HΔ111–136 and of the corresponding synthetic peptide P111–136. We have also underlined the binding of HARP to the high affinity receptor ALK through its C-terminal part. It is noteworthy that proteolysis of the C-terminal tail of HARP could be achieved in vitro by plasmin, suggesting that such a mechanism could exist in vitro and participate in the regulation of HARP biological activities. Considering the importance of HARP as a rate-limiting autocrine growth factor in pancreatic and breast cancer cells (9, 35) and its low expression in normal adult tissues, HARP appears as a promising target for cancer therapy. Synthetic HARP-related peptides or controlled expression of dominant negative mutant proteins into target cells could be used to block HARP growth tumor-promoting activity and now have to be evaluated.

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