The Lysine-rich C-terminal Tail of Heparin Affin Regulatory Peptide Is Required for Mitogenic and Tumor Formation Activities*

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Heparin affin regulatory peptide (HARP) is a 18-kDa heparin-binding polypeptide that is highly expressed in developing tissues and in several primary human tumors. It seems to play a key role in cellular growth and differentiation. *In vitro*, HARP displays mitogenic, angiogenic, and neurite outgrowth activities. It is a secreted protein that is organized in two β-sheet domains, each domain containing a cluster of basic residues. To assess determinants involved in the biological activities of HARP, C-terminally truncated proteins were produced in Chinese hamster ovary-K1 cells and tested for their mitogenic, tumor formation in nude mice and neurite outgrowth activities. Our data clearly indicate that the residues 111–136 of the lysine-rich C-terminal domain are involved in the mitogenic and tumor formation activities of HARP. Correlatively, no signal transduction was detected using the corresponding mutant, suggesting the absence of HARP binding to its high affinity receptor. However, this C-terminal domain of HARP is not involved in the neurite outgrowth activity. We also demonstrate that HARP signal peptide cleavage could lead to two matured forms that are both but differentially mitogenic.

Heparin affin regulatory peptide (HARP)† (1) is a secreted protein that belongs to the superfamily of heparin-binding growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also referred to as pleiotrophin (2), heparin-binding growth-associated molecule (3), and osteoblast specific growth factor. Also refere

HARP displays several biological activities *in vitro*. Originally isolated as a neurite outgrowth-promoting protein (13), further studies have shown that this protein stimulates the cellular proliferation of a wide variety of cells, including fibroblast, epithelial, and endothelial cells (1, 9, 14, 15). In agreement with its role in angiogenesis, HARP can induce migration of aortic bovine endothelial cells in collagen (16) and enhances plasminogen activator activity of the same cells (17). Until now, except for the neurite outgrowth activity (18, 19), the cell surface molecules involved in these biological activities are still poorly documented. However, it seems that low and high affinity binding sites could be involved in its mitogenic activity. The existence of high affinity receptors is suggested by the phosphorylation of a 200-kDa protein in NIH-3T3 and NB41A3 cells treated with HARP (20) and is strongly supported by the ability of HARP to transduce a mitogenic signal through MAPK and phosphatidylinositol 3-kinase pathways in BEL cells (21). HARP is a 18-kDa protein that contains 24% of basic residues (18% of lysines), mainly arranged in two clusters at the N- and C-terminal regions, and five intrachain disulfide bonds, clearly demonstrated using NMR experiments by the Rauvala group (22). The molecule is organized in two β-sheet domains linked by a flexible linker, and each of these two domains includes one heparin-binding site. At least one heparin-binding site is involved in the dimerization of this growth factor (23) and is important for HARP mitogenic activity, because this activity on BEL cells is modulated by exogenous addition of glycosaminoglycans (24). Furthermore, treatment of BEL cells with heparinase III abolished HARP mitogenic activity, which could be restored by the addition of soluble heparin.

Post- or cotranslational maturations of HARP have been considered as a possible mechanism involved in HARP mitogenic activity. The cleavage site of the signal peptide of HARP has been more particularly subject to controversies between several groups. The N-terminal sequence of the originally purified mature peptide from uterus or from conditioned media of epithelial cells was GKKEKP corresponding to a 136-amino acid protein (HARP136) (14, 25, 26). However, we have identified a second cleavage site of the signal peptide leading to a 3-amino acid extension AEA (HARP139), which seemed to be recognized by antibodies against endoglycosidase H (18). A recent study using a 139-amino acid recombinant protein (HARP139) has shown that the AEA cleavage site is constitutive (27). HARP139 was shown to be mitogenic and more efficiently internalized than HARP136 (18, 27). The N-terminal sequence of HARP139 was shown to be identical to that of HARP136, indicating that the AEA extension is added posttranslationally. The AEA extension has been also shown to be involved in the secretion of HARP139 (28). However, the biological significance of this extension remains to be determined.
To clarify the importance of N- and C-terminal maturations in HARP mitogenic activity, C-terminally truncated mutants of HARP were produced and purified, and the determinants playing a role in the biological activity, including mitogenic, neurite outgrowth, and tumor formation activities were investigated.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture medium, fetal calf serum (FCS), and G418 were supplied by Life Technologies (Cergy Pontoise, France). Superblock solution (Pierce) and horseradish peroxidase-conjugated rabbit anti-mouse antibodies (Amersham Pharmacia Biotech, Orsay, France), Immobilon-P from Millipore Corp. (Saint-Quentin en Yvelines, France), BM chemiluminescence and Fugene6 from Roche Molecular Biochemicals Mannheim (Meylan, France), and mouse antimouse phospho-p42/p44 MAPK from New England BioLabs (Saint-Quentin en Yvelines, France). [methyl-3H]Thymidine was provided by ICN (Orsay, France).

**Mutagenesis**—The human HARP cDNA was subcloned into the EcoRI site downstream of the cytomegalovirus promoter of the eucaryotic expression plasmid pCDNA3 (Invitrogen, Leek, Netherlands). The mutated pCDNA3-HARP plasmid was used to transfect CHO-K1 cells (a generous gift from T. Melot, Institut Curie, en Yvelines, France). Oligodeoxyribonucleotides were synthesized by Eurogentec (Belgium). The presence of the mutations was confirmed by double-stranded DNA sequencing.

**Cell Culture and Transfection**—NIH-3T3 cells expressing c-Myc/6His-tagged HARP (clone HMM-C9) were maintained in Dulbecco's modified Eagle's medium supplemented by 10% FCS and 400 μg/ml G418 (23). CHO-K1 cells (a generous gift from T. Melot, Institut Curie, Paris) were cultured in Ham's F-12 medium supplemented by 10% FCS and 800 μg/ml G418 (23). CHO-K1 cells were cultured in Ham's F-12 medium supplemented by 10% FCS and transfected 24 h later with 4 μg of pCDNA3 or pCDNA3-HARP plasmids using the liposomal system Fugene6 according to the manufacturer's protocol. 48 h after transfection, cells were selected for G418 resistance (800 μg/ml). Medium was changed every 2 days until colonies formed. Each G418-resistant clonal population was scrapped with a tip, resuspended, and cultured in the selective medium. The clones secreting the largest amount of HARP were selected. Several clones transfected with pCDNA3 were also selected for control experiments (Mock cells).

**Purification of the HARP from Conditioned Media**—Tagged, mutated, or wild type HARP proteins were purified as previously described (16). Briefly, 8 × 10⁵ NIH-3T3 or CHO-K1 cells were plated in 600-cm² dishes and cultured for 72 h in complete medium. For CHO-K1 cells, the culture medium was supplemented 24 h after seeding with 5 mM sodium butyrate, which increased by 100-fold the expression level of recombinant protein (29). Conditioned medium containing secreted HARP proteins was buffered to pH 7.4 with 20 mM Hepes, ionic strength adjusted to 0.5 mM NaCl and then loaded onto a 10-ml heparin-Sepharose column. Bound proteins were eluted with 20 mM Hepes, 2 mM NaCl, pH 7.4, and further purified using a cation-exchange Mono-S column. The purification was carried out in 50 mM Tris-HCl, pH 7.4, and proteins were eluted using a 0.4 to 2 mM NaCl gradient. Determination of the N-terminal sequence was performed using the Protein Microsequencing Service at the Pasteur Institute (Paris, France). The amount of HARP proteins in each peak was evaluated using an immunometric assay described in Soulé et al.²

**Thymidine Incorporation Assay**—3 × 10⁶ NIH-3T3 wild type cells per well were seeded in 48-well plates for 24 h in DMEM supplemented by 10% FCS. Cells were then serum-starved for 24 h, and samples were added. Cells were then incubated at 37 °C and 7% CO₂ for 18 h and incubated further for 6 h with 0.5 μCi of [methyl-3H]Thymidine. Cells were then fixed with 10% trichloroacetic acid, washed with water, and lysed with 0.1 N NaOH. Total incorporated radioactivity was counted using a micro-beta scintillation counter (LKB, PerkinElmer Life Sciences, Courtaboeuf, France). Similar protocol was used with BEL cells as previously described (27).

**Western Blotting Procedure**—Purified proteins were run on SDS-15% polyacrylamide gel and electrotransferred to Immobilon-P membrane in 10 mM CAPS (3-[cyclohexylaminol]-1-propanesulfonic acid), pH 11, containing 10% methanol. Nonspecific binding was prevented by incubating the membrane for 20 min in the Superblocker solution at room temperature. The membrane was then incubated overnight at 4 °C with goat anti-human HARP antibodies (250 ng/ml) diluted in PBS containing 0.2% Tween 20 (PBS-T) and 3% Superblocker. After three washes with PBS-T, the membrane was incubated 30 min at room temperature with the peroxidase-conjugated anti-immunoglobulin goat antibodies diluted in PBS-T, and the enzyme was detected using the BM chemiluminescence reagent.

**Phosphorylation of MAPK**—2.5 × 10⁵ NIH-3T3 cells were seeded in 35-mm culture dishes for 24 h, serum-starved for 24 h, and stimulated for 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% β-mercaptoethanol), and the presence of phospho-p42/p44 MAPK was detected by Western blot using the procedure described above except that proteins were run on SDS-10% polyacrylamide gel, electrotransferred in 25 mA Tris, pH 8.3, containing 200 mM glycine, and 10% ethanol, and Immobilon-P membrane incubated in PBS-T supplemented with 2% (w/v) powdered milk. The monoclonal anti-phospho-p42/p44 antibodies was diluted to obtain a 1 μg/ml concentration.

**Neurite Outgrowth Assays**—Cells from cerebral hemispheres of 18-day-old rat embryos were dissociated as described previously (3). Brain was dispersed into individual cells in DMEM containing 10% FCS, 6 mg/ml glucose, 100 units/ml penicillin, and 0.1 mg/ml streptomycin using a 10-ml sterile syringe and centrifuged at 100 × g for 10 min. The pellet was suspended in the same medium without FCS and containing 1 mg/ml bovine serum albumin. 2.5 × 10⁵ cells were seeded in a 96-well EIA plate (Costar, Brumath, France) precoated with 0.8, 1.6, or 3.2 μg/ml wild-type (wt) HARP or mutant proteins. After 48 h, cells were stained with May-Grünwald's solution and Giemsa stain. The number of neurites per well was evaluated using phase contrast microscopy. Each experiment was carried out in duplicate and representative results are shown.

**Tumor Formation in Nude Mice**—Tumor formation in 5-week-old female nude mice (Balb/c nu/nu, Iffa-Credo Lab, L'Arbresle, France) was tested by subcutaneous injection of 10⁶ cells suspended in 100 μl of Ham's F-12 at a unique site. Tumor size was measured twice a week, starting from the second week following injection. Mice were sacrificed 6 weeks after injection.

**RESULTS**

Contradictory results have been reported concerning the mitogenic activity of HARP. Although only neurite outgrowth activity was originally reported by Rauvala (13), further studies have suggested that the mitogenic activity of HARP was dependent either on the presence of three amino acids at the N terminus (16) or related to a C-terminally truncated form (21). To clarify this point, recombinant C-terminal-tagged molecules with the c-Myc/6His peptide, which is useful to detect C-terminal processing, were produced from NIH-3T3 cells (23). The recombinant proteins were purified, characterized, and tested for their mitogenic activity.

**N-terminal Processing of HARP and Mitogenic Activity**

Purification of HARP from NIH-3T3 Cells—Two liters of conditioned medium were collected after a 72-h culture, and proteins were purified using sequential heparin-Sepharose Mono-S chromatographies, as described under “Experimental Procedures.” Using a 0.4 to 2 mM NaCl gradient for the elution of the Mono-S column, five major fractions were collected (data not shown) and analyzed using SDS-polyacrylamide gel (Fig. 14, lanes 1–5) and Western blot experiments (Fig. 1B). In fractions 2 and 4, two proteins with an apparent molecular mass of 21 kDa were mainly purified, in good agreement with the size of the C-terminal tag extension. Both polypeptides were immunodetected by the anti-HARP (Fig. 1B, lanes 2 and 4) and anti-c-Myc (data not shown) antibodies. The fraction eluted with 0.61 mM NaCl from the Mono-S column with an apparent molecular weight of 21,000 (lane 2) corresponded to the tagged-HARP₁₂₀ molecule, whereas the fraction eluted
with 0.67 mM NaCl (lane 4) was determined to be tagged-HARP136. Those two tagged-HARP molecules, therefore, revealed N-terminal processing identical to what we previously described (16). In fraction 4, two additional bands of 18 and 15 kDa were slightly silver-stained, immunodetected with anti-HARP antibody (Fig. 1B, lane 4), and could correspond to C-terminal proteolytic cleavage, because they were not recognized by the anti-c-Myc antibody (data not shown). Two 18-kDa proteins eluted with 0.65 mM NaCl (Fig. 1A, lane 3) and 0.69 mM NaCl (Fig. 1A, lane 5) were also purified and immunodetected with the anti-HARP antibody but not using anti-c-Myc antibody (data not shown). They might correspond, respectively, to HARP139 and HARP136 produced endogenously by NIH-3T3 cells, but we cannot exclude a cleavage of the c-Myc/6His epitope, even if the purification was carried out in the presence of protease inhibitors. During this experiment, two proteins with apparent molecular masses of 65 and 30 kDa were isolated in fraction 1 (arrows in Fig. 1A, lane 1). N-terminal sequencing and Western blot experiments with an anti-HGFα antibody (inset of Fig. 1B) identified them to be the α and β chains of the hepatocyte growth factor. We can also note that bovine serum albumin was identified in fraction 2 (Fig. 1A, lane 2).

The mitogenic activity of the recombinant HARP isolated as described above was then assayed on serum-starved NIH-3T3 cells (Fig. 2A). Aliquots of each fractions, as mentioned in the figure legend, were added to the cells, and mitogenic activity was determined as described under “Experimental Procedures.” As shown in Fig. 2A, the fractions containing the HARP139 (fraction 2, tagged-HARP139; fraction 4, HARP139) as well as HARP139 form (fraction 3, tagged-HARP139; fraction 5, HARP139) induced cell proliferation in a dose-dependent manner. A 4-fold increase of tritiated thymidine incorporation in NIH-3T3 cells as compared with the control was observed for the highest concentration of each fraction and was similar to what was obtained with 10% FCS (Fig. 2A). Specific activities were calculated by estimating the concentration of HARP in the different fractions using an immunometric assay that we have developed.2 The ED$_{50}$ level for HARP139 was higher than for HARP136, i.e. 2 and 6 nM, respectively. Similar results were obtained using BEL cells (data not shown). However, although HGF was only immunodetected in the first eluted fraction from Mono-S chromatography, it was important to rule out the possibility of a cross-contamination by HGF in other eluted fractions containing HARP. In this respect, NIH-3T3 cells were stimulated with a saturating dose of HGF (10 μl of fraction 1) and 1 μl of each mono-S HARP fraction (described in Fig. 1). Purified recombinant FGF-2 (10 ng/ml) was used as a positive control of overstimulation. The results are the means of three separate experiments carried out in triplicate and the standard errors are indicated. For more details concerning the thymidine incorporation, see “Experimental Procedures.”

Fig. 1. Purification of recombinant HARP from NIH-3T3 cells. Recombinant HARP was purified from conditioned medium of NIH-3T3 cells expressing HARP tagged with the c-Myc/6His epitope, using sequential heparin-Sepharose and Mono-S chromatographs. A, 20-μl aliquots of each fraction (lanes 1–5), collected from the Mono-S column using a 0.4 to 2 M NaCl gradient, were electrophoresed using a 15% SDS-PAGE, and proteins were detected using silver staining. HGFα and HGFβ correspond to the two subunits of this growth factor. B, Western blot of 5 μl of each fraction using an anti-human HARP antibody after 15% SDS-PAGE electrophoresis and transferred to Immobilon-P membrane. Inset, similar experiment to B but using an anti-HGF antibody.

Fig. 2. Mitogenic activity of recombinant HARP produced from NIH-3T3 cells. A, stimulation of [3H]thymidine incorporation of serum-starved NIH-3T3 cells during 18 h by 0.1, 1, and 10-μl aliquots of HARP fractions obtained from the Mono-S chromatography described in Fig. 1. FGF-2 and FCS are used as positive controls. B, overstimulation experiments. Serum-starved NIH-3T3 cells were stimulated by a combination of a saturating dose of HGF (10 μl of fraction 1) and 1 μl of each mono-S HARP fraction (described in Fig. 1). Purified recombinant FGF-2 (10 ng/ml) was used as a positive control of overstimulation. The results are the means of three separate experiments carried out in triplicate and the standard errors are indicated. For more details concerning the thymidine incorporation, see “Experimental Procedures.”
Production of Recombinant HARP from CHO-K1 Cells—The N-terminal maturation of HARP was also studied using a CHO-K1 cell expression system. After transfection with the cDNA encoding HARP protein, CHO-K1 clonal populations overexpressing HARP were selected using G418, and HARP was purified from the conditioned medium as described under “Experimental Procedures.” Under these conditions, only HARP136 was isolated. Neither HARP139 nor HGF proteins have been identified. HARP135 isolated from CHO-K1 cells displayed mitogenic activity for NIH-3T3 cells (see below) and BEL cells (not shown). However, HARP136 produced by CHO-K1 cells was about three times less active than HARP136 produced in NIH-3T3 cells (ED50 of 20 nM versus 6 nM). As control, purification steps were carried out from the conditioned medium of CHO-K1 cells transfected with vector alone, and no mitogenic activity was yielded in the collected fractions from the Mono-S chromatography (not shown).

Involvement of C-terminal Determinants for the Mitogenic Activity of HARP

As mentioned in the introduction, truncated forms of HARP at the C-terminal region have been identified in SW13 transfected with HARP cDNA and in Swiss 3T3 cells (21, 28). Involvement of this C-terminal maturation in HARP mitogenic activity has not been clearly established, and, to further investigate this point, C-terminal truncated HARP proteins were produced in CHO-K1 cells.

Two different C-terminal truncated mutants were constructed by adding a stop codon (Fig. 3A): (i) HΔ129–136 in which the last 8 amino acids of HARP were suppressed but the C-terminal cluster of lysines was conserved and (ii) HΔ111–136 in which the consensus sequence, possibly involved in angiogenic activity (30) and containing the cluster of lysines, was deleted. However, all the cysteines involved in disulfide bridges were conserved. As described above, expression plasmids coding for the mutant proteins were transfected in CHO-K1 cells, selected using G418 and the HARP expression levels of each stably transfected cell line were determined by HARP immunometric assay2 (data not shown). HARP proteins were purified from 0.4 liter of transfected CHO-K1 conditioned media according to the procedure described above, and isolated proteins were analyzed by Western blotting experiment with an anti-HARP antibody (Fig. 3B). For each mutant proteins, the molecular weight observed was in agreement with the size of the deletion (17,200 and 14,500 for HΔ129–136 and HΔ111–136, respectively).

Mitogenic Activity of C-terminal Truncated HARP Proteins—Mitogenic activity of the C-terminal truncated HARP proteins was tested on serum-starved NIH-3T3 cells. A dose-response curve was performed for mutant proteins as well as wt HARP (Fig. 3C). Both wt HARP and HΔ129–136 mutant proteins presented similar mitogenic activities with an ED50 ~ 20 nM, whereas HΔ111–136 mutant protein was not mitogenic. The mitogenic activity profile was identical for BEL cells (data not shown), and a similar result was obtained with another HΔ111–136 expressing clone. HARP mitogenic activity had been associated to tyrosine kinase activation and involvement of MAPK and phosphatidylinositol 3-kinase pathways in BEL cells (21). The ability of wt HARP and HΔ111–136 mutant protein to induce MAPK phosphorylation was therefore tested on NIH-3T3 cells (Fig. 4). As observed in BEL cells (21), stimulation of NIH-3T3 cells with wt HARP led in a dose-dependent manner to the phosphorylation of ERK1 and ERK2 (Fig. 4, lanes 3 and 4). However, as expected from cell stimulation experiments, no phosphorylation was observed with the HΔ111–136 mutant (Fig. 4, lanes 5 and 6). Amino acids 111–136, therefore, seemed to play a key role in the mitogenic activity of HARP, and we then decided to compare the HΔ111–136 mutant and the wt HARP protein upon different biological activities known for HARP.

Tumor Formation in Nude Mice—HARP had been previously described to induce tumor formation in nude mice (31). Hence, 1 × 10^6 CHO-K1 cells expressing wt HARP or HΔ111–136 mutant were injected subcutaneously in nude mice. pCDNA3-transfected or parental cells were used as controls. Two weeks after the injection, two mice injected with cells expressing wt HARP had developed tumors and four mice (out of seven) had tumors 4 weeks later (Table I). No mice injected with parental cells or HΔ111–136-expressing cells had tumors, and only one injected with pCDNA3-transfected cells started to develop a tiny tumor after 6 weeks. To verify that tumors had derived from injected cells, tumor fractions were dispersed in culture medium and cultured in the presence of 800 μg/ml G418. Under these conditions, most of the cells from the tumors
Figure 4. HARP-induced phosphorylation of ERK1 and ERK2 in NIH-3T3 cells. Lysate from control cells (lane 2) or from cells stimulated 5 min with 10% SVF (lane 1), wt HARP (40 and 16 nM, respectively) and HΔ111–136 (40 and 16 nM, lanes 5 and 6, respectively) were separated by a 10% SDS-PAGE. ERKs and phosphorylated ERKs present in the lysate were, respectively, detected by Western blotting with an anti-phosphorylated p42/p44 MAPK antibody and an anti-p42/p44 MAPK antibody (A).

**TABLE 1**

Tumor formation in nude mice by parental or transfected CHO-K1 cells

| Clone            | Time         | 2 weeks | 3 weeks | 4 weeks | 5 weeks | 6 weeks |
|------------------|--------------|---------|---------|---------|---------|---------|
| Parental         | 0/7          | 0/7     | 0/7     | 0/7     | 0/7     | 0/7     |
| Control          | 0/7          | 0/7     | 0/7     | 0/7     | 0/7     | 0/7     |
| wt HARP          | 2/7 (20.4)   | 2/7 (25.7) | 3/7 (24.7) | 4/7 (54.9) | 4/7 (105.9) | 0/7     |
| HΔ111–136        | 0/7          | 0/7     | 0/7     | 0/7     | 0/7     | 0/7     |

The incidence of tumors in nude mice following the injection of 1 × 10⁶ cells was investigated for parental CHO-K1 cells and for clones isolated from cells transfected with pCDNA3 (control), pCDNA3-HARP (wt HARP), or pCDNA3-HARPΔ111–136 (HΔ111–136). The average of the surface of tumors obtained for each group of animals at the end of each week is expressed in millimeters squared in brackets.

**DISCUSSION**

Since the description of HARP as a mitogenic factor in 1989 (25), the essential objective of several groups, including ours, has been the molecular identification of cell surface macromolecule(s) linked to this activity. Until now, no candidate has been characterized, and, in this context, structure-function studies of HARP have been considered as a major development in the understanding of the mechanism involved in its mitogenic activity.

In previous reports, we described the existence of two different processing sites of the signal peptide for recombinant proteins from HARP cDNA-transfected NIH-3T3 cells (16) and for native HARP expressed by BEL cells (27) leading to the production of two molecular forms, HARP₁₃₆ and HARP₁₃₉, which differ by an extension of 3 amino acids at the N-terminal part of the molecule. To confirm these results, recombinant HARP was produced by two different cell lines, including NIH-3T3 cells and CHO-K1 cells. As expected, both HARP₁₃₆ and HARP₁₃₉ proteins were expressed by NIH-3T3 cells. However, only HARP₁₃₆ was detected in conditioned medium of CHO-K1 cells. The absence of HARP₁₃₉ had also been observed in the conditioned medium from MDA-MB231 cells (26) that endogenously produced HARP and from SW13 transfected with HARP cDNA (14). The cleavage of the signal peptide seems to be dependent on the cells that produce HARP and could explain the discrepancy between the results related to the existence of HARP₁₃₉ obtained by various laboratories in different tissues or cell lines. In contrast to our previous studies (9), both molecular forms of HARP were mitogenic for NIH-3T3 and BEL cells, demonstrating that the presence of the 3 amino acids AEA at the N part of the molecule cannot be considered as the main determinant for HARP mitogenic activity. This suggestion is strongly supported by the production of HARP₁₃₉Δ₁–₃ mutant protein expressed in CHO-K1 cells, which displayed a mitogenic activity for NIH-3T3 cells. However, we showed that the different processing of HARP, yielding the HARP₁₃₆ or HARP₁₃₉ could be a possible mechanism involved in the modulation of the mitogenic activity of this growth factor. This possibility was suggested by the difference between specific activities registered for each molecular form of HARP. The absence of mitogenic activity for HARP₁₃₉ that we have previously described could be due to structural or/and post-translational modifications, which could be different from one clone to another or depend on the mammalian expression system used. This last hypothesis is reinforced by the difference in the specific activity of HARP₁₃₆ observed with NIH-3T3 and CHO-K1 cells. Indeed, the value of ED₅₀ of HARP₁₃₆ is about three times higher when the protein is purified from NIH-3T3 cells as compared with CHO-K1 cells.

In contrast to a previous report, proposing that the mito-
Neurite outgrowth of HARP in embryonic rat neurons. Embryonic rat neurons were seeded in a plate coated with 0.8, 1.6, or 3.2 \( \mu \)g/ml of wt HARP or H111–136. Cells were stained after 48 h using May-Grünwald’s solution and Giemsa’s stain, and the number of neurites per well was determined using phase contrast microscopy (E). Data are the means of triplicate determinations and standard errors are indicated. *, 0.01 < \( p < 0.05; **, 0.001 < \( p < 0.01; *** p < 0.001. \) A–D represent phase contrast micrographs obtained in the absence of recombinant proteins (A) or in the presence of wt HARP (C) or H111–136 (B and D). Scale bar, 25 \( \mu \)m (A–C) and 7 \( \mu \)m (D).

The correct folding of mutant proteins is a complex problem of mutagenesis experiments, but recent structural data suggest that folding of the HARP mutants described here could be very similar to the wt protein (22). The Rauvala group reported that HARP is organized as two \( \beta \)-sheets domains connected by a flexible linker. In H111–136 mutant proteins, the absence of these two structural domains is unlikely, for the following reasons: (i) All the cysteines known to be involved in disulfide bridges (33, 34) are conserved and may confer a tridimensional structure closed to the wild type protein; (ii) The C-terminal part of the molecule that we have deleted appeared to form random coils (22); (iii) HARP111–136 is still recognized by a polyclonal antibody raised against wt HARP in the immunometric assay; (iv) HARP111–136 is able to induce a neurite outgrowth (see above).

Despite the lack of a detectable secondary structure in NMR and CD experiments (22), the C-terminal part of this molecule appears to be very important for the mitogenic activity and could be involved in the binding to high affinity receptors. This hypothesis was supported by signal transduction studies, because stimulation of the MAPK pathway, known to be involved in HARP mitogenic activity in BEL cells (21), was abolished for H111–136.

Taken together, these results suggest that multiple cell-specific receptors could then mediate diverse biological activities. A more complex pattern could be considered if the cell surface molecule involved in biological activities of HARP depends on the cells or tissue.
