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Interaction of HIPPI with putative promoter sequence of caspase-1 *in vitro* and *in vivo*

P. Majumder, B. Chattopadhyay, S. Sukanya, T. Ray, M. Banerjee, D. Mukhopadhyay, N.P. Bhattacharyya *

Structural Genomics Section and Crystallography and Molecular Biology Division, Saha Institute of Nuclear Physics, 1/AF Bidhan Nagar, Kolkata 700 064, India

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Abstract

To investigate the mechanism of increased expression of caspase-1 in Hippi expressing HeLa and Neuro 2A cells, reported earlier, we report here that HIPPI directly interacted with upstream sequence of caspase-1 gene (−700 to +17, 717 bp). Deletion of this 717 bp sequence and further analysis by electrophoretic mobility shift assay and fluorescence quenching revealed that HIPPI interacted with 60 bp (−151 to −92) upstream sequence of caspase-1. We also observed by chromatin immunoprecipitation assay that HIPPI interacted with the 717 bp sequence *in vivo*. In luciferase assay, when expression of the reporter gene was driven by either 717 bp or 60 bp caspase-1 upstream sequences, luciferase activity was increased in GFP-Hippi expressing HeLa cells in comparison to that obtained with parental HeLa cells with the same constructs. Similar result was obtained in Neuro2A cells with 717 bp caspase-1 upstream sequence. In summary, we showed that HIPPI could interact with the putative promoter sequence of caspase-1 and increased the expression of the downstream gene suggesting that HIPPI could act as transcription regulator.

Keywords: HIPPI; Caspase-1; Transcription regulator; EMSA; ChIP; Promoter activity; HIP1; Huntington's disease

Huntington's disease (HD) is caused by the expansion of polymorphic CAG repeats (beyond 37 repeats) at the exon1 of Huntingtin (Htt) gene [1]. Large numbers of proteins are known to interact with Htt (reviewed in [2]). Biological consequences of such interactions are not known fully. On the basis of interactions of a number of transcription factors with Htt and alterations of the expressions of large number of genes in HD, it has been proposed that the pathology of HD is mediated through alteration of transcription (reviewed in [3]). In several studies using cellular and animal models of HD (where mutated Htt gene or gene fragments are expressed by *knock in*), increase in expressions of caspase-1 and caspase-3 genes have been reported [4,5]. The molecular basis of this observation remains elusive.

HIPPI has been identified as an interactor of Htt-interacting protein HIP1. Interaction of HIPPI with HIP1 is through the "pseudo" death effector domain (pDED) present in both the proteins [6]. Strength of HIP1-Htt interaction is inversely related to the numbers of Gln in Htt [7]. Pro caspase-8 has been shown to interact with the HIP1–HIPPI hetero-dimer. It is proposed that weaker interaction of HIP1 with mutated Htt in HD enhances the propensity of HIP1–HIPPI hetero-dimer. This hetero-dimer recruits pro caspase-8 activating caspase-8 and initiating the "extrinsic" pathway of cell death [6,8].

We have previously reported that exogenous expression of Hippi induced apoptosis. It is also observed that endogenous expressions of caspase-1, caspase-3, and caspase-7 are up regulated in GFP-Hippi expressing cells [8]. Mechanisms by which the caspase-1 and other caspases expressions are increased in GFP-Hippi cells remain unknown.

* Corresponding author. Fax: +91 33 23374637.
E-mail address: nitai_sinp@yahoo.com (N.P. Bhattacharyya).

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To decipher how exogenous Hippi expression up regulated caspase-1, we tested the hypothesis that HIPPI interacts with the putative promoter sequences of caspase-1 *in vitro* and *in vivo*. We presented evidence that HIPPI interacted with upstream regulatory sequence of caspase-1 *in vitro* and *in vivo* and increased the expression of reporter gene.

Materials and methods

Reagents and cell culture. Electrophoretic mobility shift assay (EMSA) kit and luciferase promoter assay kit were purchased from Promega, USA. Anti-HIPPI antibody was purchased from (Abcam, USA). Lipofectamin 2000 transfection reagent was purchased from Invitrogen, USA. Other molecular biology grade fine chemicals were procured locally. Sources of the cells and conditions of their growth are similar to that had published earlier [8].

Cloning and transfection of Hippi. Methods of cloning of Hippi in pEGFP C3 and transfection into HeLa and Neuro2A cells were similar to that described earlier [8].

Cloning of Hippi in pGEX4T-1, expression in bacteria and protein purification. Hippi was sub cloned in pGEX 4T-1 vector from GFP-Hippi [8] after PCR amplification using primers (Forward: 5′-CGGGATCCCGGTCGGGTTTGGAAGATGG-3′ and the Reverse: 5′-ACGCGTCGACGTCTTTAATAAAAGCCTGTG CTGG-3′), following restriction enzyme (RE) digestion of the PCR product with *Bam*HI and *Sal*I. Underlined sequences are adaptors for the REs. Clones were characterized by sequencing and RE digestion. For protein expression and purification standard methods were used. The size of the GST-tagged protein was checked on 10% SDS gel (79 kDa as expected).

Electrophoretic mobility shift assay (EMSA). EMSA was essentially carried out using the methods described earlier [9]. In brief, using primers (Forward: 5′-CGGGGTACCAAGCCTAGGAAACACAAGGAGA-3′, Reverse: 5′-GGAAGATCTGGCTTTTCTCTCTCCCTTC-3′) for 717 bp (−700 to +17, 1st transcribed base being considered as '+1') and for 60 bp (−151 to 92) [Forward: 5′-CCTGATGCAGGCTACAGTTCT-3′, Reverse: 5′-GCATA TGCATGTCTTTATTTTTCTTC-3′] caspase-1 upstream sequences were amplified by PCR from genomic DNA either in the presence of [α-^{32}P]dCTP (hot PCR) or in the absence of [α-^{32}P]dCTP (cold PCR). Annealing temperature for PCR amplification of 717 bp product was 57 °C and that for 60 bp product was 50 °C. Hot and cold PCR products were purified using QIAquick purification kit (Qiagen, Germany) and used as the probe in EMSA.

Different concentrations of GST-HIPPI (130 nM to3 μM) were added to the probe (300 nM for 717 bp and 500 nM for 60 bp) in binding buffer [9] containing 50 ng/μl poly(dI:dC) and incubated at room temperature for 40 min. At the end of incubation, product was run on 5% non-denaturing PAGE at 200 V for 4.5 h at 4 °C. Gel was dried at 80 °C for 45 min and exposed to X-ray film (Kodak, India) over night at −80 °C. After developing the film, positions of bands on the film were indicative of the positions of the probe. Specific (717 bp caspase-1 upstream sequence) and non-specific (700 bp sequence from the unrelated genomic sequence) cold (without [α-^{32}P]dCTP incorporation) products were used to determine the specificity of the interaction.

Fluorimetric quenching assay. Concentrations of DNA was increased gradually (3–200 nM) to the fixed amount of purified GST-HIPPI protein (25 μg, 0.8 μM) in reaction buffer (100 mM Tris–HCl, pH 8.0, and 50 mM NaCl) and the fluorescence intensities were measured at 340 nm exciting at 295 nm in HITACHI 4010 Spectrofluorimeter. Changes in fluorescence intensities (ΔF) due to addition of upstream sequences of caspase-1 were calculated. From double reciprocal plot of ΔF and concentrations of DNA in the ranges where the decrease in fluorescence intensities reached to saturation, apparent dissociation constants (K_d) for each DNA–protein binding reaction were calculated following the methods described by Sing and Rao [10].

Chromatin immunoprecipitation (ChIP) assay. About 80–90% confluent GFP-Hippi expressing HeLa cells were used to cross link proteins with DNA by formaldehyde (2%) treatment and the cross linking reaction was stopped by glycine (150 mM) after 4 min. Cells were scraped and spun down at 13,000 rpm for 2 min, washed with 1× PBS twice and the pellet was frozen in dry ice for 20 min. Buffer C (20 mM Hepes, pH 7.9, 25% Glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) and 1 mM PMSF were added to the pellet after thawing it at 4 °C to lyse the cells. Nuclei were spun down at 13,000 rpm for 10 min and the pellet was resuspended in breaking buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% SDS, and 2% Triton X-100) and sonicated twice (two pulses of 10 s each). Contents were then spun down. The debris was discarded. Triton buffer (50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100) was added to the supernatant (nuclear extract). Anti-HIPPI antibody was added to a part of the nuclear extract (+Ab) and incubated overnight at 4 °C. Other part of the nuclear extract was allowed to bind with secondary antibody Rabbit IgG at 4 °C (−Ab). Next day, Protein G Agarose beads were added to these fractions and left on a shaker for 6 h at 4 °C. Beads were then washed four times with Triton buffer and twice with buffer [10 mM Tris–HCl (pH 8.0)].

Next, SDS–NaCl–DTT buffer (62.5 mM Tris–HCl, pH 6.8, 200 mM NaCl, 2% SDS, and 10 mM DTT) was added to a part of the beads and incubated at 65 °C overnight for reverse cross linking. DNA was purified by ethanol precipitation and was used to amplify by PCR using caspase-1 upstream 717 bp sequence specific primers as described above. Another part of the beads were heated with 1× SDS protein loading dye at 100 °C, the protein fraction was run on 10% SDS–PAGE and Western blot analysis was carried out following methods described previously [8] using anti-HIPPI (1:500) and anti-RNA pol II (1:1000) antibodies.

Luciferase reporter assay. Caspase-1 upstream 717 bp DNA (−700 to +17) along with the adaptors containing *Kpn*I and *Bgl*II recognition sites were amplified using primers described above and cloned into pGL3 enhancer vector using standard methods. Caspase-1 upstream 60 bp sequence (−151 to −92) was amplified by PCR primers mentioned above, cloned into T/A vector, checked by sequencing and subcloned into the pGL3 enhancer vector using standard methods. The pGL3 vectors containing 717 bp and 60 bp caspase-1 upstream sequence and control pGL3 plasmid were transfected separately in either HeLa cells expressing GFP-Hippi and parental HeLa or Neuro2A cells expressing GFP-Hippi and parental Neoro2A cells. The transfection was carried out using Lipofectamine 2000 reagent following the procedure provided by the manufacturer. At the time of transfection, more than 90% of the cells were expressing GFP-Hippi [8]. After 48 h of transfection, cells were harvested, lysed, and luciferase substrate was added to it. Luciferase activity was measured in Sirius tube luminometer (Berthold Detection Systems, USA). Transfection efficiency of pGL3 containing upstream sequences of caspase-1 was monitored by co-tranfecting β-galactosidase gene containing vector pSV-β-Galactosidase (Promega, USA) and measuring the β-galactosidase activity along with luciferase activity. Appropriate correction was made for equal transfection, using result obtained with the β-galactosidase activity.

Results

Interactions of GST-HIPPI with caspase-1 upstream sequences

Caspase-1 upstream sequence 717 bp (−700 to +17) was amplified (Fig. 1A, panel I) in the presence of [^{32}P]-dCTP (probe) and purified and mixed with purified GST-HIPPI (Fig. 1A, panel II) for EMSA as described above. Result of a typical experiment ($n = 4$) is shown in Fig. 1A (panels III and IV). Results revealed that GST-HIPPI interacted with the upstream sequence of caspase-1 as evident from the shift in the mobility of the probe (panel III, lanes 1, 3, and 4). When only GST protein was used, no such shift

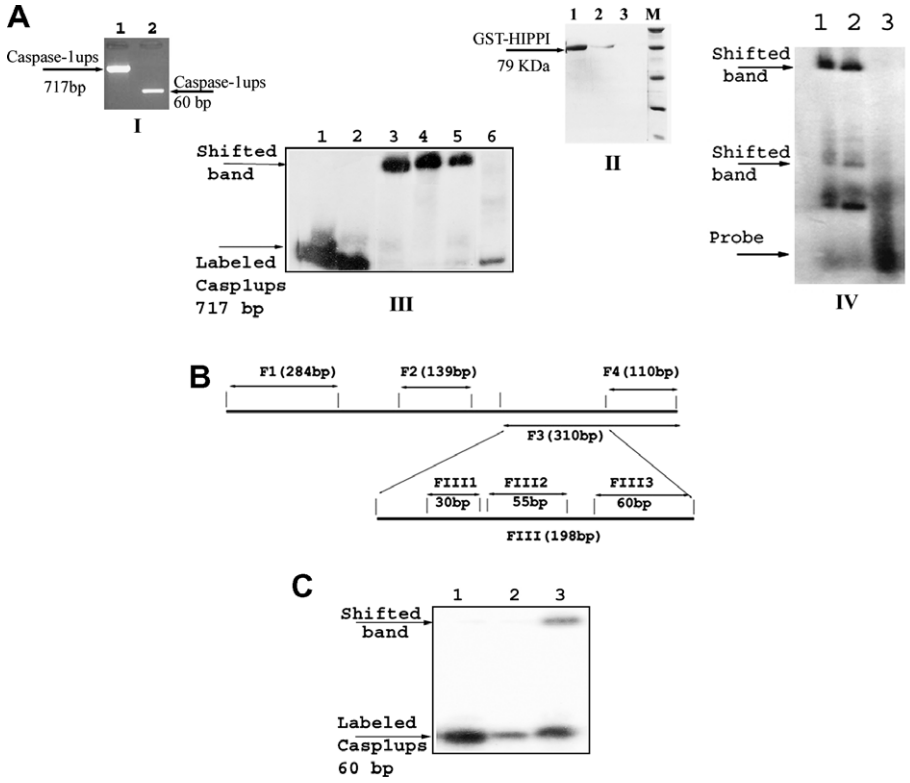

Fig. 1. Electrophoretic mobility shift due to physical interaction of PCR amplified caspase-1 upstream sequences with purified GST-HIPPI protein. (A) Panel I represents PCR amplified products of caspase-1 upstream 717 and 60 bp regions run on 1.5% agarose gel and panel II represents purified GST-HIPPI protein run on 12.5% polyacrylamide gel. Three hundred nanomolars of 717 bp [α-^{32}P]dCTP-labeled caspase-1 upstream sequence (probe) was used in each of EMSA reaction. Products were separated on polyacrylamide gel, dried and exposed to X-ray film. Representative autoradiographs are shown in panels III and IV. Panel III, lane 1: probe only; lane2: probe + 5.2 μM GST protein, lanes 3 and 4: probe + 1.7 and 2.7 μM GST-HIPPI protein, respectively, lane 5: probe + 2.7 μM GST-HIPPI protein in presence of 600 nM unlabelled non-specific DNA (700 bp PCR products) and lane 6: probe + 2.7 μM GST-HIPPI protein in presence of unlabelled 400 nM caspase-1 upstream DNA (717 bp). Panel IV represents similar analysis with lower amount of GST-HIPPI. Lane 3: probe only; lane 2: probe + 126.6 nM of GST-HIPPI and lane 1: probe + 300 nM of GST-HIPPI. (B) Pictorial representation of different deletion fragments of Caspase1 upstream 717 bp (−700 to +17) sequence generated which were used for EMSA studies. F1, F2, F3, FIII, FIII1, FIII2, and FIII3 are these fragments designated by double headed arrows. Size of each fragment was mentioned in parenthesis. (C) Result of EMSA done with 400 nM of 60 bp [α-^{32}P]dCTP-labeled caspase-1 upstream sequence as probe. Lane 1: probe only; lane 2: probe + 6.3 μM GST protein, lane 3: probe + 2.7 μM GST-HIPPI.

of the probe was observed (Fig. 1A, panel III, lane 2). Addition of non-specific DNA to the reaction mixture did not change the intensity of the shifted band, while addition of unlabeled 717 bp upstream sequence of caspase-1 decreased the intensity of the shifted band (panel III, lanes 5 and 6). Lower amounts of HIPPI (126.6 nM, 200 ng) also decreased the mobility of the 717 bp upstream sequence of caspase-1 gene (Fig. 1A; panel IV, lanes 1, 2, and 3). This result revealed specific binding of GST-HIPPI to caspase-1 upstream sequences containing the putative promoter region. Further analysis, with various deletion constructs of this 717 bp fragment (Fig. 1B) using different PCR primers and subsequent EMSA revealed that the GST-HIPPI did not interact with fragments namely F1, F2, and F4 but fragment F3, more precisely fragment FIII showed mobility shift in presence of GST-HIPPI (data not shown). Deletion of the fragment FIII and subsequent EMSA with the fragments FIII1, FIII2, and FIII3 revealed that only fragment FIII3 comprising 60 bp (−151 to −92) interacted with purified GST-HIPPI protein. This result is shown in Fig. 1C (lanes 1 and 3). Thus GST-HIPPI interacted with 60 bp (−152 to −92) upstream sequence of caspase-1 gene.

Fluorescence quenching assay

The fluorescence quenching of GST-HIPPI due to binding of increasing concentrations of PCR amplified 717 bp caspase-1 upstream sequence was studied. Fluorescence intensity of GST-HIPPI ($\lambda_{exc} = 295$ nm and $\lambda_{em} = 340$ nm) in binding buffer and in the absence of any DNA was 10.4 U. Addition of increasing amount of 717 bp caspase-1 upstream DNA sequence decreased the intensities and reached the saturation level though addition of non-specific DNA to GST-HIPPI did not decrease this fluorescence intensity considerably. Similarly, increasing concentrations of 60 bp caspase-1 upstream sequence decreased the fluorescence intensity of GST-HIPPI and reached the plateau. Apparent dissociation constants (K_d) calculated from reciprocal plots using the plateau regions were 0.4 and 0.44 nM for GST-HIPPI binding with 717 and 60 bp

fragments, respectively. Addition of caspase-1 upstream sequences to the GST protein alone or addition of non-specific DNA (55 bp) to GST-HIPPI did not decrease the fluorescence of either of the proteins. The result of these experiments is shown in Fig. 2, panel I. Reciprocal plots for GST-HIPPI binding with 717 and 60 bp regions of caspase-1 are shown in Fig. 2, panels II and III.

In vivo interaction of GFP-Hippi with caspase-1 upstream sequences

Result obtained with ChIP assay is shown in Fig. 3. It was evident from the figure that the 717 bp (−700 to +17) of caspase-1 upstream region was cross linked with HIPPI protein in the nucleus [nuclear localization of HIPPI was reported earlier [8] and immunoprecipitated with anti-HIPPI antibody (Fig. 3A, lane 1). This result further emphasized that HIPPI could bind to caspase-1 upstream sequences *in vivo*. Western blot analysis with immunoprecipitated proteins using anti-RNA polymerase II (Imgenex, USA) identified the 40 kDa band corresponding to human RNA polymerase II (Fig. 3B). This result indicated that HIPPI, bound to the putative promoter sequence of caspase-1, was also associated with RNA polymerase II.

Promoter activity of caspase-1 upstream sequences and influence of GFP-Hippi expression

When the reporter gene was driven by 717 bp caspase-1 upstream sequence the luciferasce activity was increased

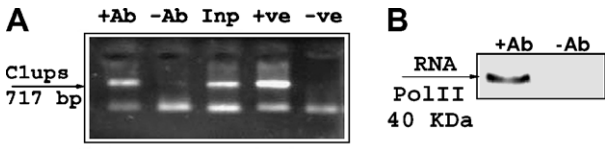

Fig. 3. *In vivo* interactions of caspase1 DNA sequences with GFP-HIPPI by ChIP assay. (A) PCR amplification using caspase1 upstream 717 bp sequence specific primers from immuoprecipitated DNA using anti-HIPPI antibody. Lanes +Ab: PCR carried out with immunoprecipitated DNA using anti-HIPPI anibody, PCR was also carried out with chromatin prepared from the same cell extract using only Rabbit IgG secondary antibody but in absence of anti-HIPPI antibody (lane: −Ab), lane Inp: PCR was carried out with DNA from total cell extract of GFP-Hippi expressing cells without any prior immunoprecipitation reaction, +ve: PCR carried out with DNA isolated from HeLa cells, −ve: PCR carried out where no template was added. (B) Western blot analysis using anti-RNA polymerase II antibody with the immunoprecipitated protein. Lane +Ab: Immunobloting carried out with protein immunoprecipitated by anti-Hippi anibody, the band represents 40 kDa RNA polymerase II, −Ab: chromatin immunoprecipitation reaction carried out similarly in absence of HIPPI antibody.

(2.1 ± 1.3)-fold in comparison with that obtained in control (only pGL3 without any insert) in HeLa cells. When the same construct was transfected in GFP-Hippi expressing HeLa cells, this value increased to (12.3 ± 2.5)-fold in comparison with the control. So this promoter activity is ~6-fold higher in GFP-Hippi cells than that obtained in HeLa cells. In Neuro2A cells, luciferase activity driven by 717 bp upstream sequence of caspase-1 was increased 2.1 ± 0.5 in comparison with that obtained in the same cells expressing the control plasmid pGL3 (without any

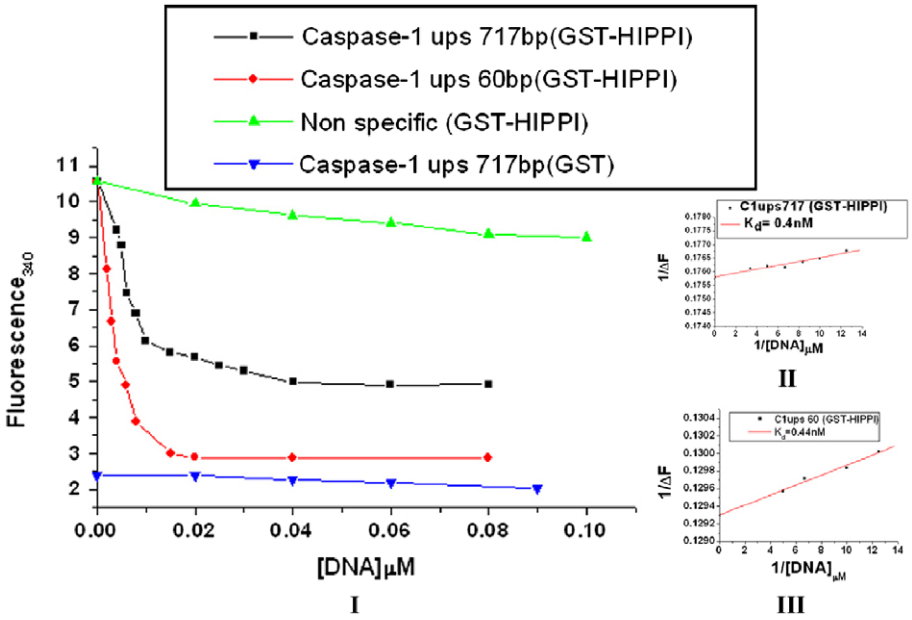

Fig. 2. Quenching of intrinsic fluorescence of GST-HIPPI in presence of caspase-1 upstream DNA sequences. Panel I: Graph shows gradual change in fluorescence intensity of GST-HIPPI (0.8μ M) at 340 nm ($\lambda_{exc} = 295$ nm) due to addition of caspase-1 upstream 717 bp sequence (solid square), caspase-1 upstream 60 bp sequence (solid diamond), non-specific DNA (solid triangle); change in fluorescence intensity of only GST protein at 340 nm ($\lambda_{exc} = 295$ nm) due to addition of caspase-1 upstream 717 bp sequence (solid inverted triangle). Linear plot of $1/\Delta F$ vs $1/c$, where 'ΔF' is the change in fluorescence with respect to protein intrinsic fluorescence due to addition of DNA with concentration 'c' expressed in micro molar. K_d values calculated from such plots where 'ΔF' is of GST-Hippi vs 'c' of Caspase1 upstream 717 bp sequence (panel II) and 'ΔF' is of GST-Hippi vs 'c' of Caspase1 upstream 60 bp fragment (panel III).

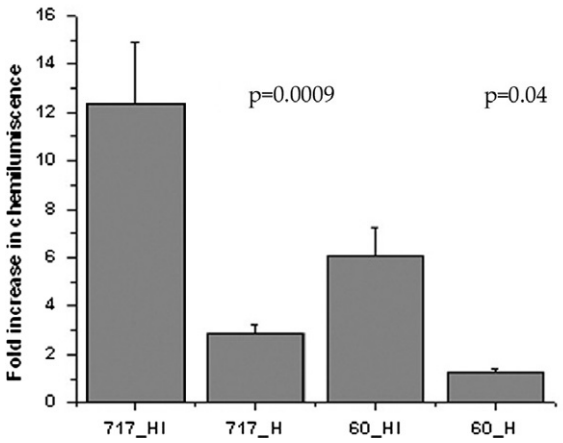

Fig. 4. Promoter activities of caspase-1 upstream sequences in Parental HeLa cells and cells expressing GFP-Hippi. Caspase-1 upstream sequence 717 and 60 bp cloned in pGL3 were transfected (4 μg each) in GFP-Hippi expressing cells and parental HeLa cells. Transfected cells are denoted as 717_Hi, 60_Hi and 717_H and 60_H, respectively. Luciferase activities relative to control (pGL3 transfected in HeLa cells) are expressed in terms of average ($n = 3$) fold increase in chemiluminescence values.

insert). However, this value was increased significantly ($p = 0.03$) further to (4.5 ± 0.3)-fold in Neuro2A cells expressing GFP-Hippi. When the luciferase gene was driven by 60 bp (−151 to −92) upstream sequence of caspase-1, the luciferase activity was increased in HeLa cells as well as in GFP-Hippi expressing HeLa cells. However, this increase was lower in comparison to that observed with the 717 bp upstream sequence of caspase-1. All these results are shown as bar diagrams in Fig. 4.

Discussions

In the present communication, we showed that HIPPI, a molecular partner of Htt-interacting protein HIP1 interacted with 717 bp (−700 to +17) upstream sequence of caspase-1 *in vitro* and *in vivo*. This sequence could act as promoter in luciferase gene in reporter assay. Luciferase activity increased about ~6-fold and ~2-fold in GFP-Hippi expressing HeLa and Neuro2A cells, respectively, in comparison to the parental cells (without exogenous Hippi expression).

Purified GST-HIPPI interacted *in vitro* with the upstream sequences of caspase-1 as revealed by EMSA (Fig. 1). Decrease in the fluorescence emission of aromatic amino acid tryptophan in GST-HIPPI due to addition of caspase-1 upstream sequences (Fig. 2) further indicated an interaction of GST-HIPPI with the upstream DNA sequences. Decrease in fluorescence intensities due to addition of specific caspase-1 upstream sequence could be attributed by the alteration of surface availability of the aromatic amino acids of GST-HIPPI (eight tryptophans; four in GST and four in HIPPI). Deletion of 717 bp of caspase-1 upstream sequence to different sizes followed by interaction studies by EMSA and fluorescence quenching revealed that HIPPI actually interacted with a 60 bp (−151 to −92) region of caspase-1 upstream sequence. ChIP assay showed that GFP-Hippi interacted with the upstream sequences of caspase-1 *in vivo* (Fig. 3A). Western blot analysis using anti-HIPPI antibody from protein fraction of the immunoprecipitated product revealed that GFP-HIPPI is actually present in the DNA–protein complex (data not shown). Presence of RNA polII in the same fraction (Fig. 3B) indicated presence of it in the HIPPI–DNA complex which indicated that HIPPI might be associated with transcription complex. As there is no detectable expression of endogenous HIPPI in HeLa cell [8], ChIP assay was also carried out with Neuro2A cells, where endogenous HIPPI is present [8]. Result showed that endogenous HIPPI could interact with caspase-1 putative promoter *in vivo* (data not shown).

Caspase-1 upstream sequence 717 bp (−700 to +17) increased the luciferase activity in luciferase (pGL3 vector) reporter assay in both HeLa (Fig. 4) and Neuro2A cells. It was increased further in GFP-Hippi expressing cells indicating that binding of HIPPI to this region might increase the transcription of down stream reporter gene. In addition, 60 bp (−151 to −92) region also increased the luciferase activity, although in lesser extent ($p = 0.007$) than that observed with the 717 bp region (Fig. 4). This result indicated that there might be other transcription regulatory proteins that bind with this sequence. Three transcription factors namely p53, p73, and ETS1 are known to bind in this 717 bp region [11–13]. Within 60 bp region, binding sites for p73 and ETS1 are missing. This may explain the reduced expression of luciferase gene obtained with the 60 bp construct.

Similarity between the increased expressions of caspase-1 in GFP-Hippi expressing cells [8] and several models of HD [4,5] indicated that HIPPI might play a role in the increased expression of the gene in HD affected cells. Our results, if replicated in HD cells or animal models or in HD patients might provide an explanation for this increase in caspase-1 expression. HIPPI normally is localized in the cytoplasm [6]. However, we have earlier shown that in HeLa cells larger proportion of exogenously expressed HIPPI is present in the nucleus [8]. The difference in localization of exogenously expressed HIPPI in HeLa cells could be mediated through HIP1. Recently it has been shown that HIP1 can interact with androgen receptors (AR), translocates it to the nucleus and increases the expression of AR target genes [14]. Thus it can be hypothesized that HIP1 can play similar role to translocate HIPPI into nucleus. The hypothesis is now being investigated. In HD, due to weaker interactions of HIP1 with the mutated Htt, the free HIP1 pool increases, that in turn can form more HIP1–HIPPI complex initiating apoptosis by activating caspase-8 and its down stream pathway [6,8], translocate HIPPI into the nucleus and increased expression of caspase-1.

In conclusion, we described here results to show a novel function of HIPPI as a transcription regulator in addition

to earlier reports of its involvement in apoptosis [6,8]. However, the role of HIPPI, if any, in HD remains unknown and requires further studies.

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