Cloning of hHRI, Human Heme-regulated Eukaryotic Initiation Factor 2α Kinase: Down-regulated in Epithelial Ovarian Cancers

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Protein synthesis is regulated in response to environmental stimuli by covalent modification, phosphorylating the components of the translational machinery. Phosphorylation of the α subunit of eIF-2 is one of the best-characterized mechanisms for down-regulating protein synthesis in higher eukaryotes in response to various stress conditions. One of mammalian eIF-2α kinases is a heme-regulated inhibitor kinase (HRI), which is activated by heme deficiency and plays an important role in translational control. In this work, we have analyzed the differentially expressed genes between epithelial ovarian cancer and normal ovary. We have screened a total of 1,408 genes isolated from a human dermal papilla cell cDNA library by cDNA array hybridization. Among many differentially expressed genes, eIF2α kinase, a heme regulated inhibitor was down-regulated in ovarian epithelium cancer. The down-regulation of hHRI was also confirmed in other ovarian cancer tissues by Northern blot hybridization. The hHRI gene is 2,887 bp in length and the amino acid sequence deduced from the cDNA clone encodes a protein of 630 amino acids with molecular mass of 73 kDa. It contains all 12 catalytic domains of the protein kinases with consensus sequences of the protein-serine/threonine kinases. The expression pattern of hHRI mRNA showed approximately 3.0 kb bands which were expressed ubiquitously in all human tissues examined, which indicates that eIF-2α kinase could play an important role in the translational regulation of nonerythroid tissues.

Keywords: cDNA Array; Human HRI Cloning; Ovarian Cancer.

Introduction

The phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) is one of the best character-
ized mechanisms for down-regulating protein synthesis in mammalian cells in response to various stress conditions (Hershey, 1991; Samuel, 1993; Wek, 1994). The phosphorylation of eIF2α was first detected in rabbit reticulocyte lysates deprived of hemin. The absence of hemin resulted in the activation of a highly specific eIF2α kinase, called the heme-regulated inhibitor (HRI) (Chen and London, 1995). Phosphorylation of eIF2α in reticulocyte lysates results in the binding and sequestration of reversing factor (RF), also designated as the guanine nucleotide exchange factor or eIF2B, to make an RF · eIF2α (P) complex. Since RF is required for the exchange of GTP for GDP in the recycling of eIF-2 and in the formation of the eIF-2 · Met-tRNA Met · GTP ternary complex, its unavailability results in cessation of the initiation of protein synthesis (Amesz et al., 1979; Matts and London, 1984).

Another mammalian eIF2α kinase, the double-stranded RNA-activated kinase PKR, is constitutively expressed in reticulocytes and inducible by interferon in other mammalian cells (Porud, 1995). At present, only these two distinct mammalian eIF2α kinases have been cloned (Chen and London, 1995; Proud, 1995). A third eIF2α kinase, termed GCN2, has been characterized from Saccharomyces cerevisiae (Hinnebusch, 1997) and Drosophila melanogaster (Santoyo et al., 1997). It is activated by uncharged tRNA in response to amino acid starvation in yeast (Hinnebusch, 1997).

All three kinases phosphorylate eIF-2α on serine residue 51. The amino acid sequence surrounding serine 51 is highly conserved among the eIF-2α factors from human, rat, and yeast, and residues between 41 and 59 are identical (Cigan et al., 1989; Ernst et al., 1987). Although PKR, HRI, and GCN2 display a similar specificity with yeast and mammalian eIF-2α (Dever et al., 1992), some differences in specificity with peptide

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Abbreviations: GCN2, yeast general amino acid control eIF-2α protein kinase; hHRI, human heme-regulated inhibitor; PKR, double-stranded RNA-dependent eIF-2α protein kinase.
substrates are observed between the PKR and HRI enzymes (Proud et al., 1991).

Activation of HRI in reticulocytes is mediated by various stimuli in addition to hemin deficiency, including heat shock, sulfhydryl reagents such as N-ethylmaleimide, oxidized glutathione, and heavy metal ions (de Haro et al., 1983; Matts et al., 1992; Palomo et al., 1985). Native HRI appears to be a dimer composed of two 90 kDa polypeptides that may in part be disulfide-linked (Yang et al., 1992). Binding of heme to HRI promotes an intersubunit disulfide bond formation that may be involved in the negative regulation of HRI (Chen et al., 1992). Heat shock proteins hsp90 and hsp70 also interact with HRI (Matts et al., 1992). This interaction may be of regulatory significance. For example, heme may regulate eIF-2α HRI kinase activity by promoting the formation of an inactive HRI·hsp90 (p87) dimer (Mendez et al., 1992). The regulation of HRI by its association to heat shock proteins appears to be similar to regulation of the steroid hormone receptor (Pratt, 1993).

In this study, we have screened the genes differentially expressed in ovarian cancer, using the strategy of cDNA array. One of the down-regulated genes in ovarian cancer was homologous to the mouse HRI (heme regulated inhibitor). We designated human HRI as hHRI and full-length cloned it for further study.

Materials and Methods

Construction of a cDNA library and sequence analysis A cDNA library was constructed by using a ZAP cDNA synthesis kit (Stratagene, La Jolla, USA) with 4 μg of poly A+ RNA obtained from primary cultured human papilla cells. The phage library was converted into a pBlue-script phagemid cDNA library by in vitro excision using the ExAssist/SOLR system (Stratagene). Randomly selected clones were sequenced from the 5' end of an insert using a T7 sequencing version 2.0 DNA sequencing kit (Amersham Pharmacia biotech.). Approximately 150 bases for each clone were compared with nonredundant GenBank data using BLASTN. Sequences were also translated and used to search the protein data base using the BLASTX sequence analysis program (Altschul et al., 1990).

cDNA array preparation A total of 1,408 cDNAs were arrayed on the positive charged nylon membranes (Amersham Pharmacia biotech) in a 96-well format. Two identical blots were produced for each set of cDNA samples. The membrane was placed into the manifold (Bio-Rad, California, USA) and two hundred nanograms each of a denatured plasmid DNA sample per dot was loaded into the manifold. The membranes were dried in air, and fixed by UV crosslinking at 125 mJ using a UV Stratatalinker 1800 (Stratagene).

Preparation of probe and hybridization Total RNAs were extracted from Ovarian tumor tissues and corresponding normal tissues from 13 patients by a modified acid guanidium thiocyanate/phenol/chloroform extraction method using the TRI reagent (Molecular Research Center Inc., Cincinnati, USA). Ten-micrograms of total RNA, which had been treated with human placental RNase inhibitor (Gibco BRL), were reverse-transcribed with an oligo-dT primer by using the SuperScript preamplification system (Gibco BRL). The first-stranded cDNAs were used for random primed radio-labelling (Megaprime DNA labelling system, Amersham Pharmacia biotech) to make the probe.

The DNA dot blots were hybridized with the radioactive probe using a ExpressHyb hybridization solution (Clontech, Palo Alto, USA), and washed according to the manufacturer’s instructions. The membranes were analyzed after autoradiography.

Northern blotting analysis Ten micrograms of total RNA prepared from normal or cancer tissue were subjected to electrophoresis on a denaturing formaldehyde-agarose gel. The RNA was capillary transferred to a Hybond-N+ membrane, baked for 2 h at 80°C under vacuum, and fixed by UV crosslink at 120 mJ using UV Stratatalinker 1800 (Stratagene).

The purified 2.2 kb of an EcoRI-XhoI fragment of the h-HRI cDNA was used as a probe. The membranes were hybridized for 1 h at 68°C in ExpressHyb Solution (Clontech Laboratories Inc., USA) containing 2 x 10⁶ cpm of radiolabelled probe per 1 ml of solution. After hybridization, the membranes were washed in 2x SSC/0.05% SDS at room temperature for 40 min and 0.1x SSC/0.1% SDS at 50°C for 40 min. The wet membrane was wrapped and exposed to X-ray film at −70°C for 4 d. Equal loading of mRNA was verified by rehybridizing the blots with a 32P-labeled beta-actin cDNA probe.

Screening of a human dermal papilla cDNA library To obtain the full-length sequence of hHRI, the recombinant plaques of the dermal papilla and human prostate cDNA libraries (Clontech, Palo Alto, USA) were screened as described by Sambrook et al. (1989). Plaques were lifted onto nitrocellulose filters, and hybridized with 1 x 10⁶ cpm/ml of a 32P-labelled probe prepared by the Megaprime DNA labelling system (Amersham, Buckinghamshire, England), in 5x Denhardt’s solution, 0.1% SDS, and 100 μg/ml of denatured sonicated salmon sperm DNA, at 42°C overnight. The hybridized filters were washed at room temperature in 2x SSC (1x SSC; 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0, 0.5% SDS for 20 min, and twice at 65°C in 1x SSC, 0.1% SDS for 1 h. Positive plaques were further purified by replating and hybridization. The isolated λ ZAP phagemid clones were converted into plasmids by in vitro excision using the ExAssist/SOLR system (Stratagene), the resulting plasmids pBluescript SK (−) having the cDNA of interest between the EcoRI and XhoI sites. Sequence analysis was performed as described previously.

Multiple Tissue Northern blots To examine the tissue-specific expression of hHRI, the expression of HRI mRNA in different human tissues was analyzed by Northern blot analysis using Multiple Tissue Northern (MTN) blots (Clontech). The MTN blots contained approximately 2 μg of poly A+ RNA per lane from different tissues or cell lines. The blots were probed with a 32P-labeled hHRI cDNA probe containing the open reading frame.