Binding of the glucocorticoid and estrogen receptors to the human H-ras oncogene sequences

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Abstract. There is evidence that hormone regulation of cellular oncogenes plays an essential role in human cancer. The c-H-ras gene is implicated through both mutation and abnormal gene expression in many types of human cancer. Computer scanning of this gene has revealed two putative hormone response motifs: A possible Glucocorticoid Response Element (GRE) at position 1261 of the first intron of the H-ras1 gene and a putative Estrogen Response Element (ERE), at position 3007 of the fourth intron of the gene. In DNA binding assays, using the HeLa and LATK⁻ cell lines, we showed specific binding of the corresponding receptors at both putative H-ras glucocorticoid and estrogen response sequences, suggesting that hormones could be contributing to H-ras transcriptional regulation through interaction with their corresponding Hormone Response Elements (HREs).

Introduction

Nuclear receptors, such as those for steroid/thyroid hormones, vitamin D, and retinoic acid, are known to act as gene regulators, controlling the transcription of target genes by binding to cis-acting DNA elements referred to as hormone responsive elements (HREs) (reviewed in ref. 1). Negative HREs, which repress expression of specific genes, have been characterized (2,3), however, the mechanism of their action is still poorly understood. Consensus steroid hormone REs correspond to palindromic sequences (4,5), however, either imperfect palindromic HREs (6,7) or their interference with other cis-acting regulatory elements (8,9) or factors (10), may also generate efficient HREs.

Glucocorticoids regulate transcription of a number of genes by the direct interaction of the hormone receptor complex with GREs. GREs contain an imperfect palindromic consensus sequence 5'-GGTACANNNTGTTCT-3', (1) with highly conserved TGTCTC oligonucleotide and quite variable upstream hexanucleotide (11,12). Interference between the glucocorticoid receptor and other transcription factors has also been shown (13,14). The hormone response elements in the conserved 5'-AGGTCANNNTGACCT-3' sequence is the ERE consensus oligonucleotide (5). Additionally, 5'-TGACCT-3' half palindromic oligonucleotides may act synergistically conferring estrogen response to a gene, even at a long distance from the gene promoter (15,16), possibly through estrogen receptor-associated proteins (17). GREs and EREs have been identified and characterized in a number of cellular genes (18-21), as well as in viral regulatory regions (22-25), but little is known about the presence of glucocorticoid and estrogen response elements in cellular oncogenes.

The H-ras gene, a member of the ras family of proto-oncogenes, is readily implicated in many types of human cancer both through mutation (26) and abnormal gene expression and is involved in multiple signal transduction pathways, under normal cellular growth as well (27). The fact that overexpression of ras genes may cause oncogenic transformation (28,29) and that expression of the normal H-ras gene can act as an onco-supressor (30), indicate the importance of understanding the way ras genes are regulated. Elevated expression of the ras genes has also been found in a variety of human tumors (for a review see ref. 31).

Considering the possible role of hormonal steroids as tumor promoters (32), we computer scanned the human c-H-ras1 sequence for potential hormone response elements. We observed a putative GRE motif with 100% similarity in the half palindromic consensus oligonucleotide at position 1261 (GGCCAGACCTGTTCT) of the conserved 3' end of the first intron (33) and a possible ERE motif, with 90% similarity, at position 3007 (GGGCCACCCTGACCT) of the fourth intron of the H-ras gene. Using gel retardation experiments with nuclear extracts from HeLa and LATK⁻ cell lines, which possess hormone receptors, we showed that both glucocorticoid and estrogen receptors bind their cognate oligonucleotides of H-ras in a specific way, thus suggesting a role for glucocorticoids and estrogens in regulation of the H-ras gene expression.

Materials and methods

Cells and culture conditions. Human cervical epithelial tumor cells (HeLa) and tumor mouse fibroblasts (LATK⁻)
were grown exponentially in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 μg/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO2 and 95% air. The cells were collected by scraping and used for preparation of nuclear extracts.

**Nuclear extract preparation.** The cells were homogenized in hypotonic buffer (25 mM Tris HCl, pH 7.5, 50 mM KCl, 0.5 mM MgCl2, 0.5 mM DTT, 0.5 mM PMSF). The nuclei were collected, washed with isotonic buffer (25 mM Tris HCl, pH 7.5, 5 mM KCl, 0.5 mM MgCl2, 0.5 mM DTT, 1 mM PMSF), 0.2 mM superose, and lysed with an extraction buffer (25 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1% Triton, 0.5 mM DTT, 0.5 mM PMSF). Nuclear debris was removed by centrifugation at 25,000 rpm for 1 h at 4°C. The protein concentration of the supernatant was measured by the method of Bradford.

**Preparation of double stranded oligonucleotides and labelling.** The PAS GRE probe was obtained by annealing the following two oligonucleotides: 5'-AGCGTTCGGCCCGTCGCCACCGCCGCTTCAGAACACAGTCTGGCCAGCGAA-3'; 5'-AGCTTTGCCCCCATGACTGGGAA-3'. The RAS ERE probe was generated by the oligonucleotides: 5'-AGACTGTGGGCCCCGCTTCACCGCCGGGAGCAGCC-3'; 5'-AGCTGTTCCATGACTGGGAA-3'. These probes contain the consensus and complemenary consensus sequences.

**Results**

**Computer scanning.** We used Cyborg/Pustell sequence analysis programs to search the nucleotide sequence of the human H-ras gene (36). For identifying possible GREs, we scanned the human c-H-ras sequence for the highly conserved hexanucleotide 5'-TGTTCT-3' and the complementary 5'-AGAACA-3' sequence. For EREs, the 5'-TGACCT-3' and the complementary 5'-AGGTCA-3' motifs were used.

**Table 1. Possible GRE and ERE elements in the c-H-ras gene.**

<table>
<thead>
<tr>
<th>Consensus sequence</th>
<th>Putative HREs in c-H-ras</th>
</tr>
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<tbody>
<tr>
<td>GRE</td>
<td>GRE</td>
</tr>
<tr>
<td>TGTTCT</td>
<td>TGTTCT</td>
</tr>
<tr>
<td>AGAACA</td>
<td>AGAACA</td>
</tr>
<tr>
<td>ERE</td>
<td>ERE</td>
</tr>
<tr>
<td>TGTTCT-3'</td>
<td>TGTTCT-3'</td>
</tr>
<tr>
<td>AGAACA-3'</td>
<td>AGAACA-3'</td>
</tr>
</tbody>
</table>

*The position of the elements in the H-ras gene sequence and homology to GRE consensus and complementary consensus sequences are also shown.*

**Gel retardation assay.** DNA binding reactions were carried out in binding buffer (50 mM Hepes, pH 7.9, 500 mM NaCl, 0.5 mM PMSF, 0.5 mM EDTA, 150 μM palmitate) at 4°C. Specific components were incubated for 1 h at 4°C in the presence of 5 μg of the radiolabelled probe. After incubation at 4°C for 1 h, the samples were layered on top of glycerol gradients (40% in 4°C) and run at 28,000 rpm for 15 h at 4°C. The position of the DNA-binding sites was monitored by autoradiography.
Figure 1. Organization of the human c-H-ras1 gene-sequences and positions of the H-ras glucocorticoid response element (GRE) and estrogen response element (ERE). Some possible regulatory elements in the vicinity of the H-ras GRE are also shown. The coding sequences are represented by black boxes, the untranslated sequences by open boxes, the VTR by a crosshatched box and the regulatory elements by shadowed boxes. The direction of transcription is indicated by arrows. Asterisks indicate homology between hormone response elements and the corresponding consensus oligonucleotides.

HeLa and LATK- nuclear extracts were used as a source of GR. Fig. 2 shows the interaction of GR and H-ras GRE. The labelled hMTIIA GRE and RAS GRE probes were incubated with HeLa nuclear extract in the absence (lanes 1,7) or presence of unlabelled oligonucleotide competitors. The labelled probes were competed for protein binding with a molar excess of unlabelled oligonucleotide competitors. A common retarded band was seen in lanes 1 and 7, indicating the presence of a GR-DNA complex. Binding of GR on RAS GRE probe was abolished in the presence of excess of GRE-containing competitors, but was unaffected by the other competitors such as SP-1, NF-1 or HIV-1LTR AP-1. A common retarded band was seen in lanes 1 and 7, indicating the presence of a GR-DNA complex. Binding of GR on RAS GRE probe was abolished in the presence of excess of GRE containing competitors, but was unaffected by the other competitors such as SP-1, NF-1 or HIV-1LTR AP-1. An equivalent picture resulted from the hMTIIA GRE probe. Fig. 2 showed a strong sequence specificity of a protein for the H-ras DNA target and suggested that this protein was the GR, as it was shown by the common band in lanes 1 and 7 and was displaced only by GRE-related competitors.

A further, more direct demonstration of the GR-H-ras GRE interaction is shown in Fig. 3. 32P-labelled RAS GRE probe was incubated with HeLa nuclear protein, in the absence (lane 3), or presence (lanes 4-6) of increasing amounts of polyclonal anti-human glucocorticoid receptor antibody. Control hMTIIA GRE probe was also incubated, in the absence (lane 1) or presence (lane 2) of polyclonal anti-human GR antibody. Inclusion of the antibody into the reaction mixture led to the disappearance of the specific retarded band, clearly indicating that this was due to GR-DNA interaction.

The hMTIIA GRE and RAS GRE radiolabelled probes were incubated with increasing amounts of GR-containing HeLa (lanes 6-11) and LATK- (lanes 1-5) extracts (Fig. 4). A retarded band was seen and a stronger GR-DNA complex was observed, in both cell lines, with increasing amount of nuclear extract.

RAS ERE probe, encompassing the H-ras ERE sequence and Vitellogenin ERE probe, encompassing the estrogen response sequence from Xenopus Vitellogenin A2 gene were used for ER binding experiments. The labelled Vitellogenin ERE and RAS ERE probes were incubated with HeLa nuclear extract in the absence (lanes 1,5) or presence of an excess of unlabelled oligonucleotide competitors (Fig. 5). The labelled probes were competed for protein binding with related (lanes 2,6) and unrelated (lanes 3,4,7,8) unlabelled oligos. A common retarded band was seen in lanes 1 and 5, indicating the presence of an ER-DNA complex. Binding of ER on the RAS ERE probe was abolished only in the presence of excess of ERE related competitors, indicating specific binding of estrogen receptor on the H-ras ERE.
The steroid hormone receptors are one of the most extensively studied group of the known regulatory factors, however, little is still known about hormonal regulation of cellular oncogenes (32,37,38). Using gel retardation assays we observed specific binding of hormone receptors in the H-ras gene, at positions which were previously predicted through computer scanning analysis. We showed that glucocorticoid receptor binds a consensus motif at position 1261 of the conserved 3' end of the first intron of the H-ras and that estrogen receptor binds a sequence at position 3007, at the fourth intron of the gene. These results provide data for hormone regulation of the human H-ras1 oncogene. Furthermore, potentially active HREs are found in sequences of the H-ras intron, suggesting a regulatory role for introns in the H-ras gene expression. Moreover, the end of the first intron is evolutionarily well conserved (33), suggesting a functional role for the H-ras GRE in human and other organisms as well. The existence of other possible regulatory elements in the vicinity of the H-ras GRE as shown in Fig. 1 (39-41), also implies a functional role for the H-ras GRE.

Previous data for regulation of the murine H-ras gene by glucocorticoids has been reported (42). The cooperation between glucocorticoid hormones and the T24 H-ras oncogene in cell transformation (43) suggests the involvement of the H-ras gene in hormonal promotion of breast tumors through abnormal hormonal regulation. In addition, development of breast carcinomas in rats has been shown to be initiated by mutations in the H-ras and K-ras genes and to depend on promotion by estrogens (44). The latency of ras oncogenes to induce cell transformation in the absence of hormonal stimulation (44) might be due to the inability of ras oncogenes to induce proliferation of the mammary precursor cells. Our experimental results suggest a more direct implication of H-ras in hormonal tumor promotion by proposing hormonal regulation of the H-ras gene (normal or mutated), also providing an explanation for the specificity of tumor development in systems involving ras genes and sexual development.

Using ras p21 as a possible marker for the disease may be of prognostic significance. Depending on the way the H-ras gene is regulated by hormones, an explanation of indications correlating the levels of hormone receptors and prognosis of the disease may also be provided (45-47).

In conclusion, more data on the hormone regulation of the H-ras gene through specific receptor binding could be provided by combining in vitro CAT assay experiments and
Figure 5. Binding of the ER to H-ras ERE. Binding reaction mixtures contained 0.01 pmol of Vitellogenin ERE (lanes 1-4) or RAS ERE radiolabelled probe and 20 μg of nuclear protein. Unlabelled RAS ERE (lane 2) and Vitellogenin ERE (lane 6) probes, as well as the unrelated probes NF-1 (lanes 3,7) and HIV-1 LTR-AP-1 (lanes 4,8) were used in competition experiments. Protein-DNA complexes were separated as indicated in the Fig. 2 legend. ER-H-ras ERE complex is indicated by the arrow.

in vivo determination of the ras p21 levels in stable transfectants, when adding the corresponding hormone. Moreover, comparing the levels of the receptor binding in corresponding HREs in human tumors with the adjacent normal tissues, through gel retardation assays, may give additional evidence for the role of hormonal regulation of the H-ras gene in human tumor progression.

References