experiments to analyze the mechanism of HCV genomic replication.

Replicon RNA and NS Proteins Were Retained in Digitonin-permeabilized Replicon Cells—Previous papers suggested that all HCV NS proteins are directly or indirectly associated with the inner cellular membranes, especially rough ER membranes, and form replication complexes on the membranes (10, 40). This suggested to us that functional HCV replication complexes could be retained in the replicon cells whose plasma membranes had been permeabilized with digitonin. To test this possibility, first, the fate of NS proteins in the replicon cells was investigated after digitonin treatment by Western blotting. In this experiment, ectopically expressed mouse DHFR was used as a cytoplasmic protein marker. After digitonin treatment, DHFR was not detected in the permeabilized cells (Fig. 1*A*, *lanes 2* and *3*), indicating that cytoplasmic soluble proteins were washed efficiently out of the cells under these conditions. On the other hand, HCV NS proteins (NS3-NS5B) were detected just like BiP/Grp78, an ER marker, in the permeabilized replicon cells as in the intact cells that were not treated with digitonin, as expected (Fig. 1*A*, *lanes 2* and *3*). Moreover, when we analyzed the RNA by Northern blotting, the retention of replicon RNA in the permeabilized replicon cells was observed (Fig. 1*B*, *lanes 2* and *3*). Treatment of the permeabilized replicon cells with the high salt buffer containing 2 M KCl did not greatly influence the amount of replicon RNA and NS proteins retained in the cells (data not shown). To investigate whether newly synthesized replicon RNA in the intact replicon cells was retained after permeabilization with digitonin, we performed metabolic labeling of the cells with [^{32}P]orthophosphate. As shown in Fig. 1*C*, newly synthesized replicon RNA was detected after permeabilization (Fig. 1*C*, *lane 3*), although the amount was slightly decreased compared with that in the intact cells (Fig. 1*C*, *lane 2*). The localization of NS5A and NS5B in the permeabilized replicon cells was also analyzed by indirect immunofluorescence. These proteins were seen to accumulate around the perinuclear region and to be mostly colocalized with protein-disulfide isomerase, an ER marker, in the permeabilized replicon cells (Fig. 1*D*, *panels i–p*), just as in the intact replicon cells (Fig. 1*D*, *panels a--h*). This indicated that treatment with digitonin did not markedly affect subcellular localization of these proteins. From all of these results, it seemed likely that the replication complexes including replicon RNA were retained in the permeabilized replicon cells just like in the intact cells.

The Replication Complexes in the Permeabilized Replicon Cells Functioned to Synthesize the HCV Subgenomic RNA—To see whether the replication complexes in the permeabilized replicon cells were active in HCV RNA synthesis, permeabilized or intact cells were incubated in reaction mixtures including [α-^{32}P]UTP for 4 h. Actinomycin D, which showed no inhibitory effect on the RNA-dependent RNA polymerase activity of HCV NS5B (15), was also added to the reaction mixture to inhibit the cellular activities of DNA-dependent DNA and RNA synthesis. After the reaction, total RNA of the cells and the reaction supernatants were extracted and analyzed by denaturing agarose gel electrophoresis followed by autoradiography. A radiolabeled product ~8 kb in length, which was equivalent to the subgenomic replicon RNA in size, was found in RNA from the permeabilized replicon cells but not from Huh-7 cells (Fig. 2*A*, *lanes 1–4*). When overexposed, the 8-kb band became detectable in RNA sample from the intact replicon cells, although the signal was much lower than that from the permeabilized replicon cells (data not shown). This 8-kb product, however, was not detected in all the reaction supernatants (Fig. 2*A*, *lanes 5–8*). The radiolabeled 8-kb product was degraded by treatment with RNase A (data not shown), suggesting that it was HCV subgenomic RNA synthesized by the replication complexes in the permeabilized replicon cells. To examine whether the 8-kb RNA synthesized in the permeabilized cells was actually HCV subgenomic RNA, we performed slot blot hybridization analysis using each plus and minus strand HCV

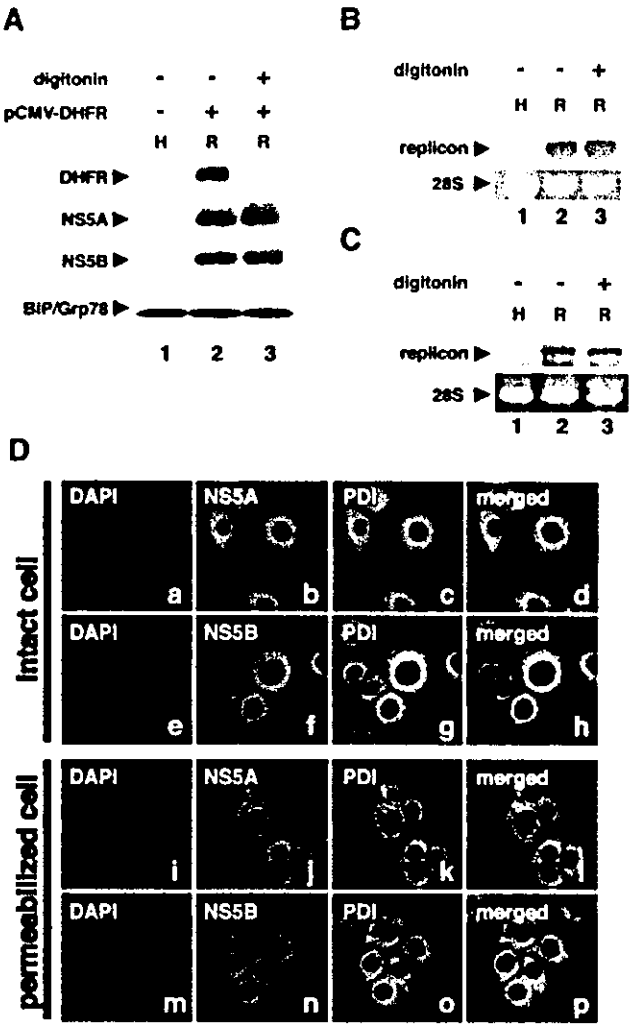

FIG. 1. **Detection of NS5A, NS5B, and subgenomic replicon RNA in permeabilized replicon cells.** *A*, total lysate from intact cells (*digitonin −*, *lanes 1* and *2*) or digitonin-treated permeabilized cells (*digitonin +*, *lane 3*) was analyzed by Western blotting with anti-DHFR, anti-NS5A, anti-NS5B, and anti-BiP/Grp78 antibodies. *H* and *R* indicate parental Huh-7 and replicon cells, respectively. DHFR produced ectopically by transient transfection of pCMV-DHFR and endogenous BiP/Grp78 were used as markers for cytoplasm and ER, respectively. *B*, total RNA extracted from the intact (*lanes 1* and *2*) or permeabilized cells (*lane 3*) was analyzed by Northern blotting using an HCV genome-specific probe (*upper panel*). The relative amount of total RNA in each lane is shown by 28 S rRNA (28S) stained with ethidium bromide (*lower panel*). *C*, the replicon RNA metabolically radiolabeled in the intact replicon cells was retained in cells after permeabilization with digitonin. After metabolic labeling of Huh-7 and replicon cells with [^{32}P]orthophosphate, radiolabeled RNA from the intact cells (*lanes 1* and *2*) or permeabilized cells (*lane 3*) was analyzed by denaturing agarose gel electrophoresis followed by autoradiography. Approximately 8-kb bands in length were indicated (replicon). *D*, the subcellular localization of NS5A (*red*), NS5B (*red*), and protein-disulfide isomerase (*PDI*) (ER marker, *green*) in the intact or permeabilized replicon cells was analyzed by indirect immunofluorescence using anti-NS5A (*panels b* and *j*), anti-NS5B (*panels f* and *n*), and anti-PDI antibodies (*panels c*, *g*, *k*, and *o*), respectively. Nucleus stained by 4′,6-diamidino-2-phenylindole (*DAPI*) is shown in *panels a*, *e*, *i*, and *m*. Merged panels are shown in *panels d*, *h*, *l*, and *p*.

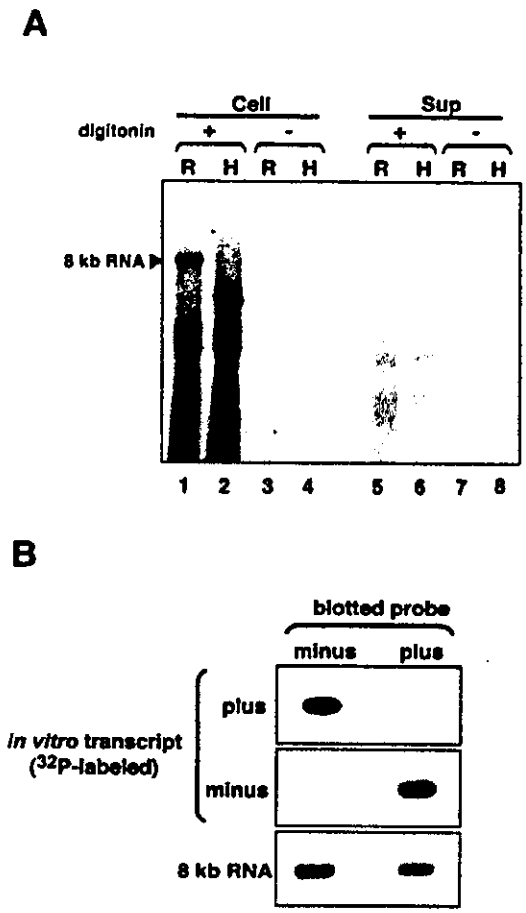

FIG. 2. **The activity for synthesis of the HCV subgenomic RNA was retained in the permeabilized replicon cells.** *A*, the replicon cells (*R*; *lanes 1, 3, 5,* and *7*) and the parental Huh-7 cells (*H*; *lanes 2, 4, 6,* and *8*) were used in this experiment after treatment with (*lanes 1, 2, 5,* and *6*) or without (*lanes 3, 4, 7,* and *8*) digitonin. Total RNA extracted from the cell fraction (*Cell*), as well as the reaction supernatant (*Sup*), of these cells after incubation in the reaction mixtures for 4 h was analyzed by denaturing agarose gel electrophoresis followed by autoradiography. The 8-kb RNA specifically found in the cell fraction of replicon cells is indicated by an *arrowhead*. *B*, characterization of the radiolabeled 8-kb RNA synthesized in the permeabilized replicon cells. Equal amounts of the unlabeled plus and minus strand replicon RNA synthesized *in vitro* were blotted on nylon membrane filter and used as specific probes for detection of the minus and plus strand replicon RNA synthesized and radiolabeled in the replicon cells, respectively, in the slot blot hybridization experiment (*8 kb RNA*). To show the strand specificity of this experiment, the results of the experiment performed by using the plus and minus HCV RNA ^{32}P radiolabeled *in vitro* instead of those in the replicon cells are presented (*in vitro* transcript, plus and minus).

RNA synthesized *in vitro* as probes on the nylon membrane filter. After denaturing agarose gel electrophoresis, the radiolabeled 8-kb RNA derived from the permeabilized replicon cells was eluted from the gel and hybridized with the probes on the filter. Then the radioactivity hybridized with either the plus or minus strand-specific probe on the filter was detected by autoradiography (Fig. 2*B*, *8 kb RNA*), whereas radiolabeled RNA prepared from permeabilized Huh-7 cells in the same way did not show any hybridization signals on the filter (data not shown). By the same procedure, we also confirmed that the metabolically radiolabeled 8-kb RNA in Fig. 1*C* was actually replicon RNA (data not shown). The ratio of plus to minus strand RNA synthesized in the permeabilized replicon cells was estimated to be 2.7 ± 0.4 (average ± S.D.) by three independent experiments. These results indicated that the radiolabeled 8-kb RNA was actually HCV subgenomic RNA synthesized in the permeabilized replicon cells and contained both plus and minus strands of the HCV RNA, implying that the functional HCV replication complexes were present in the permeabilized replicon cells.

Replicon RNA in the Permeabilized Replicon Cells Was Resistant to Nuclease—The production of radiolabeled HCV subgenomic RNA in the permeabilized cells seemed to continue until 4 h and reached a maximum until ~5 to 6 h (data not shown). Then the amount of radiolabeled HCV RNA was stably maintained even after 8 h. One possible explanation for this stability was the removal of RNase from the cell by permeabilization. Therefore, we examined the sensitivity of the newly synthesized HCV RNA in the permeabilized replicon cells to exogenously added nuclease. After the reaction for RNA synthesis, the permeabilized replicon cells were treated with micrococcal nuclease. As shown in Fig. 3*A*, the radiolabeled HCV RNA remained almost intact even after nuclease treatment (*lanes 1* and *2*), although 28 S rRNA was efficiently degradated under the same condition (*lanes 2* and *3*). This lower sensitivity of the replicon RNA to nuclease suggested that one of the reasons for the stability of the RNA in the permeabilized cells was its resistance to RNase activity. On the other hand, the radiolabeled HCV RNA was sensitive to nuclease in the presence of a nonionic detergent, Nonidet P-40 (Fig. 3*A*, *lane 3*), although a small portion of the HCV RNA was likely to remain resistant to nuclease. When the permeabilized replicon cells were treated with only Nonidet P-40, the radiolabeled HCV RNA in the cells was still detectable but apparently diminished. However, the radiolabeled molecules became detectable in the reaction supernatant, possibly because of leakage from the cells because of Nonidet P-40 (Fig. 3*A*, *lanes 4* and *8*). From these results, HCV subgenomic RNA newly synthesized in the permeabilized cells seemed resistant to nuclease in a cellular lipid membrane-dependent manner. Moreover, we also observed that the replicon RNA synthesis was equally carried out when the permeabilized cells were pretreated with nuclease before the reaction of the RNA synthesis (data not shown). This suggested that the HCV RNA used as a template in the viral RNA synthesis was also resistant to nuclease and present in the same environment as the newly synthesized RNA products mentioned above. Furthermore, we investigated the replicon RNA that was newly synthesized in the intact replicon cells. After metabolic labeling of the replicon cells with [^{32}P] orthophosphate, the cells were permeabilized with digitonin and treated with micrococcal nuclease as above. As shown in Fig. 3*B*, about 30% of radiolabeled replicon RNA in the intact replicon cells was found to be lost after permeabilization (*lanes 1* and *2*), probably implying that a part of the newly synthesized RNA flowed out with the cytoplasm from the permeabilized cells (see discussion). A large part of newly synthesized replicon RNA retained in the permeabilized cells showed resistance to the nuclease action in the absence of Nonidet P-40 (Fig. 3*B*, *lane 3*) but was tuned to be sensitive to nuclease in the presence of Nonidet P-40 (Fig. 3*B*, *lane 4*). This suggested that the fate of the newly synthesized replicon RNA in the permeabilized cells was similar to that retained in intact cells after permeabilization. Then we analyzed the nuclease sensitivity of total replicon RNA in replicon cells by Northern blotting. After permeabilization, replicon cells were treated with nuclease in the presence or absence of Nonidet P-40 as described above. As shown in Fig. 3*C*, we found that most of the replicon RNA was intact even after treatment (Fig. 3*C*, *lanes 2* and *3*). As in the case of the newly synthesized replicon RNA, the replicon RNA pre-existing in the replicon cells was sensitive to nuclease in the presence of Nonidet P-40 (Fig. 3*C*, *lane 4*). From these

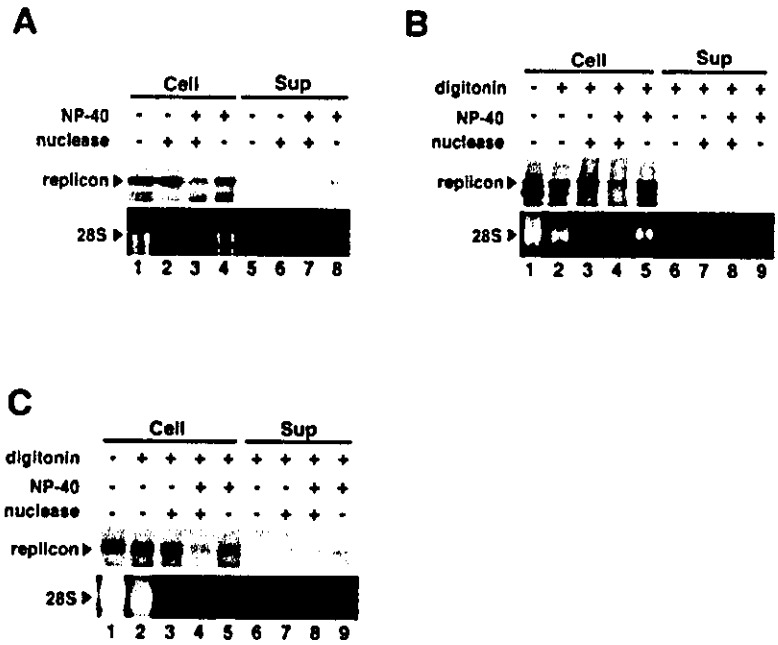

FIG. 3. **A large part of the replicon RNA was located in the nuclease-resistant environment in the replicon cells.** *A*, resistance of the newly synthesized HCV RNA in the permeabilized replicon cells to nuclease action. After the RNA synthesis reaction in the presence of [^{32}P]UTP, the permeabilized replicon cells were treated with (*nuclease +, lanes 2, 3, 6,* and *7*) or without micrococcal nuclease (nuclease −, *lanes 1, 4, 5,* and *8*). The reactions were performed in the presence (*Nonidet P-40 +, lanes 3, 4, 7,* and *8*) or absence of 0.5% Nonidet P-40 (*Nonidet P-40 −, lanes 1, 2, 5,* and *6*). Total RNA extracted from the cells (Cell, *lanes 1–4*) and the reaction supernatant (Sup, *lanes 5–8*) was similarly analyzed as described in the legend to Fig. 2. *B*, resistance of the newly synthesized replicon RNA in the intact replicon cells to nuclease action. The intact replicon cells were metabolically labeled with [^{32}P]orthophosphate. After incubation, the cells were permeabilized and treated with (*lanes 3, 4, 7,* and *8*) or without micrococcal nuclease (*lanes 2, 5, 6,* and *9*), in the presence (*lanes 4, 5, 8,* and *9*) or absence of Nonidet P-40 (*lanes 2, 3, 6,* and *7*), as described above. *C*, resistance of the replicon RNA in replicon cells against nuclease action. After permeabilization of the replicon cells with digitonin, the cells were treated with (*lanes 3, 4, 7,* and *8*) or without micrococcal nuclease (*lanes 2, 5, 6,* and *9*), in the presence (*lanes 4, 5, 8,* and *9*) or absence of (*lanes 2, 3, 6,* and *7*) Nonidet P-40 as above. Total RNA from the cell fraction or the reaction supernatant after treatment was analyzed by Northern blotting using a HCV genome-specific probe to detect the replicon RNA (indicated by the *arrowhead* with replicon, *upper panel*). 28 S rRNA (*28S*) was detected by staining with ethidium bromide (*A–C, lower panels*). In *A*, the amount of 28 S rRNA seemed to be low probably because of degradation during incubation in the reaction mixture for 4 h.

results, it was suggested that replicon RNA existed in a subcellular compartment that was formed with cellular lipid membranes.

A Large Amount of Each NS Protein Was Not Required for Nuclease Resistance of Replicon RNA—To investigate the contribution of HCV NS proteins to the resistance of replicon RNA against nuclease, the sensitivity of the replicon RNA to nuclease was examined in permeabilized replicon cells after treatment with proteinase K at various concentrations. The condition of the replicon RNA and NS proteins in the cells after treatment was analyzed by Northern and Western blotting, respectively. An endogenous Calnexin, a type I transmembrane protein of ER, was detected by using an antibody specific to its NH_2-terminal region present in the ER lumen (Calnexin-NT) as a control for monitoring the efficiency of proteinase K digestion. At the dose of proteinase K that affected the amount of full-length Calnexin, a fragment, the size of which matched that of the NH_2-terminal region of Calnexin was detected (Fig. 4, *lanes 4–11*), indicating that proteins or segments on the cytoplasmic side of ER membranes were efficiently digested and those in the lumen were protected from the digestion by proteinase K as expected. Under these conditions, a large part of each NS protein was digested by proteinase K at higher concentrations, although the sensitivity of each NS protein to treatment varied (Fig. 4, *lanes 4–7* and *8–11*). On the other hand, we also observed that a small part of each NS protein remained resistant to even prolonged treatment of the permeabilized replicon cells with proteinase. Under these conditions, we observed that nuclease treatment did not affect replicon RNA despite efficient degradation of 28 S rRNA (Fig. 4, *lanes 8–11*), although amounts of 28 S rRNA and replicon RNA were not influenced by only the proteinase treatment (Fig. 4, *lanes 4–7*). These results indicated that the stability replicon RNA was not dependent on the large parts of the NS protein. This raised the possibility that the precise subcellular localization of replicon RNA was different from that of the majority of each NS protein.

The Active Replication Complex Was in a Similar Environment to the Replicon RNA—As shown above, the majority of each NS protein in digitonin-permeabilized replicon cells were sensitive to proteinase treatment. Furthermore, all NS proteins (NS3-NS5B) in replicon cells were detectable by indirect immunofluorescent analysis after permeabilization only with digitonin following fixation with 4% paraformaldehyde, although the proteins such as protein-disulfide isomerase and BiP/Grp78 that are present in the ER lumen were not detected under this condition (data not shown). These results indicated that a large part of each NS protein was exposed on the cytoplasmic side of the inner cellular membranes. To see whether these NS proteins function in the replication of HCV, the synthesis of the HCV subgenomic RNA was investigated in the permeabilized replicon cells pretreated with proteinase K. After treatment with proteinase at higher concentrations, each NS protein was almost degradated by the treatment as shown in Fig. 4 (Fig. 5*A*, *lanes 2–5*). Under these conditions, however, the HCV RNA was synthesized in a quite similar manner to that in the cells not treated with proteinase (Fig. 5*A*, *lanes 1–5*). These results suggested that the majority of active replication complex existed in the proteinase-resistant environment and a large part of each NS protein did not participate in the synthe-

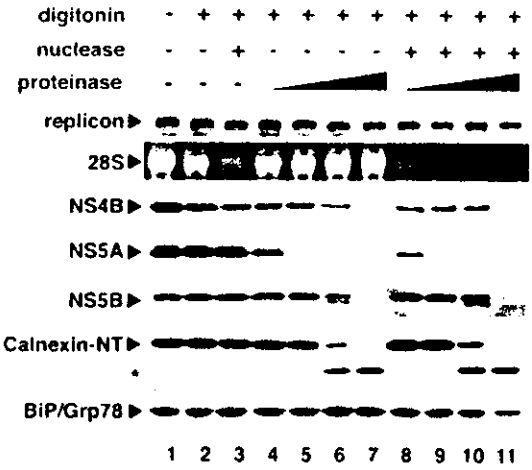

FIG. 4. **A large part of each NS protein was not required for the nuclease resistance of the replicon RNA.** The digitonin-permeabilized replicon cells were treated with proteinase K at various concentrations (0 μg/ml for *lanes 2* and *3*, 0.01 μg/ml for *lanes 4* and *8*, 0.1 μg/ml for *lanes 5* and *9*, 1 μg/ml for *lanes 6* and *10*, 10 μg/ml for *lanes 7* and *11*), followed by treatment with (*lanes 3* and *8–11*) or without (*lanes 2* and *4–7*) micrococcal nuclease. The samples from intact replicon cells without any treatments were similarly analyzed as shown in *lane 1*. After these treatments, total RNA and protein in the cells were analyzed by Northern and Western blotting, respectively. Antibodies used in the Western blotting were anti-NS4B (*NS4B*), anti-NS5A (*NS5A*), anti-NS5B (*NS5B*), anti-Calnexin (*Calnexin-NT*), and anti-KDEL (*BiP/Grp78*) antibodies. Each protein with original size is indicated by an *arrowhead*. An *asterisk* denotes the position of the Calnexin NH_2-terminal segment that is located in the lumen of the ER. BiP/Grp78, which located on the lumenal side of the ER membrane, was used as a negative control for proteinase digestion.

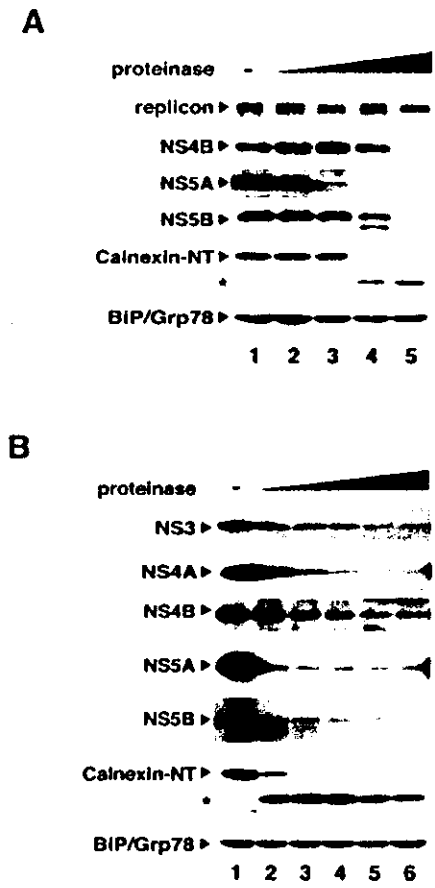

FIG. 5. *A*, the activity for the synthesis of HCV RNA in the permeabilized replicon cells was intact even after degradation of a large part of each NS protein by proteinase K treatment. After treatment of the permeabilized replicon cells with proteinase K as described in the legend to Fig. 4, the RNA synthesis reaction was performed. The concentrations of proteinase K used in this experiment were 0 (*lane 1*), 0.01 (*lane 2*), 0.1 (*lane 3*), 1 (*lane 4*), or 10 μg/ml (*lane 5*). Total protein of the cells prior to the RNA synthesis reaction was analyzed by Western blotting using anti-NS4B (*NS4B*), anti-NS5A (*NS5A*), anti-NS5B (*NS5B*), anti-Calnexin (*Calnexin-NT*), and anti-KDEL (*BiP/Grp78*) antibodies. Newly synthesized replicon RNA that was labeled with [^{32}P]UTP was indicated by the *arrowhead* with *replicon*, *upper panel*. *B*, a small part of each NS protein was located in the proteinase-resistant environment in the replicon cells. After permeabilization of the replicon cells with digitonin, fractions of the cells were treated with 0 (*lane 1*), 1 (*lane 2*), 5 (*lane 3*), 10 (*lane 4*), 50 (*lane 5*), and 100 μg/ml (*lane 6*) proteinase K. After the reaction, total protein was analyzed by Western blotting using anti-NS3 (*NS3*), anti-NS4A (*NS4A*), anti-NS4B (*NS4B*), anti-NS5A (*NS5A*), anti-NS5B (*NS5B*), anti-Calnexin (*Calenxin-NT*), and anti-KDEL (*BiP/Grp78*) antibodies.

sis of HCV RNA in the cells. This implied that a small part of each NS protein resistant to proteinase should be present in a similar situation to the active replication complexes in the cells. To clarify the existence of such proteins, we examined carefully the proteinase resistance of each NS protein in the permeabilized replicon cells. After treatment of the permeabilized replicon cells with proteinase K at various concentrations, total protein in the cells was analyzed by Western blotting. Under the conditions that the COOH terminus of Calnexin was efficiently digested by treatment as shown in Figs. 4 and 5*A*, we found that a small part of each NS protein was resistant to the treatment with proteinase K at even higher concentrations (Fig. 5*B*, *lanes 1–6*). These results strongly suggested that such proteinase-resistant NS proteins formed the functional replication complex in the cells. Furthermore, we did not detect any activity for the synthesis of the replicon RNA in the permeabilized replicon cells after treatment with nonionic detergents (data not shown). Summing up the above results, it was suggested that pre-existing replicon RNA and a small portion of each NS protein, which were resistant to nuclease and proteinase, respectively, form the replication complexes in lipid membrane structures to participate in the synthesis of replicon RNA in replicon cells.

DISCUSSION

By monitoring the synthesis of HCV RNA in permeabilized HCV replicon cells, we obtained results suggesting that the active HCV replication complexes function to synthesize the replicon RNA in subcellular compartments, which are probably formed by cellular lipid membranes. Recently, electron microscopic analysis showed that the expression of HCV proteins in Huh-7 cells induced the formation of a distinct membrane structure, designated a "membranous web," and all HCV proteins were found in the structure (40). Moreover, a similar web-like structure in livers of HCV-infected chimpanzees and HCV subgenomic replicon cells was reported (41, 42). Therefore, it seems likely that this membrane structure is a candidate for the site where the HCV RNA genome is mainly located and replicated. Until now the genomes of many positive-strand RNA viruses, such as brome mosaic virus, murine hepatitis virus, and Kunjin virus have been reported to replicate on inner cellular membranes in association with vesicles or other membrane structures (43–45). These membrane structures seemed to be constructed by viral proteins. For example, it was reported that brome mosaic virus 1a, the multifunctional RNA replication protein, selectively recruits brome mosaic virus 2a polymerase and viral RNA and forms membrane-bound spherules (43, 46). A subcellular site for the genome replication of these viruses including HCV has been suggested by the localization of brome uridine-incorporated genomic RNA, which is a

product of the replication reaction, in the cells (43, 47–49). It has not been biochemically analyzed, however, whether viral RNA is actually synthesized in or around such a membrane structure. In this paper, we showed that a quite similar activity for the synthesis of HCV subgenomic RNA was present in the permeabilized replicon cells even after proteinase treatment, which digested almost all the NS proteins including NS5B, to that in the cells not treated with proteinase. Furthermore, we found that a small part of each NS protein is actually present in the proteinase-resistant environment in the replicon cells. This suggested, therefore, that a small part of each NS protein forms the replication complex and functions in the membranous compartment.

A fairly large amount of each HCV protein accumulated in the perinuclear fraction in the replicon cells, which is exposed to the cytoplasmic environment. In this protein complex, no association of the HCV subgenomic RNA was observed and there was no activity to synthesize the viral RNA. Thus, the significance of the HCV protein complex regarding multiplication of the virus genome remains to be clarified. The replicon cells originating from Huh-7 may produce large numbers of HCV proteins in the perinuclear fraction as a consequence of overproduction and these proteins may play less important roles in the replication of the HCV genome than the active replication complex that we have noted in this paper. Conversely, the protein complex may play important roles in the regulation of not only the multiplication of the HCV genome but also further processes during the maturation of the virus. In this regard, it is important to analyze the presence as well as the function of the HCV protein complexes in cells where HCV proliferates with different degrees of multiplication. To date, several cellular proteins that interact with particular NS proteins have been reported. For example, double strand RNA-dependent protein kinase, soluble NSF attachment protein receptors-like protein, and karyopherin β3 are reported to interact with NS5A (50–52), although the physiological importance of these interactions has been obscure. This larger part of each NS protein might, therefore, participate in several cellular events and/or modulate the replication of the HCV genome through interactions with these cellular proteins.

The ratio of plus to minus strand RNA synthesized in the permeabilized replicon cells was estimated to be 2.7 ± 0.4 by slot blot hybridization analysis as shown in Fig. 2. On the other hand, that in the intact replicon cells was estimated to be 11.9 ± 2.0 as reported previously (data not shown and Ref. 15), when intact replicon cells were metabolically labeled with [^{32}P]orthophosphate and the newly synthesized and radiolabeled replicon RNA in the cells was analyzed by slot blot hybridization in a similar manner. The discrepancy in the ratio between the permeabilized and intact cells may be explained by the release of the replicon RNA synthesized in the permeabilized cells from the membranous compartment and degradated in the reaction mixture, although the possibility that the regulation of the plus to minus ratio of newly synthesized replicon RNA may be lost in the permeabilized replicon cannot be completely ruled out. Approximately 50% of the replicon RNA newly synthesized in the intact cells was actually lost by nuclease treatment following permeabilization (Fig. 3*B*, *lanes 1* and *3*), suggesting that some parts of replicon RNA newly synthesized in intact replicon cells is present in the cytoplasm. Slight degradation of the replicon RNA was also seen in permeabilized cells in the presence of Nonidet P-40 (Fig. 3). We also observed that *in vitro* synthesized replicon RNA added exogenously to the permeabilized cells was unstable in the reaction mixture (data not shown), as was observed in a recent report in which the replication activity of the HCV replicon was detected using the cell lysate fraction of replicon cells (30). These observations seem to support the former possibility indirectly. As the radiolabeled nucleotide substrate was incorporated in the newly synthesized replicon RNA in the permeabilized replicon cells, a channel-like structure should be present in the membranous compartment including the replication complex. In the case of spherules of brome mosaic virus, the channel-like structure connecting to the cytoplasm with the inside of the spherule was actually detected by the electron microscopic technique (43). This supported the idea that the replicon RNA that was present in the cytoplasm was probably exported from the membranous structure through the channel-like structure. A similar phenomenon was already reported for reovirus in that relatively large reoviral mRNA was exported from the viral core particles to the cytoplasm through the channel formed by a viral protein (53). The pore size of the putative channel of HCV, however, seemed to be limited, because nuclease and proteinase did not pass through the channel. The replicon RNA may be specifically recognized by some viral proteins and exported through the channel post- or co-replicationally. These mechanisms, however, have remained to be elucidated.

During the preparation of this article, Shi *et al.* (54) reported that almost all of the NS5A and part of the NS5B proteins were present in the membrane fraction that was resistant to treatment with 1% Nonidet P-40. Because the replicational activity in that fraction from the cell lysate was not demonstrated, we do not know whether that kind of membrane fraction from the cell lysate would include the replication activity observed here. Furthermore, a part of replicon RNA was reported to localize in the non-ionic detergent-insoluble membrane fractions (54), whereas we showed that the RNase resistance of both total and newly synthesized replicon RNA was reduced in the presence of Nonidet P-40. This discrepancy may be explained by the different methods used for the detection of the replicon RNA, reverse transcriptase-PCR and Northern blotting in that paper and ours, respectively. We also showed data indicating that a negligible amount of replicon RNA was released from the permeabilized cells into the reaction mixture by the detergent as shown in Fig. 3. It may also be relevant to this discrepancy that we and others have observed instability of the free replicon RNA in the reaction mixture (30). We also observed the existence of nuclease-resistant replicon RNA, even in the presence of nonionic detergent (Fig. 3). Such RNA may represent the replicon RNA in the non-ionic detergent-insoluble fractions.

Here we showed the stable nature of the active HCV RNA replication complex in the replicon cells by permeabilization of the cells. Our data suggests that only a small part of each NS protein contributes to RNA synthesis in the replicon cells. This implied that careful investigation would be required for identification of the precise subcellular sites of replication in the replicon cells. Further investigation to reveal how and where this complex is formed in the cells and what is essential for its activity is required for understanding the mechanism of viral replication and the life cycle of HCV.

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Transforming Growth Factor-β-mediated Signaling via the p38 MAP Kinase Pathway Activates Smad-dependent Transcription through SUMO-1 Modification of Smad4*

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Takayuki Ohshima‡ and Kunitada Shimotohno

From the Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

Post-translational modifications such as ubiquitination, phosphorylation, and acetylation play important roles in the regulation of Smad-mediated functions. Here, we demonstrate that Smad4 is covalently modified by SUMO-1, which was characterized recently as a key modulator of many transcription factors. Sumoylation of Smad4 mainly occurs at lysine 159, located in the linker region, and facilitates Smad-dependent transcriptional activation. Furthermore, we show that the PIAS family proteins, PIAS1 and PIASxβ, function as E3 ligase factors for Smad4. Intriguingly, sumoylation of Smad4 was strongly enhanced by TGF-β-induced activation of the p38 MAP kinase pathway but not the Smad pathway. Activation of p38 not only stabilized PIASxβ protein but also enhanced PIASxβ gene expression, suggesting that PIAS-mediated sumoylation of Smad4 is regulated by the p38 MAP kinase pathway. These findings illustrate a novel regulatory mechanism by which Smad-dependent transcriptional activation cooperatively modulates Smad proteins through receptor-mediated phosphorylation and sumoylation.

Members of the transforming growth factor-β (TGF-β)[1] superfamily regulate diverse biological functions including cell differentiation, growth inhibition, migration, survival, and apoptosis (1–3). The cellular effects of TGF-β are mediated by binding type I and type II serine/threonine kinase receptors. Upon ligand binding and activation, the type II receptor kinase phosphorylates the type I receptor kinase. The activated type I receptor then phosphorylates receptor-regulated Smads (R-Smad), Smad2 and Smad3, in the TGF-β/activin pathways. Smads, the central molecules in TGF-β signaling, act as transcription factors or coactivators for regulating target gene expression (4). Receptor-mediated phosphorylation of R-Smad additionally creates a complex with the common-mediator Smad (Co-Smad; Smad4). These complexes translocate and accumulate in the nucleus where they are directly involved in the transcriptional regulation of various target genes (5, 6).

TGF-β also activates members of the mitogen-activated protein (MAP) kinase family, including TGF-β-activated kinase, c-Jun N-terminal kinase, and extracellular signal-regulated kinase (7–10). TGF-β-activated kinase then activates the stress-activated kinase p38, which plays an important role in regulating cellular processes such as inflammation, cell differentiation, and apoptosis (11–14). A recent study (15) using a mutant type I receptor, which was incapable of activating the Smad pathway but still retained signaling via the MAP kinase pathway, showed that TGF-β receptor-activated p38 is involved in TGF-β-induced apoptosis but not growth arrest in mouse mammary gland epithelial (NmuMG) cells (15). Thus, the p38 pathway seems to be sufficient to induce apoptosis in these cells, whereas a fully epithelial-to-mesenchymal transition response requires additional TGF-β signaling and possibly requires Smad-mediated transcriptional activation (16). These observations suggest that diverse biological responses regulated by TGF-β are mediated by different downstream signaling pathways, dependent on either Smad or MAP kinase or both.

Post-translational modifications regulate the function of many proteins. In the case of Smads, hetero-oligomeric formation by Smads is dependent on phosphorylation of R-Smads. Furthermore, recent findings have underscored critical functions of ubiquitination and acetylation in the control of Smad-dependent gene regulation. Ubiquitination plays a role in regulating Smad function as Smads are rapidly targeted for degradation by the proteasome (17, 18). The ultimate degradation of Smads after ligand stimulation has been firmly established as a mechanism to terminate Smad signaling. However, acetylation of Smad7 by coactivator p300, one of the inhibitory Smads (I-Smad), occurs on two lysine residues that are also targeted by ubiquitination and thereby prevents ubiquitin-mediated degradation (19).

Recently, a number of ubiquitin-like proteins (Ubl) have been found, which become covalently linked to lysine residues in target proteins (20, 21). One Ubl, SUMO-1, also known as PIC1, UBL1, sentrin, GMP1, and SMT3, is an 11-kDa protein that is structurally homologous to ubiquitin and is highly conserved from yeast to humans (22–24). Several proteins modified by SUMO-1 have been identified such as RanGAP1 (25), PML (26–28), IκB (29), p53 (30, 31), LEF-1 (32), and viral proteins. SUMO-1 modification of protein plays an important role in modulation of transcriptional activity, mediation of nuclear import, recruitment of transcriptional regulators in nu-

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‡ To whom correspondence should be addressed. Tel.: 81-75-751-3997; Fax: 81-75-751-3998; E-mail: tohshima@virus.kyoto-u.ac.jp.

[1] The abbreviations used are: TGF-β, transforming growth factor-β; GFP, green fluorescent protein; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; HA, hemagglutinin; RT, reverse transcriptase; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; BMP, bone morphogenetic protein; IFN, interferon; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SUMO, small ubiquitin-like modifier; PIAS, protein inhibitors of activated STAT.

clear domains, protection from ubiquitination, and regulation of mitosis (33, 34). Furthermore, E3 ligases for SUMO-1 conjugation have been reported recently (35–37). These ligases can be classified into two types based on their structural relationships. One type of E3 ligases, the PIAS (protein inhibitor of activated signal transducers and activators of transcription) family, which is homologous to the yeast Siz family, have a conserved RING finger domain that regulates transactivation by conjugating SUMO-1 in molecules including p53 (30, 31), LEF-1 (32), and SP3 (38, 39). Another type of E3 ligase, structurally unrelated to the PIAS/Siz family, is the nucleoporin RanBP2, which sumoylates SP100 (40) and HDAC4 (41) and regulates nuclear translocation.

To understand the molecular mechanisms of Smad transcriptional function, we explored the possible modification of Smads by SUMO-1 through post-translational modifications and attempted to define the biological significance of cross-talk between Smad activation and TGF-β signaling. Here, we report that Smad4 is a target for SUMO-1 modification both *in vivo* and *in vitro*. Additionally, we identified PIAS proteins as E3 ligases for sumoylation, which activate Smad4-dependent transcriptional function. Furthermore, the TGF-β-mediated p38 MAP kinase pathway regulates PIAS gene expression and protein stabilization, which thereby enhances Smad4 sumoylation.

MATERIALS AND METHODS

Cell Culture, Transfections, and Luciferase Reporter Assay—Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Transfections were performed using FuGENE 6 (Roche Diagnostics Corp.), according to the manufacturer's instructions. Luciferase activities were normalized to Renilla luciferase activities derived from cotransfected pRL-CMV-Luc (Promega). All reporter assays were performed in triplicate, and standard errors (S.E.) are denoted by the bars in figures.

Antibodies and Reagents—Rat anti-HA (3F10; Roche Applied Science), mouse anti-Myc (9E10; Santa Cruz Biotechnology, Inc.), mouse anti-FLAG (M2; Sigma), mouse anti-GFP (Clontech), mouse anti-α-tubulin (Ab-1; Oncogene), rabbit anti-phospho-Smad2, rabbit anti-TGF-β receptor I, rabbit anti-p38, rabbit anti-phospho-p38, and rabbit anti-phospho-Elk-1 (Cell Signaling Technology) antibodies were purchased commercially. Horseradish peroxidase-linked goat antibodies to rat IgG were from Jackson ImmunoResearch Laboratory. Horseradish peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences. TGF-β, BMP-4, and SB203580 were from Calbiochem.

Plasmid Construction—pcDNA3 (Invitrogen)-based plasmids expressing FLAG-tagged human Smad2, Smad4, HA-tagged constitutively active TGF-β-type I receptor (TβR-I(T/D)), and p3TP-Luc have been described elsewhere (42). An expression plasmid of Myc-tagged Smad2 was generated by inserting the Smad2 gene into pcDNA3-Myc. cDNAs encoding human SUMO-1, human UBC9, mouse PIASxβ, and mouse PIAS1 were isolated by RT-PCR with total RNA from HeLa cells or mouse testis. Primer sequences used were as follows: human SUMO-1, 5′-AAAGGATCCCCATGTCTGACCAGGAGGCAAAACC-3′ (forward) and 5′-AAAGGATCCCTAAACTGTTGAATGACCCCCCG-3′ (reverse); human UBC9, 5′-AAAGGATCCCCATGTCGGGGATCGCCCTCAGCAG-3′ (forward) and 5′-AAAGGATCCTTATGAGGGCGCAAACTTCTTGG-3′ (reverse); mouse PIASxβ, 5′-AAAGAATTCATGGTTTCTAGTTTTAGGGTTTCTG-3′ (forward) and 5′-AAAGAATTCTCACTGTTGCACAGTATCAGAAGAT-3′ (reverse); mouse PIAS1, 5′-AAAGAATTCATGGCGGACAGTGCGGAACTAAAG-3′ (forward) and 5′-AAACTCGAGTCAGTCCAATGAGATAATGTCTGG-3′ (reverse). Bold letters in the primers denote restriction sites. The PCR products were digested with restriction enzymes and subcloned into epitope-tagged pcDNA3, pGEX-6P-1 (Amersham Biosciences), or pEGFP-C2 (Clontech) to generate pcDNA3-FLAG-PIASxβ, pcDNA3-HA-SUMO-1, pcDNA3-Myc-Ubc9, pGEX-6P-1-Ubc9, -PIASxβ, -pEGFP-PIASxβ, and -PIAS1. The cDNAs for mutant Smad4 with substitution of Lys-159 to Arg, Smad4(K/R), and the PIASxβ RING mutant with substitution of Cys-353 to Ser, PIASxβ(C/S), were created using site-directed mutagenesis and subcloned into expression vectors to obtain pcDNA3-FLAG-Smad4(K/R) and pcDNA3-FLAG-PIASxβ(C/S), or pEGFP-PIASxβ(C/S) and pGEX-6P-1-PIASxβ(C/S). Expression plasmids of Smad4-Gal4 DNA binding fusion proteins were generated by inserting these genes into pM (Clontech). MKK6(DE), a constitutively active form of MKK6, and p38-HA expression plasmids have been described elsewhere (43). The combined mutant type I receptor, TβR-ImL45(T/D), which has a constitutively active kinase domain but lacks the ability to phosphorylate Smads, was generated by PCR-based mutagenesis as described previously (15). For *in vitro* sumoylation assays, expression plasmids expressing GST-Myc-Smad4 fusion proteins were generated by inserting these genes into pGEX-6P-1-Myc. The pGEX-6P-1-SUMO(GG), which expresses a form of SUMO-1 with the four carboxyl-terminal amino acids (HSTV) deleted to facilitate conjugation, was generated by using a PCR-based approach as described previously (44). pGEX-6P-1-GFP-SUMO(GG) was constructed by inserting the PCR product of a GFP-encoding fragment from pEGFP-C2 into pGEX-6P-1-SUMO(GG). Recombinant baculoviruses expressing GST-Sua1 and His-Uba2 fusion proteins have been described elsewhere (36).

Protein Purification and in Vitro Sumoylation Analysis—GST and GST fusion proteins were expressed in the *Escherichia coli* strain BL21 (DE3) and affinity purified with glutathione *S*-Sepharose beads according to the manufacturer's instructions (Amersham Biosciences). Expression and purification of GST-Sua1 and His-Uba2 in the baculovirus system were performed as described previously (44). For *in vitro* sumoylation assays, GST-fused Smad protein substrates were incubated with E1 (GST-Sua1/His-Uba2) and E2 (GST-Ubc9) enzymes and recombinant SUMO(GG) in reaction buffer consisting of 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 3 mM $MgCl_2$, and 5 mM ATP at 30 °C for 30 min. For Western blot analysis, proteins were fractionated by SDS-PAGE and electroblotted onto an Immobilon-P membrane (Millipore). The blot was incubated sequentially with mouse anti-Myc antibody and horseradish peroxidase-conjugated anti-mouse IgG and detected using enhanced chemiluminescence (ECL; Amersham Biosciences).

Immunoprecipitations—COS7 cells (1×10^5 per 6-cm-diameter dish) were transfected using FuGENE 6 according to the manufacturer's instructions. After incubation, cells were lysed in 1 ml of RIPA buffer (25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 5 mM EDTA, 10 mM *N*-ethylmaleimide, 200 μM indole-3-acetic acid, and a complete protease inhibitor mixture tablet (Roche Applied Science)) for 30 min on ice. Cell debris was removed by centrifugation for 15 min. Lysates were first cleared with protein G beads for 30 min, followed by incubation with antibodies for 1 h at 4 °C. Finally, the antibody complexes were captured with protein G beads for 1 h. Beads were washed four times with the same buffer, and immunoprecipitates were eluted and analyzed by Western blot.

Semiquantitative RT-PCR Analysis—Total RNA was extracted using ISOGEN (Nippon Gene) according to the manufacturer's instructions. First-strand cDNA was synthesized with Superscript II (Invitrogen) using 2.5 μg of total RNA and an oligo(dT) primer in a 20-μl reaction volume. PCR was performed using 1 μl of the reaction mixture for cDNA synthesis in a 10-μl reaction volume of PCR buffer containing 200 μM of each dNTP, 2 μM of each set of primer, and 0.2 units of AmpliTaq at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The sequences of the primers used are mouse PIASxβ, 5′-AGAGAGCTCTTCTGATGAAGAGG-3′ (forward) and 5′-TCTCACTCCTGCTGCTGGATGAAC-3′ (reverse); SP6, 5′-ATTTAGGTGACACTATAGAATAG-3′ (reverse); human GAPDH, 5′-ATG GGGAAGGTGAAGGTCGG-3′ (forward) and 5′-TGGAGGGATCTCGCTCCTGG-3′ (reverse). An aliquot of PCR products was electrophoresed on 2% agarose gels containing ethidium bromide and quantitated by UV-illumination.

Pulse-Chase Analysis—COS7 cells were plated to 50% confluency in 6-well dishes, 1 day before transfection. Approximately 24–30 h post-transfection, cells were cultured with methionine/cysteine-deficient Dulbecco's modified Eagle's medium (ICN) for 30 min and then metabolically labeled with 0.1 mCi of [^{35}S]methionine/cysteine (ICN) in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum for 1 h. After washing with phosphate-buffered saline, cells were chased for the indicated time intervals in complete medium. Cells were then lysed in RIPA buffer as described above, and soluble extracts were subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography, and the relative intensity of each band was quantitated by scanning densitometry.

RESULTS

SUMO-1 Modification of Smad4 at Lys-159 in Vivo and in Vitro—SUMO-1 modification of certain transcriptional regulators is stimulated by extracellular environmental insults in-

cluding cytokines, hormones, heat shock, and irradiation (31, 45–47), and this modification regulates transcriptional function. We investigated whether the SUMO-1 modification system acts on the TGF-β signaling pathway. We first examined whether the transcription factors Smad2, Smad3, and Smad4, which are elements of the TGF-β-dependent pathway, are modified by SUMO-1 in TGF-β-treated NIH3T3 cells transiently expressing FLAG-Smads and HA-SUMO-1. Western blot analysis using anti-FLAG antibody revealed production of every Smad form in the cell lysates. However, in cells producing ectopic Smad4, an additional more slowly migrating band was observed when cells coexpressed HA-SUMO-1 (Fig. 1*A*, *lane 6*). This more slowly migrating band was not detected in cells producing ectopic Smad2 and Smad3 (*lanes 1–4*), suggesting that Smad4 may be specifically targeted for SUMO-1 modification.

To further examine whether Smad4 is indeed a substrate for SUMO-1 modification, we performed an *in vitro* sumoylation assay using recombinant Smad4 as a substrate. Smad4 has a consensus sumoylation sequence, VKDE, which is conserved among other species (Fig. 1*B*). We speculated that the Lys-159 in human Smad4 is a likely target for sumoylation. To assess this hypothesis, mutant Smad4, in which lysine 159 was converted to Arg, and wild-type Smad4 were prepared as GST-Myc-Smad4(K/R) and GST-Myc-Smad4 fusion proteins, respectively. These Smad4 proteins were incubated with various combinations of recombinant E1 and E2, together with GST-SUMO(GG) or GFP-SUMO(GG), as shown in Fig. 1*B*. Analysis of the reaction products by Western blot with anti-Myc antibody revealed a more slowly migrating Smad4 band only when E1 and E2, as well as GST-SUMO(GG), were present. However, the reaction containing Smad4(K/R) did not give rise to the slower migrating form (*lane 5*), indicating that Lys-159 is a target for modification. To confirm that this slower migrating band was indeed sumoylated Smad4, GFP-SUMO(GG) was substituted for GST-SUMO(GG) in the reaction. As expected, a slightly higher molecular weight form of Smad4 was detected, reflecting the difference in molecular weight of GFP-SUMO(GG) and GST-SUMO(GG) (*lanes 6* and *7*). These data strongly suggest that Smad4 is a substrate for SUMO-1 and that Lys-159 is a major SUMO-1 conjugation site.

To evaluate the state of Smad4 sumoylation in response to TGF-β signaling, expression plasmids containing FLAG-Smad4, FLAG-Smad4(K/R), Myc-Smad2, HA-SUMO-1, and the constitutively active form of the TGF-β type-I receptor, TβR-I(T/D), were cotransfected in various combinations into COS7 cells, as shown in Fig. 1*C*. Western blotting with anti-FLAG antibody revealed the presence of Smad4 in all cells transfected with plasmid expressing FLAG-Smad4 (Fig. 1*C*, *top panel*). When SUMO-1 was also expressed, a higher molecular weight form of Smad4 was detected, along with a substantial amount of unmodified Smad4 (Fig. 1*C*, *top panel*, *lanes 4–7*, *white arrowhead*). This form was not present in cells expressing Smad4(K/R) (*lanes 8* and *9*). Moreover, to determine whether this higher molecular weight form representing Smad4 was conjugated to SUMO-1, cell extracts were subjected to immunoprecipitation with anti-FLAG antibody and analyzed by Western blot using either anti-FLAG or anti-HA antibodies. The slower migrating form of Smad4 (about 90 kDa) was detected when SUMO-1 was coproduced (Fig. 1*C*, *second panel* from *top*, *lanes 4–7*, *white arrowhead*). The filter was then reprobed with anti-HA antibody, demonstrating that the slower migrating form of Smad4 (about 90 kDa) was indeed the sumoylated form (Fig. 1*C*, *third panel* from *top*, *white arrowhead*). Several SUMO-1-reactive bands migrating slower than SUMO-1-conjugated Smad4 were observed in cells producing Smad4 but not in cells producing Smad4(K/R) (Fig. 1*C*, *third panel* from *top*, *lanes 4–7*). These bands were not likely to be sumoylated forms of Smad4,because the anti-FLAG antibody did not recognize them (Fig. 1*C*, *second panel* from *top*). Intriguingly, the amount of sumoylated Smad4 was increased when a TβR-I(T/D) expression plasmid was cotransfected, and this was not changed by expressing ectopic Smad2 (Fig. 1*C*, *top three panels*, *lanes 5* and *7*), suggesting that TGF-β signaling plays a role in controlling Smad4 sumoylation, possibly through a pathway independent of TGF-β-mediated Smad activation.

Smad4 is a common mediator for members of the TGF-β superfamily, including TGF-β, activins, and BMPs (3). To investigate whether SUMO-1 modification of Smad4 has any effect on the TGF-β superfamily-mediated Smad pathway, we examined the effects of wild-type and mutant Smad4(K/R) on expression of a p3TP-Luc reporter gene, which contains a Smad-responsive element in its promoter. HepG2 cells were cotransfected with the reporter plasmid, together with wild-type or Smad4(K/R) expression plasmids, and then treated with TGF-β or BMP-4. Reporter activity was enhanced by treatment with TGF-β or BMP-4, even in cells not expressing ectopic Smad4 (Fig. 1*D*). Cells ectopically expressing wild-type Smad4 showed higher reporter gene activity than those expressing Smad4(K/R). This suggests that SUMO-1 modification of Smad4 plays a role in activation of the transcriptional response by the TGF-β superfamily.

PIAS Family Proteins Act as E3 Ligases for Smad4 Sumoylation—Recently, two distinct SUMO-1 E3 ligases, PIAS family proteins and RanBP2, have been identified (35–37). PIAS family proteins act as either positive or negative regulators for many transcriptional factors or cofactors (48). Therefore, we investigated whether PIAS family proteins function as E3 ligases for Smad4. We first examined the effect of PIASxβ and PIAS1 expression on SUMO-1 modification of Smad4 in COS7 cells. Sumoylation of Smad4 was enhanced by both PIASxβ and PIAS1 (Fig. 2*A*, *lanes 3* and *4*), indicating that these PIAS proteins targeted Smad4 for SUMO-1 modification. PIAS family proteins also sumoylate p53 (49), androgen receptor (50), and cytomegalovirus IE2 protein (51). Based on the data here, Smad4 is also likely to be a target for PIAS family proteins.

To further clarify the importance of PIAS protein enzymatic activity on Smad4 sumoylation, we generated mutant PIASxβ, PIASxβ(C/S), in which the conserved cysteine residue at position 353 within the RING finger domain has been changed to serine. This mutant completely lacks E3 ligase activity. Either mutant or wild-type PIASxβ were produced in cells, and Smad4 sumoylation was analyzed. Small amounts of Smad4 were covalently conjugated upon expression of SUMO-1, even in the absence of ectopic PIASxβ expression (Fig. 2*B*, *lane 5*, *top panel*), but Smad4 sumoylation was enhanced by ectopic expression of PIASxβ (*lane 6*). Sumoylated Smad4 was scarcely detected when SUMO-1 was not ectopically produced, suggesting a limiting amount of free endogenous SUMO-1 in COS7 cells. Immunoprecipitation with anti-FLAG antibody followed by Western blotting with the same antibody revealed the slower migrating form of Smad4 (Fig. 2*B*, *lane 6*, *second panel* from *top*). Reprobing this filter with anti-HA antibody, after stripping the anti-FLAG antibody, showed slower migrating bands, one of which coincided with the band observed in the second panel and thus was likely to be SUMO-1-conjugated Smad4. We also observed a band migrating even more slowly than sumoylated Smad4 (Fig. 2*B*, *white arrowhead*, *third panel* from *top*). Because this band was not detected with anti-FLAG antibody, we believe this represents sumoylated cellular protein(s) that coimmunoprecipitated with wild-type and/or

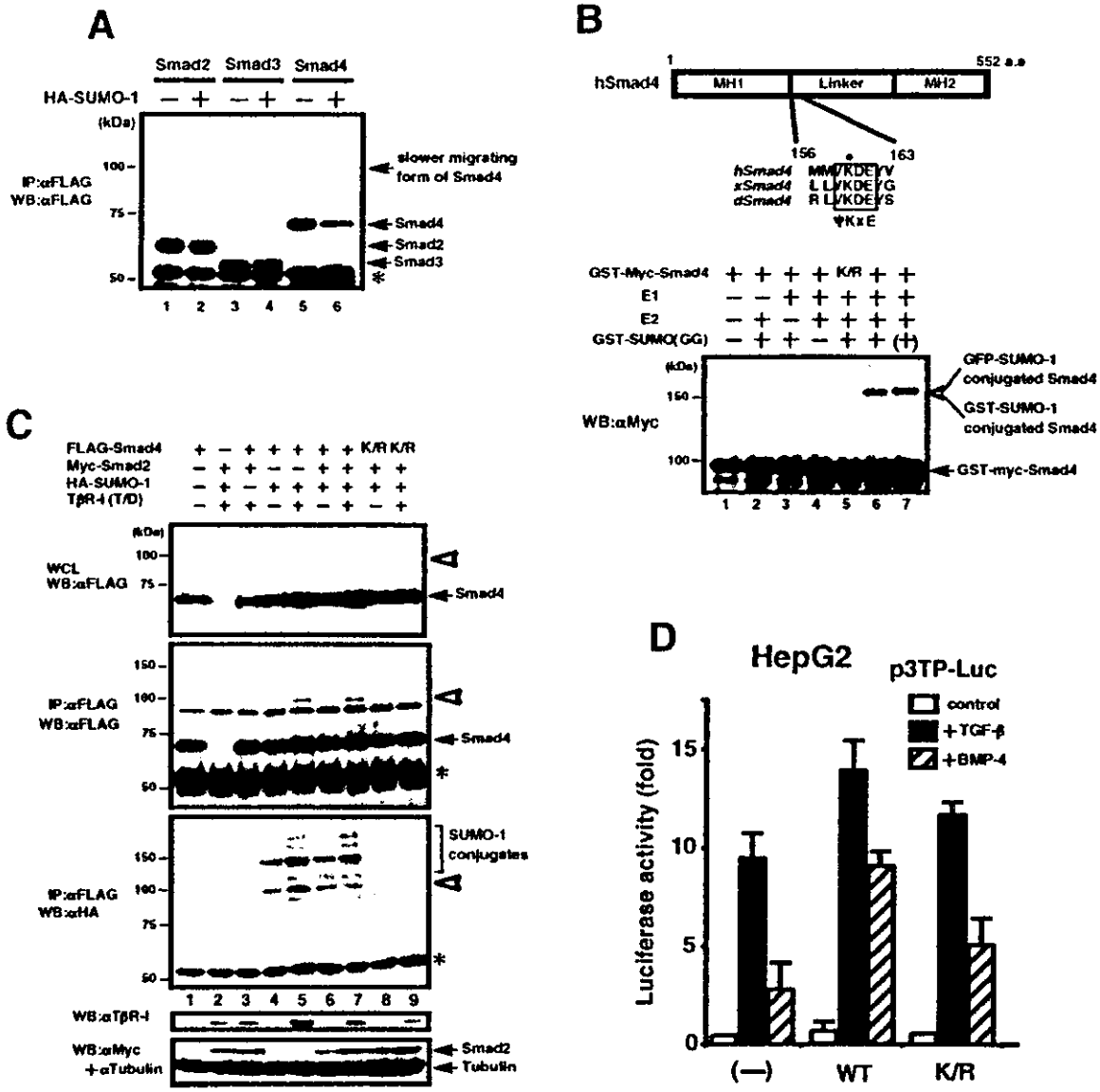

FIG. 1. **Sumoylation of Smad4 *in vitro* and *in vivo*.** *A*, sumoylation of Smad4, but not Smad2 and Smad3, *in vivo*. NIH3T3 cells were transfected with plasmids expressing FLAG-Smad2, -Smad3, or -Smad4 with (+) or without (−) HA-SUMO-1. Twelve h after transfection, cells were treated with 2 ng/ml of TGF-β for 24 h, and cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody. The immunoprecipitates were subjected to 7.5% SDS-PAGE and analyzed by Western blotting (*WB*) with anti-FLAG antibody. The *asterisk* indicates the immunoglobulin heavy chain. *B*, SUMO-1 is conjugated at lysine 159 of Smad4 *in vitro*. *Upper panel*, schematic representation of human Smad4. The MAD homology domain 1 (*MH1*), Linker domain (*Linker*), and MAD homology domain 2 (*MH2*) are shown. The lysine residue in the putative SUMO-1 acceptor site is indicated by a *black dot* within the consensus sumoylation sequence, ψ*KXE* (where ψ is a large hydrophobic amino acid, K is the SUMO-1 modified lysine residue, *X* is any amino acid, and E is glutamic acid), which is conserved in human (*hSmad4*), *Xenopus* (*xSmad4*), and *Drosophila* Smad4 (*dSmad4*). *Lower panel*, sumoylation of wild-type Smad4 but not mutant Smad4(K/R) *in vitro*. Fifty ng of recombinant GST-Myc-wild-type-Smad4 and GST-Myc-Smad4(K/R) were incubated in the presence (+) or absence (−) of 200 ng of recombinant E1 (GST-Sua1/His-Uba2 heterodimer), 50 ng of E2 (GST-Ubc9), 100 ng of GST-SUMO(GG) (or GFP-SUMO(GG); *lane 7*) in 10 μl of reaction buffer containing 5 mM ATP at 30 °C for 30 min. Proteins were fractionated by SDS-PAGE and analyzed by Western blotting with anti-Myc antibody. *C*, sumoylation of Smad4 by TGF-β signaling. COS7 cells were cotransfected with (+) or without (−) 2 μg each of plasmids expressing FLAG-Smad4 (or FLAG-Smad4K/R in *lanes 8* and *9*), Myc-Smad2, HA-SUMO-1, and TβR-I(*T/D*). Equivalent amounts of whole cell lysates (*WCL*) were prepared 36 h post-transfection. An aliquot of the lysates was fractionated by SDS-PAGE followed by Western blotting using anti-FLAG antibody (*top panel*). Another aliquot of lysate was immunoprecipitated (*IP*) with anti-FLAG antibody. The immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting with anti-FLAG antibody (*second panel*). After ECL development, the filter shown in the *second panel* was stripped and reprobed with anti-HA antibody (*third panel*). The levels of proteins expressed in whole cell lysates were analyzed and shown as indicated. *Asterisks* indicate the immunoglobulin heavy chain. Sumoylated forms of Smad4 are indicated with *white arrowheads*. *TβR-I(T/D)* denotes the constitutively active TGF-β-type I receptor. *D*, HepG2 cells were cotransfected with 25 ng of p3TP-Luc, together with 500 ng of plasmid expressing FLAG-Smad4 (*WT*), mutant Smad4(K/R) (*K/R*), or an empty vector (−). Twenty-four h after transfection, TGF-β and BMP-4 were added to culture medium to a final concentration of 2 and 20 ng/ml, respectively. Luciferase activities were then measured 12 h after treatment. *White bars* denote no treatment, *black bars* are TGF-β, and *hatched bars* are BMP-4 treatment. The activity of the reporter plasmid alone in control cells was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this assay. *a.a.*, amino acid(s).

sumoylated Smad4 as described above. In contrast, Smad4 sumoylation was not observed in cells expressing PIASxβ(C/S) (Fig. 2*B*, *lane 7*).

To confirm that PIASxβ functions as an E3 ligase factor for Smad4, we performed *in vitro* sumoylation assays. Recombinant GST-Myc-Smad4 was incubated with limited amounts of

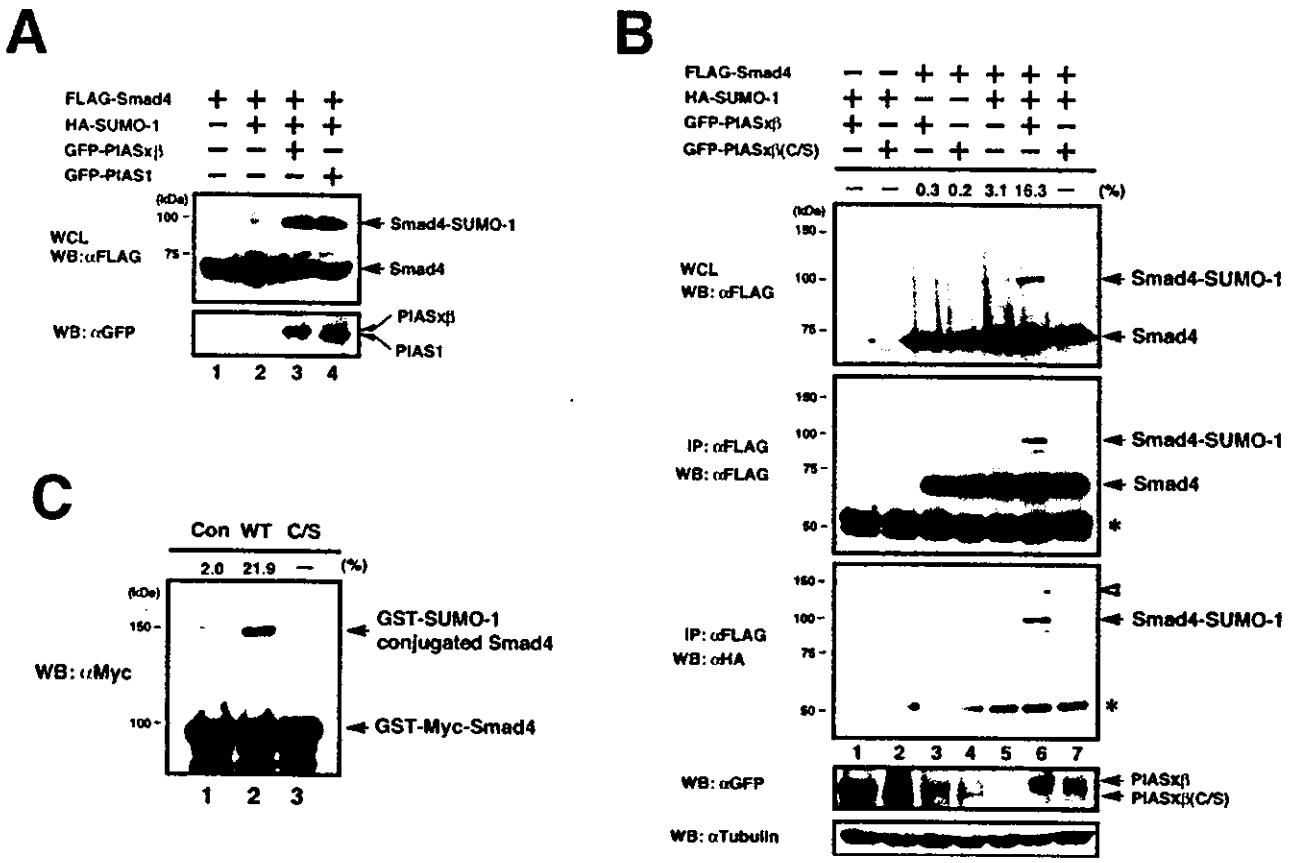

FIG. 2. **PIAS family proteins as candidate E3 ligases for Smad4.** *A*, PIASxβ and PIAS1 promote sumoylation of Smad4 *in vivo*. COS7 cells were cotransfected with (+) or without (−) 2 μg of plasmid expressing FLAG-Smad4, 1 μg of plasmid expressing HA-SUMO-1, and 4 μg of plasmids expressing GFP-PIASxβ or GFP-PIAS1. Whole cell lysates (*WCL*) were prepared 36 h post-transfection and analyzed by Western blotting with anti-FLAG antibody. Levels of PIASxβ and PIAS1 were examined by Western blotting using anti-GFP antibody. *B*, the RING domain mutant of PIASxβ abrogates the function as a SUMO-1 E3 ligase for Smad4. COS7 cells were cotransfected with (+) or without (−) 2 μg each of plasmids expressing FLAG-Smad4, HA-SUMO-1, GFP-PIASxβ, and GFP-PIASxβ(C/S). Equivalent amounts of whole cell lysates (*WCL*) were prepared 36 h post-transfection. An aliquot of the lysates was fractionated by SDS-PAGE followed by Western blotting using anti-FLAG antibody (*top panel*). Another aliquot was immunoprecipitated (*IP*) with anti-FLAG antibody. The immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting with anti-FLAG antibody (*second panel*). After ECL development, the filter shown in the *second panel* was stripped and reprobed with anti-HA antibody (*third panel*). The levels of proteins expressed in whole cell lysates were analyzed and shown as indicated. *PIASxβ(C/S)* indicates the RING domain mutant form of PIASxβ. *Asterisks* indicate the immunoglobulin heavy chain. Sumoylated cellular protein(s) coimmunoprecipitated with Smad4 is indicated by a *white arrowhead*. The relative sumoylated ratio of Smad4 at the *top panel* was indicated as a percent of total Smad4, based on densitometric quantitation. (−) shows below the detection limit. *C*, PIASxβ enhances sumoylation of recombinant Smad4 *in vitro*. Fifty ng of recombinant GST-Myc-Smad4 was incubated with 10 ng of recombinant E1 (GST-Sua1/His-Uba2 heterodimer), 5 ng of E2 (GST-Ubc9), 100 ng of GST-SUMO-1, and 500 ng of control GST (*Con*) or GST-wild-type-PIASxβ (*WT*) or GST-PIASxβ(C/S) (*C/S*), in 20 μl of reaction buffer at 30 °C for 30 min. Proteins were fractionated by SDS-PAGE followed by Western blotting with anti-Myc antibody. The relative sumoylated ratio of Smad4 was indicated as a percent of total Smad4, based on densitometric quantitation. (−) shows below the detection limit.

E1 and E2 (10 ng of E1 and 5 ng of E2 per 20-μl reaction volume) in the presence or absence of bacterially expressed GST-PIASxβ or GST-PIASxβ(C/S) and analyzed by Western blotting using anti-Myc antibody. Small amounts of sumoylated Smad4 were detected in the reaction without PIASxβ addition (Fig. 2*C*, *lane 1*). As expect, addition of GST-PIASxβ (*lane 2*), but not GST-PIASxβ(C/S) (*lane 3*), enhanced Smad4 sumoylation. Taken together, these results clearly indicate that PIAS family proteins, specifically via their RING finger domains, function as E3 ligases for Smad4, both *in vivo* and *in vitro*.

SUMO-1 Conjugation to Smad4 Positively Regulates the Expression of Genes Downstream of TGF-β—We showed previously that wild-type Smad4 has higher transcriptional activity than Smad4(K/R) on a reporter gene that responds to TGF-β and BMP-4 (Fig. 1). Thus, we speculated that SUMO-1 modification of Smad4 enhances Smad-mediated transcription. To investigate the physiological significance of SUMO-1 conjugation of Smad4 on TGF-β signaling, we analyzed the effects of Smad4 sumoylation on TGF-β-responsive transcription. For this purpose, we used cells expressing TβR-I(T/D), a constitutively active form of the TGF-β type I receptor, which mimics TGF-β signaling. COS7 cells were transfected with various combinations of plasmids expressing Smad2, Smad4, Smad4 (K/R), SUMO-1, PIASxβ, and PIASxβ(C/S), together with p3TP-Luc and a plasmid expressing TβR-I(T/D) (Fig. 3*A*). Coproduction of SUMO-1 and Smad2/4 increased the activity by about 1.5-fold relative to cells producing Smad2/4. Additional production of PIASxβ increased the activity about 3-fold relative to cells producing Smad2/4 alone. However, expression of PIASxβ(C/S) did not confer any further activation. In contrast, cells producing Smad2/Smad4(K/R) did not show significant differences in luciferase activity, even when they coproduced SUMO-1 and PIASxβ or PIASxβ(C/S). These results suggest that Smad4 sumoylation by PIASxβ, in a RING finger domain-dependent manner, enhances Smad-dependent signaling. Similar data were observed using pSBE-Luc as a reporter plasmid, which contains four Smad binding elements in its promoter (data not shown).

To verify the SUMO-1 conjugation-dependent transcrip-

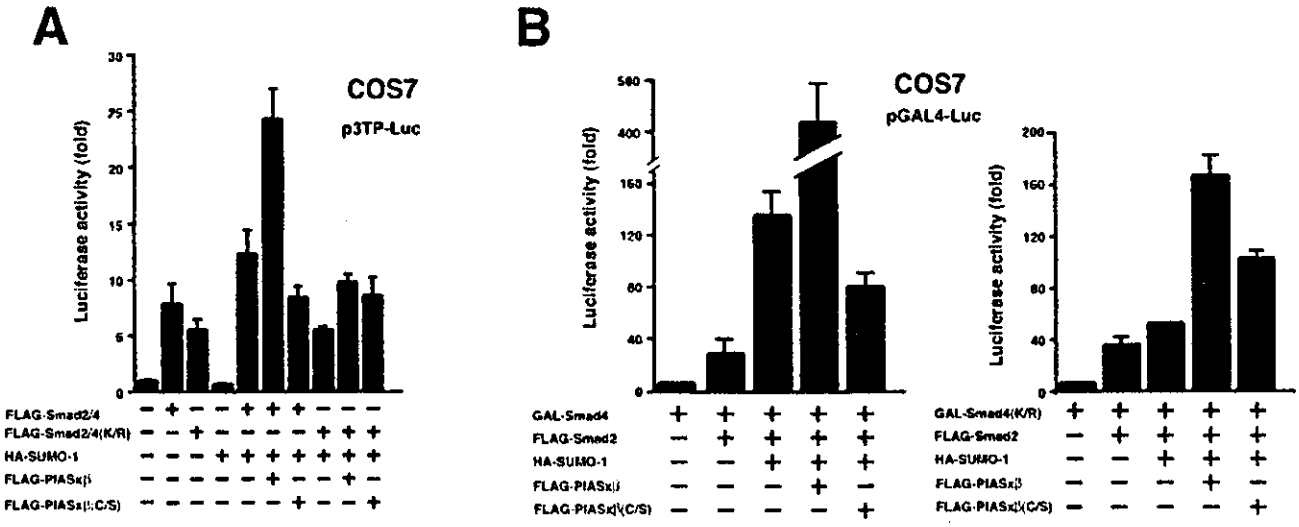

FIG. 3. **Involvement of sumoylation by PIASxβ in Smad-mediated transactivation.** *A*, COS7 cells were cotransfected with 25 ng of p3TP-Luc and 100 ng of plasmid expressing TβR-I(T/D) in the presence (+) or absence (−) of 50 ng of plasmids expressing FLAG-Smad2 plus FLAG-Smad4, or FLAG-Smad2 plus Smad4(K/R), and HA-SUMO-1, and 100 ng of plasmids expressing FLAG-PIASxβ or FLAG-PIASxβ(C/S). Luciferase activities in lysates prepared 24 h post-transfection were measured. The activity of the reporter plasmid alone was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this assay. *B*, enhancement of Smad4-dependent transactivation by PIASxβ requires sumoylation of Smad4. COS7 cells were cotransfected with 25 ng of a GAL4-luciferase reporter plasmid (*pGAL4-Luc*) and 50 ng of plasmids expressing fusion proteins of the GAL4 DNA binding domain to Smad4 (*GAL4-Smad4*; *left*) or (*GAL4-Smad4(K/R)*; *right*) in the presence (+) or absence (−) of 25 ng of FLAG-Smad2, 50 ng of HA-SUMO-1, 100 ng of FLAG-PIASxβ, and FLAG-PIASxβ(C/S). The luciferase activities are shown relative to the GAL4-Smad4 fusions, which are set at 1.

tional activity of Smad4, we utilized GAL4-Smad4 or -Smad4(K/R) to analyze gene expression from pGL2-Luc, which contains five Gal4 binding sites and an SV40 minimal promoter. COS7 cells coexpressing Gal4-Smad4 and Smad2 showed about 30-fold greater luciferase activity than cells expressing Gal4-Smad4 alone. Moreover, luciferase activities were enhanced about 130-fold and more than 400-fold in cells coproducing SUMO-1 and SUMO-1 plus PIASxβ, respectively. However, coproduction of PIASxβ(C/S) suppressed the activity slightly, suggesting that PIASxβ(C/S) has a dominant negative function (Fig. 3*B*, *left*). On the contrary, in cells expressing GAL4-Smad4(K/R), Smad2 and SUMO-1 showed only 50-fold activation relative to cells expressing GAL4-Smad4(K/R) alone, and an additional expression of PIASxβ resulted in 160-fold activation (Fig. 3*B*, *right*). Taken together, these results suggest that the SUMO-1 conjugation system positively regulates TGF-β-mediated transactivation through Smad4 sumoylation. However, mechanisms other than Smad4 sumoylation may also be involved in Smad-dependent transactivation.

Activation of p38 MAP Kinase by TGF-β-Signaling Enhances SUMO-1 Conjugation to Smad4—We observed that Smad4 sumoylation was enhanced by ectopic expression of TβR-I(T/D). To verify the involvement of TGF-β signaling, we carried out experiments to dissect the role of downstream signaling through TGF-β. As in a previous report (15), we generated a combined mutant type I receptor, TβR-ImL45(T/D), which has a constitutively active kinase domain but lacks the ability to phosphorylate Smads. This mutant molecule provides a useful tool for dissecting the molecular mechanisms underlying different TGF-β intracellular signaling pathways. In fact, in contrast to TβR-I(T/D), TβR-ImL45(T/D) was unable to reconstitute Smad-mediated transcription because of defective Smad2 phosphorylation but was able to activate p38 MAP kinase in COS7 cells (Fig. 4*A*, *lanes 2* and *3*), in agreement with a previous report (15). We examined the requirement of downstream elements of the TGF-β signaling pathway in Smad4 sumoylation by using these mutant receptors. Plasmids expressing FLAG-Smad4, HA-SUMO-1, TβR-I(T/D), and TβR-ImL45(T/D) were transfected in various combinations into COS7 cells, and Smad4 sumoylation was examined. Sumoylation was not detectable in cells lacking ectopic SUMO-1 (Fig. 4*B*, *lanes 1*, *4*, and *5*) but became detectable in cells coexpressing SUMO-1 (*lane 6*). Smad4 sumoylation was enhanced by coexpression of TβR-I(T/D) or TβR-ImL45(T/D) (Fig. 4*B*, *lanes 7* and *8*). These results indicate that TGF-β-dependent Smad activation is not required for Smad4 sumoylation. Interestingly, the amount of sumoylated Smad4 was higher in cells producing TβR-ImL45(T/D) than in those producing TβR-I(T/D), which is likely related to higher p38 phosphorylation activity in TβR-ImL45(T/D) (Fig. 4*A*). This observation led us to speculate that p38 may play a role in activation of SUMO-1 conjugation to Smad4.

We examined the effect of p38 on Smad4 sumoylation by using an inhibitor of p38. COS7 cells plated in duplicate were transfected with plasmids expressing FLAG-Smad4, HA-SUMO-1, GFP-PIASxβ, and TβR-ImL45(T/D) in various combinations as shown in Fig. 4*C*. One set was then treated with the p38 inhibitor, SB203580, for 24 h before cell harvest. In the other set of cells, not treated with inhibitor, phosphorylated p38 was detected at varying levels, with highest amounts in lysates producing TβR-ImL45(T/D) (Fig. 4*C*, *lanes 4–6*, *fourth panel* from the *top*). SUMO-1-conjugated Smad4 was detected in cells producing SUMO-1, SUMO-1 plus PIASxβ, SUMO-1 plus TβR-I(T/D), SUMO-1 plus TβR-ImL45(T/D), or SUMO-1, PIASxβ, and TβR-ImL45(T/D), although the level of sumoylated Smad4 varied. Levels were highest in the lysate coproducing SUMO-1, PIASxβ, and TβR-ImL45(T/D) (Fig. 4*C*, *lane 6*). In addition to sumoylated Smad4, several bands reactive to the anti-HA antibody were detected, which may correspond to cellular protein(s) interacting with wild-type and/or sumoylated Smad4 (Fig. 4*C*, *white arrowhead*, *third panel* from the *top*). In lysates prepared from cells treated with SB203580, the amount of SUMO-1-conjugated Smad4 was lower than in cells not treated with SB203580. In particular, levels of SUMO-1-conjugated Smad4 were dramatically enhanced when both TβR-ImL45(T/D) and GFP-PIASxβ were expressed together (*lane 6*), whereas SB203580 treatment of cells coproducing TβR-ImL45(T/D) and PIASxβ were reduced to nearly one-quar-

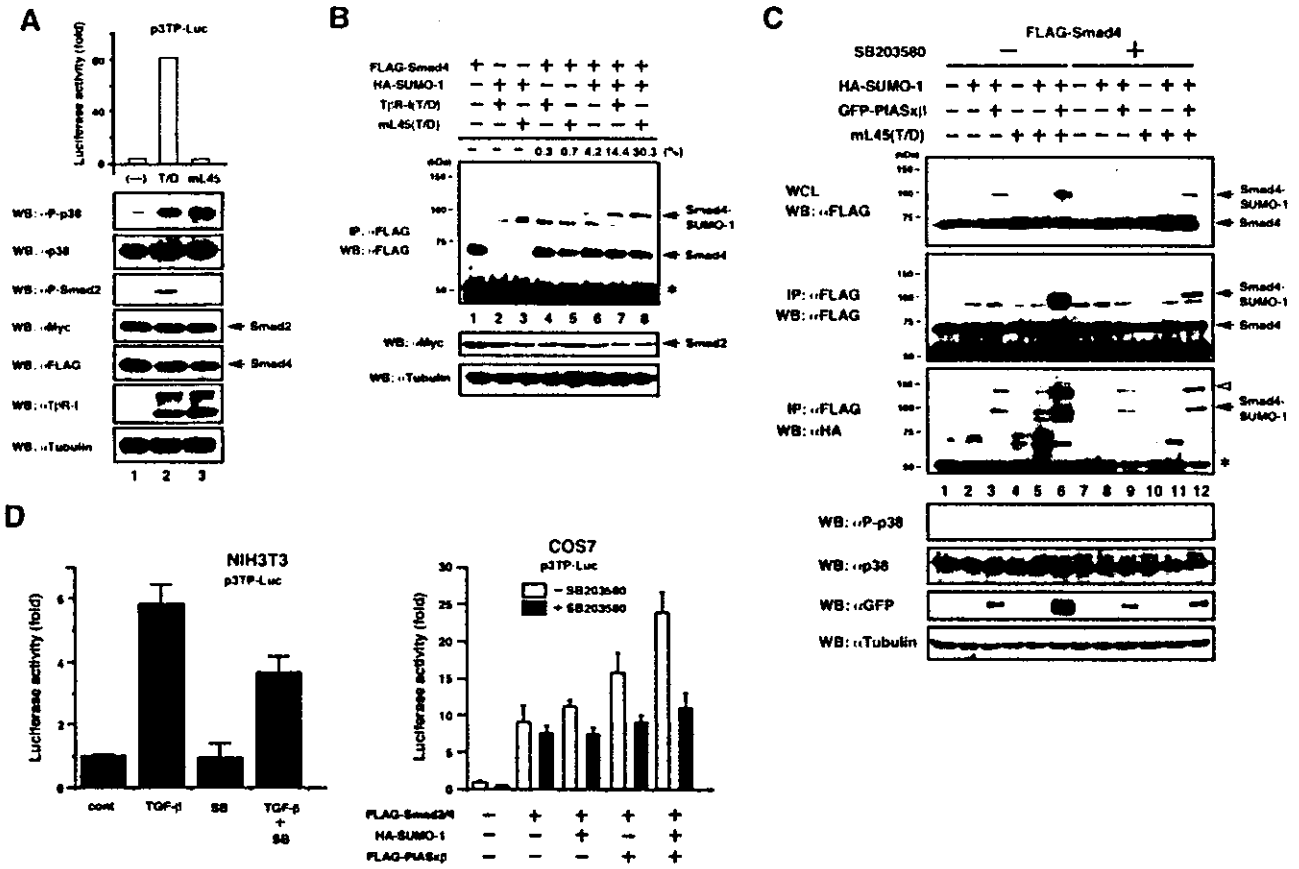

FIG. 4. **Role of p38 MAP kinase pathway in TGF-β signaling in sumoylation and transactivation of Smad4.** *A*, a mutant TβR-ImL45(T/D) that no longer activates the Smad pathway but still stimulates the p38 MAP kinase pathway in COS7 cells. COS7 cells were cotransfected with 25 ng of p3TP-Luc, 50 ng of plasmids expressing FLAG-Smad2 and FLAG-Smad4, together with 100 ng of plasmids expressing TβR-I(T/D) (*T/D*) or TβR-ImL45(T/D) (*mL45*). Luciferase assay was performed as described in the legend for Fig. 1. Phospho-p38 (*P-p38*) and phospho-Smad2 (*P-Smad2*) were analyzed by using specific antibodies. The levels of proteins expressed in whole cell lysates were analyzed and shown as indicated. *B*, Smad activation pathway in TGF-β signaling is not required for sumoylation of Smad4. COS7 cells were cotransfected with (+) or without (−) plasmids expressing FLAG-Smad4 (3 μg), HA-SUMO-1 (1 μg), or TβR-I(T/D) or TβR-ImL45(T/D) (3 μg), respectively. A plasmid expressing Myc-Smad2 was also transfected into cells in all dishes. Lysates prepared from cells 36 h after transfection had their total protein concentration adjusted to the same level. Then, lysates were immunoprecipitated (*IP*) with anti-FLAG antibody followed by Western blotting with anti-FLAG antibody. The relative sumoylated ratio of Smad4 was indicated as a percent of total Smad4, based on densitometric quantitation. (−) shows below the detection limit. The protein levels of Smad2 and α-tubulin in each lysate were shown as indicated. The *asterisk* indicates the immunoglobulin heavy chain. *C*, treatment with a p38 MAP kinase inhibitor reduces Smad4 sumoylation. COS7 cells were cotransfected with (+) or without (−) plasmids expressing FLAG-Smad4 (3 μg), HA-Smad4 (1 μg), GFP-PIASxβ (2 μg), and TβR-ImL45(T/D) (3 μg), respectively. Twelve h after transfection, cells were treated with or without 30 μM SB203580 for 24 h, and cell lysates were prepared. Immunoprecipitation and Western blot analysis were performed as shown in Fig. 1*C*. The levels of protein in whole cell lysates are shown as indicated. Sumoylated cellular protein(s) coimmunoprecipitated with Smad4 are indicated as a *white arrowhead*. *D*, inhibition of p38 MAP kinase activity suppresses Smad-mediated transactivation. *Left*, NIH3T3 cells were transfected with 50 ng of p3TP-Luc. Twenty-four h after transfection, cells were treated with solubilized solution without TGF-β (control; *cont.*), 2 ng/ml TGF-β, 30 μM SB203580 (*SB*), and TGF-β plus SB203580 (*TGF-β + SB*). Luciferase activities in cell lysates prepared after 12 h of treatment were measured. The activity of the treatment with control medium was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this assay. *Right*, COS7 cells were cotransfected with 25 ng of p3TP-Luc and 50 ng of plasmid expressing TβR-I(T/D) in the presence (+) or absence (−) of 50 ng of plasmids expressing FLAG-Smad2, together with FLAG-Smad4, and HA-SUMO-1, and 100 ng of FLAG-PIASxβ. Twenty-four h after transfection, cells were treated with (*black bars*) or without (*white bars*) 30 μM SB203580 for 12 h, and luciferase assays were performed. The activity of the reporter plasmid alone was arbitrarily given a value of 1, and relative activities were calculated.

ter that in untreated lysates (*lane 6 versus 12*). Taken together, these findings suggest that activation of p38 MAP kinase, but not Smads, by TGF-β signaling enhances PIAS-mediated Smad4 sumoylation.

We further examined the effect of SB203580 on Smad-mediated transcription in TGF-β signaling. NIH3T3 cells were transfected with a p3TP-Luc reporter plasmid and were treated with either TGF-β or SB203580, or both (Fig. 4*D*, *left*). Treatment with SB203580 significantly repressed Smad-mediated transcription. Furthermore, we performed the reporter assay with various combinations of TβR-I(T/D), Smad2 and Smad4, SUMO-1, and PIASxβ (Fig. 4*D*, *right*). COS7 cells were then treated with or without 30 μM SB203580 for 12 h, 24 h after transfection, and luciferase activities were measured. As expected, luciferase activity was inhibited by treatment with SB203580 (*black bars*). Collectively, these findings suggest that enhanced Smad4 sumoylation by TGF-β signaling is regulated by modulating PIAS functions through p38 MAP kinase activation, which thereby promotes Smad-mediated transactivation.

PIASxβ Protein Is Stabilized, and Its mRNA Is Up-regulated by p38 MAP Kinase Activation—The preceding data suggested that activation of p38 MAP kinase by TGF-β signaling might promote the function of PIAS family proteins and subsequently enhance SUMO-1 conjugation to Smad4. To investigate the molecular link between p38 MAP kinase and PIAS function, we first examined the effect of TGF-β-mediated MAP kinase acti-

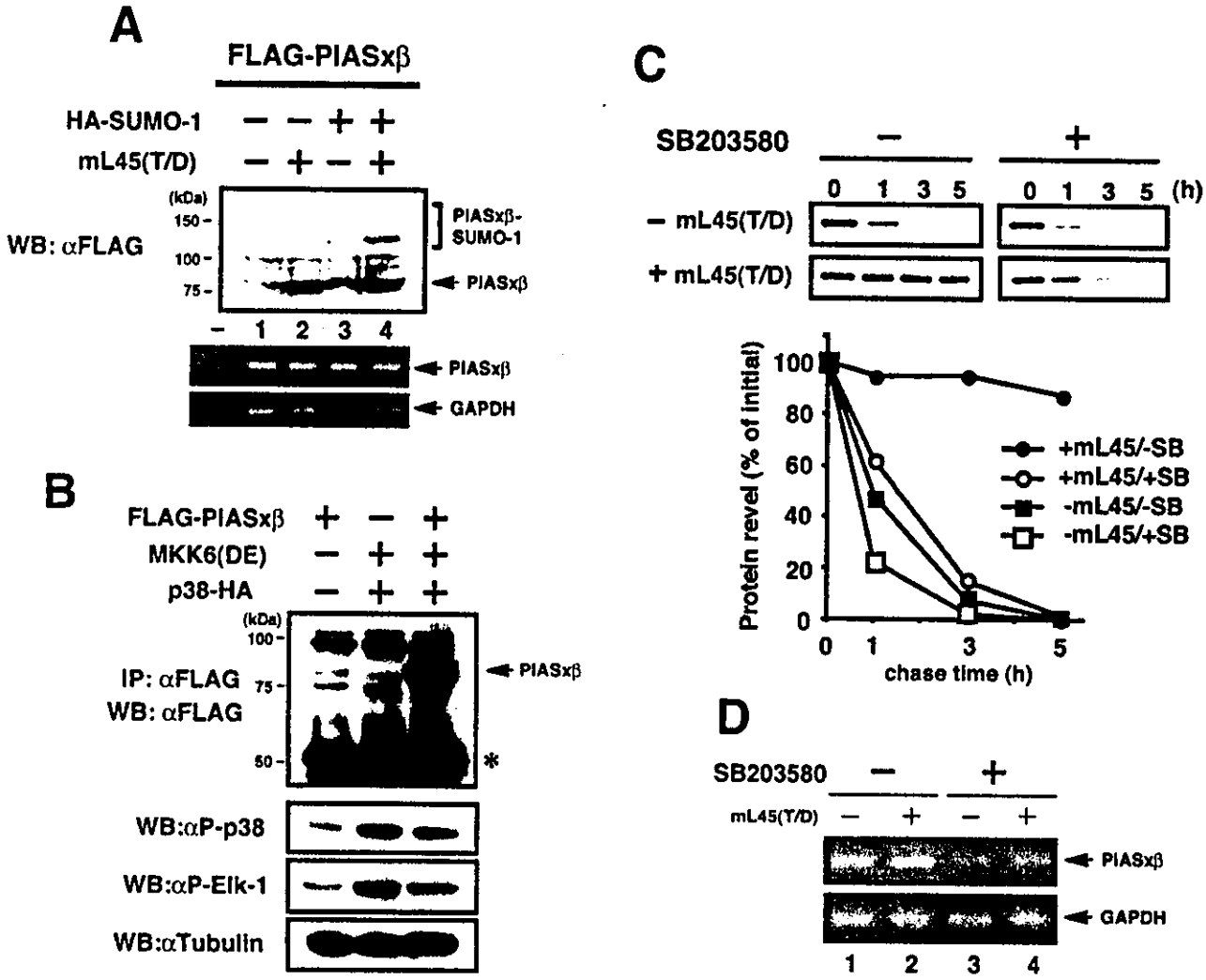

FIG. 5. **Activation of p38 enhances PIASxβ mRNA expression and protein stability.** *A*, accumulation of PIASxβ by activation of the MAP kinase pathway in TGF-β signaling. COS7 cells were transfected with 3 μg of plasmid expressing FLAG-PIASxβ, together with (+) or without (−) 1 and 3 μg of plasmids expressing HA-SUMO-1 and TβR-ImL45(T/D), respectively. Cell lysate was prepared 36 h after transfection, and equivalent amounts of each lysate were analyzed by Western blotting with anti-FLAG antibody. The levels of mRNA expressed from the FLAG-PIASxβ expression plasmid were analyzed by semi-quantitative RT-PCR using a forward primer in the PIASxβ coding region and a reverse primer in the region downstream of the multiple cloning site in the pcDNA3 vector (SP6). The signals for FLAG-PIASxβ at 12 cycles of PCR and GAPDH at 15 cycles of PCR are shown. A control lacking RT is shown (−). The levels of mRNA for GAPDH are shown as an internal control. *B*, the activation of p38 MAP kinase increased PIASxβ protein levels. COS7 cells were cotransfected with (+) or without (−) 2 μg of plasmids expressing FLAG-PIASxβ, MKK6(DE), a constitutively active form of MKK6, and p38-HA, respectively. Cell lysates were prepared 36 h after transfection, and equivalent amounts of each lysate were immunoprecipitated with anti-FLAG antibody, followed by Western blotting using anti-FLAG antibody. Phospho-p38 (*P-p38*) and phospho-Elk-1 (*P-Elk-1*) were analyzed using specific antibodies. The *asterisk* indicates the immunoglobulin heavy chain. *C*, effect of p38 inhibitor on degradation of PIASxβ. COS7 cells were cotransfected with 2 μg of plasmid expressing FLAG-PIASxβ, together with or without 3 μg of plasmid expressing TβR-ImL45(T/D). Twenty-four h after transfection, cells were treated with (+) or without (−) 30 μM SB203580. After an additional 12 h of incubation, cells were metabolically labeled with [^{35}S]methionine/cysteine for 1 h. Cells were then chased for 0, 1, 3, and 5 h with unlabeled methionine/cysteine. Cells were lysed, immunoprecipitated with anti-FLAG antibody, and analyzed by autoradiography. The amount of PIASxβ was quantified by densitometry and plotted as a percent of the zero time values under each condition. *D*, p38 MAP kinase up-regulates the mRNA level of endogenous PIASxβ. NIH3T3 cells were transfected with (+) or without (−) 5 μg of plasmid expressing TβR-ImL45(T/D). Twenty-four h after transfection, cells were treated with (+) or without (−) 30 μM SB203580 for 12 h. Total RNA was prepared, and mRNA levels of the endogenous PIASxβ were determined by semi-quantitative RT-PCR using the set of primers described under "Materials and Methods." The signals for PIASxβ at 27 cycles of PCR and GAPDH at 15 cycles of PCR are shown. The levels of mRNA for GAPDH are shown as an internal control.

vation on PIASxβ production. COS7 cells were cotransfected with plasmids expressing FLAG-PIASxβ in combination with HA-SUMO-1 and TβR-ImL45(T/D). Lysates prepared from these cells were then subjected to Western blotting using anti-FLAG antibody (Fig. 5*A*). Multiple bands reactive to the anti-FLAG antibody, which migrate slower than PIASxβ and seem to be sumoylated forms of PIASxβ, were noted, in agreement with previous reports (49, 52). The amount of sumoylated, as well as unmodified, PIASxβ was significantly increased when cells were transfected with TβR-ImL45(T/D) (*lane 2*, *4*), whereas a weak induction in response to SUMO-1 expression was observed (*lane 3*), because the amount of the transcript was the same in each case from the plasmid expressing FLAG-PIASxβ (Fig. 5*A*, *lower panel*). This suggested that activation of the MAP kinase pathway by TGF-β-signaling contributes to PIASxβ protein stability.

To further examine whether p38 MAP kinase is involved in increasing PIASxβ levels, COS7 cells were transfected with

plasmids expressing MKK6(DE), a constitutively active form of MKK6 that has the ability to phosphorylate p38, p38-HA, and FLAG-PIASxβ. Cell lysates obtained from these cells were subjected to Western blotting using anti-FLAG antibody (Fig. 5*B*). Coproduction of MKK6(DE) and p38-HA dramatically enhanced the level of PIASxβ protein, indicating that p38 MAP kinase activation is likely to be a critical determinant of PIASxβ levels in cells. Cell lysates were subjected to Western blotting using anti-phospho-p38 and anti-phospho-Elk-1 antibodies to verify the function of MKK6(DE) and p38-HA (Fig. 5*B*). Based on these observations, we hypothesized that activation of p38 MAP kinase by TGF-β signaling is important for PIASxβ stability. To address this possibility, we performed pulse-chase analysis to determine the half-life of PIASxβ, together with or without mL45(T/D), in the presence or absence of SB203580. PIASxβ was relatively stable upon coexpression of mL45(T/D) in the absence of SB203580 but was rapidly degraded upon treatment with SB203580 (Fig. 5*C*). These results suggest that PIASxβ is stabilized by TGF-β-mediated activation of p38 MAP kinase. Next, to determine whether p38 MAP kinase is involved in regulating the expression of endogenous PIASxβ, TβR-ImL45(T/D) was produced in NIH3T3 cells, and cells were analyzed with or without SB203580. mRNA levels of PIASxβ were then analyzed by semi-quantitative RT-PCR (Fig. 5*D*). Interestingly, expression of PIASxβ was up-regulated by expression of TβR-ImL45(T/D) in the absence of SB203580 (compare *lane 1 versus lane 2* in Fig. 5*D*) and repressed by treatment with SB203580 (*lanes 3* and 4). These results indicate that activation of p38 MAP kinase not only enhances PIASxβ protein stability but also up-regulates the expression of endogenous PIASxβ mRNA.

DISCUSSION

In this study, we found that SUMO-1 modification of Smad4 is a novel mechanism of gene regulation in TGF-β signaling. Smad4 is predominantly modified by SUMO-1 at Lys-159, within the linker region between MH1 and MH2, *in vivo* and *in vitro*. We identified PIAS1 and PIASxβ as E3 ligase factors for SUMO-1 conjugation to Smad4. Our observations in luciferase reporter assays indicate that PIASxβ activated SUMO-1 conjugation to Smad4 in a RING finger domain-dependent manner and that this modification is necessary for full activation in response to PIAS-mediated transactivation. On the other hand, the transcriptional activity of the sumoylation-deficient mutant Smad4(K/R) was weakly potentiated by expressing wild-type PIASxβ but not the RING finger mutant (Fig. 3), suggesting that PIASxβ functions to not only sumoylate Smad4 but also to conjugate SUMO-1 to other cellular factor(s) involved in Smad-mediated transactivation. Thus, SUMO-1 conjugation by PIASxβ at positions other than Lys-159 on Smad4 may also play a role in transcriptional activation. Further studies to clarify the molecular basis of transcriptional activation of Smad4-dependent transcription by PIASxβ are warranted. During review of this manuscript, two reports showed that Smad4 was sumoylated at multiple sites and that Smad-mediated transactivation was up-regulated by the modification (53, 54). However, we could not clearly detect an additional sumoylated form of Smad4(K/R) in COS7 cells and could not to determine whether sumoylation site(s) of Smad4 is only Lys-159 or not. Although we do not know the reasons for differences in the results at present, they may be because of cell line variation and/or expression levels of SUMO-1.

Our data clearly show that sumoylation of Smad4, despite affecting a relatively small proportion of Smad4, had a significant function in TGF-β-mediated gene expression. SUMO-1 modification is reversed by specific proteases that were still enzymatically active in cell lysates prepared for analysis of SUMO-1-conjugated Smad4. Supplementation of lysates with the SUMO-1 protease inhibitor, *N*-ethylmaleimide, often restored the level of SUMO-1 conjugation, suggesting that the level of sumoylated Smad4 in these studies is underrepresented and that the dynamics of equilibrium between sumoylation and de-sumoylation of Smad4 may regulate Smad-dependent transcription *in vivo*. Alternatively, SUMO-1-conjugated Smad4 may recruit the transcriptional coactivator(s) complex by providing novel interaction sites. Recent studies showed that the linker region adjacent to the SUMO-1 conjugation site of Smad4, known as the Smad4 activation domain (SAD), is required for activation of Smad4-dependent signaling responses and plays a role in recruiting the transcriptional coactivator CBP/p300 (55, 56). Our findings have revealed that sumoylated cellular protein(s) were coimmunoprecipitated with Smad4, but not Smad4(K/R), from cells expressing ectopic SUMO-1 (Fig. 1*C*). SUMO-1-conjugated Smad4 may recruit additional cellular factors, in addition to CBP/p300, which enhance Smad4-dependent transcription. Further identification and characterization of these protein(s) should provide significant insight into the transactivation of Smad4 by SUMO-1 modification.

Certain types of cytokine/receptor signaling can activate downstream elements using plural pathways (57). For example, type-I IFNs, such as IFN-α, IFN-β, and IFN-γ, that activate the Jak/Stat pathway, also referred to as the canonical IFNs pathway, also activate the non-canonical IFNs pathway including p38 MAP kinase. The TGF-β superfamily also activates two signaling pathways, the Smad family and MAP kinase cascade, that are referred to as canonical and non-canonical, respectively, to regulate its downstream genes. In this study, we demonstrated that the non-canonical pathway, p38 MAP kinase activation, activated by TGF-β signaling, played an important role in Smad4 sumoylation. We found enhanced PIASxβ gene expression and protein accumulation following activation of p38 MAP kinase. An inhibitor of p38, SB203580, inhibited Smad4 sumoylation and, simultaneously, suppressed transcriptional enhancement by sumoylated Smad4. Accumulation of PIASxβ by activation of p38 MAK kinase seemed to be the result of prolonged half-life of this molecule and of increased expression of this gene. p38 has been shown to activate transcription factors by phosphorylation (58). These factor(s) are likely to mediate increased PIASxβ expression at the transcriptional level. Because stabilization of PIASxβ was not correlated with sumoylation (Fig. 5*C*), it is likely that phosphorylation of PIASxβ by p38 itself or a kinase activated by p38 may contribute to its stability. The molecular mechanisms of the prolonged half-life of PIASxβ by p38 activation need to be clarified.

Association of Smad4 sumoylation with canonical and non-canonical pathways of TGF-β signaling may be an efficient way to activate downstream Smad signaling. Previous studies have shown that transcriptional activation of target genes by TGF-β, including the collagenase-3 and biglycan genes, requires the activation of both the MAP kinase and Smad pathways (59, 60). One explanation for this cross-talk between Smad and MAP kinase pathways is that activated p38 is directly involved in enhancing transcription via Smad4 sumoylation by PIAS family proteins. It has also been shown that Smad4 functions as a transcriptional cofactor for transcription factors such as AP-1 (61) and nuclear hormone receptors (1, 62, 63), in addition to functioning in a heterodimeric form with the R-Smad-dependent transcription factor, a well characterized signaling pathway of the TGF-β superfamily. In this case, Smad4 may also contribute to its own transcriptional regulation by sumoylation through a similar mechanism to that described in this paper. In

this regard, cross-talk between two independent signaling pathways, one of which includes p38 MAP kinase activation, may play an important role in regulating gene expression through sumoylation of transcriptional or related factors.

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Review Article

The roles of hepatitis C virus proteins in modulation of cellular functions: A novel action mechanism of the HCV core protein on gene regulation by nuclear hormone receptors

Koichi Watashi and Kunitada Shimotohno[1]

Department of Viral Oncology, Institute for Virus Research, Kyoto University, Shogo-in, Sakyo-ku, Kyoto 606-8507

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Hepatitis C virus (HCV) is one of the major causative agents inducing the development of hepatocellular carcinoma. The underlying mechanism of HCV pathogenesis, however, is largely unknown. Recent reports have implicated specific HCV proteins in persistent HCV infection, reduction of interferon sensitivity, and the modulation of cell proliferation, including alterations in apoptotic responses. However, the roles of these viral proteins remain controversial, because of conflicting results. Thus, it remains necessary to elucidate the precise molecular mechanisms through which the viral proteins influence cell growth and pathogenesis. In this review, after briefly describing what is known about the roles of the HCV proteins, in particular HCV core protein (core), in the modulation of cellular functions, we propose a novel molecular mechanism of the core in modulating gene expression via activation of nuclear hormone receptors. (Cancer Sci 2003; 94: 937–943)

Primary hepatocellular carcinoma (HCC) is a common malignancy with a high mortality rate worldwide. The major risk factors include alcohol consumption, exposure to chemicals, such as vinyl chloride and dietary aflatoxin B1 (AFB1), and chronic infection with hepatitis B or C virus. The majority of blood-borne non-A, non-B chronic hepatitis is caused by hepatitis C virus (HCV) in Japan. In this country, approximately 15%, 90%, and 80% of patients with sporadic acute hepatitis, post-blood transfusion chronic hepatitis, and hepatocellular carcinoma, respectively, are positive for HCV infection. Current research estimates the HCV infection rate worldwide at approximately 3% of the population.

The mechanisms by which HCV infection promotes the development of liver disease remain largely unknown. Accumulating evidence, however, suggests the direct or indirect involvement of viral proteins, especially the core protein, in liver carcinogenesis. The roles of HCV core protein remain poorly understood, however, due to a variety of conflicting findings. The discrepancies in these reports may have resulted from different experimental conditions, such as the use of different cell lines and different external stimuli for the analysis, or different genotype sources of *HCV* genes. To resolve these controversies, it will be necessary to determine precisely the molecular mechanisms by which HCV core protein regulates cellular events. Here, we will briefly review the roles of HCV proteins insofar as they are known, and in the last part of this review, we will present a novel model of the molecular mechanisms by which the core protein acts to modulate gene expression, based on the hypothesis that the effects are at least partly mediated by nuclear hormone receptor.

Persistent Infection of HCV

The critical feature of HCV infection in the development of liver disease is the persistence of infection. HCV escapes host immune surveillance; the hypervariable region of HCV E2 envelope protein has a neutralizing epitope for humoral immunity.[1)] This heterogeneity of the hypervariable region is responsible for significant variation of the infecting virus, which contributes significantly to the evasion of immune recognition and maintenance of persistent infection. In addition to humoral immunity, minimal activation of cellular immunity to HCV-infected cells may also allow persistent infection. In acute HCV infection, cellular immune responses appear to play a role in the virologic outcome; sustained antiviral CD4 and CD8 T cell responses are strongly associated with HCV clearance. In established chronic infection, however, HCV-specific T cell responses are quantitatively weak; this weak activity exerts only minimal selection pressure to produce mutated HCV forms escaping immune surveillance.[2)] The low frequency of T cell responders may contribute to incomplete control of the virus, facilitating the progression of liver disease. Recent reports suggest that the low responses of T cells in patients with chronic hepatitis correlate with functional impairment of dendritic cells (DC).[3,4)] DC is the most potent activator of CD4 T cells *in vivo*, supporting Th1/Th2 differentiation. In HCV infection, DC activation of Th1 differentiation appears to be restricted; reduced activity of DC may also be implicated in the pathogenesis of liver disease. Moreover, HCV core protein has also been reported to suppress host immune responses to vaccinia virus by downregulating viral-specific cytotoxic T lymphosyte responses and cytokine production.[5)] While the molecular mechanisms need to be clarified, immune evasion by HCV through the dysregulation of DC function likely contributes significantly to the progression of liver disease observed during chronic HCV infection.

Interferon induces an antiviral state in virally infected cells, directly inhibiting viral replication and stimulating host adaptive responses. However, viruses often develop elaborate strategies to interfere with either or both the induction of interferon and the action of interferon effector molecules. Treatment of chronic hepatitis C patients with interferon often results in the appearance of HCV resistant to this therapy. Detailed genomic analysis of the resultant HCV strains revealed sequence variations in a certain region of non-structural protein 5A (NS5A),

[1]To whom correspondence should be addressed.
E-mail: kshimoto@virus.kyoto-u.ac.jp

now called an interferon sensitivity-determining region, ISDR.[6] Variation in the amino acid sequence of this NS5A region defines differences in interferon sensitivity. NS5A plays a role in HCV tolerance to interferon treatment, possibly through its ability to inhibit interferon-induced double stranded RNA-dependent protein kinase, PKR. Increased affinity of NS5A with PKR induces a stronger repression of cellular PKR function.[7] An additional HCV protein, E2, is also reported to interfere with interferon signaling.[8] Thus, further study of the mechanisms through which HCV inhibits interferon-induced responses is needed. This impairment of interferon response by HCV infection may provide interfere crucially with innate immune responses affecting the induction of cellular and humoral immunity.

Molecular virology of HCV

HCV, a small enveloped virion containing a positive-strand RNA genome, belongs to the Hepacivirus genus within the Flaviviridae family. The HCV RNA genome is 9600 nucleotides in length; this sequence encodes a single polyprotein that is post-translationally cleaved into about 10 polypeptides, including 3 structural (core, E1, and E2) and at least 7 non-structural proteins (p7 and NS2–NS5) (Fig. 1). The NS proteins mediate all the functions necessary for the replication of the HCV genome, including a viral protease and RNA polymerase. The virus replicates primarily in the liver, but may also utilize monocytes. The virus possesses a marked sequence heterogeneity, acquired during the evolution of the viral genome. Viral proteins are translated first as a polyprotein precursor by way of a mechanism dependent on an internal ribosome entry site (IRES) located in the 5′ non-translated region. The polyprotein is processed by both host and viral proteases to yield individual HCV proteins. These proteins are organized within the polyprotein in the following order: NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. An additional protein, F/ARFP (for Frame shift/Alternate Reading Frame Protein), may also be generated from an overlapping reading frame in the region of the gene for the core.[9] The structural proteins, C, E1, and E2, used as components of progeny virions, are cleaved from the polyprotein in a translation-coupled manner by host signal peptidases associated with endoplasmic reticulum (ER). The E1 envelope protein may serve as a fusion protein during an early step of HCV infection. E2, the other envelope protein, may interact with cellular components to allow the penetration of HCV into cells. The function of the small hydrophobic p7 protein involves ion channel permeability.[10]

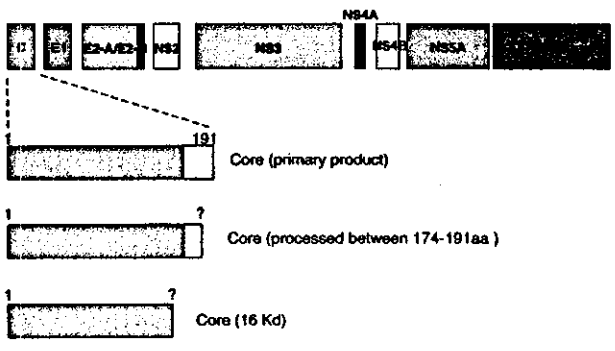

Fig. 1. HCV proteins encoded by the open reading frame of the HCV genome. The precursor polyprotein is cleaved by signal peptidase(s) and virally encoded protease(s) to produce approximately 10 viral proteins. The primary core protein, produced by the cleavage of a cellular signalase, is further cleaved in the region between aa 174 and 191. The 16 kD core is produced by only certain genotypes of HCV. The gray region within the core sequence is rich in hydrophobic amino acid residues, functioning to anchor the protein to the ER membrane.

The non-structural proteins play important roles in the viral replication.[11] The NS2 protein, together with one-third of the amino-terminal region of the NS3 protein, functions as a proteinase, which catalyzes the cleavage between NS2 and NS3. After the cleavage of this site, the amino-terminal domain of the NS3 protein functions as a serine proteinase, cleaving all other sites to produce the remaining non-structural proteins, NS4A, NS4B, NS5A, and NS5B. The carboxyl-terminal region of the NS3 protein contains an RNA helicase domain. The NS4A protein serves to enhance the activities of NS3. The function of the hydrophobic integral membrane protein NS4B remains unknown, but NS4B may modify the structure of the ER membrane. NS4B appears to form a membranous web at the locations at which viral genome replication occurs. NS5A is a phosphoprotein of unknown function that associates with ER membrane through the N-terminal region.[12] NS5A also associates with NS5B. The NS5B protein possesses an RNA-dependent RNA polymerase activity, which is indispensable for virus genome replication. A system to analyze replication of the HCV genome has recently been developed; this system may be a good tool to investigate the role of each HCV protein in viral replication.[11]

Roles of HCV proteins in modulation of cellular fuctions

During acute resolving hepatitis, HCV is cleared and ALT serum levels fall to normal. More than 50% of patients with acute hepatitis C, however, do not clear the virus, leading to the development of chronic hepatitis C. Chronic hepatitis C can be asymptomatic, but is usually associated with persistent high or fluctuating elevations in serum ALT levels. The chronic sequelae of hepatitis C include progressive hepatic fibrosis, cirrhosis, and hepatocellular carcinoma.

The mechanisms underlying HCV pathogenesis and persistence in the host are still largely unknown. The lack of an animal model or cell culture system to reproduce HCV infection has hampered detailed studies of HCV protein functions in HCV infection, modulation of cell proliferation and pathogenesis. The use of cells expressing one or a few HCV proteins together has increasingly revealed a direct influence of HCV proteins on several cellular events, suggesting the involvement of HCV proteins in the liver pathogenesis.

Many reports deal with the interactions of HCV proteins with host cell factors, including those involved in cell signaling, apoptosis, transcriptional regulation, transformation, membrane rearrangements, and immuno-modulation, which seems to be important for the dysregulation of cellular functions.[13] However, it remains uncertain which of these functions are most likely to be involved in the onset or development of cancer.

In the search for HCV proteins possessing a strong association with HCC, the core protein was found to function for the development of HCC in an analysis of transgenic mice.[14] Expression of core protein in liver cells from transgenic mice induced enhanced lipid metabolism, activation of cell signaling molecules, such as JNK, and enhanced ROS levels.[15]

Before describing the function of the core in greater detail, we would like to briefly introduce the effects of other HCV proteins on cellular functions reported thus far.

The membrane proteins, E1 and E2, are putative viral envelope proteins shown to interact with chaperone proteins, including Bip, calnexin, and calreticulin within the ER.[16] The roles of these interactions in the modulation of cellular functions, however, remain to be clarified. Cellular transformation was reported to follow exogenous expression of NS3 protein.[17] The region essential for this transformation was confined to the serine protease domain.[17] The transforming activity of NS3 may correlate with its ability to interact with the tumor suppressor p53.[18] Recently, NS3 was also shown to interact with histones H2B and H4.[19] NS3 localizes mainly to the ER

membrane through association with NS4A, another HCV nonstructural protein. In some hepatocytes derived from biopsy specimens from patients with HCV, NS3 was observed within the nucleus, suggesting that NS3 may translocate into the nucleus under certain conditions[20]; NS3 may interact with histones under such physiological conditions.

As described above, NS5A plays an important role in regulating PKR function, which appears to determine HCV interferon sensitivity. Moreover, NS5A induce IL-8 production, leading to partial inhibition of interferon-induced antiviral responses.[21]

Role of the core in virus replication and regulation of cellular functions

1) Production of the core from the HCV precursor polyprotein

The core is encoded by the N-terminal portion of the HCV precursor polyprotein. *In vitro* translational studies demonstrated that cleavage from the HCV precursor occurred only when microsomal membranes were present in the reaction mixture. The amino acid sequence upstream and proximal to the cleavage site is hydrophobic, similar to the sequences cleaved by signal peptidases associated with membranes. The primary product of the core protein cleavage, analyzed by determining the cleavage site by a signal peptidase(s), is estimated to have 191 amino acid residues (Fig. 1). As a direct analysis of the C-terminus of the core has not been performed, the possibility remains that further truncation of the C-terminal portion of the primary product may occur.[22] In fact, plasmid-based expression of the core revealed production of further truncated forms of the core, trimmed at the C-terminal region, although the efficiency of the cleavage was low. The sites of these second and third cleavages have not been precisely determined, but one lies between amino acids 174 and 191 of the primary cleaved core product. The other cleavage, upstream from amino acid residue 174, results in the production of a 16 kD core protein. The 16 kD form lacks most of the carboxy-terminal sequence, which is important for anchoring the protein to the ER membrane; thus, this truncation may result in the nuclear localization of the 16 kD core. The production of the core proteins with different sizes often varies depending on the HCV genotype. Two forms of core protein are observed in HCV-1b. Production of the 16 kD core protein fragment depends on the presence of a missense mutation at codons 9–11 of the core gene, encoded by the HCV-1a genome. The mechanism by which cells can produce the shorter protein from the core containing this missense mutation remains to be clarified.

The core protein, a component of viral particles, likely plays an important role in virus assembly. This process includes the packaging of viral RNA into virus particles through the maturation of virion formation. The core interacts with HCV RNA as well as with glycoproteins, such as the viral envelope proteins. The core proteins also associate with each other, a required step in viral particle assembly. Thus, the core protein possesses versatile intrinsic functions.

2) Subcellular localization of the core protein

Examination of the amino acid content of the core, as well as a hydropathy profile of this protein, divides the sequence into three domains[23]: an N-terminal region with high basic amino acid content (1 to 122), a central region moderately rich in basic amino acids (123–174), and a C-terminal region high in hydrophobic amino acid residues (175–191). The C-terminal hydrophobic region functions not only as a signal sequence, but also as a membrane anchoring sequence. Therefore, much of the wild-type core protein is located on cytoplasmic surface of the ER.[26] As the core influences the regulation of host gene expression, as described below, the precise subcellular localization of the core may be important to identify the underlying mechanisms of modulation of gene regulation by the core.

3) Versatile functions of the core in modulation of several cellular events

There is a large body of evidence regarding the role of the core in the modulation of several cellular events, suggesting multiple effects of the core on cells. As the core can localize to not only the cytoplasm, but also the nucleus based on structural differences, the versatile functions of the core against host cells may be exerted by differential localization. Some reports have shown that an artificial C-terminally truncated form of the core protein resided primarily in the nucleus. The core, however, is usually only retained in the nucleus in very small quantities; in addition, the 16 kD core, located mainly in the nucleus, can only be produced from limited HCV genotypes.[24] Thus, the significance of results obtained using the C-terminally truncated artificial core needs to be re-evaluated and confirmed by other approaches. At present, it is difficult to find any consensus among the multitude of published reports utilizing different model systems and constructs of the core. Reasons for the difference of these observations are not entirely clear, but the differences in experimental systems are likely the source of such conflicting results. Thus, it will be critical to evaluate these data in light of their potential physiological relevance. According to various reports, the core protein exerts several effects on cellular processes, including the following representative cases.

Effect on apoptosis: Expression of the core has been reported to affect Fas-mediated apoptosis both negatively and positively. The conditions of expression of the core, as well as the method of apoptosis induction, seem to determine the outcome. To exert a suppressive function against Fas-mediated apoptosis in HepG2 and other epithelial cells, the core activates NF-κB,[25] and this activation is correlated with anti-apoptotic function. While the mechanism by which NF-κB is activated remains unclear, the physical association of the core with the ER membrane is essential for this effect.[26] The core functioned differently in the case of TNFα-mediated apoptosis, however, as stable expression of the core in HepG2 and HeLa cells did not significantly affect the cytotoxicity induced by TNFα in the presence of cycloheximide.[27] Core expression, however, sensitized the cells to apoptotic induction by treatment of TNFα when administered together with actinomycin D.[28] These conflicting results suggest that core is not directly involved in the control of apoptosis, but instead seems to function as an indirect modulator affected by external and internal environmental conditions. In fact, the core appears to exert an anti-apoptotic function following TNFα treatment, but a pro-apoptotic effect in a cell type-specific manner after retinoid treatment in the same MCF7 cells.[29]

Cell transformation: Rat embryo fibroblasts and rodent cell lines were transformed by exogenous core expression when co-produced with a cellular oncogene, either c-Myc or activated H-Ras.[30] Other workers, however, were unable to transform rat embryo fibroblasts using the core from the HCV-1b genotype, despite transformation of these cells by the E1A oncogene of adenovirus.[31] This discrepancy is puzzling, but may be explained, at least in part, by the different sources of the core gene. In the former experiments, they utilized the core gene from HCV-1a producing the C-terminally deleted, nuclear-localized form.

Another line of inquiry suggesting that the core protein has a potential to transform cells comes from the analysis of STAT3 activation in core-expressing cell lines.[32] The authors demonstrated that the core directly interacts with and activates STAT3 through phosphorylation of a critical tyrosine residue. Activation of STAT3 by HCV core in NIH-3T3 cells resulted in rapid cellular proliferation and the upregulation of Bcl-XL and cyclin-D1 levels. Overexpression of STAT3 in HCV core-expressing cells resulted in anchorage-independent growth and tumorigenesis, suggesting that the core transforms cells in cooperation with STAT3.[32]

Development of hepatocellular carcinoma in core-expressing transgenic mice: Direct evidence of involvement of the core protein in the development of hepatocellular carcinoma was first presented using two independent core transgenic mouse lines.[14] These mice developed hepatic steatosis early in life, and subsequently developed hepatocellular carcinoma at an age of 16 months. Hepatic tumors first appeared as adenomas containing cytoplasmic fat droplets. Next, hepatocellular carcinomas developed in a "nodule-in-nodule" manner without cytoplasmic fat droplets, closely resembling the histopathological features of early hepatocellular carcinoma in patients with chronic hepatitis C. The molecular mechanisms governing the function of the core in the development of cancer, however, remain to be clarified. The authors' observation of the accumulation of lipid prior to the onset of disease suggests that aberrant lipid metabolism, which causes the overproduction of lipid peroxidation products, may be one mechanism through which the core contributes to tumorigenesis.[33] In addition, it was reported that core-expressing cells contained high levels of reactive oxygen species (ROS) and lipid peroxidation products.[15] These may result from alteration of mitochondrial function, because a mitochondrial electron transport inhibitor prevented the core-induced increases in ROS. Increases of lipid peroxidation products, however, may correlate with the activation of nuclear hormone receptor-dependent transcription induced by expression of the core, as will be described further below.

Recently, other groups also made transgenic mouse lines exhibiting core expression. One line developed malignant lymphoma at a high frequency (80%) at ages over 20 months, then subsequently developed HCC.[34] Both mRNA and protein expression of the core were detected in enlarged lymph nodes. These results clearly indicate a potential role of the core in the induction of cancer.

Dysregulation of lipid metabolism: Steatosis is a common condition observed in patients with chronic hepatitis C. Thus, HCV infection appears to be connected to affliction with this condition. This possibility was later supported by studies with transgenic mice expressing the core.[35] These mice frequently developed steatosis early after birth. Thereafter, fat droplets within the liver were observed over the next 10 months, just before the development of HCC. The core protein is thus associated with the formation of lipid droplets.[36]

Modulation of immunological function: The downregulation of T cells in patients with chronic hepatitis C may be a major factor contributing to the persistence of viral infection. Association of the complement receptor, gC1qR, with the core results in downregulation of T lymphocyte proliferation.[38] T cell activation and proliferation is suppressed by the impairment of DCs in patients with chronic hepatitis C. HCV induces the suppression of the expression of co-stimulatory molecules and production of cytokines in DCs, which are required for proper activation and proliferation of T cells. Infection of DCs from healthy individuals with a recombinant adenovirus expressing HCV core, E1, and E2 resulted in suppression of the cells' ability to activate allogenic T cells.[3] Similarly, infection of mice with a recombinant vaccinia virus expressing the core enhanced the virulence, suggesting a significant reduction in the cytotoxic T cell population.[5] These results suggest that the core plays an important role in the suppression of immunological function in individuals with chronic hepatitis C, although the mechanisms serving to downregulate DC function remain to be revealed.

Regulation of cell signaling: The core alters the expression of various cellular as well as viral genes. The suppression of HBV transcription and replication in core-expressing cells has previously been reported. Activity of cellular genes such as *c-fos*, *p21^{Waf1}*, *p53*, and *viral HIV LTR* is also suppressed by core expression.[23] In contrast, transcription from the Rous sarcoma virus, c-myc, and SV40 early promoters is upregulated by the core.[31,37] Conflicting results on alteration of activity of transcription factors, however, have also been observed. Although the core is reported to modulate expression of various genes, many of these results were obtained only by reporter gene assay. Thus, it is not clear whether this regulation of gene expression is a direct effect of the core. On the other hand, the core interacts with a variety of proteins, including heterogenous nuclear ribonucleoprotein K, lymphotoxin β, tumor necrosis factor receptor 1, DEAD box RNA helicase, p53, p21, and LZIP protein,[13] some of which are involved in transcriptional regulation. Therefore, these interactions may play a more direct role. As yet, no reports have clarified the mechanism through which the core regulates gene transcription.

4) Activation of nuclear receptor-dependent transcription and a possible molecular mechanism of activation of transcription by the core

As described earlier, the core appears to have bi-functional activities in the execution of apoptosis. This bi-functional activity may depend on both the types of external signals inducing apoptosis and the cell types tested. During our analysis of the effect of the core on apoptosis, we observed that core-expressing cells became sensitive to cell death induced by treatment with all-*trans* retinoic acid (ATRA). The induction of apoptosis was correlated with the activation of RARα-dependent transcription. Thus, we suspected that a gene downstream of RARα signaling is responsible for the induction of apoptosis. While the core protein mainly localizes to the ER membrane, RARα is a nuclear receptor functioning within the nucleus to upregulate downstream genes. After the treatment of core-expressing cells with ATRA, we did not observe any gross alterations in the subcellular localization of the core, suggesting that the core is not directly involved in RARα gene activation.

The core likely manipulates gene activation through interaction with a target molecule in the cytoplasm. A cellular protein, Sp110b, which interacts with the core, is likely to be involved in this regulation.[29] Sp110b is a newly identified protein found to associate with the core by means of studies in a yeast two-hybrid system. Sp110b suppresses RARα-dependent transcription, likely acting as a transcriptional co-repressor. Previously, Sp110 was identified as a protein associating with the promyelocytic leukemia (PML) nuclear body.[39] Sp110 is a splicing variant of the *Sp110b* gene, containing an extra amino acid sequence at the C-terminus with the conserved plant homeobox domain (PHD) and bromodomain sequences. Both proteins possess a consensus sequence motif, LXXLL, in which X indicates any amino acid, that is involved in interactions with the liganded nuclear hormone receptor. While Sp110 enhances RARα-dependent transcription following ATRA treatment, Sp110b suppresses such transcriptional activation. The suppressive effect of Sp110b is counteracted by the presence of the core. While Sp110 mRNA is expressed at high levels in peripheral blood leukocytes and the spleen, Sp110b mRNA is ubiquitously expressed throughout various tissues. The core protein associates with Sp110b, but not with Sp110. Analysis of the region within Sp110b necessary for core association identified a sequence of approximately 60 amino acid residues within the C-terminal region of Sp110b. Excess amounts of a peptide consisting of this region antagonize the function of the core in the regulation of RARα-dependent transcription. A core mutant lacking the association with Sp110b could not suppress the function of Sp110b in RARα-dependent transcription, suggesting that a physical interaction of the core with Sp110b is required for sequestration of Sp110b function. Co-expression of the core with Sp110b changes the subcellular localization of Sp110b from the nucleus to the cytoplasm, but alterations in the localization of Sp110 were not observed following the exogenous expression of the core (Fig. 2). Mutant core lacking the ability to interact with Sp110b failed to change the cellular lo-