ウイルスによる感染例も含まれている。また、試験的に行われている輸血実施例での全数調査により１例の感染例が確認されている。２００４年９月に厚生労働省から出された「輸血療法の実施に関する指針の一部改正」で、患者の輸血前後での検査が徹底されれば、この数はさらに増えるものと考えられる。ＨＣＶおよびＨＩＶ陽性例は確認されなかったが、ＨＢＶ同様個別ＮＡＴ検出限界以下のウイルスの存在は否定できない。チンパンジーの感染実験では１０コピーのＨＣＶで感染するといわれており、遡及調査の徹底が重要と考えられた。

ＮＡＴのプールサイズ縮小によりある程度の検出感度向上は期待されたが、検出された４２陽性検体の５０倍希釈による検査では、検出が困難と考えられるもの（２回検査で２回とも陰性）６例（１４．３％)、検出できない可能性のあるもの（２回検査で１回のみ陽性）１０例（２３．８％）という結果であった。５０プールＮＡＴで検出できないＨＢＶ陽性例は、そのもともとのコピー数が非常に低く、またＨＢＶは感染初期例でもウイルスの増殖速度が遅いため、プールサイズの縮小は期待以上の効果があったと考えている。さらに例数を増やしてより信頼性の高い評価としたい。

図１　ＨＢＶ症例数と解析結果

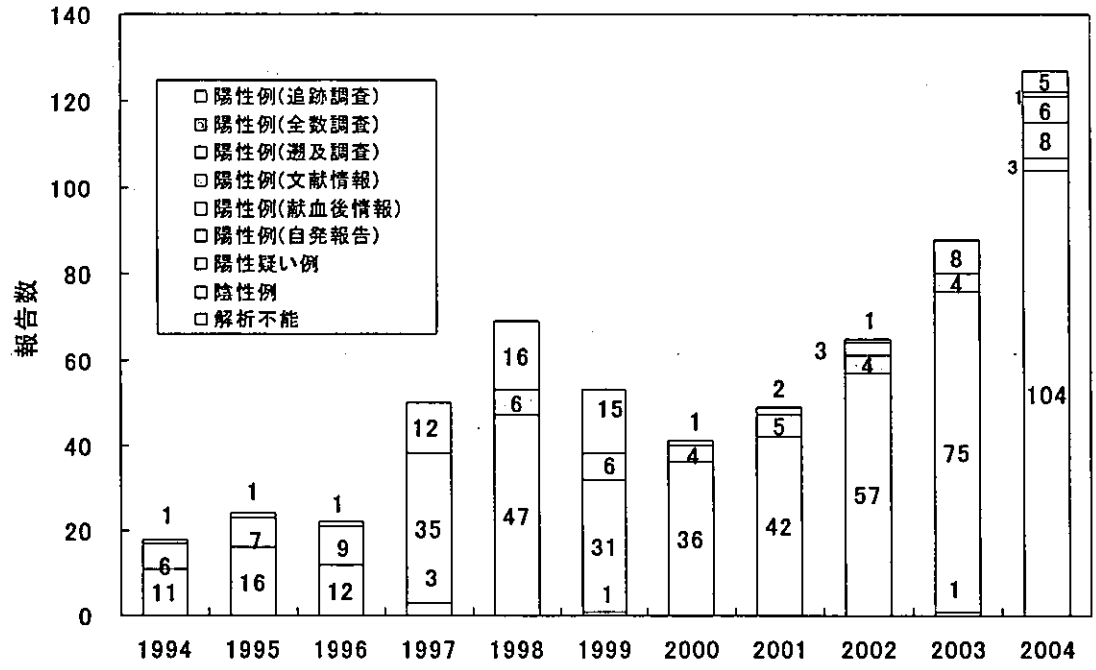

図２　ＨＣＶ症例数と解析結果

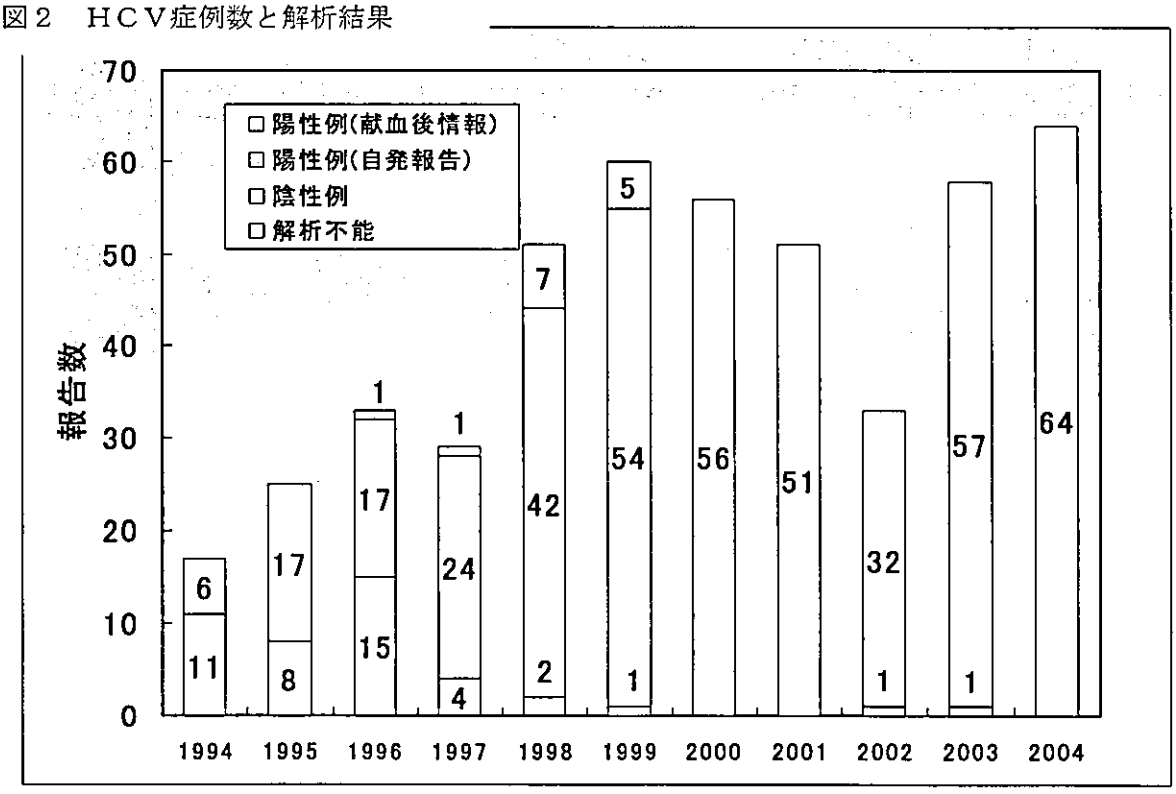

表１

低濃度HBVキャリア献血者の献血歴及び調査結果

献血日	個別NAT	受血者	受血者輸血前	受血者輸血後
04.4.12	陽　性	50代男性		sAg －、sAb －、DNA －
04.3.25	陽　性	男性	sAg －、sAb ＋、cAb ＋	sAg －、sAb ＋、cAb ＋、DNA －
04.3.10	陽　性	70代男性		sAg －、sAb ＋、cAb ＋、DNA －
04.2.23	陽　性	未使用		
04.2.6	陽　性	小児男性		原疾患にて死亡
04.1.19	陽　性	50代男性	sAg －	sAg ＋、sAb －、cAb ＋、DNA ＋*
03.12.29	陰　性	30代男性	sAg －、sAb －	sAg －、sAb －、cAb －、DNA ＋*
03.12.4	陰　性	20代男性		原疾患にて死亡
03.10.22	陽　性	小児男性	sAg －	sAg －、sAb ＋、cAb ＋、DNA －
03.9.22	陰　性	70代女性	sAg －	sAg ＋*
02.4.22	陰　性	小児女性	sAg －、DNA －	DNA ＋（塩基配列調査中）
97.5.17	陰　性	40代男性	sAg －	sAg ＋

＊塩基配列は献血者と一致

表２

20 pool NATと50 pool NAT

2004.8～2005.2

	20プール検出数	50倍希釈でのNAT結果*		
		+	+/-	-
HBV	42	26	10	6
HCV	3	3	0	0
HIV	3	3	0	0

＊ NAT陽性検体を50倍希釈した時のNAT結果
+ :2回検査して2回とも陽性
+/- :2回検査して1回だけ陽性
- :2回検査して2回とも陰性

研究成果の刊行に関する一覧表

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Keratinocyte-specific POU transcription factor hSkn-1a represses the growth of cervical cancer cell lines

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The POU transcription factor human Skn-1a (hSkn-1a) specifically promotes the proliferation of keratinocytes and enhances their differentiation. We examined the effects of hSkn-1a on cervical cancer cell lines of epithelial origin, in which the differentiation program is interrupted. From HeLa/Tet-On, a clone that can be induced to make hSkn-1a by doxycycline (HeLa/hSkn-1a) was prepared and characterized. Shortly after the induction, the cells expressed cytokeratin 10 (K10), a major marker protein in differentiating keratinocytes. While maintained for several days in the presence of doxycycline, the HeLa/hSkn-1a cells showed a slightly prolonged time of population doubling, the occasional appearance of flat cells with lowered DNA synthesis, and a low level of apoptotic DNA fragmentation. In SiHa and HeLa S3 cultures, K10 mRNA and apoptotic DNA fragmentation were detected at 48 h after infection with an adenoviral vector capable of expressing hSkn-1a. A colony inhibition assay showed that the growth of HeLa S3, SiHa, CaSki, and C-33A cells was repressed, as seen from the decreased number and average size of the drug-resistant colonies at 2 or 3 weeks after transfection with a plasmid that can express hSkn-1a and neomycin resistance gene. These results suggest that the expression of hSkn-1a represses the growth of the cervical cancer cells through the partial resumption of the differentiation pathway followed by slow suppression of cell replication and apoptosis.
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Keywords: hSkn-1a; cervical cancer; keratinocyte differentiation; POU-transcription factor

Introduction

Histopathological studies show that the interruption of differentiation pathways is a common phenotypic feature of cancer cells. It is generally believed that differentiation and malignancy are inversely correlated, but the defects in differentiation are not obligatory or causal events in carcinogenesis (Fusenig *et al.*, 1995). Many types of cancer cells retain the potential to differentiate in response to external differentiation stimuli, such as retinoic acid (Altucci and Gronemeyer, 2002). The remaining differentiation capacity of cancer cells provides a basis for a new therapeutic strategy, the differentiation therapy. For the development of this strategy, more studies are needed about the differentiation control of cells and differentiation capacity of cancer cells.

Cell differentiation is regulated by transcription factors. Mammalian proteins Pit-1 (Ingraham *et al.*, 1988) and Oct-2 (Clerc *et al.*, 1988) and *Caenorhabditis elegans* protein unc-86 (Finney and Ruvkun, 1990) induce terminal differentiation of pituitary cells, B-lymphocytes, and neuronal cells, respectively. These factors containing common DNA-binding domain, named POU domain, are grouped as the POU domain family (Herr *et al.*, 1988). Rat Skn-1a and human Skn-1a (hSkn-1a), which are members of the POU domain family, are primarily expressed in differentiating suprabasal keratinocytes, but not in proliferating basal cells (Andersen *et al.*, 1993). They activate genes encoding cytokeratin 10 (K10) (Andersen *et al.*, 1993), a major marker protein for differentiating keratinocytes, and small proline-rich protein 2A (Fischer *et al.*, 1996), and downregulate genes encoding involucrin (Welter *et al.*, 1996) and profilaggrin (Jang *et al.*, 2000). A recent study shows that hSkn-1a contributes to epidermal stratification by promoting keratinocyte proliferation and by enhancing the subsequent keratinocyte differentiation (Hildesheim *et al.*, 2001). Thus, hSkn-1a is likely to trigger and regulate the differentiation of epithelial cells.

In this study, we designed the experiments to describe how the cancer cells derived from keratinocytes respond to the expression of hSkn-1a, which triggers the process of normal keratinocyte differentiation leading to cell death. We prepared and characterized a HeLa line that can induce expression of hSkn-1a placed under the control of a tetracycline-inducible promoter (HeLa/hSkn-1a). We found that in the HeLa cells, the hSkn-1a expression upregulates K10 (a typical marker gene) expression, and, subsequently, repress cell growth and induce DNA fragmentation in some of the cells. These changes seem to be part of the differentiation, because some upregulated genes are common to the induced HeLa/hSkn-1a and the primary keratinocytes that have

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started differentiation, as reported recently (Enomoto *et al*., 2003). Thus, this study shows that HeLa cells retain part of the potential for differentiation. To extend these findings to other cervical cancer cell lines, we used a transient expression system and a colony inhibition assay. For comparison we also examined two cell lines from hepatocellular carcinoma, an immortalized keratinocyte cell line, and a transformed human kidney cell line.

Results

Doxycyline-induced expression of hSkn-1a in HeLa/hSkn-1a, a HeLa clone containing the inducible gene

To study how HeLa, a representative cell line from cervical carcinoma, responds to the expression of hSkn-1a, we prepared HeLa/hSkn-1a, a cell line that expresses hSkn-1a in the presence of doxycycline. In the lysate of HeLa/hSkn-1a cultured without doxycycline, hSkn-1a was not detected by immunoblotting using anti-Skn-1a antibody (Figure 1a). The addition of doxycycline of 0.1 μg/ml to the medium induced a detectable level of hSkn-1a and of 2 μg/ml the maximum level (Figure 1a). Therefore, doxycycline of 2 μg/ml was used for the induction of hSkn-1a in the following experiments. The parental HeLa/Tet-On cultured in the medium with doxycycline of 2 μg/ml for 2 days did not produce hSkn-1a, indicating doxycycline itself did not induce hSkn-1a (Figure 1d). After the addition of doxycycline, hSkn-1a in HeLa/hSkn-1a became detectable at 15 min and then its level raised, indicating the induction is very rapid (Figure 1b).

The immunofluorescence staining of the HeLa/hSkn-1a culture incubated with doxycycline for 4 days showed strong nuclear immunofluorescence in accordance with nuclear localization of hSkn-1a (Figure 1c). Although all the cells in the culture were positive for hSkn-1a, the levels of hSkn-1a slightly varied from cell to cell. When the HeLa/hSkn-1a culture was maintained in the growth medium containing doxycycline, the number of cells with strong immunofluorescence reached maximum at 2 days after the induction and then became fewer gradually.

In parallel to the immunofluorescence data, immunoblotting showed that the amount of hSkn-1a in cellular extracts, which was adjusted to contain a fixed amount of cellular proteins, reached maximum at 2 days after the induction (Figure 1d). The level of hSkn-1a was maintained for the following 3–5 days and then gradually lowered. Based on the above findings, we concluded that HeLa/hSkn-1a was appropriate to analyse cellular responses to hSkn-1a within 6 to 7 days after the induction.

Changes of HeLa/hSkn-1a cells resulting from the induced expression of hSkn-1a

K10, one of the major marker proteins expressed in differentiating keratinocytes, was detectable by immunochemical staining in almost all the HeLa/hSkn-1a cells cultured in the presence of doxycycline for 3 days (Figure 2a). The level of K10 in each cell varied as the level of hSkn-1a did (Figure 1d). K10 was not detected in extract from the parental HeLa/Tet-On cultured with doxycycline of 2 μg/ml for 2 days (Figure 2b). Changes of the total amount of K10 in the culture after the induction of hSkn-1a were shown by immunoblotting with anti-K10 antibody (Figure 2b). K10 was detectable at 1 day after the induction and reached the highest level at 2 days. Then the level of K10 gradually lowered and

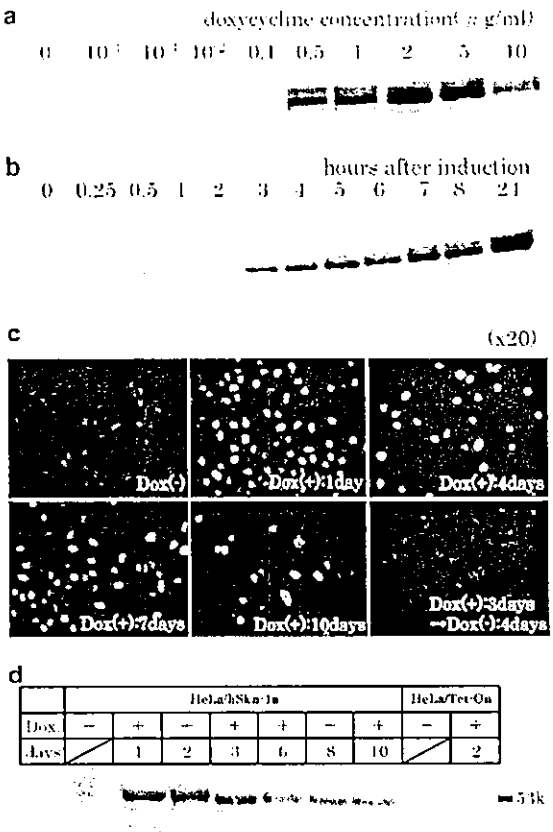

Figure 1 Doxycycline-induced expression of hSkn-1a in HeLa/hSkn-1a. (a) Dependency of hSkn-1a expression on doxycycline concentrations in the culture medium. Extracts from HeLa/hSkn-1a grown in the presence of various concentrations of doxycycline were examined for the presence of hSkn-1a by immunoblotting with anti-Skn-1a antibody. (b) Immediate expression of hSkn-1a after the induction. HeLa/hSkn-1a was cultured in the medium with doxycycline of 2 μg/ml for indicated periods. Then, extracts from the cells were examined for the presence of hSkn-1a by immunoblotting with anti-Skn-1a antibody. (c) Immunofluorescent staining of hSkn-1a in HeLa/hSkn-1a cultures grown under the presence of doxycycline. HeLa/hSkn-1a was cultured without or with doxycycline for the indicated periods. Cells were fixed and hSkn-1a was visualized by fluorescence staining with anti-Skn-1a antibody and AlexaFluor 488-conjugated anti-rabbit IgG antibody. Photos were taken under an UV microscope. (d) Levels of hSkn-1a in HeLa/hSkn-1a cultures grown under the presence of doxycycline. HeLa/hSkn-1a was cultured without or with doxycycline for the indicated periods. Cellular extracts containing fixed amount of protein were examined for the presence of hSkn-1a by immunoblotting with anti-Skn-1a antibody

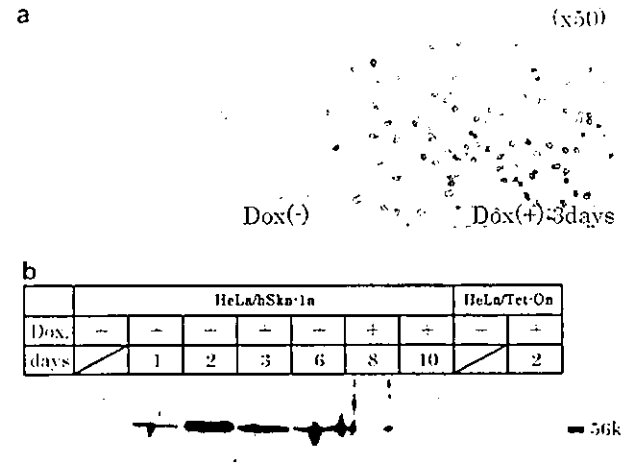

Figure 2 Expression of K10 in HeLa/hSkn-1a after the induction of hSkn-1a. (a) Immunochemical staining of K10 in HeLa/hSkn-1a cultures grown under the presence of doxycycline. HeLa/hSkn-1a was cultured without or with doxycycline for 3 days. K10 was stained with anti-K10 monoclonal antibody. (b) Levels of K10 in HeLa/hSkn-1a cultures grown under the presence of doxycycline. HeLa/hSkn-1a was cultured without or with doxycycline for the indicated periods. Cellular extracts containing fixed amount of protein were examined for the presence of K10 by immunoblotting with anti-K10 antibody

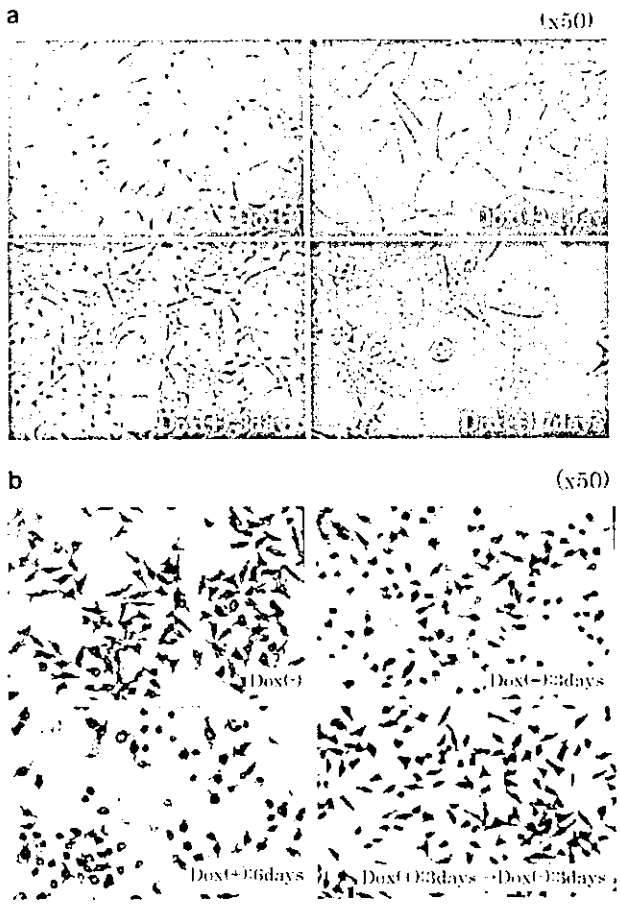

Figure 3 Changes of HeLa/hSkn-1a resulting from the induction of hSkn-1a. (a) Morphological changes of HeLa/hSkn-1a cultured under the presence of doxycycline. HeLa/hSkn-1a was cultured without or with doxycycline for the indicated periods. Photos of the cultures were taken under a phase-contrast microscope. (b) Incorporation of BrdU by HeLa/hSkn-1a cultured under the presence of doxycycline HeLa/hSkn-1a was cultured without, with doxycycline for 3 or 6 days, or with 3 days followed by without for 3 days. Then, cells were cultured in the medium containing 10 μM BrdU for 15 min at 37°C. The incorporated BrdU were visualized by immunochemically

became undetectable at 10 days. The change in the level of K10 was consistent with the level of hSkn-1a shown in Figure 1d. The expression of involucrin and filaggrin, the other marker proteins in the differentiating keratinocytes, were not detectable. Thus, the expression of hSkn-1a was followed by the expression of K10, indicating that HeLa/hSkn-1a maintained the capability of resuming a differentiation pathway leading to K10 expression.

The induction of hSkn-1a did not immediately cause any morphological changes of cells in the HeLa/hSkn-1a culture. However, at 3 days after the induction, flat and enlarged cells emerged (Figure 3a). Some of the flat cells appeared not to divide but to become larger at 7 days. The majority of flat cells disappeared when doxycycline was removed from the medium and incubated for further 3 days, indicating that the morphological changes depended on hSkn-1a and were reversible at this time point.

The incorporation of BrdU into nuclei, which is evidence of active DNA synthesis, was clearly decreased in some of the cells including the flat cells at 3 and 6 days after the induction (Figure 3b). The level of BrdU varied from almost zero to close to the maximum among the cells in the culture, suggesting that the shut down of DNA synthesis did not occur uniformly at a time. When doxycycline was removed from the medium at 3 days and incubated for further 3 days, the incorporation of BrdU recovered, indicating that the lowered DNA synthesis also depended on hSkn-1a and were reversible at this time point.

Some of the cells, which were morphologically similar to those cultured without doxycycline, grew in the medium containing doxycycline. A small number of the flat cells were always present in the culture, suggesting that they were generated occasionally from some of the dividing cells. The number of cells in the culture dish was counted everyday with appropriate passages. The periods required for population doubling of HeLa/hSkn-1a cultured in the growth medium for 6 days with or without doxycycline (2 μg/ml) were 17.8 h and 19.9 h, respectively.

The apoptotic fragmentation of chromosomal DNA was shown in total DNA extracted from HeLa/hSkn-1a culture at 3 days after the induction and it became more prominent at 6 days (Figure 4). The level of the fragmented DNA in the culture incubated with doxycycline for 3 days and then incubated without doxycycline for 3 days was similar to the level of culture incubated

Figure 4 Apoptotic DNA fragmentation in HeLa/hSkn-1a after the induction of hSkn-1a. HeLa/hSkn-1a was cultured without or with doxycycline for 3 or 6 days, or with 3 days followed by without for 3 days. Apoptotic DNA fragments in total DNA extracted from the cells were amplified by LM-PCR Ladder Assay Kit (BD Biosciences Clontech), electrophoresed in a 1.2% agarose gel, and stained with ethidium bromide

with doxycycline for 3 days, suggesting strongly that apoptotic DNA fragmentation was associated with the expression of hSkn-1a.

The response of the HeLa clone to hSkn-1a was the apparent initiation of differentiation, as indicated by the production of K10, followed by some morphological change, the slow suppression of cell growth, and apoptosis. The changes that occur after the induction of K10 expression would be slow and unsynchronized, probably causing the apparent discrepancy between the K10 expression and morphological or biochemical alterations. Since none of these changes were induced by doxycycline in parental HeLa/Tet-On cells, we concluded that the changes were specific to the expression of hSkn-1a. To know whether these findings can be extended to the parental HeLa line and other cervical cancer cell lines, we used a transient expression system using adenovirus and a colony inhibition assay in the following sections.

Responses of HeLa S3 and SiHa to transiently expressed hSkn-1a

HeLa S3 and SiHa, another cervical cancer cell line, were examined for the expression of K10 mRNA and apoptotic DNA fragmentation after infection with a newly generated recombinant adenovirus expressing hSkn-1a (Ad/hSkn-1a). For comparison, Alexander and HepG2, cell lines derived from liver cancer, were similarly infected with Ad/hSkn-1a. These cells were selected because susceptibility of these cells to infection with the recombinant adenovirus was relatively high (data not presented). The steady-state hSkn-1a was not detectable in extracts from these cells by immunoblotting with anti-Skn-1a antibody (data not presented).

The infectivity of Ad/hSkn-1a was measured with HEK293 cells that support the propagation of the defective recombinant adenoviruses. For the cells, in which the recombinant adenovirus cannot propagate, it was necessary to inoculate the virus at a higher MOI. SiHa was infected at an MOI of 10 and the other cells at 100. The recombinant adenovirus expressing lacZ (Ad/

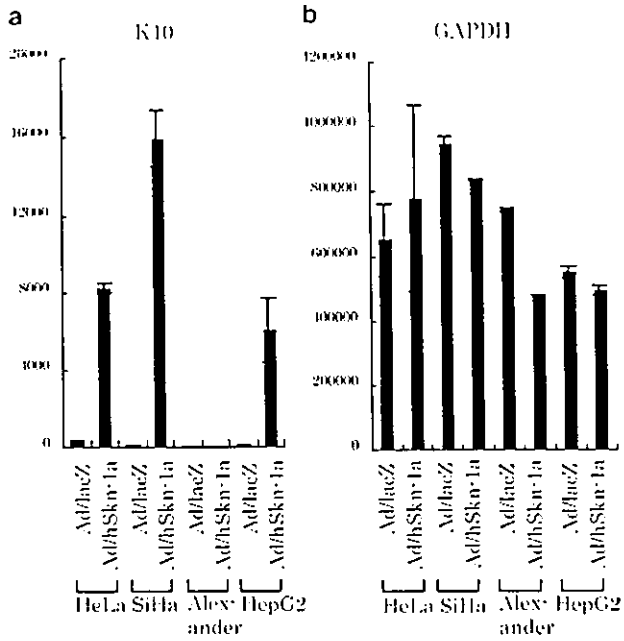

Figure 5 K10 and GAPDH mRNA in the cells infected with Ad/hSkn-1a or Ad/lacZ. HeLa S3, SiHa, Alexander, and HepG2 were infected with the recombinant adenoviruses at an MOI of 10 for SiHa and 100 for other cells. After 2 days, total RNA was extracted and levels of K10 mRNA (a) and GAPDH mRNA (b) were quantified by real-time PCR using QuantiTect SYBR Green PCR Kit (QIAGEN GmbH) by ABI PRISM 7700 Sequence Detector (Applied Biosystems). Specificity of the reaction was verified by dissociation curve analysis and agarose-gel electrophoresis of PCR products

lacZ) was similarly inoculated to the cells. All the cells in the culture inoculated with Ad/hSkn-1a or Ad/lacZ at an indicated MOI were positive for hSkn-1a or lacZ, respectively, and just after the inoculation of the viruses (before the accumulation of the transgene product), these cells did not show any morphological abnormalities presumably resulting from the nonspecific cell toxicity of the inocula (data not presented).

After the infection with Ad/hSkn-1a, K10 mRNA was readily detectable in the HeLa S3, SiHa, and HepG2 cultures, but not induced in the Alexander culture. Total RNA was extracted from the cells at 2 days after the infection and level of K10 mRNA was measured by real-time RT–PCR (Figure 5a). Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were similar in the cells infected with either Ad/hSkn-1a or Ad/lacZ (Figure 5b), indicating that basic cellular metabolism was not affected by the infection with the recombinant adenovirus.

At 2 days after the infection with Ad/hSkn-1a, the cells positive for apoptotic DNA fragmentation were present in HeLa S3, SiHa, Alexander, and HepG2 cultures stained by TdT-mediated dUTP nick end labeling (TUNEL), which is a method to label the 3′-end of intracellular fragmented DNA for cytological detection (Figure 6). Apoptosis was the most prominent in Alexander among these cell lines. The apoptotic

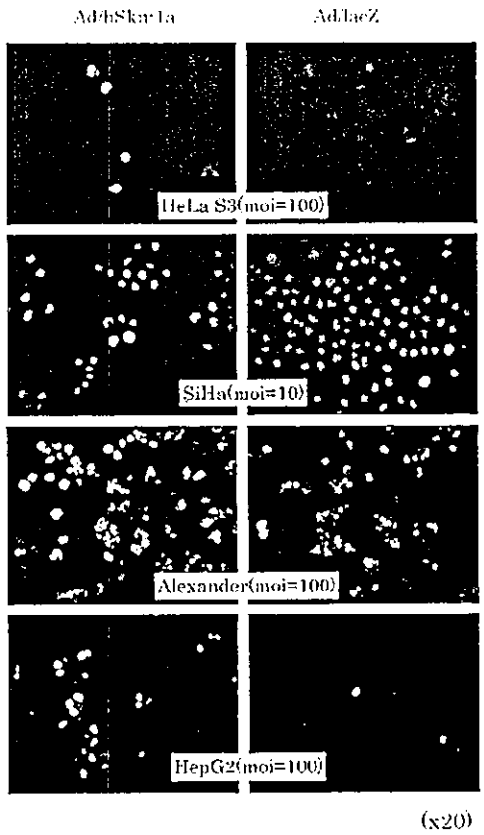

Figure 6 Apoptotic DNA fragmentation in the cells infected with Ad/hSkn-1a. HeLa S3, SiHa, Alexander, and HepG2 were infected with Ad/hSkn-1a or Ad/lacZ at the MOI indicated in figures. After 2 days, the cells containing fragmented DNA were detectable by staining the 3'-ends of DNA by TUNEL method in the culture infected with Ad/hSkn-1a

Table 1 Colony formation of G418-resistant cells after transfection with pCMV/IRESneo or pCMV-DsRed/IRESneo

Cells		*Exp. 1*	*Exp. 2*	*Exp. 3*
HeLa S3	DsRed	4133.3	5880.0	5633.3
	hSkn-1a	546.7	2313.3	2183.3
	Ratio	7.56	2.54	2.58
SiHa	DsRed	168.3	262.0	101.0
	hSkn-1a	28.3	87.0	42.0
	Ratio	5.94	3.01	2.40
CaSki	DsRed	67.0	41.5	23.0
	hSkn-1a	23.0	6.0	9.0
	Ratio	2.91	6.92	2.56
C-33A	DsRed	1181.7	3336.7	
	hSkn-1a	235.0	766.7	
	Ratio	5.03	4.35	
HaCaT	DsRed	66.7	71.0	126.0
	hSkn-1a	30.0	17.5	40.0
	Ratio	2.22	4.06	3.16
Alexander	DsRed	406.7	740.0	
	hSkn-1a	13.3	25.0	
	Ratio	30.50	29.60	
HEK293	DsRed	66133.3	46333.3	74333.3
	hSkn-1a	49800.0	45666.7	74333.3
	Ratio	1.33	1.01	1.00
COS-1	DsRed	1140.0	1446.7	3040.0
	hSkn-1a	226.7	256.7	885.0
	Ratio	5.03	5.64	3.44

Cells were transfected with pCMV-hSkn-1a/IRESneo, a plasmid encoding hSkn-1a and neomycine resistance gene, or pCMV-DsRed/IRESneo, a plasmid encoding DsRed and neomycine resistance gene. Colonies formed with G418-resistant cells were scored. Colony counts per 1 μg plasmid in two or three independent experiments were presented with ratios of the counts obtained by pCMV/IRESneo to those obtained by pCMV-DsRed/IRESneo

fragmented DNA was also present in total DNA extracted from the cells infected with Ad/hSkn-1a (data not presented).

Colony inhibition assay for cervical cancer cell lines transfected with a plasmid expressing hSkn-1a

The effects of hSkn-1a on the growth of the cervical cancer cell lines, HeLa S3, SiHa, CaSki, and C-33A, were evaluated by colony formation of cells transfected with pCMV-hSkn-1a/IRESneo (a plasmid expressing both hSkn-1a and neomycin resistance gene) or pCMV-DsRed/IRESneo (a plasmid expressing DsRed1 and neomycin resistance gene). For comparison, HaCaT (immortalized human keratinocytes), Alexander, HEK293 (transformed human fibroblast), and COS-1 (transformed monkey epithelial cell) were included. Similar to the other cell lines, the steady-state hSkn-1a was not detectable in extracts from HaCaT and COS-1 cells by immunoblotting. Owing to the extremely low transfection efficiency, HepG2 was not included in the experiment. The expression of hSkn-1a or DsRed in the cells transfected with pCMV-hSkn-1a/IRESneo or pCMV-DsRed/IRESneo was confirmed by immunoblotting or fluorescence microscopy, respectively (data not presented).

Table 1 summarizes the numbers of G418-resistant colonies of the cell lines and Figure 7 shows representative cultures with the drug-resistant colonies. All the cell lines derived from cervical cancers, HeLa S3, SiHa, CaSki, and C-33A, were found to be sensitive to colony inhibition by pCMV-hSkn-1a/IRESneo. The drug-resistant colonies formed after transfection with pCMV-hSkn-1a/IRESneo were much fewer and sometimes smaller than those formed after transfection with pCMV-DsRed/IRESneo. For example, diameters of great majority of HeLa S3 colonies with pCMV-hSkn-1a/IRESneo were less than 1 mm, although those with pCMV-DsRed/IRESneo were in the range from 1 to 3 mm. Besides the cervical cancer lines, HaCaT, an immortalized keratinocyte line, was found to be sensitive to the colony inhibition.

Whereas HEK293, an adenovirus E1A-transformed kidney cell line, was insensitive, Alexander, a hepatocellular carcinoma cell line, and COS-1, an SV40-transformed monkey kidney cell line, were sensitive to

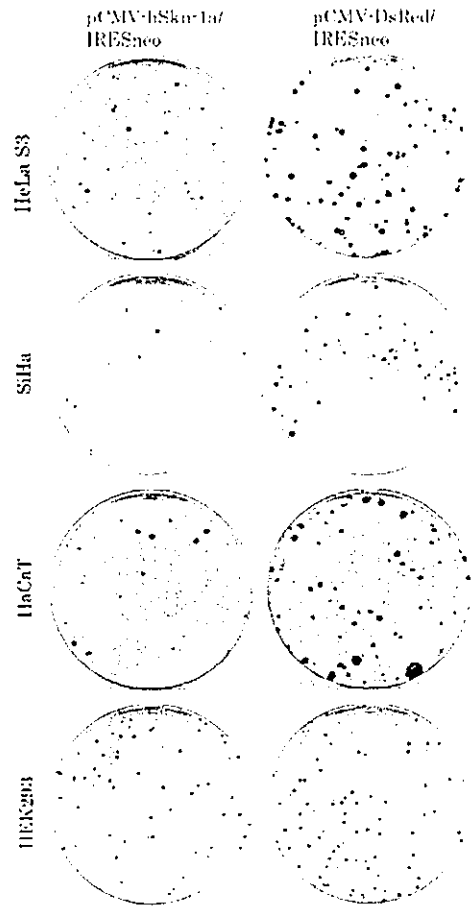

Figure 7 G418-resistant colonies formed by the cells after transfection with pCMV-hSkn-1a/IRESneo or pCMV-DsRed/IRESneo. Cells transfected with the pCMV-hSkn-1a/IRESneo or pCMV-DsRed/IRESneo (plasmids expressing hSkn-1a or DsRed1 along with neomycin resistance gene, respectively) were replated at an appropriate concentration in a 100 mm dish and cultured in the presence of G418. After 2–3 weeks, selected colonies were fixed and stained

the expression of hSkn-1a in the colony inhibition assay. The results suggest that the keratinocyte origin may not be an absolute requirement for the sensitivity to hSkn-1a.

Discussion

We were interested in the effect of hSkn-1a on cervical cancer cell lines, because hSkn-1a is a keratinocyte-specific transcription factor regulating differentiation, and because cervical cancer cells, whose differentiation capability is thought to be interrupted, are presumably of keratinocyte origin. The expression of hSkn-1a was not detectable in cervical cancer cells tested, being consistent with the previous observation that the level of hSkn-1a decreased with the progression of CIN from grades I–III (Hietala *et al*., 1997).

We prepared a HeLa clone that contains an inducible hSkn-1a gene (HeLa/hSkn-1a) and characterized the cells after the induction. HeLa/hSkn-1a expressed hSkn-1a in response to doxycycline added to the culture medium. The induction of hSkn-1a in HeLa/hSkn-1a resulted in the expression of K10 in almost all the cells in a culture (Figure 2a), indicating that hSkn-1a allowed the cells to resume the differentiation pathway leading to K10 expression. In addition, 3–6 days later, some flat cells with lowered DNA synthesis appeared in the culture (Figure 3), and fragmented DNA was detected (Figure 4). Most of the cells expressing hSkn-1a grew slowly with occasional production of the flat cells. DNA synthesis of these cells was reduced (Figure 3b). Thus, the slow growth of the cells and the occasional apoptosis strongly suggest that hSkn-1a triggers the terminal differentiation pathway leading to the growth arrest. This apparent slow process may have mimicked only partly, but reflect what occurs in nature in keratinocyte differentiation composed of multisteps starting from transient active cell growth to the final cell death. The observations with HeLa/hSkn-1a were extended to other cervical cancer cell lines. The results suggest that in cervical cancer cells hSkn-1a can trigger the initial step of differentiation.

The behaviors of the HeLa cells after induction of hSkn-1a were interpreted in this study as part of the differentiation, not as nonspecific toxicity of the protein, because lacZ alone did not cause such effects and because those behaviors were observed in the context of the partial differentiation as indicated by some upregulated genes in both the induced HeLa/hSkn-1a cells and the differentiating keratinocytes. We reported recently that transcriptions of hSkn-1a, K10, ARHH (a gene encoding a small GTP-binding protein of ras family) and Cx43 (a gene encoding a component of conexin) genes are induced in both the doxycycline-induced HeLa/hSkn-1a and the primary human keratinocytes that have started differentiation (Enomoto *et al*., 2003). This indicates that biochemical pathways for differentiation are working, at least partly, in the HeLa cells expressing hSkn-1a. On the basis of these observations, we interpreted that the morphological and biochemical changes observed in this study (Figures 3 and 4) are likely to result from the partial differentiation of the HeLa cells, although to rule out completely a possibility of nonspecific toxicity would require a hypothetical mutant hSkn-1a that cannot upregulate the K10 gene.

It is interesting that the emergence of flat cells and induction of apoptosis appear to have occurred only in a small number of HeLa/hSkn-1a cells out of many expressing hSkn-1a within a week after the induction (Figures 1c and d). The molecular mechanism controlling such an occasional phenotypic change is not clear at present. Part of the phenomenon resembles the unequal division of a stem cell, which produces one daughter stem cell and one differentiating cell in the first step of a differentiation. The level of hSkn-1a may be one of the possible factors involved in the regulation of the unique dividing.

When a HeLa/hSkn-1a culture was maintained in the presence of doxycycline, the level of hSkn-1a was lowered gradually and came down to a very low level especially after a week (Figures 1c and d). It is not likely that hSkn-1a directly inactivates the CMV promoter, from which the hSkn-1a gene is transcribed in HeLa/hSkn-1a. As is generally observed with the expression of a foreign gene integrated in cellular DNA, the cells inefficiently producing the foreign protein may be selected and come to constitute a major population after repeated cell divisions. If hSkn-1a, which is repressive on the cell growth, were cytotoxic to HeLa cells, those insensitive to or producing less hSkn-1a would have an advantage in surviving in the presence of doxycycline. We attempted to prepare a HeLa S3 clone stably expressing hSkn-1a, but could not (unpublished observation). The failure may be accounted for by the probable cytotoxicity of hSkn-1a for HeLa cells.

The experiments with transiently expressing hSkn-1a and the colony inhibition assay showed that hSkn-1a is suppressive on the growth (or triggers the resumption of partial differentiation) of the cervical cancer cell lines tested and an immortalized keratinocyte line, and also on the growth of the two liver carcinoma cell lines tested and monkey COS-1 cell line. This suggests that hSkn-1a is effective not only on those of keratinocyte origin but also on other types. Alexander is particularly interesting, in that, after the introduction of the hSkn-1a gene, it does not produce K10 mRNA but becomes susceptible to apoptosis and colony inhibition presumably caused by hSkn-1a. It is possible that the production of hSkn-1a is not directly linked to the terminal differentiation leading to apoptosis.

In view of the application to gene therapy for cancer, hSkn-1a appears to be moderate and not so dramatic in showing repressive effects on the cell growth. It is possible, however, that hSkn-1a, as a candidate for the therapeutic molecule in differentiation therapy, may be used in combination with other strategies, such as an antisense RNA strategy (Watanabe *et al.*, 1993). It is known that the growth of the cervical cancer cells harboring human papillomavirus DNA is repressed by an antisense RNA to the viral E6E7 genes. The combination of these strategies may be useful if an appropriate targeting vector is developed.

Materials and methods

Cells

Human cell lines, HeLa S3, SiHa, and CaSki (derived from cervical cancer), Alexander and HepG2 (from liver cancer), HEK293 (fibroblast transformed with adenovirus E1 gene), HaCaT (immortalized keratinocyte from skin), and a monkey epithelial cell line, COS-1, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA) with fetal calf serum (FCS) (10%). A human cell line, C-33A (derived from cervical cancer), was cultured in minimum essential medium (MEM) (Invitrogen Corp.) with FCS (10%). HeLa/Tet-On cells (BD Biosciences Clontech, Palo Alto, CA, USA) were cultured in DMEM with FCS (10%) and G418 (200 μg/ml). The cell cultures were incubated at 37°C in a humidified CO_2 incubator.

Construction of plasmids

The DNA fragment containing hSkn-1a coding region was excised from pGEM-T/hSkn-1a (Kukimoto and Kanda, 2001) by digestion at the multicloning site with *Eco*RI or with *Not*I. The *Eco*RI fragment was inserted into pIRESneo (BD Biosciences Clontech) and pTRE (BD Biosciences Clontech) at their *Eco*RI sites to generate pCMV-hSkn-1a/IRESneo and pTRE/hSkn-1a, respectively. The *Not*I fragment was inserted into pShuttle (BD Biosciences Clontech) at its *Not*I site to generate pShuttle/hSkn-1a. A *Not*I linker was inserted into pDsRed1-N1 (BD Biosciences Clontech) at the *Sma*I site, and then the DNA fragment containing DsRed1 coding region was excised by digestion with *Not*I and inserted into pIRESneo at the *Not*I site to generate pCMV-DsRed/IRESneo.

Transfection of DNA

Subconfluent culture of the cells in a 60 mm dish or 100 mm dish were transfected with 1 or 2 μg of plasmid DNA, respectively, using Effectene Transfection Reagent (QIAGEN GmbH, Hilden, Germany).

Preparation of a HeLa cell line that expresses hSkn-1a in the presence of doxycycline

The tetracycline-inducible expression system and the HeLa/Tet-On cells, which is a HeLa clone expressing reverse tetracycline-controlled transactivator, were purchased from BD Biosciences Clontech. HeLa/Tet-On (1×10^6) in a 100 mm culture plate were transfected with a mixture of *Sca*I-cleaved pTRE/hSkn-1a (2 μg) and *Hpa*I-cleaved pTK-Hyg, a plasmid expressing hygromycin resistance gene (BD Biosciences Clontech) (0.1 μg). The cells were cultured in the medium containing hygromycin (200 μg/ml), and 96 drug-resistant clones were obtained. Induction of hSkn-1a with doxycycline (10 μg/ml) was examined by immunoblotting with anti-Skn-1a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and a clone, designated as HeLa/hSkn-1a, which showed the lowest level of hSkn-1a without doxycycline and the highest with doxycycline was selected.

Construction of an adenovirus vector capable of expressing hSkn-1a

The E1/E3-deleted adenovirus vectors expressing hSkn-1a (Ad/hSkn-1a) or lacZ (Ad/lacZ) were generated according to the instruction of Adeno-X Expression System (BD Biosciences Clontech). Expression cassettes were excised from pShuttle/hSkn-1a and pShuttle/lacZ (BD Biosciences Clontech) by I-*Ceu*I and PI-*Sce*I digestion and inserted into pAdeno-X (BD Biosciences Clontech). The recombinant adenovirus genome excised from the backbone vector by digestion with *Pac*I was transfected to HEK293 using Effectene Transfection Reagent (QIAGEN GmbH). The recombinant virus propagated in HEK293 cells were obtained from the culture medium and cell lysate. The virus propagated in HEK293 inoculated with the first stock of the virus was purified by Adeno-X Virus Purification Kit (BD Biosciences Clontech) and used in the experiments. Virus infectivity was titerated by Adeno-X Rapid Titer Kit (BD Biosciences Clontech). hSkn-1a in cells infected with Ad/hSkn-1a was detected by immunohistochemistry using anti-Skn-1a antibody and LSAB2 Systems (DAKO Corp., Carpinteria, CA, USA). LacZ in cells infected with Ad/lacZ was detected by *In Situ*

β-galactosidase Staining Kit (Stratagene Cloning Systems, La Jolla, CA, USA).

Immunofluorescence staining

Cells grown in a Lab-Tek chamber slide (Nalge Nunc Internatioal Corp., Rochester, NY, USA) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. After washing with PBS, the slide was incubated with anti-Skn-1a antibody diluted at 1 to 200 in PBS containing 3% bovine serum albumin (BSA) for 30 min at room temperature. The slide was washed with PBS and incubated with AlexaFluor488-conjugated anti-rabbit IgG antibody (Molecular Probes, Inc., Eugene, OR, USA) diluted at 1 to 400 in 3% BSA/PBS for 30 min at room temperature. After washing with PBS, the slide was covered with a coverslip using Slow-Fade Antifade kit (Molecular Probes, Inc.) and examined under a UV microscope (Olympus Co Ltd., Tokyo, Japan).

Labeling with BrdU

For BrdU labeling study, HeLa/hSkn-1a grown in a Lab-Tek chamber slide were labeled in a medium containing 10 μM BrdU for 15 min at 37°C. The incorporated BrdU were visualized by immunohistochemistry using 5′-Bromo-2′-deoxy-uridine Labeling and Detection Kit II (Roche Diagnostics GmbH, Mannheim, Germany).

Detection of apoptosis

Apoptotic DNA ladder formation of HeLa/hSkn-1a was examined by agarose-gel electrophoresis. Total DNA of cells were extracted by QIAamp DNA Blood Mini Kit (QIAGEN GmbH) and assayed by LM-PCR Ladder Assay Kit (BD Biosciences Clontech).

DNA fragmentation of cells infected with Ad/hSkn-1a or Ad/lacZ was examined by TUNEL. For TUNEL assay, cells grown in a Lab-Tek chamber slide (Nalge Nunc International Corp.) were stained using *In Situ* Cell Death Detection Kit, Fluoroscein (Roche Diagnostics GmbH, Germany).

Immunoblotting

Cells were suspended in 1 × RIPA buffer containing proteinase inhibitors (Complete Mini, Roche Diagnostics GmbH, Germany), sonicated briefly, centrifuged at 15000 *g* for 30 min, and the supernatant was collected. Protein concentration of samples were measured by BCA-200 Protein Assay Kit (Pierce Chemical Company, Rockford, IL, USA). Samples were electrophoresed on 10% SDS–polyacrylamide gel and proteins were transferred onto Hybond-P membranes (Amersham Biosciences Corp., Piscataway, NJ, USA). Following incubation in blocking buffer (PBS with 0.1% Tween-20 and 5% skim milk), membranes were incubated with anti-Skn-1a antibody (1 : 1000) or anti-keratin 10 mouse monoclonal antibody (DAKO Corp.) (1 : 500) in blocking buffer for 1 h at room temperature. After washing six times using PBS with 0.1% Tween-20 (PBST), membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG goat antibody (Santa Cruz Biotechnology, Inc.) (1 : 5000) or anti-mouse IgG goat antibody (Santa Cruz Biotechnology, Inc.) (1 : 5000) in blocking buffer for 1 h at room temperature. The membranes were washed six times in PBST, and immunoreactive proteins were visualized by ECL-plus Western Blotting Detection Kit (Amersham Biosciences Corp.).

Quantitative RT–PCR

Total RNA was extracted from the cells 2 days after infection with Ad/lacZ or Ad/hSkn-1a using RNeasy Mini Kit (QIAGEN GmbH), followed by cDNA synthesis with Taqman Gold RT–PCR Kit (Applied Biosystems, Foster City, CA, USA) using random hexamers as primers. cDNAs specific for K10 or GAPDH were quantified by real-time PCR by ABI PRISM 7700 Sequence Detector (Applied Biosystems) using QuantiTect SYBR Green PCR Kit (QIAGEN GmbH). Specificity of the reaction was verified by dissociation curve analysis and agarose-gel electrophoresis of PCR products.

Colony inhibition assay

pCMV-hSkn-1a/IRESneo and pCMV-DsRed/IRESneo were linearized by *Pvu*I digestion and purified by phenol–chloroform extraction and ethanol precipitation. At 1 day before transfection, 5×10^5 or 1×10^6 cells were seeded in a 60 mm or 100 mm culture dish, respectively. The cells in the 60 mm dish or those in the 100 mm dish were transfected with 1 or 2 μg of the linearized plasmid, respectively. After 24 h, the transfected cultures were replated at a split ratio giving countable colonies per dish at the time of reading. The cells were cultured in the growth medium containing G418 (800 μg/ml for Alexander, 2000 μg/ml for C-33A, and 400 μg/ml for the other cells) with refeeding every 3 days for 2–3 weeks. Then, the cultures were fixed with formalin and stained by crystal violet for colony scoring.

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Intramembrane Proteolysis and Endoplasmic Reticulum Retention of Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein is suggested to localize to the endoplasmic reticulum (ER) through a C-terminal hydrophobic region that acts as a membrane anchor for core protein and as a signal sequence for E1 protein. The signal sequence of core protein is further processed by signal peptide peptidase (SPP). We examined the regions of core protein responsible for ER retention and processing by SPP. Analysis of the intracellular localization of deletion mutants of HCV core protein revealed that not only the C-terminal signal-anchor sequence but also an upstream hydrophobic region from amino acid 128 to 151 is required for ER retention of core protein. Precise mutation analyses indicated that replacement of Leu^{139}, Val^{140}, and Leu^{144} of core protein by Ala inhibited processing by SPP, but cleavage at the core-E1 junction by signal peptidase was maintained. Additionally, the processed E1 protein was translocated into the ER and glycosylated with high-mannose oligosaccharides. Core protein derived from the mutants was translocated into the nucleus in spite of the presence of the unprocessed C-terminal signal-anchor sequence. Although the direct association of core protein with a wild-type SPP was not observed, expression of a loss-of-function SPP mutant inhibited cleavage of the signal sequence by SPP and coimmunoprecipitation with unprocessed core protein. These results indicate that Leu^{139}, Val^{140}, and Leu^{144} in core protein play crucial roles in the ER retention and SPP cleavage of HCV core protein.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (5, 19) and has been estimated to infect more than 170 million people throughout the world (15). Symptoms of persistent HCV infection extend from chronic hepatitis to cirrhosis and finally to hepatocellular carcinoma (18, 42). HCV belongs to the genus *Hepacivirus* in the family *Flaviviridae* and possesses a viral genome consisting of a single, positive-strand RNA with a nucleotide length of about 9.4 kb (6, 48). The genome encodes a large precursor polyprotein of approximately 3,000 amino acids (6, 17). The polyprotein is processed co- and posttranslationally into at least 10 viral proteins by host and viral proteases (2, 6, 10, 45). The structural proteins of HCV are located in the N-terminal one-fourth of the polyprotein and are cleaved by host membrane proteases (10, 44). Comparison with other flaviviruses suggests that HCV core protein forms the nucleocapsid, which is surrounded by the envelope containing glycoproteins E1 and E2 (6, 48). Functional analyses suggest that HCV core protein has regulatory roles in host cellular functions. In tissue culture systems, HCV core protein regulates signaling pathways and modulates apoptosis (4, 29, 40, 41, 46, 54, 55). Moreover, transgenic mice expressing HCV core protein developed liver steatosis and thereafter hepatocellular carcinoma (34, 36). Thus, it has been suggested that HCV core protein is a multifunctional molecule that acts as a structural protein but is also involved in the pathogenesis of hepatitis C. HCV core protein has two major forms, p23 and p21 (16, 25, 31, 43, 53). HCV core protein p23 represents a 191-amino-acid product in which the C-terminal hydrophobic region also acts as a signal sequence for E1. HCV polyprotein is cleaved between residues 191 and 192 by host signal peptidase to generate C-terminal and N-terminal polypeptides encompassing the core and E1 proteins, respectively. For the full maturation of HCV core protein, the C-terminal signal-anchor sequence was thought to be further processed by an unidentified microsomal protease (25, 30, 31, 43, 53), and the 21-kDa isoform of core protein is predominantly detected both in cultured cells by transfection with expression plasmid and in viral particles obtained from sera of patients with hepatitis C (53). These results suggest that p21 is the mature form of HCV core protein (53). Immunostaining revealed that most HCV core protein is distributed diffusely throughout the cell, probably in the endoplasmic reticulum (ER) (31, 53). However, a minor population was observed in the nucleus (53).

Recently, a presenilin-related aspartic protease, signal peptide peptidase (SPP), was identified (50). SPP is located in the ER membrane and promotes intramembrane proteolysis of signal peptides. The chemical compound $(Z\text{-}LL)_2$-keton inhibits processing of signal peptides by SPP, and it was shown to suppress intramembrane proteolysis of major histocompatibility complex class I molecules, preprolactin, HCV core protein, and others (21, 30, 51). Replacement of Asp^{265} with Ala in SPP resulted in a loss of catalytic function, although this mutant could bind to TBL_4K, a derivative of $(Z\text{-}LL)_2$-keton (50). HLA-A was processed into yeast microsomes following the addition of wild-type SPP but not mutant SPP, suggesting that SPP interacts with HLA-A (50). Processing of the signal sequence of HCV core protein by SPP was inhibited by the

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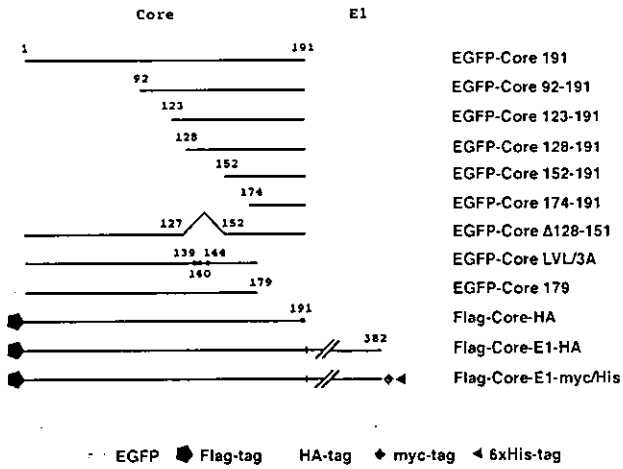

FIG. 1. Expression plasmids used in this study. The genes encoding HCV proteins and their mutants were cloned into pcDNA3.1FlagHA, pcDNA3.1/*myc*-His C, or pEGFP-C3 as described in Materials and Methods. Other plasmids are described in the text or in the other figure legends.

addition of $(Z\text{-}LL)_2$-keton, and Ser^{183} and Cys^{184} in the signal sequence of core protein were demonstrated to be important for flexibility and intramembrane proteolysis by SPP (23). Signal sequences generally have a tripartite structure, including a central hydrophobic H region and hydrophilic N- and C-terminal flanking regions (28). SPP recognizes the N- and C-terminal regions and cleaves in the middle of the H region (28). Mutational analyses suggested that the flexibility of signal peptides is generally required for substrate recognition of SPP (23). SPP contains the aspartic protease motifs YD and LGLGD, which are located in the predicted transmembrane region, and it is thought to cleave type II (N terminus in the cytosol and C terminus in the lumen)-oriented substrates (50). However, the effect of the cytoplasmic region of type II membrane substrates on intramembrane proteolysis by SPP is not known. In this study, we examined the regions of HCV core protein that are essential for ER retention and intramembrane cleavage by SPP.

MATERIALS AND METHODS

Plasmids. For expression of enhanced green fluorescence protein (EGFP)-fused HCV core proteins in culture cells, the core protein-coding region was amplified by PCR from cDNA encoding full-length HCV polyprotein type 1b (1). The PCR products were subcloned into SalI and BamHI sites 3′ of the EGFP-coding region of pEGFP-C3 (Clontech, Palo Alto, Calif.). The cDNA fragments encoding amino acids 1 to 191, 1 to 179, 92 to 191, 123 to 191, 128 to 191, 152 to 191, and 174 to 191 of HCV core proteins were amplified by PCR and then introduced into pEGFP-C3; these constructs are designated EGFP-Core 191, EGFP-Core 179, EGFP-Core 123-191, EGFP-Core 128-191, EGFP-Core 152-191, and EGFP-Core 174-191, respectively. The genes encoding core proteins with the region between amino acids 128 and 151 deleted and replacement of Leu^{139}, Val^{140}, and Leu^{144} with Ala were generated by the method of splicing by overlap extension (11, 14, 49) and introduced into pEGFP-C3; these constructs are designated EGFP-Core Δ128-151 and EGFP-Core LVL/3A, respectively (Fig. 1).

Fragments encoding Flag and hemagglutinin (HA) tags were inserted at both ends of the multicloning site of pcDNA3.1 (pcDNA3.1FlagHA). PCR products encoding either HCV core protein alone, core protein followed by E1 (Core-E1), or their mutants were cloned into pcDNA3.1FlagHA, resulting in plasmids encoding recombinant proteins sharing Flag and HA tags at the N and C termini, respectively (Fig. 1). In Flag-Core-HA and its derived mutants, Ala^{191} was replaced by Arg to avoid processing by signal peptidase for determination of cleavage by SPP, as previously shown for the processing of the E1-E2 junction (7). In addition, the region encoding Flag-Core-E1 or its mutants was cleaved from pcDNA3.1FlagHA constructs and then introduced between the SacI and XhoI sites of pcDNA3.1/*myc*-His C (Invitrogen Corp., Carlsbad, Calif.). The resulting plasmids encode HCV proteins sharing Flag and myc/His epitopes at the N and C termini, respectively (Fig. 1). Genes encoding core protein with a single amino acid (Leu^{139}, Val^{140}, or Leu^{144}), double amino acids (Leu^{139} and Val^{140}, Leu^{139} and Leu^{144}, or Val^{140} and Leu^{144}), or triple amino acids (Leu^{139}, Val^{140}, and Leu^{144}) replaced with Ala were generated by splicing by overlap extension and introduced into pcDNA3.1FlagHA and pcDNA3.1/*myc*-His C (Fig. 1; see Fig. 4).

The genes encoding the ER-targeting and ER retrieval sequences of calreticulin fused with DsRed at the N and C termini, respectively (8, 37, 39), were inserted between the EcoRV and XbaI sites of pcDNA3.1 (pcDNA ER-DsRed) to visualize the ER in culture cells. This recombinant protein is designated ER-DsRed in this study.

Cloning of SPP. The cDNA encoding SPP was amplified from human liver mRNA (Clontech) by reverse transcription-PCR and cloned into T-vector prepared from pBluescript II SK(−) (27). The gene encoding SPP with an attached HA tag and ER retrieval signal, KEKK, at the C terminus (SPP-HAER) was cloned into pcDNA3.1 to eliminate the possibility that the HA tag suppresses the endogenous ER retrieval signal of SPP. SPP-HAER was colocalized with ER-DsRed on the ER membrane and glycosylated upon transfection into cells (data not shown).

Subcellular localization of wild-type and mutant HCV core proteins. HeLa cells were maintained in the Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HeLa cells were seeded on an eight-well chamber slide at 2×10^4 cells per well 24 h before transfection. The cells were transfected with the various plasmids by lipofection with Lipofectamine 2000 (Invitrogen). To determine protein subcellular localizations, transfected cells were fixed with phosphate-buffered saline (PBS) containing 3% paraformaldehyde at 18 h post-transfection and then observed with a confocal laser-scanning microscope (Bio-Rad, Tokyo, Japan). To confirm subcellular localization of the core proteins, transfected cells were fractionated with a subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany). Stepwise extraction resulted in four distinct fractions, which contain mainly cytosolic, membrane-organelle, nuclear, and cytoskelton proteins, respectively. Each fraction was precipitated with trichloroacetic acid and analyzed by immunoblotting, and the densities of the bands were measured with Multi Gauge version 2.2 (Fujifilm, Tokyo, Japan).

Immunoblotting. After transfection, 293T cells were harvested, washed twice with PBS, and lysed in 20 mM Tris-HCl (pH 7.4) containing 135 mM NaCl, 1% Triton X-100, and 10% glycerol (lysis buffer) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 1 mM Na_2VO_3. The lysate was centrifuged at 6,500 × *g* for 5 min at 4°C. The resulting supernatants were subjected to sodium dodecyl sulfate (SDS)–13.5% polyacrylamide gel electrophoresis. The separated proteins were electroblotted onto a Hybond-P polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, N.J.). These membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 (Sigma, St. Louis, Mo.) and incubated with mouse monoclonal anti-Flag M2 (Sigma), anti-HA 16B12 (HA.11; BabCO, Richmond, Calif.), or monoclonal mouse anti-His_6-AD1.1.10 (Genzyme/Techne, Tokyo, Japan) immunoglobulin G (IgG) at room temperature for 30 min and then with horseradish peroxidase-conjugated anti-mouse IgG antibody at room temperature for 30 min. Immunoreactive bands were visualized by using the enhanced chemiluminescence Super Signal West Femto substrate (Pierce, Rockford, Ill.).

Immunoprecipitation. Immunoprecipitation analysis was carried out as described previously (32). Plasmids were transfected into 293T cells by lipofection. Transfected cells were harvested at 18 h posttransfection and lysed in lysis buffer with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid (CHAPSO) (Dojindo, Kumamoto, Japan). Cell lysates were incubated with monoclonal anti-HA, anti-Glu-Glu (anti-EE) (BabCO), or anti-Flag antibody at 4°C for 1.5 h and then with protein G–Sepharose CL-4B (Amersham Bioscience) at 4°C for 1.5 h. After centrifugation at 6,500 × *g* for 3 min at 4°C, the pellets were washed five times with lysis buffer. Immunoprecipitates were subjected to immunoblotting.

Deglycosylation. Plasmids encoding core and E1 proteins were transfected into 293T cells by lipofection, and cell lysates were immunoprecipitated with anti-HA antibody at 18 h posttransfection. Immunoprecipitates were eluted from protein G–Sepharose CL-4B in 0.5% SDS and 1% 2-mercaptoethanol and digested with endo-β-*N*-acetylglucosaminidase H (Endo H) or peptide-*N*-glycosidase F

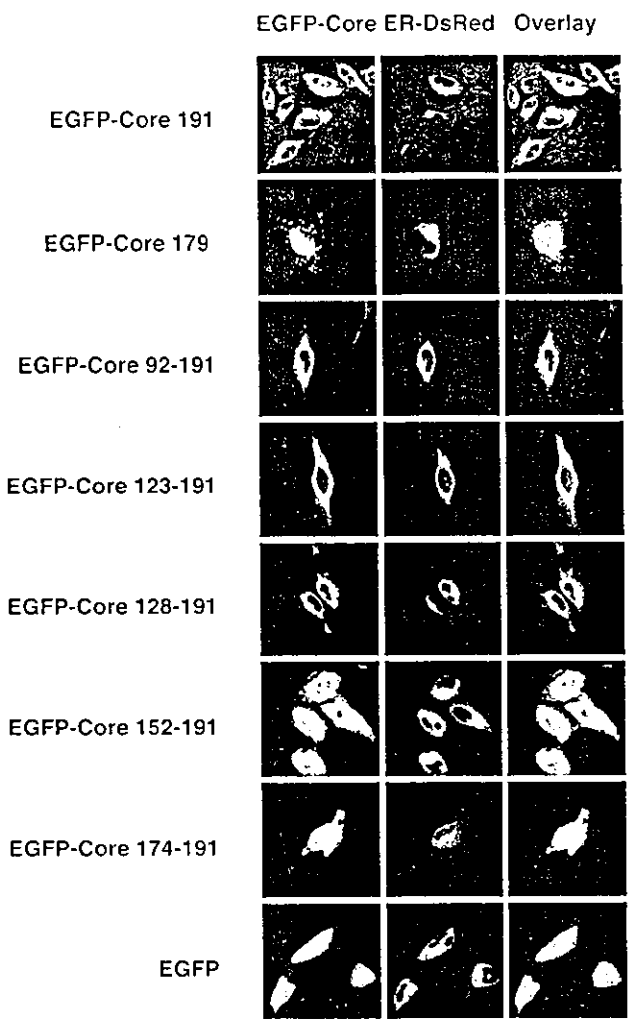

FIG. 2. Intracellular localization of EGFP-Core mutants. EGFP-Core and its deletion mutants were coexpressed with ER-DsRed in HeLa cells, and the localization of core proteins was examined by confocal microscopy.

(PNGase F) according to the protocol of the manufacturer (Roche, Mannheim, Germany). The resulting mixtures were subjected to immunoblotting.

RESULTS

Region required for ER retention of HCV core protein. To determine the regions within HCV core protein that are responsible for ER retention, EGFP-fused, N-terminally truncated HCV core protein (Fig. 1) was coexpressed with the ER marker ER-DsRed. EGFP-Core 191 colocalized with ER-DsRed to the ER (Fig. 2), whereas EGFP-Core 179 was localized primarily to the nucleus as reported previously (3, 33, 43, 47), suggesting that the C-terminal signal sequence is essential for anchoring HCV core protein to the ER membrane. However, EGFP-Core 174-191 exhibited diffuse staining similar to that of EGFP, suggesting that the signal sequence alone is not sufficient for ER localization. EGFP-Core 92-191, EGFP-Core 123-191, and EGFP-Core 128-191 were colocalized with ER-DsRed in the ER, but EGFP-Core 152-191 stained similarly to EGFP-Core 174-191 and EGFP. These data suggest that not only the C-terminal signal sequence but also the region from amino acids 128 to 151 is required for ER retention of HCV core protein.

Region essential for processing of the signal sequence of HCV core protein by SPP and signal peptidase. Based on hydrophobicity and a cluster of basic amino acids, HCV core protein was proposed to possess three regions (domains 1 to 3) (Fig. 3A, upper panel) by Hope and McLauchlan (12). To assess the involvement of the region encompassing amino acids 128 to 151 in proteolysis of the signal sequence of HCV core protein by signal peptidase and SPP, three hydrophobic amino acids, Leu^{139}, Val^{140}, and Leu^{144}, in the most hydrophobic peak in domain 2 were replaced with Ala to reduce hydrophobicity, and Ala^{191} was replaced with Arg to eliminate processing by signal peptidase (Fig. 3A, lower panel). When a wild-type Flag-Core-HA construct was expressed in 293T cells, a single band of 23 kDa was detected by blotting with anti-Flag, but not with anti-HA, suggesting that the HA-fused signal sequence was properly processed by SPP and that Flag-core protein of 23 kDa was generated (Fig. 3B, lanes 2 and 11). In cells expressing the substitution mutants, 25- and 23-kDa bands were detected by the anti-Flag antibody (Fig. 3B, upper panel, lanes 3 to 9) and 25-kDa bands were detected by the anti-HA antibody (Fig. 3B, lower panel, lanes 3 to 9), indicating that the 25- and 23-kDa bands correspond to core proteins that are unprocessed and processed by SPP, respectively. Cleavability of the signal sequence of mutant core proteins by SPP was suppressed in accordance with the number of substitutions, and almost no processing of the signal sequence was observed in cells expressing Flag-Core LVL/3A-HA, which has three amino acid substitutions (Fig. 3B, lane 9). These results indicate that Leu^{139}, Val^{140}, and Leu^{144} play crucial roles in the processing of the signal-anchor of HCV core protein by SPP. Furthermore, deletion of the hydrophobic region including amino acids 128 to 151 from HCV core protein completely eliminated processing by SPP, and this species was seen only as a single band of 23.5 kDa which was detected by both the anti-Flag and anti-HA antibodies (Fig. 3B, lane 10). Taken together with the observation that Ala^{180}, Ser^{183}, and Cys^{184} in the signal sequence of HCV core protein of the type 1a Glasgow strain were demonstrated to be essential for SPP proteolysis (13, 23), these results indicate that the hydrophobic region from amino acid 139 to 144 in domain 2 of HCV core protein also participates in the processing of the signal sequence by SPP.

To examine the role of the region from amino acid 139 to 144 in the cleavage of the HCV core protein signal sequence by signal peptidase and SPP in more detail, substitutions of Leu^{139}, Val^{140}, and/or Leu^{144} with Ala were introduced into the Flag-Core-E1-HA polyprotein (Fig. 1). Flag-Core-E1-HA protein was cleaved to the expected molecular mass of 23 kDa of Flag-Core protein by signal peptidase and SPP (Fig. 3C, lanes 2 and 11), whereas slightly larger bands corresponding to a core protein unprocessed by SPP were detected in cells expressing polyproteins possessing mutations within amino acids 139 to 144 (Fig. 3C, lanes 3 to 9). A lack of processing by SPP was detected mainly in core proteins containing double amino acid changes of Leu^{139}, Val^{140}, and/or Leu^{144} to Ala

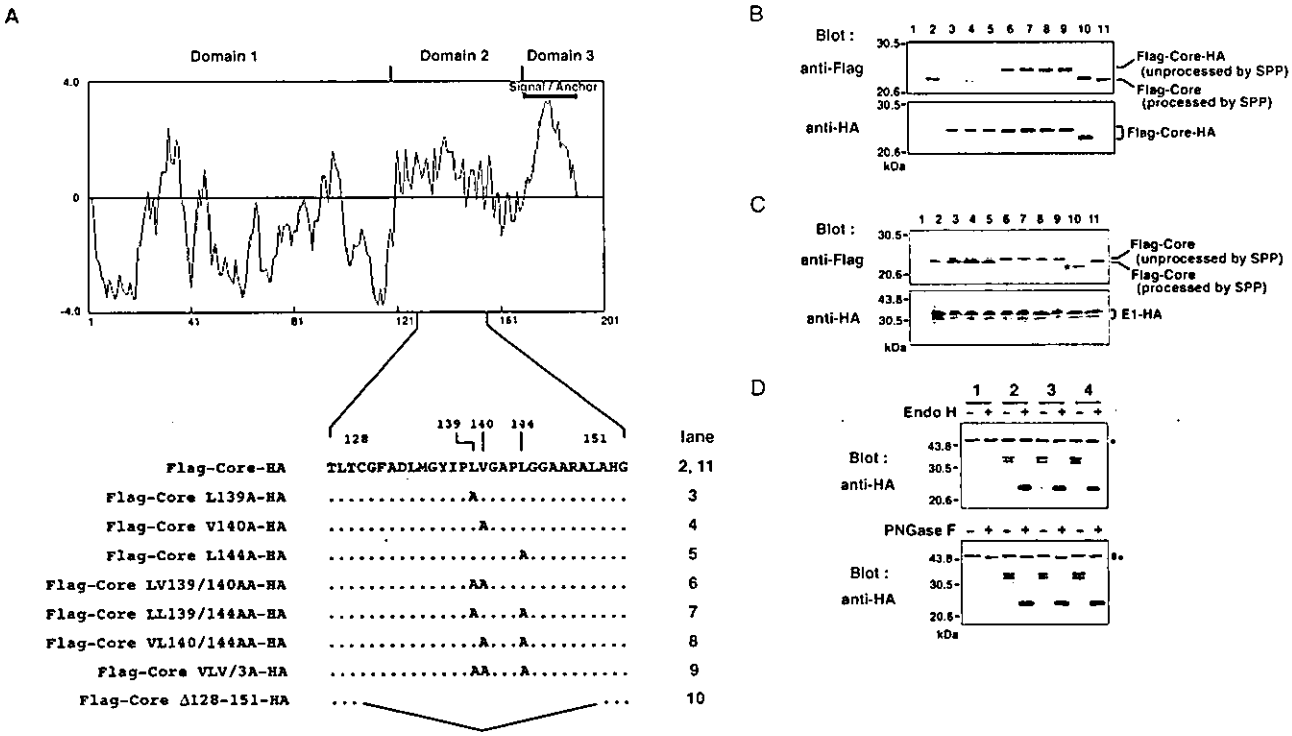

FIG. 3. Identification of the region responsible for processing of the signal sequence of HCV core protein by SPP and signal peptidase. (A) The hydrophobicity profile of HCV core protein was predicted by the method of Kyte and Doolittle (20). Hope and McLauchlan separated the HCV core protein into three regions, domains 1 to 3 (12). Two hydrophobic regions are predicted in the regions from amino acid 128 to 151 and from amino acid 164 to 186 in the C terminus of the HCV core protein. Mutations and deletions in the region from amino acid 128 to 151 of Flag-Core-HA and Flag-Core-E1-HA constructs are indicated. Dots indicate unchanged amino acids. (B) Expression of Flag-Core-HA polyproteins with changes of Ala^{191} to Arg in 293T cells. Flag-Core-HA (lanes 2 and 11), Flag-Core L139A-HA (lane 3), Flag-Core V140A-HA (lane 4), Flag-Core L144A-HA (lane 5), Flag-Core LV139/140AA-HA (lane 6), Flag-Core LL139/144AA-HA (lane 7), Flag-Core VL140/144AA-HA (lane 8), Flag-Core LVL/3A-HA (lane 9), and Flag-Core Δ128-151-HA (lane 10) were analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. Cells transfected with an empty plasmid were used as a negative control (lane 1). (C) Expression of Flag-Core-E1-HA mutants in 293T cells. Flag-Core-E1-HA (lanes 2 and 11), Flag-Core L139A-E1-HA (lane 3), Flag-Core V140A-E1-HA (lane 4), Flag-Core L144A-E1-HA (lane 5), Flag-Core LV139/140AA-E1-HA (lane 6), Flag-Core LL139/144AA-E1-HA (lane 7), Flag-Core VL140/144AA-E1-HA (lane 8), Flag-Core LVL/3A-E1-HA (lane 9), and Flag-Core Δ128-151-E1-HA (lane 10) were analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. The asterisk indicates unprocessed Flag-Core Δ128-151. Cells transfected with an empty plasmid were used as a negative control (lane 1). (D) The deglycosylation procedure is described in Materials and Methods. After transfection, cell lysates were immunoprecipitated with anti-HA antibody and immunoprecipitates were digested with Endo H (upper panel) or PNGase F (lower panel). Following digestion, proteins were separated by SDS-polyacrylamide gel electrophoresis, and material from cells transfected with vector (lane 1), Flag-Core-E1-HA (lane 2), Flag-Core LVL/3A-E1-HA (lane 3), and Flag-Core Δ128-151-E1-HA (lane 4) was detected by blotting with anti-HA. Nontreated and Endo H- or PNGase F-treated samples are indicated by − and +, respectively. Asterisks indicate mouse IgG heavy chains.

(Fig. 3C, lanes 6 to 8), and only an unprocessed band was detected in a triple amino acid substitution mutant (Fig. 3C, lane 9) and a deletion mutant lacking amino acids 128 to 151 (Fig. 3C, lane 10). In contrast to the processing of core protein, E1 protein processed from the mutant polyproteins exhibited the same molecular mass of 32 to 35 kDa and the same deglycosylation patterns following digestion with Endo H or PNGase F (Fig. 3D). These results indicate that the internal hydrophobic region from amino acid 139 to 144 of HCV core protein is essential for processing by SPP but not for cleavage of the core-E1 junction by signal peptidase and the subsequent translocation of E1 protein into the ER. It was suggested that signal peptides must be liberated from the precursor protein by cleavage with signal peptidase in order for them to become substrates for SPP (23). Our data indicate that processing by SPP is not a prerequisite for cleavage of the core-E1 junction by signal peptidase.

Amino acid sequence essential for SPP cleavage of the signal sequences of HCV core proteins of genotypes 1a and 1b. Martoglio and colleagues reported that HCV core protein is processed by SPP after cleavage by host signal peptidase and that Ala^{180}, Ser^{183}, and Cys^{184} residues in the signal sequence of HCV core protein of type 1a Glasgow strain are essential for SPP proteolysis, as they maintain the structure of the breaking α-helix (23, 30). To determine the amino acids essential for SPP cleavage of the signal sequence of type 1b HCV core protein, Flag-Core-E1-HA and its substitution mutants were expressed in 293T cells (Fig. 4). Mutation of one, two, or three amino acids, except for Flag-Core IF176/177AL-E1-HA (Fig. 4B, lane 9), did not affect the processing of the core protein signal sequence. Flag-Core IF176/177AL-E1-HA exhibited the same molecular size as Flag-Core LVL/3A-E1-HA (Fig. 4B, lane 2), suggesting that Ile^{176} and Phe^{177} in the signal sequence of core protein are essential for cleavage by SPP in our system.

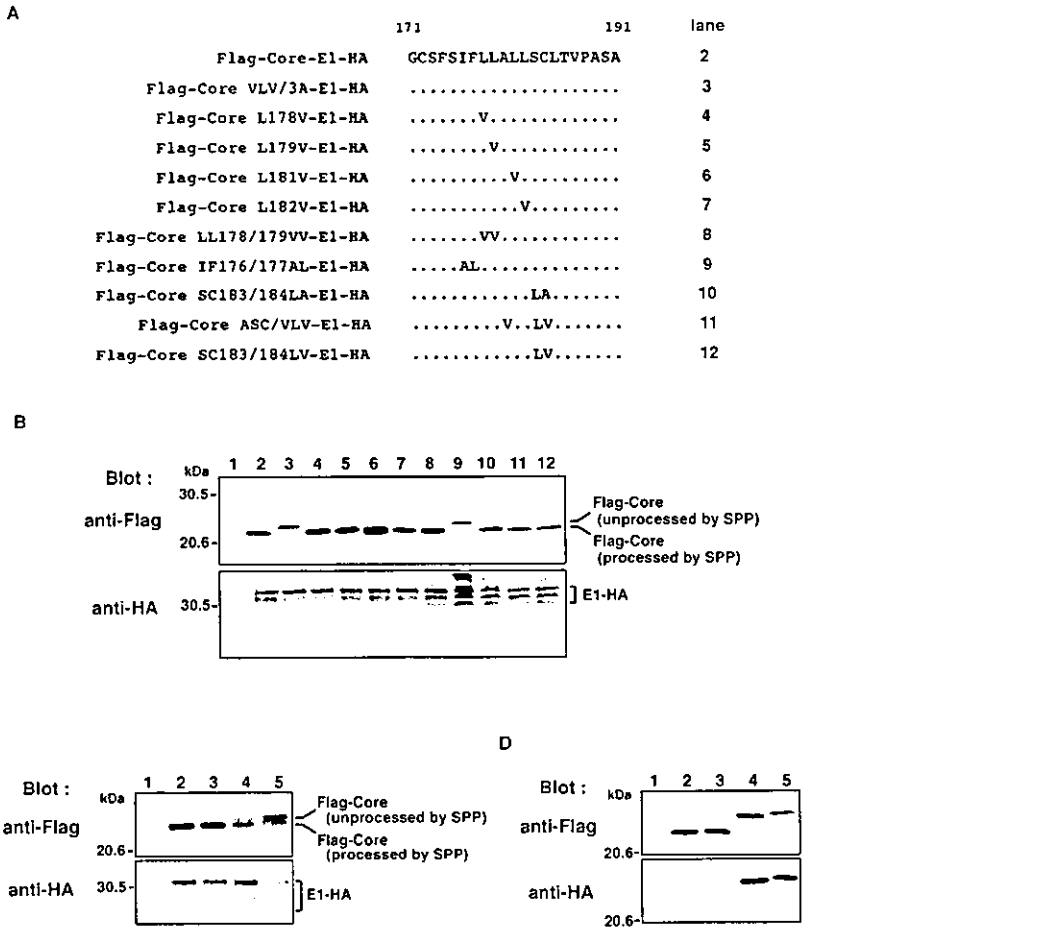

FIG. 4. Amino acid residues essential for SPP cleavage of the HCV core protein signal sequence of genotype 1a and 1b strains. (A) Mutations in the amino acid residues in the signal sequence of Flag-Core-E1-HA are indicated. Dots indicate unchanged amino acids. (B) Flag-Core-E1-HA (lane 2), Flag-Core LVL/3A-E1-HA (lane 3), Flag-Core L178V-E1-HA (lane 4), Flag-Core L179V-E1-HA (lane 5), Flag-Core L181V-E1-HA (lane 6), Flag-Core L182V-E1-HA (lane 7), Flag-Core LL178/179VV-E1-HA (lane 8), Flag-Core IF176/177AL-E1-HA (lane 9), Flag-Core SC183/184LA-E1-HA (lane 10), Flag-Core ASC/VLV-E1-HA (lane 11), or Flag-Core SC183/184LV-E1-HA (lane 12) was expressed in 293T cells. (C) The gene encoding core and E1 polyprotein of the genotype 1a H77c strain of HCV was introduced into pcDNA3.1FlagHA. Flag-H77c Core-E1-HA (lane 2), Flag-H77c Core ASC/VLV-E1-HA (lane 3), Flag-H77c Core LVL/3A-E1-HA (lane 4), or Flag-H77c Core IF176/177AL-E1-HA (lane 5) was expressed in BHK cells. Cell lysates were analyzed by immunoblotting with anti-Flag (upper panel) and anti-HA (lower panel) antibodies. (D) Expression of Flag-Core 191-HA mutants in 293T cells. The gene encoding core protein with a change of Ala^{191} to Arg was introduced into pcDNA3.1FlagHA. Flag-Core-HA (lane 2), Flag-Core ASC/VLV-HA (lane 3), Flag-Core LVL/3A-HA (lane 4), and Flag-Core IF176/177AL-HA (lane 5) were analyzed by immunoblotting with anti-Flag (upper panel) and anti-HA (lower panel) antibodies. Cells transfected with an empty plasmid were used as a negative control (lanes 1 in panels B, C, and D).

However, the triple amino acid substitution (Ala^{180}, Ser^{183}, and Cys^{184}) in the type 1b J1strain (Flag-Core ASC/LVL-E1-HA) (Fig. 4B, lane 11), which is the same as the spmt mutant of the type 1a Glasgow strain (23, 30), did not affect the processing of the signal sequence of HCV core protein by SPP. All derived E1 proteins exhibited a molecular mass of 32 to 35 kDa irrespective of the presence of mutations, and deglycosylation by digestion with endoglycosidases generated uniform 22-kDa bands of E1 proteins (data not shown). These results indicate that Ile^{176} and Phe^{177}, but not Ala^{180}, Ser^{183}, and Cys^{184}, in the signal sequence of type 1b HCV core protein are essential for processing by SPP and confirm that processing of signal sequence by SPP is not required for cleavage by signal peptidase and translocation of E1 protein into the ER. To determine whether the difference in cleavage of signal sequence depends on the genotype of HCV, Ala^{180}, Ser^{183}, and Cys^{184} in the HCV core protein of the genotype 1a H77c strain were replaced with Val, Leu, and Val, respectively. The spmt construct of the type 1a H77c strain did not affect the processing of core and E1 proteins in BHK cells (Fig. 4C, lane 3) and 293T cells (data not shown). In contrast, replacement of Leu^{139}, Val^{140}, and Leu^{144} by Ala and of Ile^{176} and Phe^{177} by Ala and Leu suppressed the processing of the core protein signal sequence of the type 1a H77c strain in BHK cells (Fig. 4C, lanes 4 and 5). These results indicate that three hydrophobic amino acids Leu^{139}, Val^{140}, and Leu^{144} in the hydrophobic peak in domain 2 and the two amino acids Ile^{176} and Phe^{177} in the transmembrane domain play important roles in the in-

tramembrane proteolysis of HCV core protein signal sequence of genotypes 1a and 1b by SPP.

To further examine the cleavage of the signal sequence of HCV core proteins by SPP, we prepared IF176/177AL and the spmt mutant core proteins carrying a substitution of Ala^{191} to Arg to avoid processing by signal peptidase as described above. In cells expressing a wild-type or LVL/3A mutant core protein, a 23-kDa processed or a 25-kDa unprocessed core protein was detected, as seen in Fig. 3B (Fig. 4D, lanes 2 and 4). The IF176/177AL mutant exhibited a 26-kDa unprocessed band which was detected by anti-HA antibody (Fig. 4D, lane 5). In contrast, the spmt core protein exhibited a major band at 23 kDa and a faint 24-kDa band after blotting with the anti-Flag antibody (Fig. 4D, lane 3). Detection of a small amount of the 24-kDa unprocessed band by the anti-HA antibody indicates that most of the spmt mutant core protein was processed by SPP. The unprocessed core proteins of spmt, LVL/3A and IF176/177AL exhibited different electrophoretic mobilities, estimated to be 24, 25, and 26 kDa, respectively (Fig. 4D, lower panel, lanes 3 to 5). Lemberg and Martoglio pointed out that the mobility of a protein does not necessarily correlate with its molecular mass when analyzed in a Tris-glycine gel system due to the unexpected electrophoretic mobility of the proteins (22). However, detection of HA-tagged unprocessed signal sequence in the core mutants clearly demonstrated that LVL/3A and IF176/177AL mutants substituted with Leu^{139}, Val^{140}, and Leu^{144} in domain 2 and with Ile^{176} and Phe^{177} in the transmembrane domain, respectively, have lost the ability to be cleaved by SPP.

Effect of a loss-of-function mutant of SPP on the processing of the signal sequence of HCV core protein. Although there are two reports suggesting that SPP is involved in the processing of the signal sequence of HCV core protein by using the SPP inhibitor $(Z\text{-}LL)_2$-keton (23, 30), a direct interaction of HCV core protein with SPP has not been demonstrated. To determine the direct involvement of SPP in the processing of HCV core protein signal sequence, the C-terminal HA tag in the Flag-Core-E1-HA constructs used in the experiments described above was replaced with a myc/His tag and coexpressed with wild-type SPP (SPP-HAER) or with a mutant SPP with amino acid substitutions in the putative protease active sites, i.e., Asp^{219} (SPP D219A-HAER) or Asp^{265} (SPP D265A-HAER) to Ala. The signal sequence of HCV core protein was processed in cells coexpressing Flag-Core-E1-myc/His and SPP-HAER (Fig. 5, anti-Flag, lane 3), whereas two bands corresponding to processed and unprocessed (the same size as Flag-Core LVL/3A-E1-myc/His [lane 6]) core proteins were detected in cells coexpressing Flag-Core-E1-myc/His and the mutant SPP constructs (Fig. 5, anti-Flag, lanes 4 and 5). Proper cleavage and glycosylation of E1 proteins in cells coexpressing Flag-Core-E1-myc/His and the SPP mutants (Fig. 5, anti-His, lanes 4 and 5) and those expressing Flag-Core LVL/3A-E1-myc/His (Fig. 5, anti-His, lane 6) indicates that processing of signal sequence by SPP is not required for the cleavage of the core-E1 junction by signal peptidase and translocation of E1 protein into the ER. These results indicate that loss-of-function mutants of SPP inhibit the intramembrane proteolysis of HCV core protein signal sequence and further confirm that the slightly larger bands detected in cells expressing Flag-Core LVL/3A-E1-HA or Flag-Core IF176/177AL-E1-HA are immature core proteins unprocessed by SPP (Fig. 4B, lanes 3 and 9).

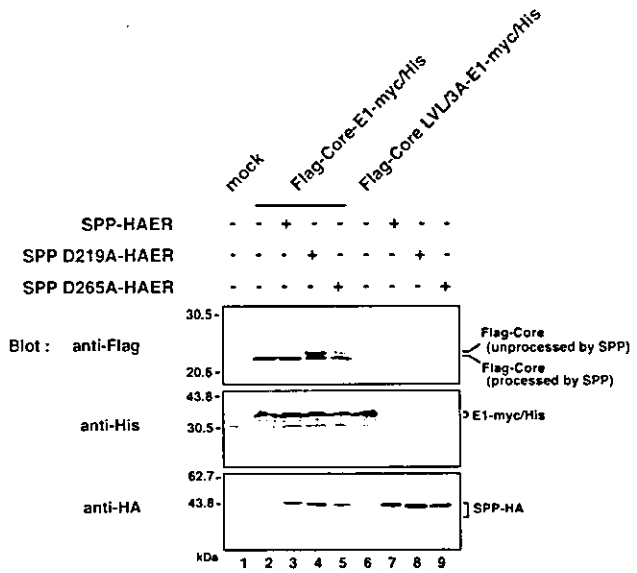

FIG. 5. Effect of loss-of-function mutants of SPP on the processing of the signal sequence of HCV core protein. SPP-HAER, SPPD219A-HAER, or SPPD265A-HAER was coexpressed with Flag-Core-E1-myc/His or Flag-Core LVL/3A-E1-myc/His in 293T cells. Cell lysates were analyzed by immunoblotting with anti-Flag (upper panel), anti-His_6 (middle panel), or anti-HA (lower panel) antibody. + and −, presence or absence of each plasmid, respectively. Lane 1, mock; lanes 2, 6, 7, 8, and 9, single expression of Flag-Core-E1-myc/His, Flag-Core LVL/3A-E1-myc/His, SPP-HAER, SPPD219A-HAER, and SPPD265A-HAER, respectively; lanes 3 to 5, coexpression of Flag-Core-E1-myc/His with SPP-HAER, SPPD219A-HAER, and SPPD265A-HAER, respectively.

Interaction of HCV core protein with SPP. To examine the specific interaction of HCV core protein with SPP, Flag-Core-E1-myc/His, Flag-Core LVL/3A-E1-myc/His, or Flag-Core IF176/177AL-E1-myc/His was coexpressed with SPP-HAER or SPP D219A-HAER in 293T cells and immunoprecipitated with anti-Flag or anti-HA antibody. In cells coexpressing the loss-of-function mutant, SPP D219A-HAER, and one of the three HCV polyprotein substrates, nonspecific bands were detected by immunoblotting with the anti-HA and anti-Flag antibodies in the immunoprecipitates (Fig. 6A, upper and second panels, lanes 3 to 5). Therefore, lysates immunoprecipitated with anti-Flag and anti-HA antibodies were evaluated by comparison with those precipitated with anti-EE. Three bands corresponding to SPP D219A-HAER were coimmunoprecipitated with core proteins by anti-Flag immunoprecipitation (Fig. 6A, upper panel, lanes 8 to 10). SPP has two glycosylation sites (50), and therefore the upper, middle, and lower bands seem to correspond to SPP possessing two glycans, one glycan, and no glycan, respectively. Deglycosylation by PNGase F treatment reduced the molecular sizes of all bands to that of the lowest band (data not shown). Only unprocessed core protein was coimmunoprecipitated with SPP D219A-HAER by anti-HA (Fig. 6A, second panel, lanes 8 to 10). Coexpression of Flag-Core LVL/3A-E1-myc/His or Flag-Core-IF176/177AL-E1-myc/His reduced the expression of SPP D219A-HAER (Fig. 6A, third panel, lanes 4 and 5), suggesting that the