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A LIM Protein, Hic-5, Functions as a Potential Coactivator for Sp1

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Abstract Hic-5 is a LIM protein with striking similarity to paxillin, and shuttles between focal adhesions and the nucleus. Our previous study suggested that Hic-5 participates in the transcriptional control of several genes such as the c-*fos* and *p21* genes. In the present study, we examined the function of Hic-5 in the nucleus using the transcriptional promoter region of the *p21* gene. When localized to the nucleus, Hic-5 was found to transactivate the p21 promoter through two of five Sp1 sites in the region proximal to the TATA box. The Hic-5 effect was mediated by a transactivation domain of Sp1 and functional interaction with p300 through the LIM4 domain. Hic-5 was also shown to interact functionally and physically with Smad3 through the LIM domains and to potentiate p21 promoter activity together with Smad3 and Sp1. These properties were confirmed in an artificial system using GAL4-fusion protein. Thus, Hic-5 was suggested to have a potential function as a cofactor in the transcriptional complex that contains Sp1, playing a role in gene transcription in the nucleus as well as in integrin signaling at focal adhesion sites. J. Cell. Biochem. 91: 633–645, 2004. © 2004 Wiley-Liss, Inc.

Key words: hic-5; transcription; Sp1; p21/WAF1

Hic-5, originally isolated as TGFβ1- and hydrogen peroxide-inducible clone [Shibanuma et al., 1994], is almost identical to ARA55, one of the androgen receptor coactivators [Fujimoto et al., 1999; Yang et al., 2000]. Hic-5 is a member of a LIM protein family whose members have four LIM domains and four or five LD motifs in the C-terminal and N-terminal, respectively, both of which are potential interfaces for protein–protein interaction [Brown et al., 1998; Bach, 2000]. Hic-5 is structurally related to paxillin, and constitutes a family including leupaxin and PaxB [Turner, 2000]. These proteins are mainly localized to a distinct structure at the cell surface, so called focal adhesion sites in fibroblasts, and are thought to be adaptor molecules, playing a role in regulating the assembly of the focal adhesion complex and actin cytoskeleton as well as in the regulation of signal transduction under integrins [Turner, 2000]. The molecular mechanisms supporting such a role have been particularly well studied in paxillin. Paxillin was shown to interact with multiple signaling molecules such as src, Crk, Csk, FAK, PYK2, ILK, PTP-PEST, and members of the Arf GAP family as well as structural proteins such as vinculin and actopaxin, recruiting them in close proximity with each other to the focal adhesion sites and thereby, integrating the actin dynamics, and integrin- and growth factor-activated signals [Turner, 2000]. Like paxillin, to which it shows the most extensive homology, Hic-5 has also been suggested to serve as an adaptor molecule at focal adhesion sites. This idea is mainly based on the recent findings that most of the molecules interacting with paxillin bind to Hic-5 [Fujita et al., 1998; Matsuya et al., 1998; Nishiya et al., 1999; Thomas et al., 1999]. However, differences were noted between Hic-5 and paxillin, particularly distinctive tissue distributions

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in vivo [Jia et al., 2001] as well as expression patterns in cultured cells [Shibanuma et al., 1994]. In addition, their expression seems to be controlled differently during the immortalization of fibroblasts [Ishino et al., 2000]. Thus, Hic-5 has been assumed not to be a simple competitor of paxillin but to have distinctive functions of its own, while Hic-5 could compete with paxillin for an integrin signal when coexpressed in the cells [Nishiya et al., 2001].

In a previous study, we occasionally found Hic-5 to be localized to the nucleus and observed an enhancement in the expression of several genes such as extracellular matrix-related genes, the c-*fos* gene and the *p21* gene in Hic-5 high-expressing clones established from human immortalized fibroblasts, suggesting a potential function of Hic-5 in the transcriptional regulation of genes [Shibanuma et al., 1997; Kim-Kaneyama et al., 2002]. In this respect, the recent identification of Hic-5 as a coactivator of estrogen receptors as mentioned above is noteworthy [Fujimoto et al., 1999; Yang et al., 2000]. On coexpression with steroid receptors, Hic-5 enhanced ligand-dependent receptor function in a transient reporter assay. The authors also demonstrated the presence of Hic-5 in the nuclear matrix fraction [Yang et al., 2000]. Most recently, we demonstrated that Hic-5 shuttles between focal adhesion sites and the nucleus via an oxidant-sensitive nuclear export signal (NES) [Shibanuma et al., 2003]. The oxidant sensitivity of the NES caused the nuclear accumulation of Hic-5 under oxidative stress. Importantly, the nuclear localized Hic-5 transactivated the c-*fos* promoter, and under oxidative stress, Hic-5 was shown to play a role in the transcriptional up-regulation of the endogenous c-*fos* gene [Kim-Kaneyama et al., 2002; Shibanuma et al., 2003] Paxillin also shuttles between the two compartments, though the process is insensitive to oxidants and appears unrelated to transcriptional regulation [Woods et al., 2002].

As described above, Hic-5 has been suggested to have a role in transcriptional regulation in the nucleus [Shibanuma et al., 1997, 2002, 2003; Kim-Kaneyama et al., 2002]. However, its precise mode of action in the nucleus remains unsolved. The aim of this study was to characterize the factors functionally or physically interacting with Hic-5 in the nucleus using a reporter system with the transcriptional promoter of the *p21* gene. The study suggested a role for Hic-5 as a cofactor for the transcription factor Sp1, acting through collaboration with p300 and Smad3.

MATERIALS AND METHODS

Cell Culture and Plasmids

C2C12 mouse myoblastic cells were grown as described previously [Nishiya et al., 1999]. COS7 cells were grown in Dulbecco's modified MEM supplemented with 10% fetal calf serum.

A series of expression plasmids for wild-type Hic-5, mutant Hic-5 (pCG-LD1m*hic-5*, pCG-LD1m*hic*/mL1, /mL2, /mL3, /delL4), those of human Hic-5 (pCG-h*hic-5*, pCG-h*hic*LIM) and paxillin (pCG-*pax*), and a nuclear targeted version (+NLS), all tagged with HA, were produced as described previously [Shibanuma et al., 2003]. The following constructs were donated; pGL2 T + I 5xGAL4, pGAL4-Sp1-BQ, and pGAL4-VP16 from Dr. X.-F. Wang (Duke University) [Li et al., 1998], vectors expressing wild-type Flag-tagged Smad1, 2, 3, and 4 proteins and deletion mutants of Smad3 from Dr. K. Miyazono (Tokyo University) [Fukuchi et al., 2001], the expression plasmid for Sp1 from Dr. J.T. Kadonaga (University of California, San Diego) [Kadonaga et al., 1988], the expression plasmid for p300 from Dr. K. Nakashima (Kumamoto University) [Nakashima et al., 1999], and wild-type and mutant E1A from Dr. K. Oda (Science University of Tokyo) [Nakajima et al., 1992]. pGL2 T + I 5xGAL4 was provided by Dr. X.-F. Wang (Duke University) [Li et al., 1998].

Luciferase Assay

The reporter and expression plasmids were introduced into C2C12 cells by a conventional calcium phosphate method. The transfected plasmid mixture included 1 μg each of the reporter and effector plasmids together with 0.02 μg of an internal control plasmid, pRL/CMV (Promega, Madison, WI), per assay. The total amount of DNA was kept constant by addition of an empty vector. The reporter containing the 2.4 kb upstream region of the p21 promoter (WWP) was provided by Dr. K.W. Kinzler, and deleted and mutated reporters were described previously [Egawa et al., 1998]. Luciferase activity was quantified 18–24 h after the transfection using a Dual Luciferase Assay kit (Promega). Each assay was done in duplicate and repeated at least three times, and values

were normalized with respect to the *Renilla* luciferase activity expressed from pRL/CMV.

Preparation of Cell Lysates, Immunoprecipitation, and Western Blot Analysis

The expression plasmids were introduced into cells by a conventional calcium phosphate method (C2C12) or using TransIT-LT1 reagent (COS-7) purchased from PanVera (Madison, WI). After being incubated in complete medium for 24 h, the cells were processed for immunoprecipitation or for immunoblotting as described previously [Ishino et al., 2000]. For immunoprecipitation, after a wash with PBS, an aliquot of cells was lysed in buffer containing 0.5% SDS, 150 mM NaCl, and 50 mM Tris (pH 6.8), and analyzed by immunoblotting as the lysate fraction, or lysed in IP buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris (pH 6.8), 10 mM NaF, 10 mM sodium pyrophosphate, 0.4 mM sodium orthovanadate, and protease inhibitor mixture (Wako Pure Chemical Industries, Ltd., Osaka, Japan)). After the preclearing of the lysate (approximately 200 μg of total protein per sample) with normal IgG (Dako, Copenhagen, Denmark) immobilized on protein A Sepharose for 1 h at 4°C, immunoprecipitation was performed with antibodies immobilized on protein A Sepharose with gentle agitation for 2 h at 4°C. After immunoprecipitation, the resins were washed with the IP buffer supplemented with 0.1% BSA three times, followed by PBS three times. The bound proteins were eluted by boiling in SDS sample buffer for immunoblotting.

The polyclonal antibody to HA (Y-11) and monoclonal anti-Flag tag (M2) antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma Chemical Co. (St. Louis, MO), respectively.

RESULTS

Transcriptional Activation of the *p21* Gene by Nuclear Localized Hic-5

Since the *p21* gene was suggested to be one of the targets of Hic-5 in the nucleus [Shibanuma et al., 1997, 2002], we first examined whether Hic-5 localized to the nucleus affected the transcriptional activity of the p21 promoter using a luciferase reporter gene driven by 2.4 kb of the p21 promoter. In brief, Hic-5 and paxillin were expressed from vectors in which a nuclear localization signal (NLS) from SV40 large T antigen was inserted at the N-terminal of the proteins, localizing them exclusively in the nucleus to exclude the contribution of the proteins in cytoplasm or at focal adhesions. Figure 1A shows their protein structures. Immunoblotting to examine the expression levels of these effectors showed that the +NLS forms of paxillin and Hic-5 were almost equally expressed, while their levels were remarkably lower than those of wild types (−NLS forms) for unknown reasons [Shibanuma et al., 2003]. The result in Figure 1B showed that the expression of nuclear Hic-5 increased the luciferase activity by threefold. Hic-5 without NLS had no effect on the activity (−NLS bar), and in contrast to Hic-5, paxillin, even when localized in the nucleus by the NLS, could only marginally affect the activity (+NLS Pax). These observations suggested that the nuclear localized Hic-5 increased the transcriptional activity of the p21 promoter.

We next determined the region of the p21 promoter responsible for the transactivation by Hic-5. By deleting the 5′ region of the p21 promoter sequentially, we found that the induction of the reporter by Hic-5 was maintained through a construct that retained only the −217 to +14 region (Fig. 2A, PstI), suggesting that this region was sufficient for the response to Hic-5. In this region, there were six clustered Sp1 sites whose importance as shown in Figure 2B has been recently noted in the transcriptional regulation of this gene by a growing list of inducers such as TGFβ1, NGF, phorbol esters, butyrate, Ca^{2+}, progesterone, androgen, and geranylgeranyltransferase I inhibitor [Datto et al., 1995; Biggs et al., 1996; Nakano et al., 1997; Prowse et al., 1997; Adnane et al., 1998; Owen et al., 1998; Billon et al., 1999; Lu et al., 2000]. With regard to the responsiveness to Hic-5, mutations at either of the Sp1 sites, designated Sp1−3 and Sp1−4, abolished the response, whereas the disruption of other Sp1 sites did not affect the response (Fig. 2A, B). These analyses implicated the involvement of specific Sp1 sites in the responsiveness to Hic-5 on the p21 promoter.

Transactivation of the Glutamine-Rich Domain of Sp1 by Hic-5 and Involvement of p300 in the Hic-5 Effect

The above assignment of Sp1 sites on the p21 promoter as the elements responsive to Hic-5

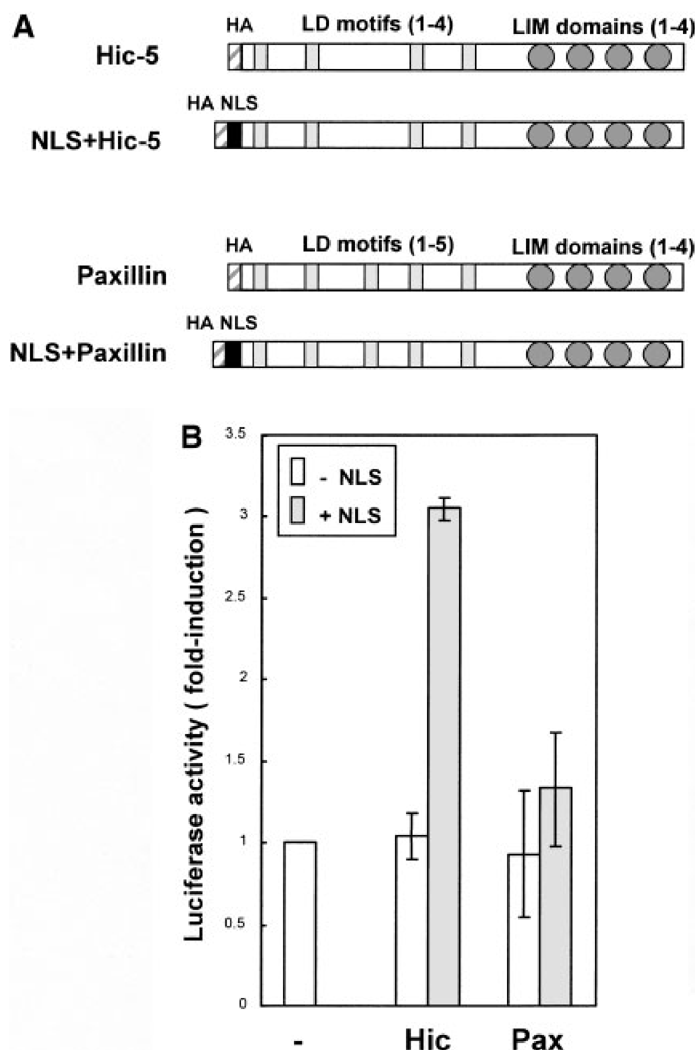

Fig. 1. Transcriptional activation of the p21 promoter by nuclear localized Hic-5. **A**: Diagrammatic representation of effector proteins used in the reporter assay. **B**: The p21 reporter plasmid (WWP in Fig. 2A) and the effector plasmids expressing Hic-5 (Hic) or paxillin (Pax) together with an internal control plasmid, pRL/CMV, were transiently cotransfected into the cells. In each experiment, equal amounts of DNA were transfected. Luciferase activities were determined 24 h after transfection and shown as a ratio to the control transfected with the pCG empty vector (−) after normalization with the internal control. Bars represent the means ± SD obtained from at least three independent experiments.

prompted us to investigate the effect of Hic-5 on the transactivating ability of the Sp1 transcription factor. For this purpose, we performed a reporter assay with the GAL4 fusion system. In the system [Li et al., 1998], the activity of the reporter (pGL2 T + I 5xGAL4) was under the control of five tandem repeats of GAL4-binding elements and regulated by an artificial transcription factor consisting of the GAL4 DNA binding domain and the transactivation domain of Sp1 (GAL-Sp1(BQ)) as illustrated in Figure 3A. BQ (amino acids from position 346 to 487 of the Sp1 protein) was a central part of the so-called B subdomain (amino acids 263–542) of the transactivation domain of Sp1 containing glutamine-rich sequences. Based on this system, previous studies have established that the B subdomain played a central role in mediating the effect of a wide spectrum of inducers [Prowse et al., 1997; Li et al., 1998; Kardassis et al., 1999]. The result in Figure 3B indicated that Hic-5 enhanced the transactivating potential of GAL-Sp1(BQ) by about sixfold. This effect appeared to be specific to the BQ domain of Sp1 in that no effect was observed on the activity of the GAL-VP fusion transcription factor.

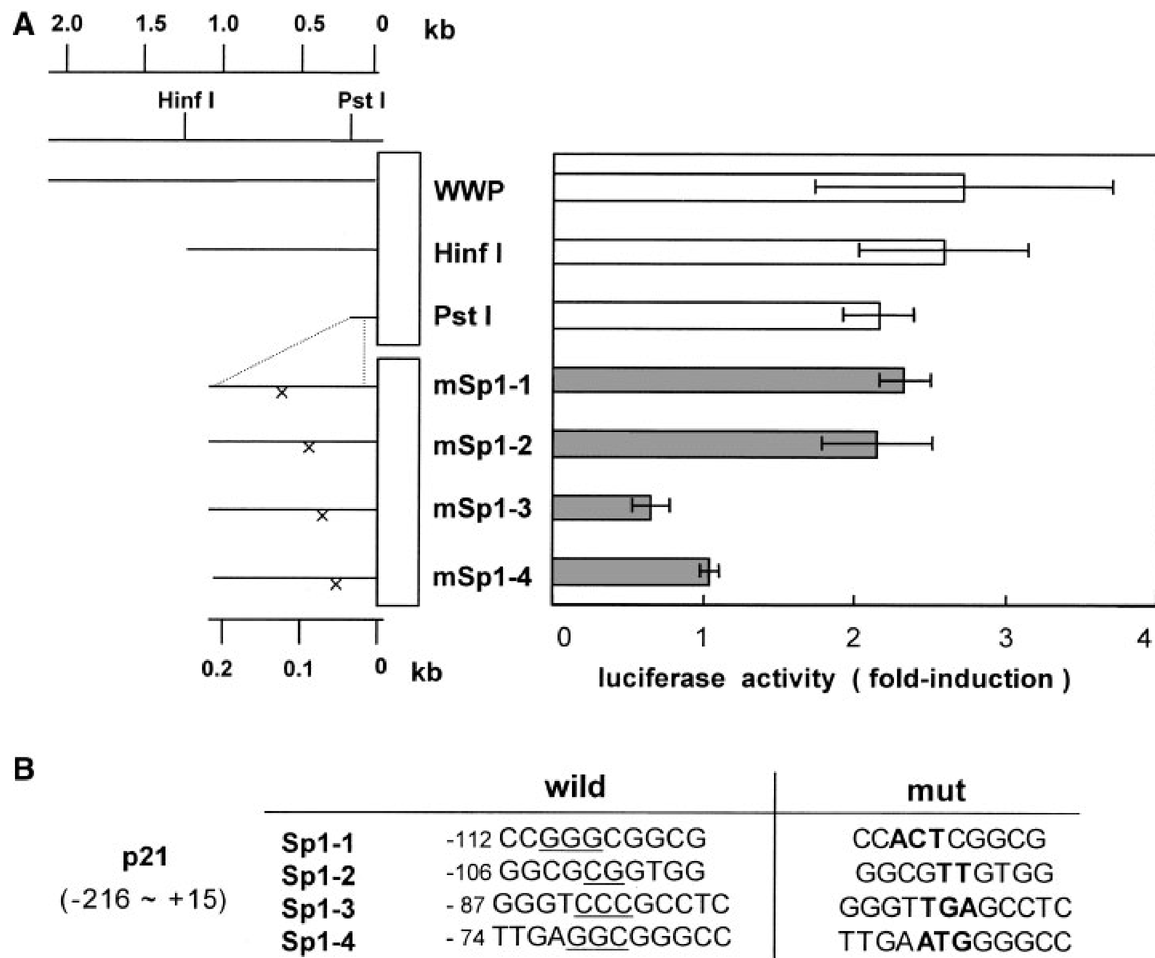

Fig. 2. The nuclear localized (+NLS) Hic-5 transactivates the p21 promoter via its Sp1 sites. **A**: Schematic representation of the p21 luciferase reporter constructs and their responsiveness to Hic-5. The indicated p21 reporters and pCG-LD1m*hic-5*(+NLS) or the pCG vector as a control were cotransfected with the internal control of pRL/CMV into the cells. Luciferase activities were determined 24 h after transfection, and transactivation of each reporter by Hic-5 is shown as fold-induction relative to the control after normalization with respect to the internal control. Bars represent the means ± SD obtained from at least three independent experiments. The crosses on the lines in the scheme mark the points mutated in the reporter as shown in (**B**). **B**: The point mutations introduced into the Sp1 sites are shown. The mutations indicated in boldface were introduced into the corresponding wild-type sequence (underlined) in the reporters.

Thus, Hic-5 was suggested to potentiate the transactivating function of Sp1 by acting on its BQ subdomain.

Sp1–3 and Sp1–4, the sites responsive to Hic-5 as demonstrated in Figure 2, were identical to those for NGF and progesterone [Biggs et al., 1996; Owen et al., 1998]. Previous investigations on the regulation of the p21 promoter by these stimuli suggested the involvement of p300, a general transcriptional coactivator, in the regulation. Thus, we next examined whether p300 was also involved in the function of Hic-5. First, we expressed the oncoprotein E1A with Hic-5 and found that the presence of wild-type E1A, which disrupts the function of endogenous CBP/p300 by binding to it, but not that of a mutant defective in the disrupting function, completely blocked the effect of Hic-5 on the p21 promoter activity (Fig. 3C). The induction of the reporter activity by Hic-5 was decreased from 3.2- to 0.9-fold by wild-type E1A but only to 2.7-fold by mutant E1A. The wild-type E1A also decreased the basal activity of the promoter, suggesting the involvement of endogenous p300 in the activity. In contrast, when p300 was coexpressed with Hic-5, the Hic-5 effect was enhanced from threefold to sixfold. Together, it was suggested that Hic-5 contributed to the gene expression through functional interaction with p300.

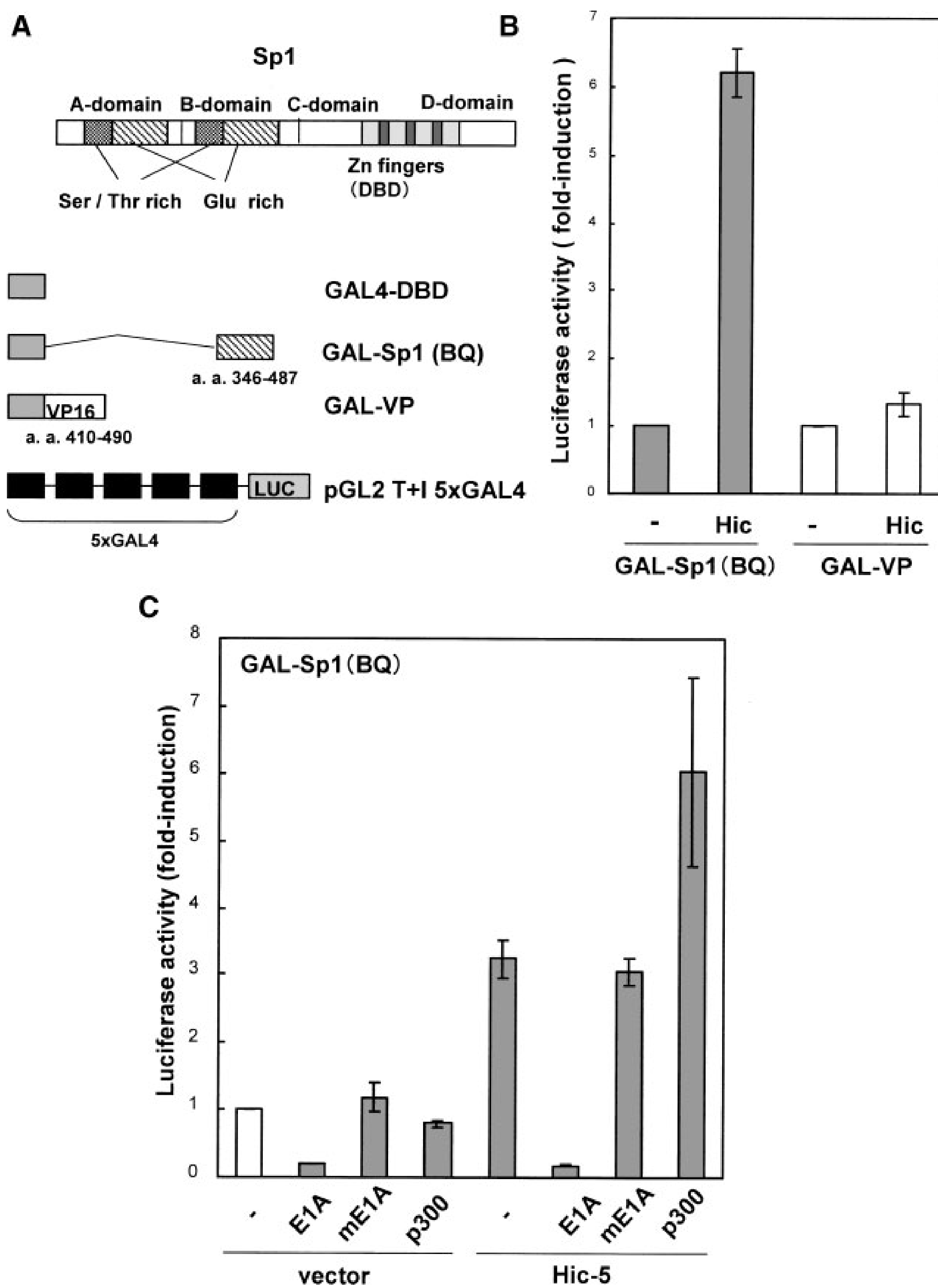

Fig. 3. Hic-5 up-regulates the transcriptional activity of the glutamine-rich domain of Sp1 fused with GAL4 DNA binding domain, collaborating with p300. **A**: Schematic representation of the reporter and GAL4 fusion protein constructs used in (**B**, **C**). **B:** The cells were cotransfected with the reporter, pGL2 T + I 5xGAL4, and either of the effectors, pGAL4-Sp1-BQ(GAL-Sp(BQ)) or pGAL4-VP16(GAL-VP), in the presence of either the pCG vector (−) or pCG-LD1m*hic-5*(+NLS) (Hic). Luciferase activities were determined as above, and the fold-induction by Hic-5 is shown. The values are means ± SD obtained from at least three independent experiments. **C:** pGL2 T + I 5xGAL4 reporter was cotransfected with pGAL4-Sp1-BQ and E1A (either wild- or mutant-type) (E1A or mE1A) or p300 expression vectors, in the absence (pCG vector) or presence of Hic-5 (pCG-LD1m*hic-5*(+NLS)).

LIM4 Is Critical for Hic-5 Function and Interaction With p300

Our previous study suggested that a particular LIM domain, LIM4, was critical for Hic-5 to transactivate the c-*fos* promoter reporter [Shibanuma et al., 2003]. In the present study, we examined the effects of the same Hic-5 mutants (mL1 to mL3 and delL4 illustrated in Fig. 4A) whose LIM domain was disrupted by point mutations or deletions on the transactivation of the p21 promoter. The deletion of LIM4 resulted in almost the complete elimination of the effect of Hic-5 on the p21 promoter (Fig. 4B), while the mutations of other LIMs were ineffective or caused a remarkable increase (mL3). As shown in Figure 4C, LIM4 was also required to stimulate the transactivating domain of Sp1 in the GAL-based system described above. Thus, these results highlighted the importance of LIM4 in the cofactor function of Hic-5. The reporter activity driven by GAL-VP was not influenced by delL4 (Fig. 4C). Importantly, this specific LIM seems to play a role in the functional cooperation of Hic-5 with p300; since the transcriptional activity of GAL-Sp1(BQ) in the presence of p300 was enhanced up to 20.8-fold by the wild-type Hic-5 but only 3.4-fold by delL4 (Fig. 4D).

Hic-5 Physically and Functionally Interacts With Smad3

We further explored other transcriptional activators, which cooperate with Hic-5 and Sp1 to transactivate the p21 promoter, and found that Smad3 was one such factor. As seen in Figure 5A, either one of the three alone, Hic-5 (lane 9), Sp1 (lane 5), or Smad3 (lane 4), increased the promoter activity by no more than twofold. In combination, however, the three proteins together achieved a sevenfold to eightfold activation (lane 16), whereas two of them, Hic-5 with Sp1 (lane 13), and Smad3 with Hic-5 (lane 12), or Sp1 (lane 8), caused at most a fourfold induction. The collaboration with Hic-5 and Sp1 was specific to Smad3, because Smad1, 2, and 4 were ineffective.

These findings suggested functional interaction of Hic-5 with a set of transcriptional regulators such as Sp1, p300, and Smad3, through which Hic-5 presumably exerted its influence on gene transcription. We next examined the physical interactions of Hic-5 with these regulators. Among the above factors, we found that Smad3 but not p300 and Sp1 physically interacted with Hic-5 under the experimental conditions. In the experiment, Flag-tagged Smad3 and HA-tagged Hic-5 (+NLS form) were coexpressed in COS7 cells, and cell lysates were immunoprecipitated with an antibody to HA tag, followed by immunoblotting with an antibody to Flag. The immunoprecipitation was performed with several independent cell lysates, and the same result was obtained. Smad3 was reproducibly detected in the immunocomplex as shown in lane 13 of Figure 5B, when the lysate was immunoprecipitated with a specific antibody to the tag but not with control IgG. Other members of the Smad family, Smad1, 2, and 4, showed no physical interaction with Hic-5, with which functional interaction was not observed, either.

We further carried out an immunoprecipitation analysis using truncated forms of Smad3 and Hic-5 proteins to determine the domains responsible for the interaction. The truncated forms of Smad3 were expressed with the wild-type Hic-5 and examined as above (Fig. 6A). Smad3-(deltaMH1), which contained MH2 and the linker sequences, exhibited detectable interaction with Hic-5 (Fig. 6A, lane 9). In contrast, Smad3-(deltaMH2), which contains MH1 and the linker sequences, failed to support the interaction (Fig. 6A, lane 7), suggesting the requirement of the MH2 region for the interaction. The result in Figure 6B showed the interaction of only LIM domains of Hic-5 with the wild-type Smad3, suggesting that these domains were sufficient for the binding to Smad3.

DISCUSSION

Hic-5, one of the LIM proteins shuttling between focal adhesion sites and the nucleus [Petit et al., 2000; Turner, 2000; Alpin and Juliano, 2001; Wang and Gilmore, 2001; Woods et al., 2002], was recently found to interact with androgen and glucocorticoid receptors and to potentiate ligand-dependent steroid receptor function in a transient reporter assay when coexpressed with the receptors [Fujimoto et al., 1999; Yang et al., 2000]. On the other hand, our previous study showed that Hic-5 played a role in endogenous c-*fos* expression under oxidative stress, which appeared to be independent of the steroid receptor function [Kim-Kaneyama et al., 2002; Shibanuma et al., 2003]. In the present

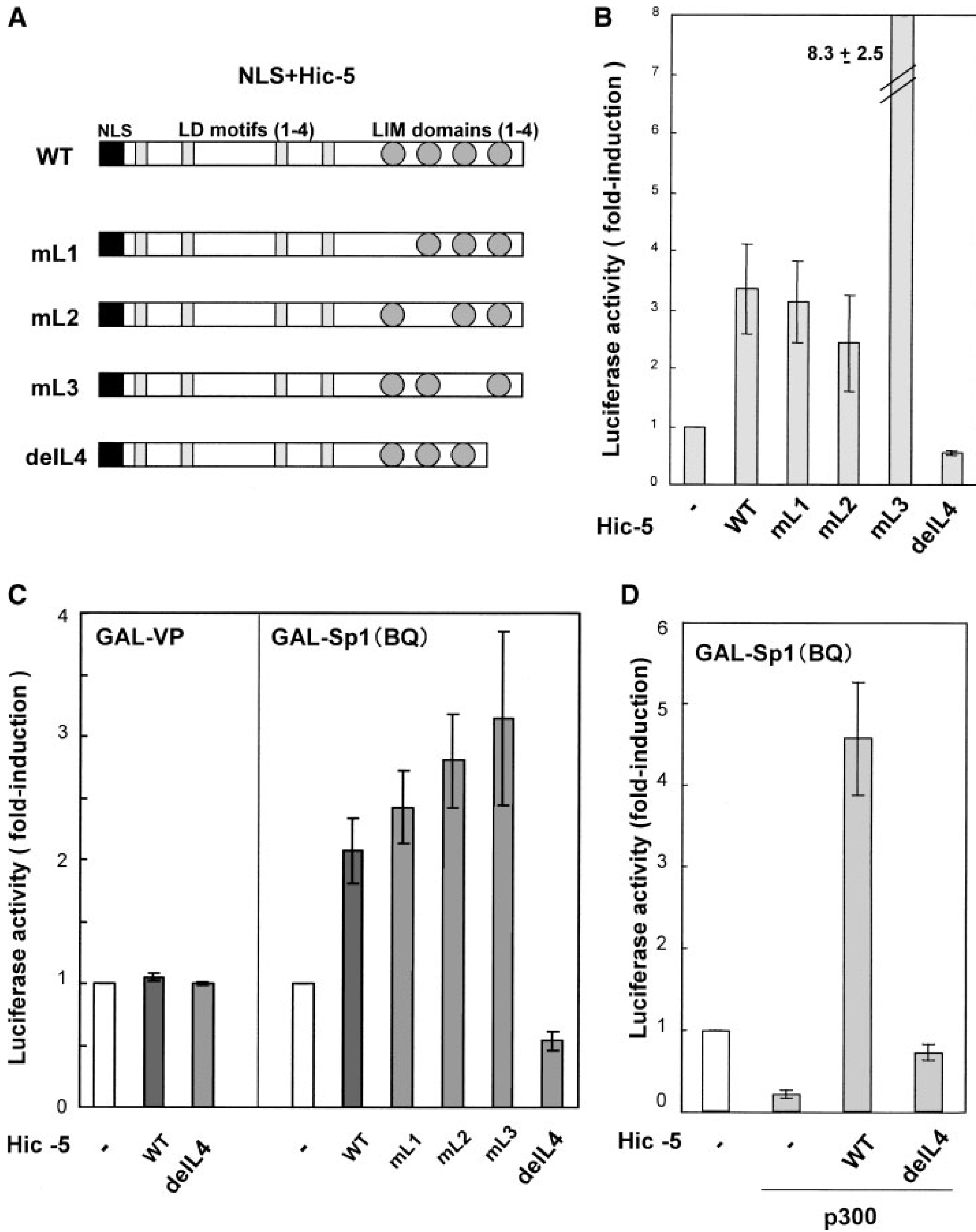

Fig. 4. A critical role for the LIM4 domain in the transactivating function of Hic-5. **A**: Schematic diagram of the Hic-5 mutants used in the following experiments. The mutants were expressed from pCG-LD1m*hic-5*/mL1, /mL2, /mL3, and /delL4(+NLS), respectively. **B**: The p21 luciferase reporter (WWP) was co-transfected with the expression vectors for the NLS + Hic-5 mutants as illustrated in (A), and luciferase assays were performed as above. **C**: The above mutants of Hic-5 were cotransfected with the pGL2 T + I 5xGAL4 reporter together with pGAL4-Sp1-BQ(GAL-Sp(BQ)) or pGAL4-VP16(GAL-VP), and the assay was done as in (B). **D**: The pGL2 T + I 5xGAL4 reporter was cotransfected with pGAL4-Sp1-BQ, p300 expression vector, and vectors for NLS + Hic-5 either of wild-type (WT; pCG-LD1m*hic-5*) or LIM4 mutant type (delI4; pCG-LD1m*hic-5*/delL4). The luciferase assay was performed as above.

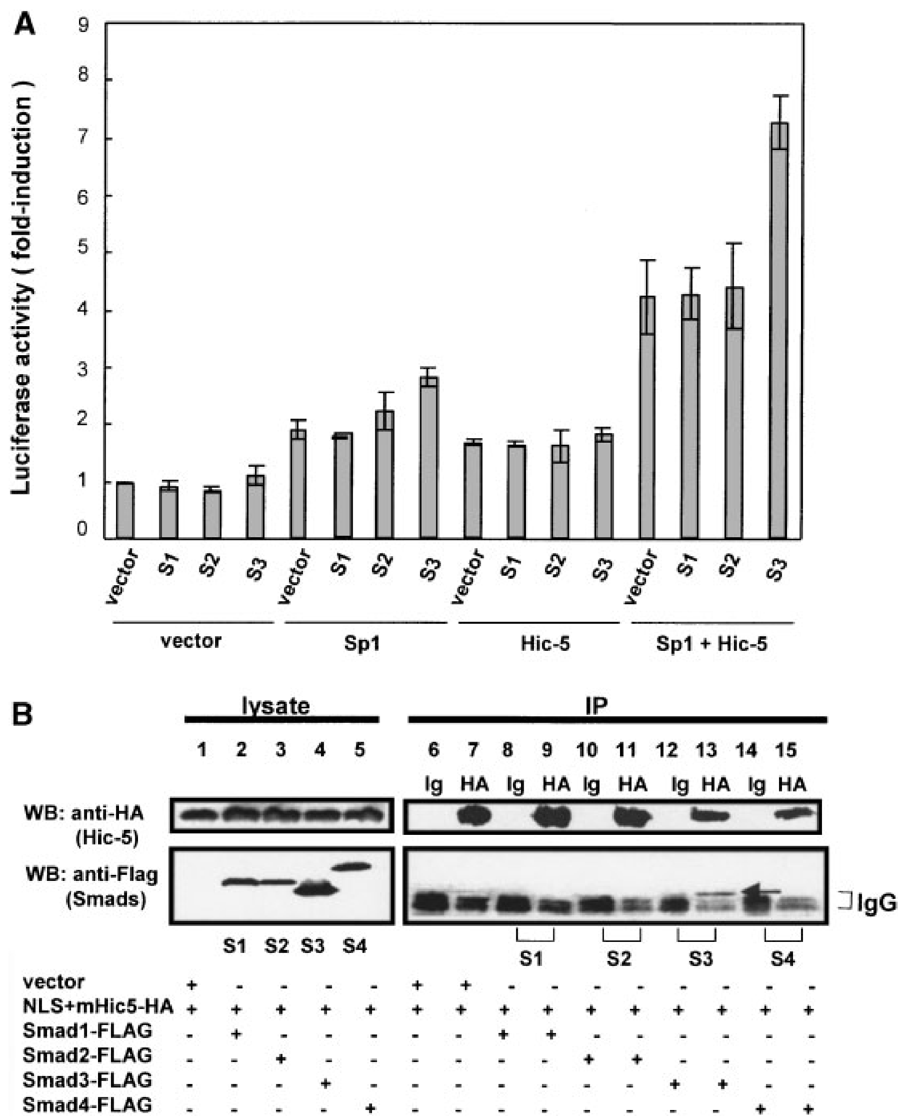

Fig. 5. The functional and physical interaction between Hic-5 and Smad3. **A**: The p21 reporter (−217 to +14; PstI) was cotransfected with an empty vector or each set of plasmids encoding Smad1–3, in the absence or presence of Sp1 and/or Hic-5 into COS7 cells. S1, S2, and S3 stand for Smad1, 2, and 3, respectively. Luciferase activities were determined as in the previous figures. **B**: Each set of plasmids was cotransfected to express the indicated proteins and after 24 h, cells were lysed and subjected to immunoprecipitation followed by immunoblotting. The plasmids used for expressing NLS + mHic-5-HA and Smads-FLAG were the same as those for Hic-5, S1–3 in (A). Antibodies against HA (HA) or normal IgG (Ig) were used for the immunoprecipitation, and against HA and Flag epitope for the immunoblotting. Total cell lysates were analyzed in parallel in the same immunoblotting. The arrow indicates immunoprecipitated Smad3.

study, we addressed the coactivator function of Hic-5 for the transcription factor of Sp1 based on the finding that Hic-5 transactivated the p21 promoter through its effect on specific Sp1 sites, potentiating the transactivating domain of Sp1.

Since Hic-5 was shown to bind to DNA in vitro [Nishiya et al., 1998], it may be directly involved in the transactivation of the promoter through binding to the Sp1 sites. However, experiments such as gel shift assays failed to detect Hic-5 in the complex on the DNA fragment encompassing the responsive region of the *p21* gene (data not shown). Instead, Hic-5 was shown to potentiate the transactivating ability of Sp1, although it appeared not to interact directly with Sp1, but in collaboration with p300 and with Smad3, to which Hic-5 bound physically. Smad3 has already been revealed to be critically involved in the up-regulation of the *p21* gene. It interacts physically and functionally with Sp1 and p300 at the Sp1–3 site of the promoter region of p21, thereby mediating TGFβ1

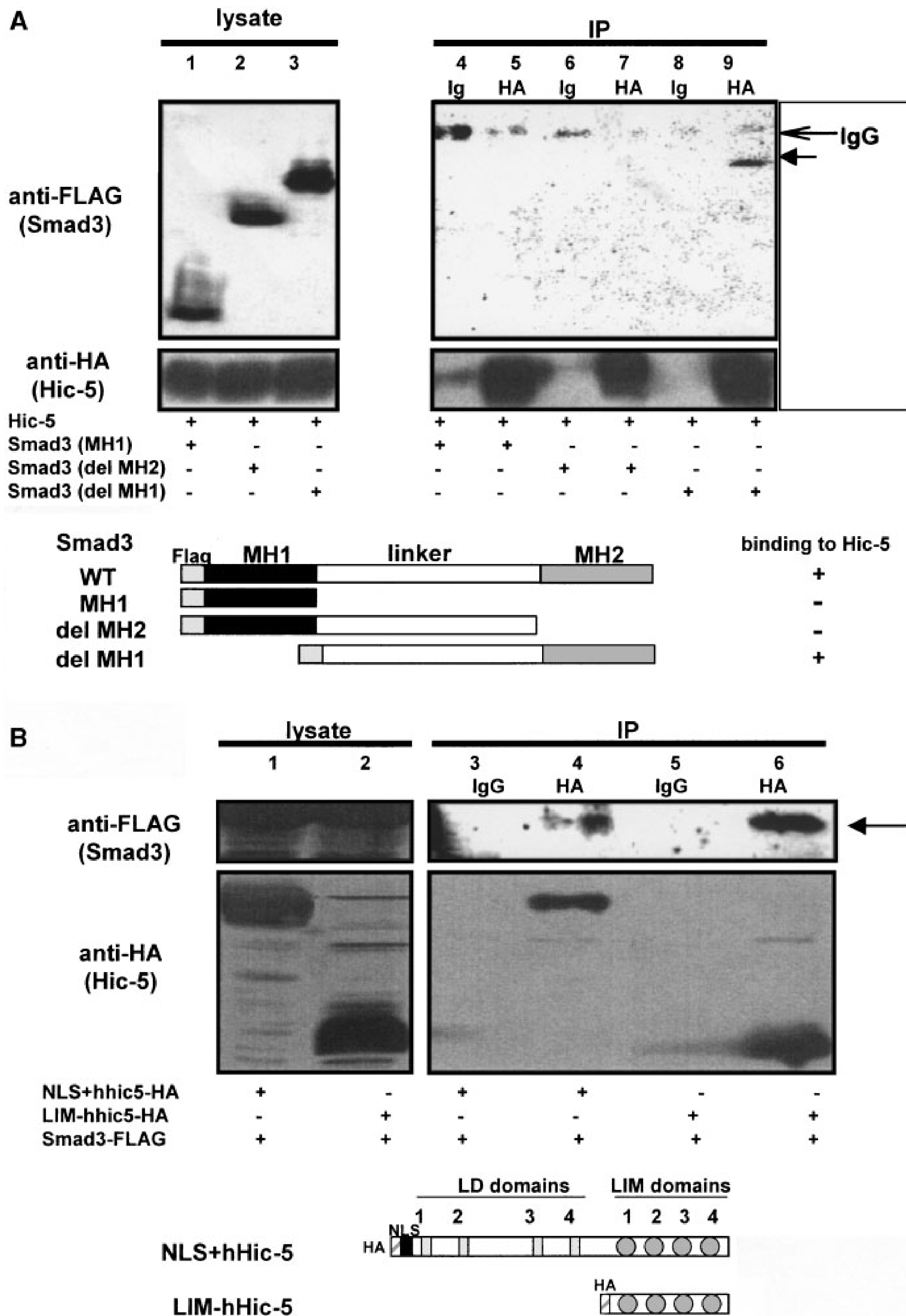

Fig. 6.

signaling [Moustakas and Kardassis, 1998; Shen et al., 1998; Pardali et al., 2000]. Physical interaction was also demonstrated between Sp1 and p300 [Suzuki et al., 2000]. These findings together made it likely that Hic-5 was incorporated in a multi-protein complex on the DNA, which included Sp1, Smad3, and p300, through direct interaction with Smad3.

The Hic-5-responsive Sp1 sites were those designated as Sp1–3 and 1–4 (Fig. 2B), which are reported to contribute to the induction of p21 expression by TGFβ1, NGF, Ca^{2+}, and butyrate [Datto et al., 1995; Biggs et al., 1996; Prowse et al., 1997; Billon et al., 1999]. Sp1–3 and Sp1–4 are also known to mediate the effect of progesterone and androgen [Owen et al., 1998; Lu et al., 2000], and in this regard, it should be noted that Hic-5 is a potential coactivator of the androgen receptor. This receptor was reported to interact physically with Sp1, resulting in the induction of p21 expression [Lu et al., 2000]. Likewise, the progesterone receptor, which could also induce p21 expression, was shown to associate with Sp1 and CBP/p300 [Owen et al., 1998]. Involvement of the estrogen receptor and Sp1 complex in transcriptional regulation was suggested in several genes [Porter et al., 1996; Duan et al., 1998; Sun et al., 1998]. In addition, cross-talk between the steroid receptor and TGFβ1 was noted in which Smad3 was a focal point [Hayes et al., 2001; Kang et al., 2001; Chipuk et al., 2002]. Because the present study was performed in the absence of ligands, it was unlikely that the effect of Hic-5 was associated with these nuclear receptor functions in gene transcription. However, it is possible that Hic-5 activated the receptors without ligands via unknown mechanisms, resulting in the transactivation of the p21 promoter under the experimental conditions, although the coexpression of androgen receptor had no effect on the activation of the promoter by Hic-5 (unpublished data).

In the experiments on physical interaction between Hic-5 and Smad3, we co-immunoprecipitated Smad3 with Hic-5 but not Hic-5 with Smad3. It seemed likely that the incorporation of both proteins in a multi-protein complex limited their accessibility to the antibodies. We made an effort to immunoprecipitate Hic-5 with Smad3 tagged not only at the N-terminal but also at the C-terminal, and tried a polyclonal antibody to Smad3 that recognized the central portion of the linker domain instead of antibody to the tag. In either case, however, Hic-5 was not detected in the immunocomplex containing Smad3.

In a previous study, we pointed out the importance of LIM4 to the function of Hic-5 in transcriptional regulation [Shibanuma et al., 2003]. In the present study, LIM4 was revealed to be required to transactivate the p21 promoter and BQ domain of Sp1 as well as c-*fos* promoter, implicating this particular LIM in the interaction with a factor generally required for the transcription. In fact, this domain was suggested to be an interface for functional interaction with p300. Besides, Hic-5 has been shown to interact with a set of transcription factors, including steroid receptors [Fujimoto et al., 1999; Yang et al., 2000] and Smad3 (the present study). Thus, Hic-5 is a potential scaffold on which to assemble the transcriptional complex at Sp1 sites and to couple the transcriptional activity with that of p300 through LIM4, although the precise mechanisms and other molecules associated with the Hic-5 function need to be clarified in more detail. In this regard, the mutation in LIM3 dramatically augmented the effect of Hic-5 on the activity of the p21 reporter as well as on the c-*fos* reporter, suggesting the interaction of this LIM domain with a negative regulator for transcription. PTP-PEST tyrosine phosphatase is the only molecule currently known to interact with this domain [Nishiya et al., 1999]. For the interaction with Smad3, the C-terminal region including the four LIM domains were suggested to be required as a whole.

A new molecular function as a coactivator for transcriptional control has recently been demonstrated for other LIM proteins that are

Fig. 6. Hic-5 interacts with the MH2 domain of Smad3 through the LIM domains. **A**: Expression plasmids for wild-type or mutant Smad3 as illustrated were cotransfected with the Hic-5 expression plasmid (pCG-LD1m*hic-5*(+NLS)) into COS7 cells, and the interaction of the Smad3 proteins with Hic-5 was analyzed by immunoprecipitation and immunoblotting as in Figure 5B. Antibodies against HA (HA) or normal IgG (Ig) were used for the immunoprecipitation, and against HA and Flag epitope for the immunoblotting. Total cell lysates were analyzed in parallel in the same immunoblot. The arrow indicates immunoprecipitated Smad3. **B**: Full-length or only LIM domains of Hic-5 as illustrated were coexpressed with the wild-type Smad3, and the interaction was analyzed as in (A). The expression plasmids for NLS + hHic-5 and LIM-hHic-5 were pCG-h*hic-5*(+NLS) and pCG-h*hic*LIM, respectively. The arrow indicates immunoprecipitated Smad3.

localized at actin-based cellular structures such as focal adhesion sites and stress fibers as well as the nucleus. FHL2, which belongs to the LIM-only protein family, has been recently identified as a coactivator of the androgen receptor and demonstrated to be translocated to the nucleus in response to Rho signals, resulting in the activation of FHL2- and androgen receptor-dependent genes [Müller et al., 2000]. Another LIM protein, Trip6, which is a member of the zyxin family shuttling between focal adhesion sites and the nucleus, was a likely coactivator of thyroid hormone receptor [Wang and Gilmore, 2001]. LPP was also shown to have the potential to regulate transcription [Petit et al., 2000]. Further study of the mechanisms underlying the function of these LIM-containing cofactors and their regulation should provide information on how dynamism of the actin cytoskeleton could be coupled with transcriptional regulation.

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