Accumulation of Focal Adhesion Protein Hic-5 in the Nucleus by Hydrogen Peroxide

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Reactive oxygen species (ROS) are produced in cultured cells upon stimulation with cytokines and tumor promoters, and participate in signal transduction pathways leading to gene expression. The hic-5 gene was isolated as a hydrogen peroxide-inducible clone by differential screening, and was shown to encode a paxillin-related protein localized in the focal adhesion plaques. In mouse osteoblastic and fibroblastic cells treated with low concentrations of hydrogen perox-

ide, Hic-5 protein was found to be reversibly localized to the nucleus within 30 min. Hic-5 was also translocated to the nucleus in cells treated with leptomycin B, an inhibitor of nuclear export of proteins. Hic-5 protein with mutations in the nuclear export signal and the LIM-3 domain accumulated in the nucleus. These results indicated that Hic-5 shuttles between the cytoplasm and the nucleus, and may participate both in surface signals and in gene expression.

Key words: Hic-5, Nuclear translocation, NES, Hydrogen peroxide

I. Introduction

Reactive oxygen species (ROS) including superoxide anion, hydroxy radicals, hydrogen peroxide and lipid peroxides are produced in various types of cells in response to various signals. Harmful ROS appear to be trapped mostly by intracellular or extracellular scavenging substances, but there is accumulating evidence of some important functions of ROS in signaling pathways and the regulation of cellular phenotypes. The signals participate in normal physiological responses as well as in aging or pathological disorders.

The essential roles of ROS and derived species in signal transduction were reviewed by Lander [9] and Finkel [3]. Extracellular ligands such as hormones and cytokines trigger activation of cytoplasmic signals that lead to nuclear events through a cascade of protein-protein interactions. Recent studies indicate that many extracellular ligands generate and/or require ROS or derived species to transmit their signals to the nucleus [11, 16, 18]. TGF β 1 treatment activates production of hydrogen peroxide from osteoblastic cells, and hydrogen peroxide participates in the induction of *egr-1* gene expression [16]. The CARG box present upstream of the *egr-1* gene was essential for induction. Platelet-derived

growth factor also stimulated smooth muscle cells to produce hydrogen peroxide, which is involved in MAP kinase activation [18]. Phorbol 12-myristate 13-acetate induced MCP-1/JE gene transcription that was dependent on active oxygen species, and NF κ B was required for the induction [11].

During the course of our studies to identify signaling molecules that participate in the ROS cascade, we isolated a novel gene, *hic-5*, by differential screening of a cDNA library from mouse osteoblastic cells [17]. The *hic-5* gene encodes a polypeptide of about 50 kDa with a marked similarity to paxillin, a well known component of the focal adhesion complex. Hic-5 protein is co-localized in the focal adhesion complex with vinculin and paxillin in fibroblastic cells [7, 19]. The precise biological functions of Hic-5 are not known, and in the present study we examined the effects of hydrogen peroxide on subcellular localization of Hic-5.

II. Materials and Methods

Cell culture

Mouse myoblastic C2C12 cells were cultured in Dulbecco's modified MEM supplemented with 5% fetal bovine serum and 10% horse serum. Mouse fibroblastic C3H10T1/2 and osteoblastic MC3T3 cells were cultured in Dulbecco's modified MEM supplemented with 10% fetal bovine serum. Plasmid DNA was transfected into cells using

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Fig. 1. Subcellular distribution of Hic-5 in C2C12 cells. Cells were either untreated or treated with 2 mM hydrogen peroxide for 30 and 60 min, and lysed in the presence of Triton X-100. Soluble (S) and insoluble (P) fractions were separated and Western blotted using the antibodies indicated.

Fig. 2. Nuclear localization of Hic-5 in hydrogen peroxide-treated MC3T3 cells. Cells were treated with 0.5 mM hydrogen peroxide, fixed and stained with anti-Hic-5 or anti-vinculin antibody followed by the second fluorescently labeled antibody.

Fig. 3. Nuclear translocation of exogenously expressed Hic-5 in hydrogen peroxide-treated MC3T3 cells. Cells were transfected with pCG-LD1mHic-5, and 24 hr later treated with 0.5 mM hydrogen peroxide for 60 min, fixed and stained with anti-HA antibody.

Fig. 4. Effect of leptomycin B on subcellular localization of Hic-5. MC3T3 cells were treated with 10 ng/ml of leptomycin B for 2 hr, fixed and stained with anti-Hic-5 or anti-vinculin antibody.

Fig. 5. Mutations introduced into Hic-5. Mutations at the nuclear export signal (NES)-like sequence and LIM3 domain were introduced by site-directed mutagenesis, and expression vectors were constructed in pCG-N-BL. Consensus sequences of NES in Hic-5 are boxed. Two leucine residues in NES and two cysteine residues in LIM3 were mutated.

LipofectAMINE PLUS (Life Technologies, Inc) according to the manufacturer's protocol or by microinjection using a micromanipulator (Eppendorf).

Plasmid construction

pCG-LD1hic-5 was the hemagglutinine (HA)-tagged mouse Hic-5 expression plasmid as described previously [13]. Nuclear export signal (NES)-like site (L191H, L193/Q) and LIM 3 (C352G, C355/G) mutants of hic-5 were constructed by site-directed mutagenesis using a PCR-based kit (Mutan-Super, Takara, Co. Kyoto), and confirmed by sequencing.

Subcellular fractionation

Cells were lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 10 mM KCl, and 1% Triton X-100 on ice, and lysates were centrifuged at 10,000 rpm for 5 min in a Microfuge to separate the supernatant and pellet fractions. Samples were separated by SDS-PAGE and Western blotted using anti-Hic-5 (#1024 [7]), anti-vinculin (Sigma Chemical Co.), anti-paxillin (Transduction Laboratories) or anti-FAK (Focal adhesion kinase, Transduction Laboratories) antibody.

Immunostaining

Transfected cells were fixed in 3.7% formaldehyde in phosphate buffered saline followed by permeabilization in 0.2% Triton X-100 in saline. Cells were treated with anti-Hic-5, anti-vinculin or anti-HA (12CA5) antibody, and then treated with FITC-labeled monoclonal anti-mouse IgG or rhodamine-conjugated anti-rabbit IgG (Dako). Fluorescence microscopy was carried out using an Axioscope microscope (Carl Zeiss).

III. Results and Discussion

Change in subcellular localization of Hic-5 following oxidative stress

Cells were treated with non-toxic levels of hydrogen peroxide, and subcellular fractionation was carried out using C2C12 cells. The western blotting data shown in Fig. 1 indicated that most Hic-5 protein was recovered in the soluble fraction of Triton X-100-treated cell lysate, and only a small amount was detected in the pellet fraction of untreated cells. However, in cells treated with 2 mM hydrogen peroxide for 30 to 60 min (Fig. 1), a significant increase in the level of Hic-5 was observed in the pellet fraction. On the other hand,

Fig. 6. Subcellular localization of Hic-5 mutants. Expression vectors of Hic-5 with mutation at the NES-like site (a) or NES-like site/LIM3 (b) were introduced into C3H10T1/2 cells, and cells were fixed and stained 24 hr later.

the Hic-5 homologue, paxillin was detected only in the soluble fractions in cells treated with hydrogen peroxide.

Precise subcellular localization of Hic-5 under the above conditions was examined by immunocytochemistry with anti-Hic-5 antibody. MC3T3 cells were either untreated or treated with 0.5 mM hydrogen peroxide, and immunostained using anti-Hic-5 antibody. The results shown in Fig. 2 indicated that Hic-5 began to dislodge from the focal adhesions 10 min after treatment with hydrogen peroxide, and almost all Hic-5 was accumulated in the nuclei within 30 min after treatment. Vinculin, which was colocalized in the focal adhesion complex with Hic-5 in untreated cells, did not accumulate in the nuclei. These results indicated that nuclear Hic-5 accumulation was specific.

The accumulation of Hic-5 in the nuclei was further confirmed by transfection of HA-tagged Hic-5 expression vector and staining with anti-HA antibody (Fig. 3). Staining with anti-HA and anti-Hic-5 antibodies showed similar patterns, and hence the possibility was excluded that the results obtained using anti-Hic-5 antibody was due to cross-reaction with other proteins.

Nuclear export signal of Hic-5

It was reported that oxidative stress affected cytoskeletal structure and cell-matrix interactions in cells from ocular tissue [21]. The change in the cytoskeletal structure may cause redistribution of focal adhesion protein. Hydrogen

peroxide has been shown to regulate cytoplasmic localization of yeast transcription factor Pap1, and this process was dependent on the nuclear export factor Crm1 (exportin) [20].

Leptomycin B inhibits nuclear export of proteins through binding to Crm1 [2]. Treatment of MC3T3 cells with this agent resulted in nuclear accumulation of Hic-5, whereas vinculin remained at the focal adhesions (Fig. 4). This result indicates that Hic-5 contains a NES [12]. Examination of the amino acid sequence of Hic-5 revealed an NES-like sequence in the third LD domain (Fig. 5). Mutation was introduced in this NES-like sequence, and its subcellular localization was examined by immunofluorescent microscopy. The mutant protein, however, was localized mostly in the focal adhesions. We previously reported that the LIM3 domain participates in retention of Hic-5 in the focal adhesions [14]. Therefore, a mutation was also introduced at LIM3, and the double mutant exhibited nuclear localization, although the protein was not exclusively located in the nucleus (Fig. 6). It appears that release from the focal adhesion complex and association of Hic-5 with nuclear import factor are required for the nuclear translocation of Hic-5.

Many signaling molecules are known to translocate to the nucleus upon growth factor stimulation [1, 4–6, 8, 10]. A focal adhesion protein, zyxin, shuttles between the nucleus and cytoplasm [15], but the conditions that stimulate the shuttle have not been identified. Hic-5 is localized in focal

adhesions in normal cells through interactions with other components of the focal adhesion complex [14], and oxidative stress may either directly or indirectly modify Hic-5 itself or interacting factors and permit its nuclear translocation. Further studies are necessary to determine the molecular mechanisms of the nuclear translocation of Hic-5.

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V. References

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