Overexpression of the HIP Gene Coding for a Heparin/Heparan Sulfate-binding Protein in Human Thyroid Carcinomas

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ABSTRACT

A subtractive library screening was performed to identify changes in gene expression that occur during the process of neoplastic transformation of thyroid cells. A cDNA library was constructed from a human thyroid papillary carcinoma cell line (NPA) subtracted with cDNAs from normal thyroid cells (HTC 2). The differential screening of this library lead to the isolation of 39 cDNA clones; six of them showed homology with a recently isolated gene, named HIP, that codes for a protein belonging to a novel class of heparin/heparan sulfate-binding proteins. Northern blot analysis revealed HIP gene overexpression in all of the human thyroid carcinoma cell lines analyzed, as compared to the HTC 2 cells. HIP expression was particularly abundant in the anaplastic carcinoma-derived cell lines. The analysis of surgically removed thyroid tumors showed overexpression of HIP gene in all of the carcinomas, independent of the histotype, although the largest increase in HIP expression was observed in the undifferentiated forms. In contrast, none of the benign adenomas or normal thyroid tissues showed HIP overexpression.

To establish the role of HIP overexpression in cell transformation, the NPA cell line was transfected with an eukaryotic expression vector carrying the HIP gene in the antisense orientation. Stable transfectants expressed reduced HIP mRNA levels and showed morphological changes, such as becoming spindle-shaped and growing scattered. The growth rate of the antisense clones was greatly reduced compared to the NPA cells transfected with the backbone vector. Taken together, these results indicate that HIP gene overexpression is associated with thyroid carcinogenesis and strongly suggest its involvement in thyroid cell growth regulation.

INTRODUCTION

Thyroid carcinomas comprise a broad spectrum of tumors with different clinical behaviors (1). Activation of the RET/PTC oncogene, caused by rearrangements of the RET proto-oncogene, represents the main genetic alteration in papillary carcinomas because it is detectable in 40% of the cases (2). TRK gene rearrangements and MET gene overexpression are often found in carcinomas of the papillary type (3, 4). Conversely, ras gene mutations are frequently detected in tumors of the follicular type. Impairment of the p53 protein function represents a typical feature of the anaplastic carcinomas (5, 6). Overexpression of other genes, such as c-myc (7), NFkB p65 (8), HMGI-C and HMGI Y (9), galectin-1 (10), and thymosin b-10 (11), is associated with thyroid malignant tumors. The isolation of other genes preferentially expressed in thyroid tumors, as compared to normal thyroid tissue, may contribute to the comprehension of the mechanisms involved in thyroid carcinogenesis and to the identification of genetic markers for each step of thyroid carcinogenesis.

Subtractive hybridization has been successfully used to identify changes in gene expression occurring during the progression of different types of cancer. Recently, we have demonstrated, by means of a subtractive library between an anaplastic cell line (ARO; Ref. 12) and a papillary carcinoma cell line (NPA; Ref. 12), that the thymosin b-10 gene is overexpressed in thyroid carcinomas, particularly in the undifferentiated forms (11).

Here, we describe the construction and differential screening of a subtraction library between a human thyroid papillary carcinoma cell line (NPA) and normal HTC 2 human thyroid cells (13). By this approach, we were able to enrich the NPA cDNA library with sequences expressed preferentially in thyroid carcinomas, making likely the isolation of unique genes, possibly involved in thyroid carcinogenesis, that are expressed in NPA cells but not in normal thyroid cells.

By the differential screening of this subtractive cDNA library, we isolated 39 phage clones expressed more abundantly in NPA cells than in HTC 2 cells. Here, we report the characterization of one of these cDNAs. Sequence analysis showed that cDNA corresponded to the HIP gene coding for a HP/HS-binding protein (14, 15). The heparan sulfate proteoglycans can be located on cell surfaces and/or in extracellular matrices, found in nearly all mammalian tissues (16). Functionally, heparan sulfate proteoglycans interact with HP/HS-binding proteins and their effectors and play a key role in extracellular matrix structure and function, cell adhesion, growth, and differentiation (17). HP/HS-binding effectors comprise a variety of proteins that include growth factor (18), extracellular matrix components (19, 20), cytokines (21), and cell adhesion molecules (22). The HIP protein has been demonstrated to play a pivotal role in the initial attachment of human trophoblast cells to uterine epithelial cells (14). Here, we report that the HIP gene is overexpressed in all of the differentiated and undifferentiated thyroid carcinomas examined. In contrast, its expression is weak or almost undetectable in all of the adenomas and normal thyroid tissues analyzed. Finally, NPA cells in which the synthesis of the HIP protein was suppressed by transfection with a HIP antisense construct decreased their growth rate, became spindle-shaped, and grew scattered. These data suggest that the HIP protein plays an important role in the control of cell proliferation and in the mechanisms related to cell-cell contact inhibition.

MATERIALS AND METHODS

Cell Lines. The human thyroid carcinoma cell lines studied were: TPC-1 (23), WRO (24), NPA (12), ARO (12), FRO (25), NIM1 (26), and B-CPAP (27). They were grown in DMEM (Life Technologies, Inc.) containing 10% fetal bovine serum (Flow Laboratories). Human thyroid cell cultures (HTC 2...
were established as described (13). The NPA cell line was transfected according to the calcium phosphate transfection protocol (28). Transfected cells were selected in a medium containing G418 (400 μg/ml). For each transfection, G418-resistant clones and the mass cell population were isolated and grown for further analysis.

**Human Thyroid Tissues.** Neoplastic human thyroid tissues and normal adjacent tissue were obtained from surgical specimens and frozen immediately in liquid nitrogen. Some of the thyroid tumors were collected at the Laboratoire d’Histologie et de Cytologie, Center Hospitalier Lyon Sud, and the Laboratoire d’Anatomie Pathologique, Hôpital de L’Antiquaille (Lyon, France).

**Subtractive Library Construction and Screening.** A subtraction library was constructed enriching for NPA-specific sequences with respect to normal thyroid-specific sequences. Briefly, mRNA isolated from NPA cells was retrotranscribed to cDNA that was subsequently hybridized with a molar excess (30-fold) of mRNA isolated from normal thyroid cells. Hydroxyapatite chromatography was used to separate single-stranded cDNAs from the cDNA-RNA hybrids, and finally, the subtracted cDNAs were inserted between the EcoRI and HindIII sites of the bacteriophage vector SHix-1, which allows automatic plasmid excision (29). The NPA sequence enrichment was verified evaluating the hybridization intensity of DNA amplified by PCR from the primary NPA and normal thyroid libraries and the NPA(−NT) library probed with a 32P-labeled RNA transcribed from the NPA(−NT) library. The DNA from the NPA primary and NPA(−HTC 2) libraries yielded strong signals, whereas the normal thyroid library produced a very weak signal. Differential plaque hybridization screening of the NPA(−NT) cDNA library was performed according to a standard procedure (30). Briefly, bacteriophages were plated at the appropriate concentration to give ~3000 plaque-forming units per 150-mm dish, and duplicate blots were obtained by gently layering and successively lifting nitrocellulose filtersonto the top agar surface. The filters were alkali denatured, neutralized, and fixed by baking for 2 h in a vacuum oven at 80°C. Poly(A)+ RNA was purified from total RNA using a commercially available kit (Qiagen, Hilden, Germany). Poly(A)+ and RNA (2.5 μg) extracted from NPA and HTC 2 cells were retrotranscribed at 42°C for 1 h using reverse transcriptase in the presence of radiolabeled [32P]dCTP (400 Ci/mmol; Amer sham). For each 15-μl reaction, 1 μCi/μl [3H] thymidine (1 mCi/μl; New England Nuclear, Boston, MA) was added to the medium; cells were harvested at 10, 12, 24, 36, 48, and 72 h after serum addition. DNA was extracted by a Cell Harvester (Wallac, Oy Turku, Finland), and specific radioactivity was measured by scintillation counting. Each experiment was repeated at least three times in duplicate samples.

**RESULTS**

**Construction and Differential Screening of a cDNA Subtractive Library between NPA Cells and Normal Thyroid Cells.** A cDNA subtractive library between the NPA cell line, established from a papillary human thyroid carcinoma, and HTC 2 cells, derived from normal thyroid tissue, was constructed, with enrichment for NPA-specific sequences, as described in “Materials and Methods.” Subsequently, this subtractive library was analyzed by hybridization of duplicated filters with cDNAs deriving from total poly(A)+ mRNA s from NPA and HTC 2 cells, respectively. The plaques, showing differential hybridization between the two probes, were subsequently picked and analyzed again by hybridization. After three consecutive screenings, we isolated 39 clones that gave a much stronger signal with the NPA probe than with the HTC 2 probe. Sequence analysis of six of these clones showed 100% identity with a recently isolated gene, named HIP, which encodes a HIP/HS-binding protein (14). By Northern blot analysis, we confirmed that HIP was overexpressed in the NPA carcinoma cells (Fig. 1A, Lane 3) compared to HTC 2 cells and normal tissue (Fig. 1A, Lanes 1 and 2).

**Analysis of HIP Gene Expression in Normal and Neoplastic Thyroid Tissues and Cells.** To investigate whether the overexpression of the HIP gene was a general event in thyroid neoplastic transformation, its expression was analyzed in other thyroid carcinoma cell lines derived from different tumor histotypes. These cell lines were: NIM 1, B-CPAP, and TPC-1 from papillary carcinomas; NPA from anaplastic carcinomas; and WRO from a follicular carcinoma; and finally, FRO and ARO from anaplastic carcinomas. As shown in Fig. 1B, HIP expression was increased in all of the carcinoma cell lines (Lanes 2–8) compared to normal thyroid cells (Lane 1), in which it was almost undetectable. HIP gene expression level was particularly abundant in ARO (Lane 8; 7-fold increase) and FRO (Lane 7; 10-fold increase) cells. Interestingly, these anaplastic carcinoma lines show a highly malignant phenotype because they generate large invasive tumors with short latency period after injection in athymic mice (7).
To determine whether the increased expression in carcinoma cell lines was attributable to gene amplification or to deregulated expression of the HIP gene, we analyzed genomic DNA from normal cells and carcinoma-derived thyroid cells by Southern blotting using a HIP cDNA as a probe. The intensity of the hybridization signals was comparable in normal and carcinoma cell lines and was compatible with a single copy of the gene (Fig. 2), excluding amplification of the HIP gene in the carcinoma cell line. The same filter was hybridized with a RET proto-oncogene probe (data not shown), as a control for a single-copy gene. A RT-PCR assay was performed on total RNA extracted from surgically removed benign and malignant thyroid tumors to examine the in vivo HIP expression. There was very little, if any, HIP gene expression in normal thyroid and adenoma tissues, whereas it was evident in 16 of 20 papillary, 3 of 3 follicular, and 10 of 10 anaplastic carcinomas (Table 1). Fig. 3 shows some significant results. HIP gene expression was higher in anaplastic carcinomas (Fig. 3, A, Lanes 9–12, and B, Lanes 13–18) compared to the differentiated forms (Fig. 3, A, Lanes 5–8, and B, Lanes 7–12). Conversely, the HIP gene expression was undetectable or very low in normal thyroid (Fig. 3, A, Lanes 1 and 2, and B, Lanes 1–3) and adenoma tissues (Fig. 3, A, Lanes 3 and 4, and B, Lanes 4–6). Subsequently, the expression and distribution of HIP was examined in different human cell lines and tissues by Northern blot. A single 1.3-kb mRNA species was found in normal tissue; it was very abundant in skeletal muscle, pancreas, and heart, whereas it was almost undetectable in brain and kidney. Intermediate HIP gene expression levels were observed in placenta, lung, and liver (Fig. 4).

HIP gene overexpression was also found in several colon carcinoma cell lines and tissues with different degree of malignancy (data not shown).

**Effects of the Suppression of the HIP Protein Synthesis in the NPA Cells.** To investigate the possible role of the HIP gene in thyroid carcinogenesis, we introduced an antisense construct of the HIP cDNA into the NPA cells, pursuing the aim of blocking the synthesis of this protein. Fig. 5 shows the maps of the constructs used. The pCMV HIP sense and antisense constructs were obtained by inserting the HIP cDNA in both sense and antisense orientations in the pCMV vector under the transcriptional control of the cytomegalovirus promoter. This vector also carries the gene for G418 resistance. The transfected cells were selected from a G418-containing medium. Three sense clones (2S, 3S, and 4S), three antisense clones
Table 1 Expression of HIP gene in human thyroid neoplasias

<table>
<thead>
<tr>
<th>Histological type of thyroid specimens</th>
<th>No. of patients with elevated levels of HIP mRNA×/no. of patients analyzed</th>
<th>Average HIP mRNA levels (relative amounts)</th>
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<tbody>
<tr>
<td>Normal tissue</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>Adenoma</td>
<td>1/16</td>
<td>-</td>
</tr>
<tr>
<td>Follicular</td>
<td>3/3</td>
<td>+</td>
</tr>
<tr>
<td>Papillary</td>
<td>16/20</td>
<td>+</td>
</tr>
<tr>
<td>Anaplastic</td>
<td>10/10</td>
<td>+ +</td>
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</table>

× HIP mRNA levels were considered elevated when they were at least 3-fold higher than the level found in normal thyroid. The expression of the GAPDH gene was used to normalize the amount of RNA loaded on the gel. The relative amounts were scored as follows: −, trace amounts; +, increased level with respect to the normal counterpart (at least 5-fold); and ++, very high levels (at least 10-fold higher than the level found in normal thyroid).

(12AS, 13AS, and 14AS), and two clones transfected with the backbone vector (V1 and V2) were selected for further analysis. Western blot analysis revealed a reduction of HIP protein in the NPA cells carrying the HIP cDNA in antisense orientation in comparison with the NPA cells transfected with the backbone vector (Fig. 6A). Northern blot analysis was also performed to determine the level of expression of endogenous and exogenous HIP mRNA. As shown in Fig. 6B, the 1.3-kb band corresponding to the HIP endogenous mRNA was decreased (at least 5-fold) in the 12AS, 13AS, and 14AS NPA cell clones as compared with the V1 cell clone, as demonstrated by densitometric analysis (as shown in Fig. 6C). The presence of a lower specific band, of ~500 bp, in the sense and antisense transfected NPA cell clones accounts for the expression of the exogenous HIP mRNA. Morphological changes were observed in the NPA cells carrying the HIP antisense constructs. In fact, the 12AS, 13AS, and 14AS NPA cells, transfected with the antisense construct, became spindle-shaped and grew scattered (the 12AS clone is shown in Fig. 7C). Conversely, the S2, S3, and S4 NPA sense clones (S2 is shown in Fig. 7D) and the V1 and V2 NPA cells (V1 clone is shown in Fig. 7B) carrying the empty pCMV-neo vector were morphologically indistinguishable from parental cells (Fig. 7A). The growth rate of antisense and sense cell lines was determined as shown in Fig. 8A. Although growth rates of the different sense clones were almost identical to those of the cells carrying the vector alone, the antisense cell lines showed a longer (4-day) lag period before the onset of the logarithmic phase, as compared to control or sense clones. These results, taken together, suggest that there is a strong relationship between the level of HIP RNA and growth rate. Interestingly, the NPA antisense clones showed a lower saturation density (5 × 10^5/dish) than the sense and vector NPA cell clones (2 × 10^5/dish). This result is consistent with the scattering of the cells caused by the suppression of the HIP gene expression. Normal thyroid cells transfected with sense and antisense construct did not show any difference in morphology and in growth rate in comparison to the untransfected cells (data not shown).

Growth Kinetics of NPA Transfected with the Sense and Antisense HIP Constructs. In an attempt to cast further light on the role of HIP in growth regulation of the NPA thyroid cell line, we evaluated DNA synthesis by [3H]thymidine incorporation. The cells were forced to quiescence by a 24-h serum deprivation, and then addition of serum to the medium induced entrance into the cycle. The results are shown in Fig. 8B. The 12AS clone, expressing the antisense HIP, entered in
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Fig. 5. Schematic representation of the plasmids used in this study. The sense and antisense HIP cDNAs were subcloned into the pCMV expression vector.

Fig. 6. A. Western blot analysis of HIP expression in HIP sense and antisense clones. One hundred µg of total homogenates from NPA V1 cells (Lane 1); from 12AS, 13AS, and 14AS antisense NPA clones (Lanes 2–4, respectively); and from 2S, 3S, and 4S NPA sense clones (Lanes 5–7, respectively) were separated by SDS-PAGE, and HIP expression was detected using Western Blot analysis as described in “Materials and Methods.” B. Northern blot analysis of the expression of the HIP expression in the NPA cells transfected with the HIP cDNA in sense and antisense orientation. Twenty µg of total RNA for each cell line were size-fractionated on a denaturing formaldehyde agarose gel, blotted onto nylon filters (Hybond-N), and probed with HIP cDNA. Sources of RNAs were: Lane 1, NPA VI cells; Lane 2, NPA 12 AS; Lane 3, NPA 13 AS; Lane 4, NPA 14 AS; Lane 5, NPA 2S; Lane 6, NPA 3S; Lane 7, NPA 4S. A probe of GAPDH was used as control for uniform RNA loading.

C. relative amount of HIP mRNA in sense and antisense clones. Densitometric analysis of the experiment shown in B; the peak volume of HIP mRNA was corrected for GAPDH and plotted as an amount relative to that of the NPA cell line. Lane 1, NPA V1 cells; Lane 2, NPA 12 AS; Lane 3, NPA 13 AS; Lane 4, NPA 14 AS; Lane 5, NPA 2S; Lane 6, NPA 3S; Lane 7, NPA 4S.

S phase 48 h after the serum addition. In contrast, the 2S and V1 cell clones showed the maximum of [3H]thymidine incorporation at 24 h. Analogous results were obtained with the other transfected NPA cell clones (data not shown). Therefore, reduced expression of the HIP gene induced a delayed entrance into the S phase. These data would account for the decreased proliferation rate of antisense transfected NPA cells compared with sense and mock-transfected cells.

Analysis of the Malignant Phenotype. The neoplastic phenotype of the sense and antisense NPA cell clones was evaluated by a soft agar colony assay and by injection into athymic mice. As reported in Table 2, the NPA cells transfected with the antisense construct gave rise to colonies in soft agar with a lower efficiency compared to the NPA cells transfected with the backbone vector or the sense construct. However, no difference was observed in the ability of these cells to induce tumors in athymic mice.

Fig. 7. Morphology of the NPA cells expressing the sense and antisense HIP constructs. Photographs of the normal and transfected NPA cell lines cultured under standard conditions as described in “Materials and Methods.” Magnification, ×150. A. NPA untransfected cells. B. NPA transfected with the backbone vector, V1 cell clone. C. NPA cells transfected with the antisense HIP construct, the AS12 cell clone. D. NPA cells transfected with the HIP sense construct, the S2 cell clone.
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**DISCUSSION**

The aim of this study was to identify genes that were up-regulated in transformed thyroid cells. Toward this purpose, we have used the differential screening of a subtractive library between a papillary carcinoma cell line (NPA) and normal thyroid cells (HTC 2). One of the clones isolated corresponded to the HIP gene. This gene, coding for a novel class of HP/HS-binding protein, was found overexpressed in the NPA cells compared to the normal thyroid cells and tissues. The analysis of the HIP gene expression in seven human thyroid carcinoma cell lines deriving from different carcinoma histotypes revealed its overexpression in all of the neoplastic cell lines with respect to normal thyroid cells and tissues. The increase in HIP gene expression was particularly high in the anaplastic carcinoma cell lines ARO and FRO, which show a highly malignant phenotype. The analysis of surgically removed thyroid tumors revealed a high expression of the HIP gene in all of the anaplastic and papillary carcinomas analyzed. The expression was particularly abundant in the anaplastic carcinomas. Conversely, no or low expression was observed in benign adenomas and normal thyroid tissues. Therefore, these data indicate that the HIP overexpression is associated with thyroid tumorigenesis. Moreover, increased HIP mRNA levels were found in human colon normal and carcinoma cell lines and tissues with respect to the normal colonic mucosa (data not shown). Although previous results (14) by other groups have shown HIP overexpression in several carcinoma cell lines of different origin, this study describes, for the first time, an increase in HIP gene expression in surgically removed neoplasias.

To investigate whether the induction of HIP gene expression was a phenomenon only peripherally associated with cell transformation or it was causally related to induction of the expression of the malignant phenotype, HIP protein synthesis was blocked in the NPA cells by an antisense methodology. NPA cells were transfected with a construct carrying the HIP gene in antisense orientation. We found that the NPA cells transfected with the antisense construct grew scattered and

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**Table 2** Analysis of the transformed phenotype of the NPA cells transfected with the HIP cDNA in the sense and antisense orientation

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Colony forming efficiency (%)</th>
<th>Tumor incidence$^a$ (no. with tumors/no. tested)</th>
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<tbody>
<tr>
<td>pCMV vector</td>
<td>23</td>
<td>1/3</td>
</tr>
<tr>
<td>pCMV HIP sense</td>
<td>20</td>
<td>1/3</td>
</tr>
<tr>
<td>pCMV HIP antisense</td>
<td>6</td>
<td>1/3</td>
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$^a$ Assayed by injecting $2 \times 10^6$ into athymic mice (4–6 weeks old). The animals were monitored for the appearance of tumours at the inoculation site for 6 weeks.
showed modifications in cell growth. In fact, the HIP antisense transfected NPA cells showed a longer lag phase (~4 days), compared to the NPA cells transfected with the HIP sense construct or the β-galactosidase control. The NPA cells transfected with the HIP sense construct showed a longer lag phase (~4 days), compared to the NPA cells transfected with the HIP antisense construct or the β-galactosidase control.

Taken together, these data suggest a pivotal role of the HIP gene in thyroid carcinoma cell proliferation. It is likely that HIP plays this role by interfering in cell-cell and cell-matrix interactions. Some heparin-binding proteins (22, 35) have been shown to modulate these functions. The pivotal role of HIP gene overexpression in cell proliferation and carcinogenesis is also supported by previously published data showing that the expression of the other members of heparin binding proteins correlate with the metastatic phenotype of liver cancer (36, 37). For instance, the HIP/PAP protein, belonging to a new family of calcium-dependent C-type lectins, is abundantly expressed in hepatocarcinoma cells and in liver carcinoma biopsies but not in normal liver tissue and cells (38, 39).

The results presented here and those previously published suggest an important role of the HIP gene overexpression in thyroid carcinoma genesis. The mechanism for the enhanced HIP gene expression in thyroid is still unknown; however, there is an evident correlation between its expression and the degree of the malignant phenotype. Therefore, it is possible to predict that detection of HIP mRNA combined with that of other genes found overexpressed in thyroid neoplasms, such as HMGI(Y) and thymosin β-10, can be a valid tool for the diagnosis of thyroid tumors.

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