0·3 mg ml^{-1}, and the medium was changed twice per week. After 3 weeks, the colonies obtained on the culture dish were stained with Coomassie brilliant blue as described previously (Naganuma *et al.*, 2004).

RESULTS

Efficient replication of HCV replicons is maintained in long-term cell culture

In order to prepare the specimens for the genetic analysis of 50-1 and 1B-2R1 replicons, three people independently cultured 50-1 cells; one person cultured for 18 months (M) (K cell culture line; MK) and the two people cultured for 12 months (D and N cell culture lines; MD and MN), and one person cultured 1B-2R1 cells for 12 months. Using the specimens obtained at several time points (after 0, 4, 6, 12 and 18 months in culture), the levels of replicon RNAs and HCV proteins were examined by Northern and Western blot analyses, respectively. As shown in Fig. 1(a), replicon RNAs approximately 8 kb long were detected in all specimens except those from the cured cells, from which the replicons had been eliminated from the replicon cells by treatment with interferon-α. The number of copies of replicon RNAs in total RNA (each 3 μg) extracted from the replicon cells was estimated to be in the range of 10^7 to 10^8 by comparing these replicon RNAs with replicon RNA synthesized *in vitro*. The NS3 and NS5B were also detected in all specimens except those from the cured cells (Fig. 1b). The expression levels of replicon RNAs and HCV proteins differed somewhat among these specimens, and no strong quantitative relationship between replicon RNA and HCV proteins was observed (Fig. 1). These results suggest that the stability of replicon RNA or HCV proteins produced from the replicon RNA, or the efficiency of translation, changes during the periods of cell culture. In summary, we demonstrated that the replication efficiencies of the 50-1 and 1B-2R1 replicons remained high under the G418 selection pressure.

Sequence analysis of the 50-1 and 1B-2R1 replicon RNAs

To clarify the genetic variations and diversities of the replicons during the period of cell culture, we carried out sequence analysis of 50-1 and 1B-2R1 replicon RNAs obtained at several time points in the cultures of both replicon cells. Two separate RNA fragments (one was 2·0 kb in length, containing the 5′ UTR to the amino-terminal of the NS3 region; the other was 6·1 kb in length, containing the NS3 to NS5B regions) were amplified by RT-PCR, and three independent clones of each were sequenced after subcloning into pBR322MC, as described previously (Kato *et al.*, 2003b).

Genetic variations of 50-1 and 1B-2R1 replicons during long-term cell culture

The determined nucleotide sequences of the 50-1 and 1B-2R1 replicon RNAs were compared with those of the

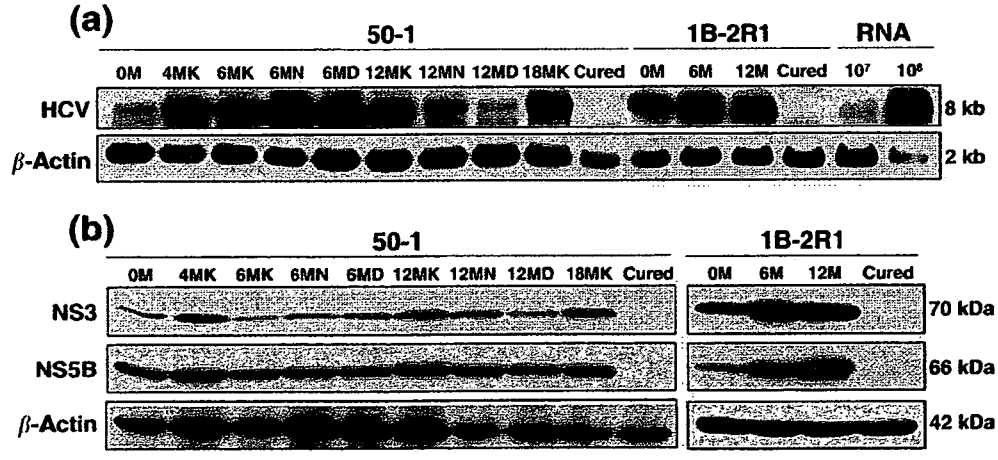

Fig. 1. Characterization of replicon cells in long-term cell culture. (a) Northern blot analysis. Total RNAs from 50-1 cells after 4 months (4MK), 6 months (6MK, 6MN and 6MD), 12 months (12MK, 12MN and 12MD) and 18 months (18MK) in culture, as well as total RNA from the parental 50-1 cells (0M) were used for the analysis. Total RNAs from 1B-2R1 cells after 6 months (6M) and 12 months (12M) in culture, as well as total RNA from the parental 1B-2R1 cells (0M) were used for the analysis. Total RNAs from each cured cells obtained from 50-1 and 1B-2R1 cells by interferon treatment were also used as a negative control. Northern blot analysis was performed using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific probe (lower panel). Synthetic RNA transcribed from pNSS1RZ2RU (10^8 and 10^7 genome equivalents spiked into normal cellular RNA) was used for the comparison of the expression level. (b) Western blot analysis. The orders of specimens were the same as in (a). Productions of NS3 and NS5B in 50-1 and 1B-2R1 cells were analysed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane.

original 50-1 (Kishine *et al.*, 2002; GenBank accession no. AB041927) and 1B-2R1 replicons (Kato *et al.*, 2003b; AB109543), respectively. The results revealed that the numbers of base substitutions in the first 2·0 kb region and in the NS region (6·1 kb) of both replicon RNAs were time-dependently increased with linearity (Fig. 2). These substitutions were considered to be mutations that occurred during the intracellular replication of replicon RNA. Based on the results after 12 months in culture, the apparent mutation rates in 50-1 replicon RNA were calculated to be $3{\cdot}1 \times 10^{-3}$ and $3{\cdot}0 \times 10^{-3}$ base substitutions/site/year in the first 2 kb region and NS region, respectively, indicating that there was no difference in mutation rate between the two regions of 50-1 replicon RNA. Interestingly, almost the same mutation rates ($3{\cdot}0 \times 10^{-3}$ base substitutions/site/year in the first 2 kb region; $3{\cdot}1 \times 10^{-3}$ base substitutions/site/year in NS region) were obtained for the 1B-2R1 replicon RNA, suggesting that the replication efficiency of the 1B-2R1 replicon was almost equal to that of the 50-1 replicon.

Fig. 3(a) shows the schematic presentation of mutations detected in the first 2 kb region by comparison with the original sequences (NNRZ2RU) of 50-1 and 1B-2R1 replicon RNAs (Kato *et al.*, 2003b; Kishine *et al.*, 2002). The results revealed that there were no common mutations among the four cell culture lines (three for 50-1 and one for 1B-2R1) over at least 12 months of cell culture. However, genetic mutations in both replicons were time-dependently increased and accumulated, and several mutations became abundant during the subsequent cell culture (Fig. 3a).

The NS regions (6·1 kb) of the 50-1 and 1B-2R1 replicon RNAs were also analysed in addition to the first 2 kb region. The mutation sites that showed amino acid substitutions are schematically presented in Fig. 3(b). Regarding the 50-1 replicon, 2 aa substitutions (P1115L and E1966A) were newly detected after 6 months in culture in all three cell culture lines, in addition to 2 aa substitutions (K1609E and V1896F) already observed when the replicon was first established. These four substituted amino acids were stably maintained over at least 12 months of cell culture. However, such amino acid substitutions were not observed in the 1B-2R1 replicon even after 12 months of culture. After more than 12 months in culture, several culture line-specific amino acid substitutions (*1–5 for the K culture line; *6–8 for the D culture line; and *9–12 for the N culture line in Fig. 3b) were observed in the 50-1 replicon. Also in the 1B-2R1 replicon, 1 aa substitution (*13 in Fig. 3b) was detected after 12 months in culture; however, no common amino acid substitutions were observed between the 50-1 and 1B-2R1 replicons. The mean numbers of amino acid substitutions occurring after 6 and 12 months in culture were 4·2 and 8·9, respectively, for the 50-1 replicon, and 4·7 and 10·0, respectively, for the 1B-2R1 replicon. These values indicate a steady genetic evolution of 50-1 and 1B-2R1 replicons during the cell culture.

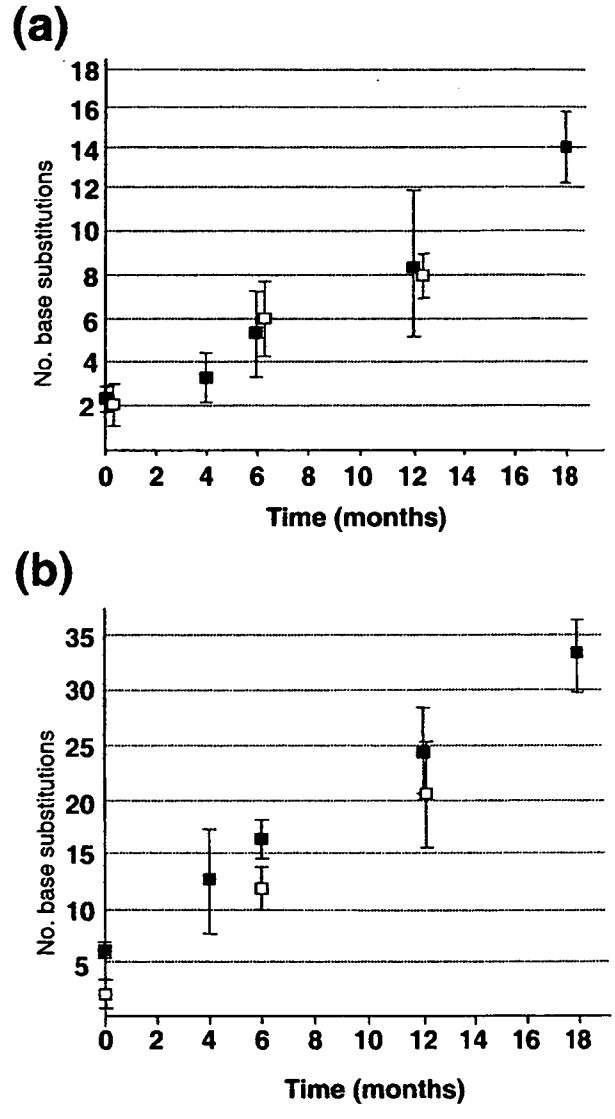

Fig. 2. Genetic variations of 50-1 and 1B-2R1 replicon RNAs. (a) First 2·0 kb region of replicon RNA. Filled squares indicate the mean numbers of base substitutions detected in nine (after 0, 6 and 12 months in culture) or three (after 4 and 18 months in culture) clones containing the first 2·0 kb region of 50-1 replicon RNA, by comparison with its original sequences (NNRZ2RU) (Kishine *et al.*, 2002). Open squares indicate the mean numbers of base substitutions detected in three clones containing the first 2·0 kb region of 1B-2R1 replicon RNA, by comparison with its original sequences (NNRZ2RU) (Kishine *et al.*, 2002). (b) NS region (6·1 kb) of replicon RNA. Filled squares indicate the mean numbers of base substitutions detected in nine (after 0, 6 and 12 months in culture) or three (after 4 and 18 months in culture) clones containing the NS region of 50-1 replicon RNA, by comparison with its original sequences (Kishine *et al.*, 2002). Open squares indicate the mean numbers of base substitutions detected in three clones containing the NS region of 1B-2R1 replicon RNA, by comparison with its original sequences (Kato *et al.*, 2003b).

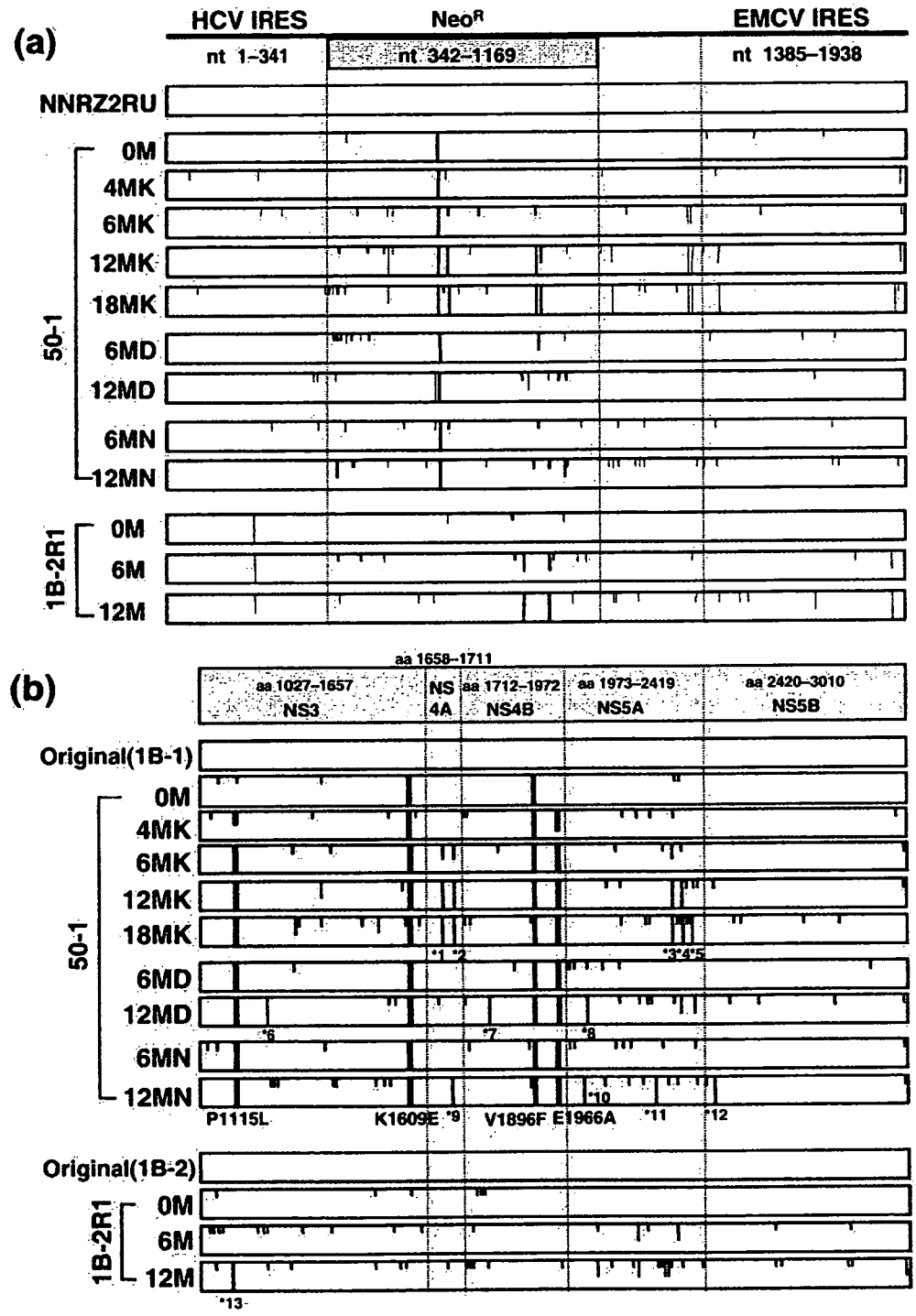

Fig. 3. Genetic variations of 50-1 and 1B-2R1 replicons in long-term cell culture. (a) Schematic presentation of mutations detected in first 2·0 kb regions of the replicon RNAs. Compared with the nucleotide sequences of the first 2·0 kb region of the original replicon RNA (NNRZ2RU), nucleotide positions mutated in all three clones, in two of three clones and in one of three clones are indicated by full-length, two-thirds and one-third vertical lines, respectively. Non-synonymous substitutions in the Neo^R region are indicated by heavy vertical lines. (b) Schematic presentation of amino acid substitutions detected in the NS regions of the replicons. Compared with the amino acid sequences of NS region of the original 50-1 (Kishine *et al.*, 2002) and 1B-2R1 replicons (Kato *et al.*, 2003b), amino acid positions substituted in all three clones, in two of three clones and in one of three clones are indicated by full-length, two-thirds and one-third vertical lines, respectively. Four amino acid substitutions (P1115L, K1609E, V1896F and E1966A) are indicated by heavy vertical lines. Culture line-specific amino acid substitutions (indicated by the numbers with asterisks) are as follows: *1, I1686V; *2, L1701R; *3, T2332A; *4, G2336E; *5, A2372T; *6, A1243G; *7, I1797V; *8, S2053G; *9, L1701R; *10, T2051N; *11, R2279G; *12, L2476M; *13, I1097V.

Classification of mutations occurring in 50-1 and 1B-2R1 replicon RNAs during the long-term cell culture

To understand the mutation mode of the replicons in long-term cell culture, we examined the numbers of synonymous and non-synonymous mutations with transition or transversion. The results are summarized in Table 1. The ratio of synonymous to non-synonymous mutations in 50-1 replicon RNA was 0·81 to 1·50 (1·38±0·14 after 6 months in culture and 1·03±0·20 after 12 months in culture), and the ratio in 1B-2R1 replicon RNA was 0·63 after 6 months in culture and 0·59 after 12 months in culture. These values indicate that amino acid substitutions in the replicons occur frequently during the cell culture. The rate of mutations with transition in the 50-1 replicon was 1·82–4·06-fold (2·00± 0·18 after 6 months in culture and 2·85±1·07 after 12 months in culture) greater than the rate of mutations with transversion. Similarly, the 1B-2R1 replicon showed a transition-to-transversion ratio of 2·69 (after 6 months in culture) or 2·86 (after 12 months in culture).

Regarding the mutation patterns over more than 12 months of culture, we observed that A→G and U→C mutations were the most and second-most common mutations, and these mutations were approximately two to three times more common than G→A and C→U mutations (Supplementary Table A, which is available as Supplementary material in JGV Online). The rarest mutation was G→U (Supplementary Table A).

Genetic diversity of the 50-1 and 1B-2R1 replicons arising during long-term cell culture

To clarify whether or not the replicons acquire a quasispecies nature during long-term cell culture, we estimated the genetic diversities of the 50-1 and 1B-2R1 replicon populations. First, based on the sequence data of all clones obtained in this study, we constructed phylogenetic trees for the first 2 kb region and the NS region. The results revealed that the genetic diversity of 50-1 replicon populations was expanded in a time-dependent manner (Fig. 4). Similar phylogenetic trees were obtained for the 1B-2R1 replicon populations as well (data not shown). Next, as another index of genetic diversity, we calculated the mean number of nucleotide differences among three independent clones at each time point. The schematic presentation of such analysis on the NS regions of 50-1 and 1B-2R1 replicon RNAs was shown in Supplementary Fig. A, which is available as Supplementary material in JGV Online. The results also showed a time-dependent expansion of genetic diversity. After 12 months in culture, 0·32 % (mean of three cell culture lines) and 0·55 % diversities in nucleotide sequences were observed in the NS region of 50-1 and 1B-2R1 replicon RNAs. A similar time-dependent expansion of genetic diversity was also observed in the first 2 kb regions of both replicon RNAs (data not shown). These results indicate that the quasispecies nature of replicon RNA was easily acquired during the replication of the replicons.

Table 1. Base substitutions occurring in 50-1 and 1B-2R1 replicon RNAs during long-term cell culture

The counting of base substitutions was performed by comparison with the consensus sequence obtained from the 0M series of 50-1 or 1B-2R1 replicon.

Replicon	series	No. base substitutions										Synonymous/ non-synonymous	Transition/ transversion
		Transition					Transversion						
		Synonymous		Non-synonymous		Non-coding region	Synonymous		Non-synonymous		Non-coding region		
		Neo^R	NS	Neo^R	NS		Neo^R	NS	Neo^R	NS			
50-1	4MK	1	13	0	8	4	0	5	0	6	2	1·36	2·00
	6MK	0	20	2	10	8	3	8	1	9	1	1·41	1·82
	12MK	3	29	6	19	13	4	9	4	9	2	1·18	2·50
	18MK	5	43	8	26	16	3	10	4	14	5	1·17	2·72
	6MD	3	20	3	9	2	0	5	4	7	1	1·22	2·18
	12MD	5	29	2	26	3	2	5	1	8	0	1·11	4·06
	6MN	2	19	2	8	3	2	4	0	8	3	1·50	2·00
	12MN	3	25	2	21	9	1	6	5	15	3	0·81	2·00
1B-2R1	6M	1	14	5	14	1	1	1	3	5	3	0·63	2·69
	12M	2	22	4	29	6	1	2	3	10	6	0·59	2·86

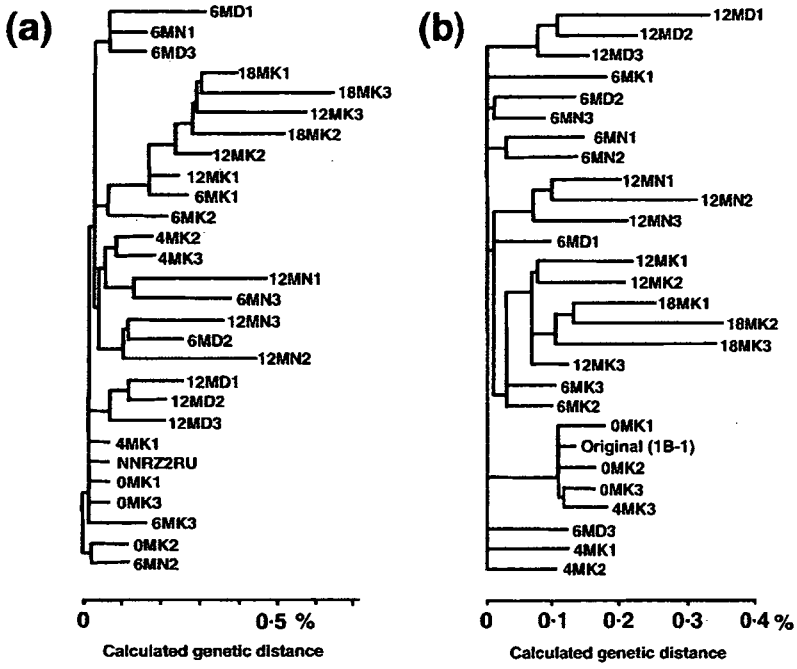

Fig. 4. Phylogenetic trees of 50-1 replicon populations obtained in long-term cell culture. The phylogenetic tree is depicted on the basis of nucleotide sequences of all replicon clones obtained by long-term culture of 50-1 cells. (a) The first 2·0 kb region of replicon RNA. NNRZ2RU indicates the original sequences of 50-1 replicon RNA, and the others indicate the names of clones. (b) The NS region of replicon RNA. Original (1B-1) indicates the original sequences of 50-1 replicon RNA, and the others indicate the names of clones.

Enhancement of HCV replication is associated with the expansion of the replicons' genetic diversity

To assess whether or not the mutations accumulating in the replicons increase the replication efficiencies of the replicons, the efficiency of colony formation (ECF) of the replicon was examined at each time point of the culture. An ECF assay was performed by transfection of total RNAs isolated from 50-1 and 1B-2R1 replicon cells at different time points into naïve HuH-7 cells. After 3 weeks of G418 selection, only a few colonies were obtained when RNAs from 50-1 replicon cells cultured less than 4 month were used (Fig. 5). However, ECF was apparently increased when RNAs from cells cultured 6 months, in particular the D and N cell culture lines, were used, and much higher numbers of colonies were obtained when RNAs from cells cultured 12 months were used (Fig. 5). Interestingly, ECFs of RNAs from D and N cell lines cultured more than 6 months were higher than those in the K cell culture line. These results indicated that ECF of the replicon was increased with the cultured periods of the replicon cells and suggested that ECF enhancement is associated with the expansion of the 50-1 replicon's genetic diversity.

In contrast to the case with 50-1 replicon cells, a number of colonies were obtained even when RNA from the initial culture of 1B-2R1 replicon cells was used (Fig. 5). In this replicon also, the ECF of RNA from cells cultured 12 months was apparently higher than those of RNA from the initial culture or 6 months of culture (Fig. 5). These results suggest that S2200R substitution, which was detected when the 1B-2R1 replicon was established (Kato *et al.*, 2003b), function as an adaptive mutation, and that the expansion of genetic diversity in the 1B-2R1 replicon also contributes to the enhancement of ECF, as was the case with the 50-1 replicon.

Effect of ribavirin and mizoribine on the genetic evolution and dynamics of the 50-1 replicon

Combined treatment of interferon plus ribavirin for patients with chronic hepatitis C has been shown to be more effective than treatment with interferon alone (McHutchison *et al.*, 1998), although it has been shown that ribavirin alone does not cause a decrease of HCV level in patients with chronic hepatitis C. Recently, several groups have reported that ribavirin might cause 'error catastrophe' of HCV genome (Contreras *et al.*, 2002; Tanabe *et al.*, 2004; Zhou *et al.*, 2003), however, controversial results have also been reported (Schinkel *et al.*, 2003). Therefore, to clarify whether or not ribavirin affects the genetic alterations of HCV, we cultured parent 50-1 cells (corresponding to 0M in Fig. 1) for 6 months in the presence of ribavirin (5 or 25 μM) or its derivative molecule, mizoribine (25 μM). As a control, the parent 50-1 cells were also cultured for 6 months in the absence of ribavirin or mizoribine. After 6 months in culture, the levels of replicon RNAs and HCV proteins were examined by Northern and Western blot analyses, respectively. As shown in Fig. 6(a), the level of replicon RNA in the cells treated with ribavirin or mizoribine was almost the same as that in the cells without ribavirin or mizoribine treatment. The NS3 and NS5B were also expressed at similar levels in the cells irrespective of ribavirin or mizoribine treatment (Fig. 6b). These results indicate that even 6 months of treatment with ribavirin or mizoribine did not prevent the replication of replicon RNA under the G418 selection pressure. Using the 50-1 cells cultured for 6 months with or without ribavirin or mizoribine, we performed sequence analysis of replicon

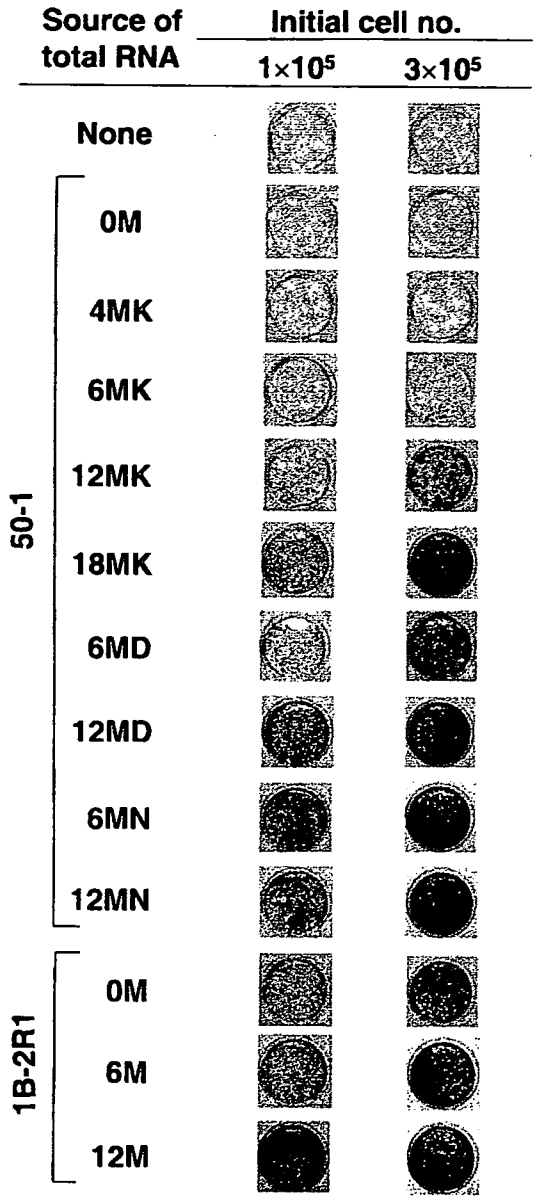

Fig. 5. ECF of the RNAs isolated from 50-1 and 1B-2R1 replicon cells at different time points in the culture. Total RNAs obtained from the replicon cells were transfected into HuH-7 cells as described in Methods. The panels show the cell colonies that were recovered after 3 weeks of G418 selection.

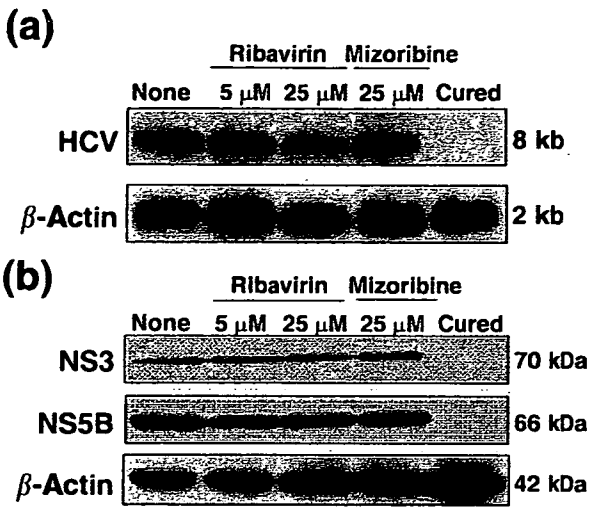

Fig. 6. Characterization of 50-1 cells cultured for 6 months in the presence of ribavirin or mizoribine. (a) Northern blot analysis. Total RNAs from 50-1 cells cultured for 6 months in the presence of ribavirin (5 and 25 μM) or mizoribine (25 μM), as well as total RNA from 50-1 cells cultured for 6 months in the absence of ribavirin and mizoribine were used for the analysis. Northern blot analysis was performed as indicated in Fig. 1(a). (b) Western blot analysis. The orders of specimens were the same as in (a). Western blot analysis was performed as indicated in Fig. 1(b).

RNAs as described above. As shown in Table 2, the results revealed that the numbers of mutations in the first 2·0 kb and NS regions of the replicon RNAs sequenced were not significantly different among the specimens, although the number in the NS region derived from the cells treated with 25 μM of ribavirin was a little lower than those of the other specimens. These results suggest that the treatment of replicon cells with either ribavirin or mizoribine does not increase the mutation rate of replicon RNA. The ratio of synonymous and non-synonymous mutations, and the ratio of transition and transversion mutations were also not altered by ribavirin or mizoribine treatment (data not shown). In addition, we did not observe any ribavirin- or mizoribine-specific common amino acid substitutions in either the first 2 kb or NS regions of the replicon RNA, although P1115L and E1966A were detected after 6 months in culture in all cell culture lines. The above-described analysis of genetic diversity among the replicon RNAs did

Table 2. Base substitutions occurred in 50-1 replicon RNA during 6 months culture in the presence of ribavirin or mizoribine

6MR5 and 6MR25 indicate the series treated with 5 and 25 μM of ribavirin, respectively. 6MM25 indicates the series treated with 25 μM of mizoribine. The counting of base substitutions was performed by the comparison with the original sequence of 50-1 replicon (Kishine *et al.*, 2002).

Series	First 2-kb region	NS region
6M (Fig. 2)	5·4±1·9*	16·4±1·8
6M	5·7±2·5	16·0±0·0
6MR5	5·7±1·5	16·3±1·5
6MR25	5·7±1·5	10·7±1·2
6MM25	3·7±0·6	18·7±4·0

*Numbers of base substitutions ± SD.

not reveal any significant differences between the specimens derived from the replicon cells with and those without ribavirin or mizoribine treatment (data not shown). Taken together, these results suggest that neither ribavirin nor mizoribine accelerated the mutation rate of HCV replicons or the development of their quasispecies nature.

DISCUSSION

In this study, we analysed the genetic evolution and dynamics of HCV replicons, and time-dependent genetic mutations of HCV replicons were observed. Time-dependent expansions of their genetic diversities were also revealed. Our results should provide useful fundamental information for understanding the remarkable genetic diversity and variation among the HCV genomes observed in patients with chronic hepatitis C.

Although RT-PCR techniques were used to amplify the replicon RNAs in this study, it is unlikely that the detected mutations were due to errors related to the use of the KOD-plus DNA polymerase in the PCR reaction, because we previously showed that KOD-plus DNA polymerase possessed a high proofreading activity (Alam *et al.*, 2002; Naganuma *et al.*, 2004). Furthermore, in the present study, we sequenced several clones (containing a 2·0 or 6·1 kb fragment) obtained by PCR using KOD-plus DNA polymerase and a single sequenced clone as a template, but no mutations were detected in these sequenced clones, indicating that KOD-plus DNA polymerase possesses extremely high fidelity. However, we are not able to completely exclude the possibility that some substitutions resulted from the erroneous use of KOD-plus DNA polymerase during the PCR. Even if such errors occurred, the error frequency is estimated to be less than one nucleotide per sequenced clone. This is explained as follows. Fig. 2 shows that the numbers of substitutions time-dependently increased with linearity in both HCV replicons. Interestingly, when these linear lines are extrapolated to zero base substitutions, the crossing points show approximately −2–−3 months in the time axis. These range of months is in accord with the time of initial electroporation of HCV replicon RNA to HuH-7 cells. Therefore, PCR-induced mutations are considered to be very rare and such mutations would have very little effect on the results shown in Fig. 2. In addition, to avoid a sampling effect, we sequenced three independent clones derived from each time point.

We showed that the mutation rates for the 50-1 and 1B-2R1 replicon RNAs were almost the same – about 3×10^{-3} base substitutions/site/year. However, the actual mutation frequency of the replicon RNAs would be higher than this value, because the mutations that occurred in positions that were critical for the replication of replicon RNA should not have been passed on to the progeny. Our observed mutation rates of the replicon RNAs were approximately two times higher than those previously obtained in chimpanzees and clinical patients with chronic hepatitis C (Major *et al.*, 1999; Ogata *et al.*, 1991; Okamoto *et al.*, 1992). Since the selective pressure of the immune system also functions *in vivo* (Kato *et al.*, 1993), the mutation rate in cell culture obtained in this study may be reasonable value as a potential mutation rate of HCV. However, direct comparison of these mutation rates would be difficult, because both the experimental model and analytical method were different in this study compared with the previous studies. It would be interesting to examine whether this mutation rate (3×10^{-3} base substitutions/site/year) would be maintained during longer-term culture of the replicon cells. If so, approximately 3 % of nucleotide sequences of the replicon RNAs might be mutated after 10 years in cell culture. Alternatively, the mutations might become saturated during further long-term culture of the replicon cells. To clarify this point, further long-term culture of replicon cells is in progress.

Although the mutations detected in this study were dispersed throughout the entire length of the replicon RNAs (Fig. 3), the mutation frequencies in the 5′ UTR and NS5B region were lower than those in other regions, and the NS5A region showed the highest mutation frequency. These observations are consistent with the genetic diversities of HCVs in patients with chronic hepatitis C reported to date (Kato, 2001). In addition, the positions in which amino acid substitutions were observed during the cell culture did not appear to be critical for replication of the HCV genome.

Time-dependent expansions of genetic diversities of HCV replicons were also found in this study. However, this finding seems to be different from the previous findings that HCV populations in the cells infected *in vitro* gradually altered with time and converged to the limited populations (Kato *et al.*, 1998; Kato, 2001). This gap may have been due to the differences in the HCV sources used: a patient's inoculum containing a quasispecies of HCV was used for the *in vitro* infection experiment, and a single HCV species was used for the replicon system. Alternatively, the gap may have been due to the overwhelming difference between the replication level of the HCV genome in the cells infected *in vitro* and that in the replicon cells.

To date, a number of amino acid substitutions belonging to adaptive mutations that enhance the frequency with which the replicon is established *in vitro* have been found in established HCV replicons (Bartenschlager, 2002; Blight *et al.*, 2000, 2003; Ikeda *et al.*, 2002; Krieger *et al.*, 2001; Lanford *et al.*, 2003; Lohmann *et al.*, 2001, 2003; Pflugheber *et al.*, 2002). Although none of the amino acid substitutions detected in the long-term cultures of the 50-1 and 1B-2R1 replicons were the same as those reported as adaptive mutations, ECF analysis of the replicons using naïve HuH-7 cells suggested that adaptive mutations accumulated in the replicon populations in a time-dependent manner. In particular, drastic enhancement of ECF was observed in the 50-1 replicon after 6 months of culture. However, this result suggests that the four common amino acid substitutions (P1115L, K1609E, V1896F and E1966A) do not contribute much to the drastic enhancement of ECF,

because the ECFs of 4MK and 6MK samples possessing these substitutions did not increase much. Therefore, we estimate that some uncommon amino acid substitutions accumulated as so-called adaptive mutations. The candidates for such adaptive mutations are culture-line-specific amino acid substitutions (Fig. 3b, *1–12), and many amino acid substitutions sporadically appeared in the replicons in the long-term cell cultures. To identify which amino acid substitution is the main contributor to the drastic enhancement of ECF, further transfection experiments using replicon RNAs possessing mutations will be needed. Based on the results of this study, S2200R substitution in the 1B-2R1 replicon is considered an adaptive mutation. This description is supported by the previous result that we were unable to obtain any G418-resistant colonies when the original 1B-2 replicon RNA library, used in the isolation of the 1B-2R1 replicon, was transfected into naïve HuH-7 cells (Kato *et al.*, 2003b). Since the ECF of 1B-2R1 replicon RNA from 12 months of culture was further enhanced, it may be that the I1097V substitution, detected commonly at 12 months of culture, functions as an additional adaptive mutation.

Interestingly, once a new mutation was observed in all three clones at a particular time point, the clones which went back to the original sequences were never obtained in the subsequent cell culture, except for one clone (a mutation in the HCV IRES region) derived from 1B-2R1 replicon cells after 12 months in culture (Fig. 3a). This finding suggests that the genetic evolution of HCV replicons is irreversibly progressing.

Although the mechanism of action of ribavirin for patients with chronic hepatitis C is ambiguous, an 'error catastrophe' theory of ribavirin has been proposed by several groups (Contreras *et al.*, 2002; Tanabe *et al.*, 2004; Zhou *et al.*, 2003). However, our results obtained in this study were not able to support this 'error catastrophe' theory, because ribavirin had no effect on the genetic variation and diversity of the 50-1 replicon. The concentration (5 and 25 μM) of ribavirin used in this study was considered to be reasonable, because the growth rate of 50-1 cells decreased at a ribavirin concentration of more than 50 μM, and approximately 10 μM of ribavirin is the maximum plasma concentration in current clinical usage (Tanabe *et al.*, 2004). Higher concentration (more than 50 μM) of ribavirin used in previous studies may be required for causation of the error catastrophe. Recently, a single amino acid substitution (F2834Y) was identified as a ribavirin-resistant NS5B mutation in genotype 1a (Young *et al.*, 2003); however, it is difficult to evaluate that finding in this study, because most of the HCV strains belonging to genotype 1b, including 1B-1 (50-1) and 1B-2 (1B-2R1), already possess a Tyr residue at position 2834. No amino acid substitution at position 2834 in NS5B was observed in the replicon cells treated with ribavirin.

This study provided the fact that the genetic diversity of HCV replicons was enlarged in a time-dependent manner during long-term cell culture. Since all the HCV replicons established to date have been shown to be highly sensitive to interferon-α, -β and -γ (Kato *et al.*, 2003b), and most of the HCV replicons established to date are able to replicate in only HuH-7 cells, the extensive genetic polymorphism of HCV replicon populations obtained by long-term cell culture may change the sensitivity against interferon or the ability of replication in the cells except for HuH-7. In the future, it will be necessary to clarify these points. Thus, HCV replicon populations obtained by long-term cell culture may be useful not only for analysis of the genetic variations and dynamics of HCV but also for analysis of the variable properties of HCV.

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Novel regulation of MHC class II function in B cells

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The presence of post-translational regulation of MHC class II (MHC II) under physiological conditions has been demonstrated recently in dendritic cells (DCs) that potently function as antigen-presenting cells (APCs). Here, we report that MARCH-I, an E3 ubiquitin ligase, plays a pivotal role in the post-translational regulation of MHC II in B cells. MARCH-I expression was particularly high in B cells, and the forced expression of MARCH-I induced the ubiquitination of MHC II. In B cells from MARCH-I-deficient mice (MARCH-I KO), the half-life of surface MHC II was prolonged and the ubiquitinated form of MHC II completely disappeared. In addition, MARCH-I-deficient B cells highly expressed exogenous antigen-loaded MHC II on their surface and showed high ability to present exogenous antigens. These results suggest that the function of MHC II in B cells is regulated through ubiquitination by MARCH-I.

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Introduction

Ubiquitination is an essential post-translational modification of proteins that 'marks' proteins with ubiquitin molecules, often resulting in degradation (Hershko and Ciechanover, 1998). This modification is achieved through the action of three enzymes: ubiquitin-activating enzyme E1 (E1), ubiquitin-conjugating enzyme E2 (E2), and ubiquitin-protein ligase E3 (E3). E1 activates ubiquitin, and the activated ubiquitin is subsequently transferred to a substrate through the interaction of E2 and E3. Whereas E2 carries the activated ubiquitin, E3 conjugates ubiquitin moieties to the target substrate (Hershko and Ciechanover, 1998).

At present, ubiquitination is thought to play an important role in the degradation of membrane proteins through the induction of endocytosis (Dupre *et al*, 2004). In yeast, an E3, Rsp5p, has been reported to induce ubiquitination of the cytoplasmic tail of substrate proteins, a step that is necessary for the endocytosis and degradation of the substrate proteins (Dupre *et al*, 2004). Similarly, we and other groups have recently identified a novel family of E3 enzymes termed MIR family, whose catalytic domain is a variant RING domain (RINGv domain). The MIR family members have been shown to induce rapid endocytosis and degradation through the ubiquitination of the cytoplasmic tail of substrate proteins in mammals (Coscoy *et al*, 2001; Coscoy and Ganem, 2003; Goto *et al*, 2003; Bartee *et al*, 2004; Lehner *et al*, 2005; Ohmura-Hoshino *et al*, 2006a). The MIR family proteins share a secondary structure and the RINGv domain located at the amino terminus. They bind to the membrane through their hydrophobic domains located at the center, and possess two intracellular regions.

Most importantly, the forced expression of MIR family members was found to be capable of degrading immune recognition-related molecules, such as MHC class I (MHC I) and MHC II (Coscoy and Ganem, 2000, 2001; Ishido *et al*, 2000a, b; Fruh *et al*, 2002; Ohmura-Hoshino *et al*, 2006b). However, the physiological substrates for these novel ubiquitin ligases remain completely unknown. Among the MIR family members, c-MIR and MARCH-I are of particular interest, because they can efficiently degrade important proteins in the immune system, and the surface expression of MHC II has been shown recently to be regulated by ubiquitination in dendritic cells (DCs) that potently function as antigen-presenting cells (APCs) (Goto *et al*, 2003; Bartee *et al*, 2004; Ohmura-Hoshino *et al*, 2006b; Shin *et al*, 2006). Within the E3 catalytic domain and transmembrane regions, the amino-acid identity between c-MIR and MARCH-I is >80%, suggesting similar functions. As MARCH-I expression was reported to be restricted to secondary lymphoid tissues such as spleen and lymph node, we have been especially interested in the functional elucidation of MARCH-I (Bartee *et al*, 2004).

In this report, we demonstrate that in B cells, the surface expression of MHC II is regulated through ubiquitination by MARCH-I, and the ubiquitination does not contribute to the internalization of surface MHC II. In parallel with the stabilization of surface MHC II, we found that MARCH-I-deficient B cells highly expressed exogenous antigen-loaded MHC II on their surface and showed high ability to present exogenous antigens. Thus, our results suggest that the function of MHC

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II on the surface of B cells is regulated through ubiquitination by MARCH-I.

Results

MARCH-I is highly expressed in B cells

To identify MARCH-I mRNA *in vivo*, we first performed Northern blot analysis in various tissues. Consistent with a previous report (Bartee *et al*, 2004), MARCH-I mRNA expression was restricted to secondary lymphoid tissues, such as spleen and lymph node (Figure 1A). Next, we generated MARCH-I-specific antibodies (Abs) to identify MARCH-I protein. Initially, we were unable to detect MARCH-I protein by Western blot analysis in all the examined tissues. As the MARCH-I protein expression level was expected to be low, we performed immunoprecipitation (IP)–Western blot analysis. Also, in order to increase the S/N ratio for detection, matrix conjugated with 13F11D12 anti-MARCH-I monoclonal antibody (mAb) was employed for IP, and a biotin-conjugated 4526 anti-MARCH-I polyclonal Ab was used for Western blot analysis. The epitopes for mAb and polyclonal Ab are different, as described in 'Materials and methods.' As shown in Supplementary Figure 1C, we found a faint band (marked with *), whose MW is similar to that of a protein transcribed from the expected full-length MARCH-I cDNA. These results suggested that the MARCH-I protein expression levels are extremely low *in vivo*. To provide some clues to determine the physiological role of MARCH-I, we further examined the expression levels of MARCH-I mRNA in various hematopoietic cells in the spleen by real-time PCR. As shown in Figure 1B, MARCH-I mRNA was highly expressed in B cells, and among splenic B cells, follicular B cells in particular highly expressed MARCH-I mRNA (Figure 1C). DCs and macrophages moderately expressed MARCH-I mRNA, whereas the expression was relatively low in splenic T cells, suggesting that MARCH-I might mainly function in APCs.

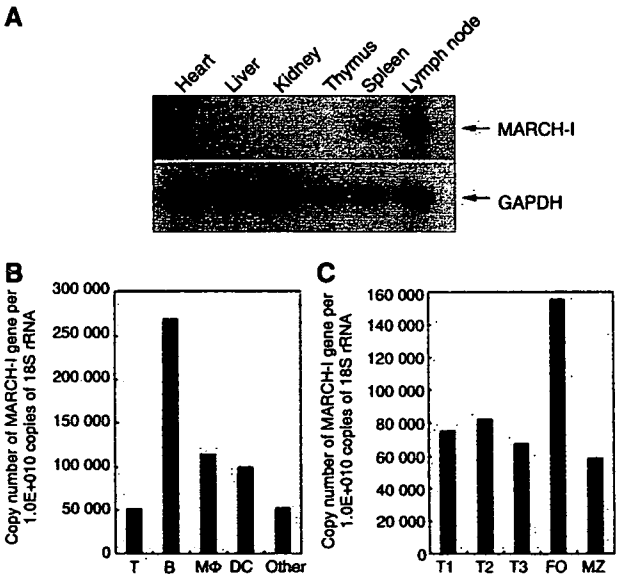

Figure 1 Expression profile of MARCH-I mRNA. (**A**) Expression profile of MARCH-I mRNA was analyzed by Northern blot in the indicated tissues. Data are representative of two independent experiments. (**B**) Expression levels of MARCH-I mRNA were compared among the indicated cell fractions in splenocytes. Data are representative of two independent experiments. (**C**) Expression levels of MARCH-I mRNA were compared among the indicated cell fractions in splenic B cells. Data are representative of two independent experiments. FO, follicular B cells; MZ, marginal zone B cells.

Downregulation and ubiquitination of MHC II by forced expression of MARCH-I

Next, we examined whether MARCH-I is functionally equivalent to c-MIR, using a 293T reconstitution system reported earlier (Ohmura-Hoshino *et al*, 2006b). As shown in Figure 2A, MARCH-I downregulated the surface expression of MHC II reconstituted with wild-type the I-A β chain. In contrast, the surface expression of MHC II reconstituted with the I-A β chain mutant, whose cytoplasmic lysine (the lysine residue at position 225 in the I-A β chain) was mutated to arginine, was not downregulated by MARCH-I. Also, a MARCH-I mutant, whose RINGv domain was disrupted by point mutation, was not able to downregulate MHC II surface expression. Consistent with these observations, MARCH-I ubiquitinated the cytoplasmic lysine residue within the I-A β chain, and its activity was dependent on the structure of the RINGv domain (Figure 2B). The downregulation of MHC II surface expression was also observed in two different murine B cell lines, A20 and M12 C3 cells (Griffith *et al*, 1988; Harding *et al*, 1995) (Figure 2C and D). Taken together, the forced expression of MARCH-I pointed to its broad functional homology with c-MIR.

MHC II expression is regulated through ubiquitination of the I-A β chain by MARCH-I

To examine whether MHC II is a physiological substrate for MARCH-I, we generated MARCH-I KO by gene targeting. As shown in Supplementary Figure 1A, exon 6 encoding the RINGv domain was flanked by two loxP sequences. After confirmation of insertion of three loxP sequences and a Neo cassette by Southern blot analysis, these mice were crossed with CAG-cre mice (Sakai and Miyazaki, 1997) to delete both exon 6 and the Neo cassette by cre-mediated recombination. Finally, two KO lines were established from two independent ES cells derived from 129 mice, as evaluated by Southern blot analysis (Supplementary Figure 1B). No MARCH-I protein was detected in these mice, demonstrating that these gene-targeted mice are indeed MARCH-I-null mice (Supplementary Figure 1C). FACS analysis did not show any significant abnormalities in the cellularity and development of T cells and B cells in the spleen (data not shown). Importantly, MHC II surface expression level was strikingly increased in the blood-circulating B cells from MARCH-I KO (Figure 3A). The extent of MHC II upregulation seems to parallel the extent of MARCH-I down-regulation. In contrast, the expression level of B7-1 was not altered (Figure 3A). Identical results were obtained from experiments with splenic B cells and splenic DCs in MARCH-I KO (data not shown). The same data were obtained from two different lines of MARCH-I KO.

To examine whether the amount of MHC II protein is increased in splenic B cells from MARCH-I KO, we analyzed the total amount of I-A β chain protein with 0.1% SDS-containing lysis buffer. As shown in Figure 3B, the amount of I-A β chain protein was significantly increased in splenic B cells from MARCH-I KO compared with control littermates. In contrast, as we expected, the expression level of I-A β chain

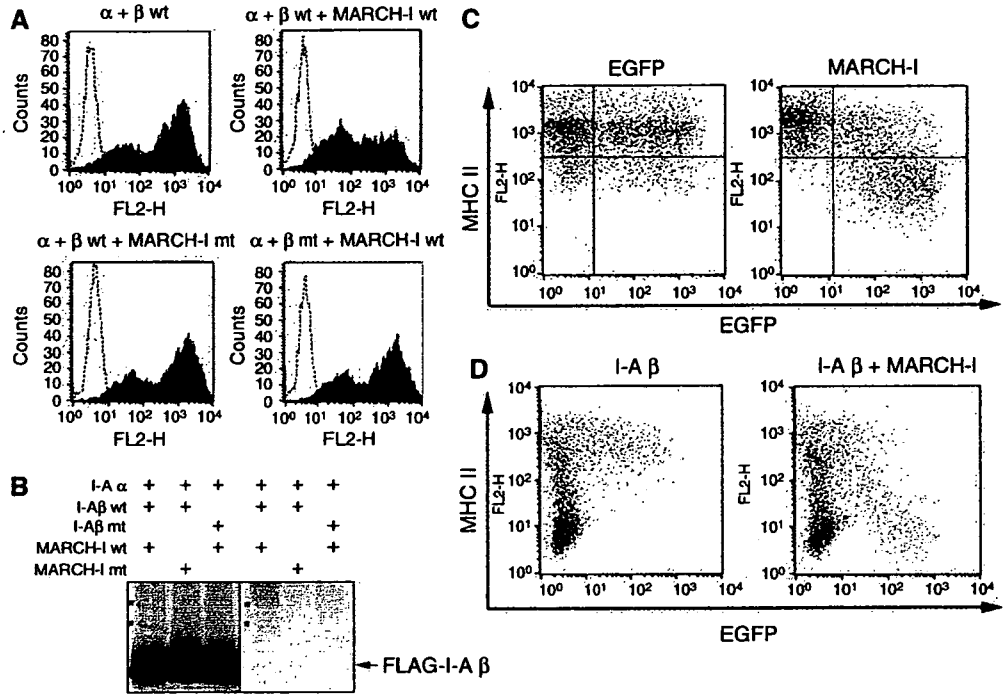

Figure 2 Downregulation and ubiquitination of MHC II by MARCH-I. (**A**) 293T cells were transfected with the expression plasmid indicated above each panel, with Fugene 6 reagent (Roche). Twenty-four hours after transfection, MHC II surface expression was analyzed by FACS. Data are representative of two independent experiments. (**B**) As indicated above each panel, 293T cells were cotransfected with several expression plasmids, and Flag-tagged I-A β chain was precipitated with anti-Flag Ab. Precipitated samples were probed with anti-Flag Ab (left) or anti-ubiquitin Ab (right). The band corresponding to the ubiquitinated I-A β chain is marked (*) as shown. Data are representative of two independent experiments. (**C**) A20 cells were transfected with plasmid expressing EGFP and MARCH-I from different promoters (right). In the left panel, only EGFP protein was expressed as control. MHC II surface expression level was examined by FACS. Data are representative of two independent experiments. (**D**) In M12 C3 cells that express the I-A α, but not I-A β, chain, I-A β chain alone (left) or MARCH-I plus I-A β chain (right) was expressed by electroporation with EGFP-coexpressing plasmid used in (C). MHC II surface expression was examined by FACS. Data are representative of two independent experiments.

mRNA was not significantly altered as judged by real-time PCR, suggesting that the upregulation of MHC II occurs at the post-transcriptional level. To confirm this, pulse–chase analysis was performed. B cells from MARCH-I KO or control littermates were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine and chased for indicated periods. At the end of the chase periods, pulse-labeled proteins were immunoprecipitated with Y-3P anti-I-A^{b} β chain mAb that preferentially recognizes mature αβ dimers (Brachet *et al*, 1997), and the precipitated samples were boiled before SDS–PAGE. At 1 h of chase, bands corresponding to the I-A β chain in both groups showed maximal and identical intensities, suggesting that both types of B cells synthesize the same amount of mature MHC II (αβ dimers). In contrast, at 6 and 9 h of chase, the amount of remaining MHC II was significantly increased in MARCH-I KO, compared with control littermates (Figure 3C). Thus, the I-A β chain of MHC II protein was stabilized in splenic B cells from MARCH-I KO.

The data from experiments with forced expression of MARCH-I and experiments with MARCH-I KO strongly suggested that MHC II is a physiological substrate for MARCH-I. To test this hypothesis, we examined the status of ubiquitination of the I-A β chain in splenocytes. In the splenocytes from control littermates, the polyubiquitinated I-A β chains were clearly detected, but the ubiquitinated I-A β chains completely disappeared in the splenocytes from MARCH-I KO, demonstrating that the I-A β chain of MHC II is indeed a physiological substrate for MARCH-I (Figure 3D).

Furthermore, to confirm that MHC II surface expression is regulated by ubiquitination of the I-A β chain, we expressed the I-A β chain wild type (I-A β wt) or the I-A β chain mutant type, whose K225 was mutated to arginine (I-A β K>R), in bone marrow (BM) cells by using retrovirus that coexpress human CD8 as a marker, and generated chimeric mice with these modified BM cells. We also generated control chimeric mice with BM cells infected with control retrovirus expressing human CD8 alone. Eight weeks after reconstitution, blood-circulating B cells expressing the same level of human CD8 were analyzed among the three groups. As K225 in the I-A β chain is responsible for the downregulation and ubiquitination of MHC II by forcibly expressed MARCH-I (Figure 2A and B), B cells generated from the I-A β K>R-expressing virus-infected BM cells are expected to highly express MHC II, compared with B cells generated from the I-A β wt-expressing virus-infected BM cells. As shown in Figure 3B and E, cells generated from the I-A β wt-expressing virus-infected BM cells (I-A β wt) highly expressed MHC II compared with B cells generated from control BM cells (Cont), and B cells generated from the I-A β K>R-expressing virus-infected BM cells (I-A β K>R) showed even higher expression of MHC II than B cells generated from the I-A β wt-expressing virus-infected BM cells. In contrast, MHC I expression levels were indistinguishable among these groups. These results strongly suggest that in B cells, MHC II surface expression is regulated by ubiquitination of the I-A β chain.

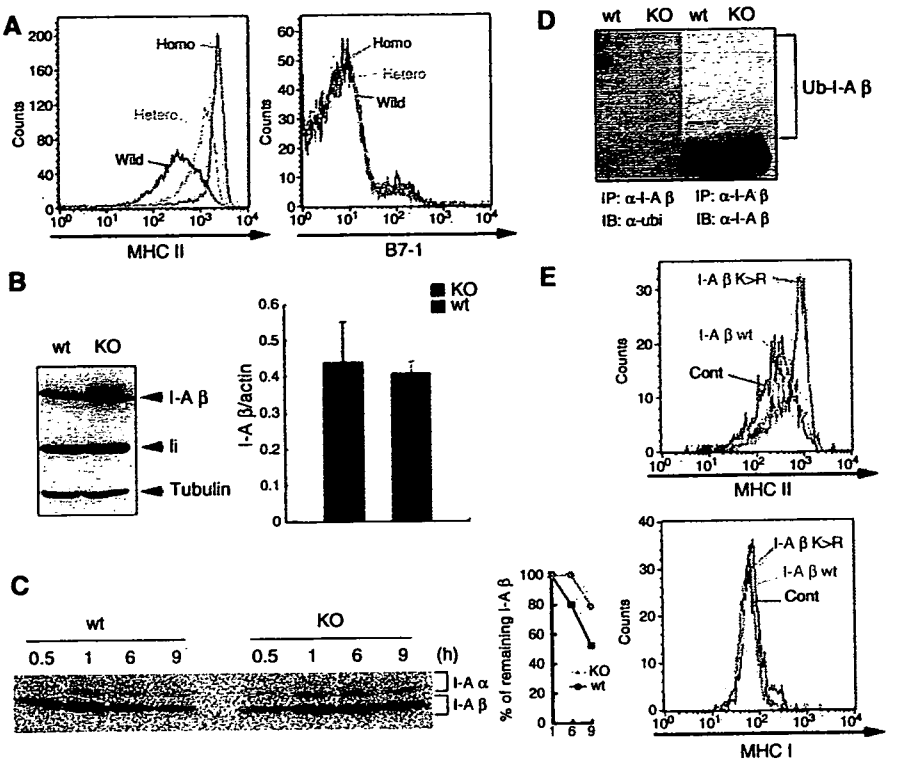

Figure 3 MHC II as a physiological substrate of MARCH-I. (**A**) MHC II (left) or B7-1 (right) surface expression was examined in blood-circulating B cells. Data from control littermates (wild), heterozygous MARCH-I KO (Hetero), and homozygous MARCH-I KO (Homo) are shown. Data are representative of two independent experiments. (**B**) In the left panel, I-A β chain protein or invariant chain (Ii) protein expression was examined in splenic B cells from respective mice. To show that the two samples have the same amount of loaded proteins, each sample was probed with anti-tubulin Ab. Data are representative of two independent experiments. Wt, control littermates; KO, homozygous MARCH-I KO. In the right panel, I-A β chain mRNA expression levels were compared between MARCH-I-deficient B cells (KO) and control B cells (Wt) by real-time PCR. Data are expressed as the mean ± s.d. of triplicate samples, and values are representative of two independent experiments. (**C**) Splenic B cells from each mouse were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min and chased for 0.5–9 h. Labeled protein samples were extracted and precipitated with Y-3P anti-I-A β chain Ab and analyzed by SDS–PAGE. At each point, the percentage of remaining I-A β chain was calculated relative to the amount of labeled I-A β chain at 1 h of chase (right panel). Data are representative of three independent experiments. (**D**) MHC II molecules were purified with Y-3P anti-I-A β chain Ab, and subjected to Western blot analysis with FK2 anti-ubiquitin Ab (left) or KL295 anti-I-A β chain Ab (right). Data are representative of two independent experiments. (**E**) Sca-1$^+$ BM cells were infected with retrovirus that expresses the I-A β chain wild type (I-A β wt), I-A β chain mutant type (I-A β K > R), or human CD8 alone (Cont). Chimeric mice were generated with these modified BM cells. Eight weeks after reconstitution, blood-circulating B cells expressing the same level of human CD8 were analyzed in terms of MHC II expression level by FACS.

Surface MHC II molecules are stabilized in MARCH-I-deficient B cells

As shown in Figure 3A, MHC II surface expression was remarkably increased in B cells from MARCH-I KO. The next question was how the MHC II surface expression was enhanced in MARCH-I KO. MHC II surface expression level is regulated by several steps: the assembly between α and β chains, peptide loading onto αβ dimers, trafficking to the cell surface, and internalizing from the surface. As Figure 3C shows that the assembly of MHC II was not impaired in MARCH-I KO, we examined whether the formation of peptide-loaded αβ dimers was altered in MARCH-I KO. B cells from MARCH-I KO or control littermates were pulse-labeled as in Figure 3C. At the end of the chase periods, pulse-labeled proteins were immunoprecipitated with Y-3P anti-I-A^b β chain mAb, and the precipitated samples were analyzed by SDS–PAGE without boiling the samples before electrophoresis. As shown in Figure 4A, in both groups, bands corresponding to SDS-stable compact dimers, which reflect peptide-loaded αβ dimers (c(α/β)), were equally detected at 6 h of chase, suggesting that the step for peptide loading was not impaired.

Further, we examined whether the transport of MHC II to the surface was modified in MARCH-I KO. B cells from MARCH-I KO or control littermates were pulse-labeled as in Figure 4A, and at the end of the chase periods, the labeled cells were biotinylated with a membrane-impermeable reagent. The pulse-labeled and biotinylated MHC II molecules were immunoprecipitated with Y-3P mAb, followed by purification with streptavidin–agarose. As shown in Figure 4B, the transport efficiency of αβ dimers to the cell surface was slightly low in MARCH-I KO. Together, these findings did not explain how MHC II surface expression was increased in MARCH-I KO; rather, they suggest that the molecular events at the cell surface might be altered in MARCH-I KO. As the half-life of MHC II was prolonged (Figure 3C), we examined the half-life of surface MHC II in both types of mice. Surface MHC II molecules on splenic B cells were biotinylated and the stability of the biotinylated I-A β chains of MHC II was examined by IP–Western blot analysis. As MHC II surface expression was significantly upregulated in MARCH-I KO (Figure 5B), we used one-fifth of the total volume of each sample from MARCH-I KO at each chase point; otherwise,

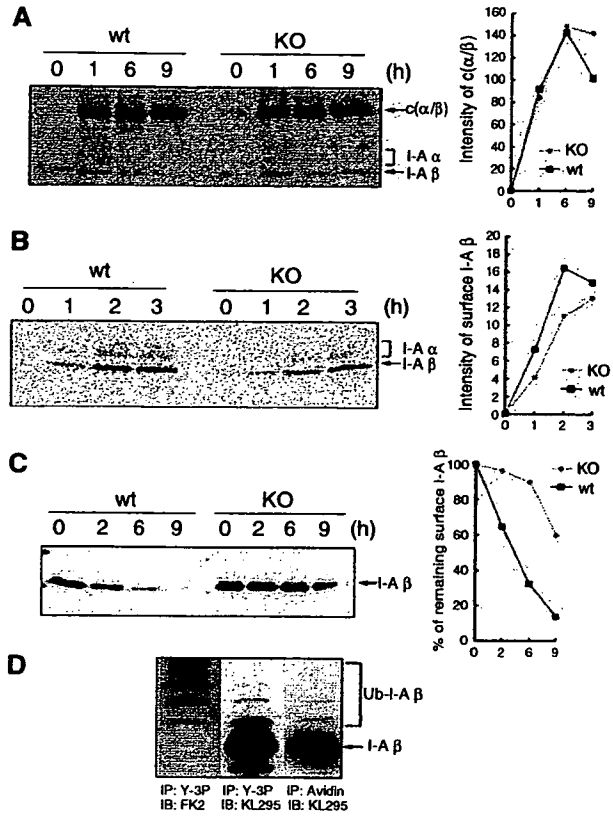

Figure 4 Stabilization of surface MHC II in MARCH-I-deficient B cells. (**A**) Splenic B cells from each mouse were pulse-labeled as shown in Figure 3C. Labeled protein samples were extracted and precipitated with Y-3P anti-I-A β chain Ab and analyzed by SDS–PAGE, without boiling the samples before electrophoresis. At each point, the intensity of SDS-stable compact dimer was measured with an image analyzer and presented far right from the panel. c(α/β) represents the SDS-stable compact dimer. Data are representative of two independent experiments. (**B**) Splenic B cells were pulse-labeled and chased as in (A). At the end of the chase periods indicated above the panel, each cell type was biotinylated with NHS-SS-biotin in PBS. The samples were precipitated with Y-3P anti-I-A β Ab, followed by precipitation with streptavidin–agarose. At each point, the intensity of the I-A β chain was measured with an image analyzer and presented far right from the panel. Data are representative of four independent experiments. (**C**) Surface MHC II molecules of splenic B cells were biotinylated and chased for 0–9 h. At each point, the amount of biotinylated MHC II was determined by Western blot analysis with KL295 anti-I-A β chain Ab, and the percentage of remaining surface I-A β was calculated relative to the value at 0 h (right panel). Data are representative of two independent experiments. (**D**) Surface MHC II molecules of splenic B cells from control littermates were biotinylated, purified with streptavidin–agarose, and analyzed with KL295 MHC II mAb (right panel). The same samples were incubated with Y-3P MHC II mAb and precipitated MHC II proteins were eluted with SDS buffer and analyzed with FK2 ubiquitin mAb (left panel) or KL295 MHC II mAb (middle panel). Data are representative of two independent experiments.

signals from the biotinylated I-A β chains from MARCH-I KO would have been too strong to enable precise comparison with those from control littermates. As shown in Figure 4C, the half-life of MHC II was significantly prolonged in B cells from MARCH-I KO. Collectively, these results indicate that MARCH-I modulates MHC II surface expression presumably through ubiquitination at the B cell surface. To investigate this hypothesis, we examined the status of ubiquitination of surface MHC II in B cells from control littermates. Surface MHC II molecules in B cells from control littermates were biotinylated, purified with streptavidin–agarose, and analyzed with KL295 MHC II mAb. The same samples were incubated with Y-3P MHC II mAb and precipitated MHC II proteins were analyzed with FK2 ubiquitin mAb or KL295 MHC II mAb. As shown in Figure 4D, in the sample purified with streptavidin–agarose, above the band corresponding to the unmodified I-A β chain, we detected additional bands showing similar MWs to those corresponding to the ubiquitinated I-A β chain. Thus, surface MHC II molecules in B cells were ubiquitinated.

MHC II molecules are internalized and recycled in MARCH-I-deficient B cells

We previously found that B7-2 was rapidly degraded in lysosome by c-MIR, suggesting that MHC II might be degraded in the same manner by MARCH-I (Goto *et al*, 2003). To investigate this possibility, the rate of degradation of surface MHC II was examined in the presence of bafilomycin A1, which raises endolysosomal pH through the inhibition of vacuolar H^+-ATPase. As shown in Figure 5A, the degradation of the surface I-A β chain was completely blocked in the presence of bafilomycin A1. Next, we examined whether the internalization of MHC II is inhibited in MARCH-I-deficient B cells, as we also found that surface MHC II molecules were rapidly internalized by c-MIR (Ohmura-Hoshino *et al*, 2006b). Surface MHC II molecules of MARCH-I-deficient B cells were biotinylated with a membrane-impermeable derivative of biotin. After incubation for 30 min, the remaining cell-surface biotin was cleaved with glutathione, and internalized proteins were collected using streptavidin-conjugated beads. The internalization rate of MHC II was analyzed with KL295 MHC II mAb. Unexpectedly, in MARCH-I KO, MHC II molecules were clearly internalized, as observed in control littermates (Figure 5B). These results demonstrate that ubiquitination is not essential for the internalization of MHC II in B cells.

These results suggest that ubiquitination mainly contributes to the sorting of MHC II into acidic organelles such as lysosome, for degradation, and in the absence of MARCH-I, internalized MHC II might be efficiently recycled to the surface, as MARCH-I-deficient B cells highly expressed MHC II on their surface (Figure 3A). To confirm these, we examined where the internalized MHC II molecules are localized in B cells from MARCH-I KO or control littermates. Surface MHC II molecules were labeled with FITC-conjugated AF6-120.1 anti-MHC II mAb at 4°C and chased for 60 min at 37°C. LysoTracker and transferrin were used to viably label the lysosomal compartment and the recycling compartment, respectively. To clearly visualize the internalized MHC II, labeled MHC II molecules remaining on the surface were removed with acidic solution. As shown in Figure 5C, MHC II molecules were internalized in both types of cells, and the amount of labeled MHC II detected inside MARCH-I-deficient B cells was larger than that detected inside B cells from control littermates. As expected, in B cells from control littermates, most internalized MHC II molecules were sorted into the lysosomal compartment (left panel in Figure 5C). In contrast, most internalized MHC II molecules were sorted into the recycling compartment in MARCH-I-deficient B cells (right panel in Figure 5C). Collectively, these results suggest

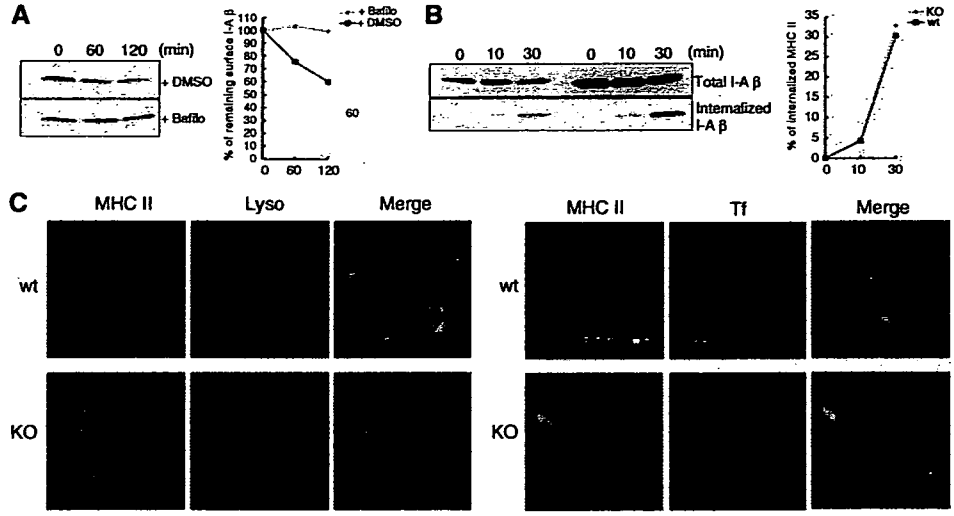

Figure 5 Internalization and recycling of MHC II in MARCH-I-deficient B cells. (**A**) Purified B cells were pretreated with 10 μM bafilomycin A1 (Bafilo) or DMSO at 37°C for 30 min. Surface MHC II molecules of pretreated cells were biotinylated and chased for 0–120 min. At each point, the amount of biotinylated MHC II was determined by Western blot analysis with KL295 anti-I-A β chain Ab, and the percentage of remaining surface I-A β chain was presented far right from the panel. Data are representative of two independent experiments. (**B**) Surface molecules of the same number of splenic B cells from MARCH-I KO or control littermates were biotinylated and incubated at 37°C for 10 or 30 min. At each point, the remaining cell-surface biotin was cleaved by reducing its disulfide linkage. Upper panel shows the total amount of biotinylated surface I-A β chains and lower panel shows the amount of internalized I-A β chains at each incubation time. At each point, the percentage of internalized MHC II was calculated relative to the total amount of biotinylated surface I-A β chains, and presented far right from the panel. Data are representative of four independent experiments. (**C**) Purified B cells were stained with FITC-conjugated AF6-120.1 anti-I-A β chain mAb at 4°C and washed with 2% calf serum-containing PBS. Labeled B cells were cultured in RPMI with 10% fetal calf serum in the presence of 50 nM LysoTracer Red DND-99 or 5 μg/ml Alexa 594-conjugated transferrin (Invitrogen) for 60 min at 37°C. Cells were washed in an acidic solution to remove uninternalized Abs, fixed, and subjected to examination with a LEICA DMIRE2 confocal laser scanning microscope.

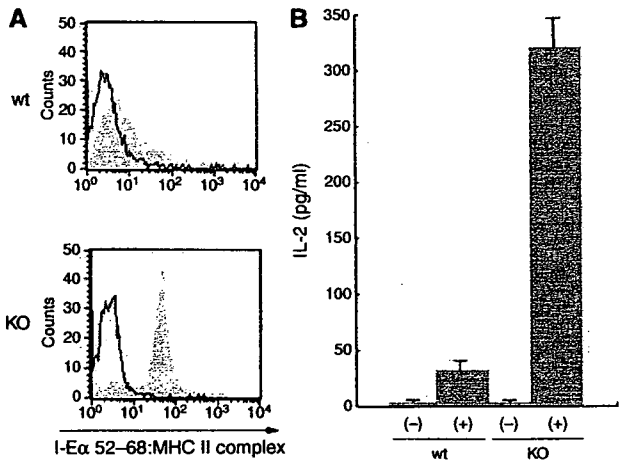

Figure 6 Enhanced antigen presentation by MARCH-I-deficient B cells. (**A**) Splenic B cells from each mouse type were incubated with 10 μM I-Eα peptide 52–68 for 20 h, and surface expression of I-Eα peptide-loaded MHC II was analyzed using Y-Ae mAb. Data are representative of two independent experiments. Open and shaded histograms show the values for non-incubation and incubation with peptide, respectively. (**B**) Splenic B cells incubated as in (A) were fixed with 0.5% paraformaldehyde and incubated with 20.6 T-T hybridoma overnight. IL-2 production from 20.6 T-T hybridoma was determined by enzyme-linked immunosorbent assay. Data are expressed as the mean±s.e.m. of triplicate samples, and values are representative of two independent experiments. (–) and (+) indicate non-incubation and incubation with peptide, respectively.

that in B cells, MARCH-I-mediated ubiquitination of the I-A β chain leads to the sorting of MHC II into acidic organelles such as lysosome to regulate MHC II surface expression.

MARCH-I-deficient B cells highly present exogenous antigens through MHC II

As shown in Figure 3A, in MARCH-I-deficient B cells, the amount of surface MHC II was remarkably increased. The same result was obtained using Y-3P MHC II mAb that preferentially recognizes mature αβ dimers of MHC II, suggesting that surface MHC II expressed in MARCH-I-deficient B cells would be functional. To investigate this possibility, we examined whether MARCH-I-deficient B cells can express exogenous peptide-loaded MHC II on their surface. B cells from MARCH-I KO or control littermates were incubated with I-Eα peptide 52–68 for 20 h, and the surface expression of I-Eα peptide-loaded MHC II was monitored with Y-Ae mAb that specifically recognizes the complexes of I-A^b MHC II presenting the I-Eα peptide 52–68 (Rudensky *et al*, 1991). As shown in Figure 6A, MARCH-I-deficient B cells highly expressed I-Eα-peptide-loaded MHC II relative to B cells from control littermates. To confirm the enhanced presentation of the I-Eα peptide 52–68 in MARCH-I-deficient B cells, peptide-loaded B cells from MARCH-I KO or control littermates were fixed with 0.5% paraformaldehyde, and incubated with 20.6 T-T hybridoma that recognizes the MHC II–peptide complex detected with Y-Ae mAb (Ignatowicz *et al*, 1996). As shown in Figure 6B, IL-2 production from 20.6 T-T hybridoma was significantly enhanced in MARCH-I-deficient B cells. These results demonstrate that the expression of functional MHC II is increased in MARCH-I-deficient B cells.

Discussion

In this report, we demonstrated two important novel findings in B cells: the presence of post-translational regulation of

functional MHC II by ubiquitination, and the indispensable role of MARCH-I in MHC II ubiquitination. These important findings were based on the fact that the forced expression of MARCH-I downregulated MHC II surface expression through ubiquitination of the lysine residue at position 225 in the I-A β chain, and that surface functional MHC II molecules were stabilized owing to complete loss of ubiquitination of the I-A β chain of MHC II in MARCH-I-deficient B cells. Given that MHC II molecules are indispensable for T-cell-mediated immunity (Cosgrove *et al*, 1991), these novel findings serve as clues to understand the molecular basis of immune regulation.

MARCH-I was also expressed moderately in DCs and macrophages (Figure 1B), suggesting that MHC II is also regulated by ubiquitination in other APCs. Indeed, we found that MHC II expression was also augmented on the surface of bone-marrow-derived DCs (BMDCs) and splenic DCs in MARCH-I KO (Supplementary Figure 2A). In addition, the amount of ubiquitinated MHC II was decreased in BMDCs from MARCH-I KO, compared with those from control littermates (Supplementary Figure 2B). Interestingly, we found that in MARCH-I-deficient BMDCs, ubiquitinated MHC II did not completely disappear, indicating that other E3s might play a role in the ubiquitination of MHC II in BMDCs (Supplementary Figure 2B). In relation to this, we previously showed that c-MIR was expressed in DCs. c-MIR is a homologue of MARCH-I and acts to effectively downregulate and ubiquitinate MHC II (Ohmura-Hoshino *et al*, 2006b). Therefore, in DCs, c-MIR and MARCH-I might work together in ubiquitinating MHC II. On the other hand, in B cells, the expression level of c-MIR was extremely low (Ohmura-Hoshino *et al*, 2006b); thus, MARCH-I is thought to be a unique E3 for MHC II in B cells.

What is the role of MHC II ubiquitination in B cells? We showed that ubiquitination is crucial for the turnover of functional MHC II at the surface of B cells. In the absence of MARCH-I, an E3 for MHC II, the turnover of mature surface MHC II was decreased. As the results of experiments with Y-Ae mAb and I-Eα-specific T cell hybridoma indicated that stabilized MHC II molecules are capable of presenting exogenous antigens, MHC II ubiquitination might prevent excessive antigen presentation. In this connection, an important observation has been reported recently (Kitamura *et al*, 2006), strongly suggesting that MHC II surface expression is regulated by zinc-requiring enzymes. The catalytic domain of MARCH-I is a variant type of RING domain that requires zinc to maintain its structure and function as E3. DCs treated with TPEN, a zinc-chelating reagent, highly expressed MHC II on the surface and highly presented peptide antigens. Thus, in TPEN-treated DCs, MARCH-I might be inactivated owing to zinc deficiency, leading to an increase in MHC II surface expression. It would be interesting to investigate the status of MHC II ubiquitination in TPEN-treated DCs.

In order to answer the question of the role of MHC II ubiquitination in B cells, the results from MARCH-I KO have to be carefully interpreted, because other molecules might be targeted by MARCH-I *in vivo*. For example, the interpretation of the results showing increased ability of antigen presentation by MARCH-I-deficient B cells should be done with caution. Indeed, the forced expression of MARCH-I has been reported to downregulate transferrin receptor, B7-2, and Fas (Bartee *et al*, 2004). Therefore, to answer the question raised above, we are going to generate MHC II knock-in mice in which mutant I-A β, whose lysine residue at position 225 was mutated to arginine, is expressed in APCs. As shown in Figure 2B, mutant MHC II was not ubiquitinated by MARCH-I, so that in this knock-in mouse, the ubiquitination-mediated regulation of MHC II is expected to be disrupted.

In our experiments, we found that MHC II ubiquitination was not necessary for the internalization of MHC II in B cells. Previously, we demonstrated that the forced expression of c-MIR induced the rapid internalization of MHC II using A20 cells (Ohmura-Hoshino *et al*, 2006b). As c-MIR and MARCH-I are thought to share the molecular machineries for the downregulation of MHC II, we expected that MARCH-I contributes to the initiation of MHC II internalization from the plasma membrane. Unlike primary B cells used in this study, A20 cells might have different features of MHC II traffic. Indeed, we could not observe the spontaneous internalization of MHC II in A20 cells (Ohmura-Hoshino *et al*, 2006b). Thus, ubiquitination might play an important role in MHC II internalization in the situation where spontaneous internalization does not take place, such as in A20 cells. However, in the situation where spontaneous internalization takes place, ubiquitination might be no longer necessary for internalization. Recently, the contribution of the AP2 clathrin adaptor complex to the rapid internalization of MHC II was reported (Dugast *et al*, 2005; McCormick *et al*, 2005). It would be interesting to examine whether the AP2 complex is involved in the spontaneous internalization of MHC II in primary B cells.

MARCH-I is highly expressed in B cells. Why do B cells need MARCH-I? What happens when MHC II molecules are highly expressed in B cells? As long as the development of B cells was examined on the basis of the expression level of surface markers by FACS analysis, striking abnormalities could not be observed and a more fine-grained analysis is in order. In primary B cells, the molecular machinery for MHC II trafficking remains largely unknown. Our findings suggest that in native B cells, surface MHC II molecules are replaced with newly synthesized molecules through the degradation of pre-existing molecules, as is the case in immature DCs (Villadangos *et al*, 2005). Is this 'MHC II metabolism by MARCH-I' necessary for B cell homeostasis? We are going to answer numerous open questions that are yet to be addressed.

Materials and methods

Molecular cloning

Murine MARCH-I cDNA was generated from total RNA of the spleen by RT–PCR. The gene-targeting vector was generated using murine BAC clones. Substitutions were generated into the cytoplasmic tail of the I-A β chain by PCR-based mutagenesis (Promega). To construct retroviral vectors, I-A β chain cDNA was subcloned into pMX-IRES-hCD8 (Yamasaki *et al*, 2006).

Mice

The *Xho*I-linearized gene-targeting vector was electroporated into R1 embryonic stem (ES) cells, and targeted ES cells were selected with G418. ES cell colonies were screened by Southern blot analysis. Four independent clones were injected into blast cysts and two independent clones gave rise to chimeric mice that transmitted the desired mutation to the germ line. Two independent gene-targeted mice were mated with CAG-Cre mice to generate MARCH-I KO. All mice were maintained under specific pathogen-

free conditions according to RIKEN's guidelines for animal facilities and used for analysis at 8–12 weeks of age.

Northern blot analysis and quantification by real-time RT–PCR
For Northern blot analysis, 2 μg of poly (A) + RNA was extracted from various tissues, transferred to Hybond-N + membrane (GE Healthcare Bio-Sciences), and probed with ^{32}P-labeled cDNA probes. For MARCH-I mRNA quantification, the TaqMan Gene Expression Assay (Applied Biosystems) was employed, as described previously (Ohmura-Hoshino *et al*, 2006b). Quantitative analysis of I-A β chain expression was performed as described previously (Goto *et al*, 2003).

Pulse–chase analysis
Cells were labeled in medium containing 50 μCi of [^{35}S]methionine and [^{35}S]cysteine (Perkin Elmer) for 30 min and chased for the indicated time. Labeled proteins were extracted with 1% NP 40 buffer (1% NP 40, 300 mM NaCl, and 50 mM Tris buffer (pH 7.4)) containing protease inhibitors, and incubated with Y-3P anti-I-A^b Ab. Precipitated labeled MHC II molecules were subjected to quantitative analysis using Fuji BAS 2500 (FUJIFILM Corporation).

Detection of the ubiquitinated I-A β chain
In order to detect ubiquitination of the exogenous I-A β chain, we employed a previous method (Ohmura-Hoshino *et al*, 2006b). For detection of endogenous MHC II ubiquitinated *in vivo*, cell lysate was obtained by extraction with 1% NP 40 buffer containing 5 mM ethylmaleimide, and MHC II molecules were precipitated with Y-3P anti-I-A^b Ab coupled with CNBr-activated Sepharose 4B (GE Healthcare Bio-Sciences). The precipitated MHC II molecules were eluted with 0.1 M glycine–HCl (pH 2.7). The eluted samples were subjected to Western blot analysis with KL295 anti-I-A β Ab (ATCC) or FK2 anti-ubiquitin Ab (AFFINITI).

Detection of MARCH-I protein
Two Abs to MARCH-I were generated. The 4526 polyclonal Ab was generated with a synthetic peptide, GCETKLKPLRKWEKLQMTTS, corresponding to amino acids 123–140 of MARCH-I. 13F11D12 mAb was generated with a synthetic peptide, GCLNMWKKSKISTMYYLNQD, corresponding to amino acids 18–35 of MARCH-I. Protein sample was extracted with 0.1% SDS–Tris buffer from the spleen, and MARCH-I protein was precipitated with 13F11D12 anti-MARCH-I Ab coupled with protein G Sepharose (GE Healthcare Bio-Sciences). The precipitated MARCH-I protein was eluted with 2% SDS-containing PBS, and the eluted samples were analyzed with biotinylated 4526 anti-MARCH-I Ab.

Immunofluorescence microscopy
Purified B cells were stained with FITC-conjugated AF6-120.1 anti-I-A β chain mAb (BD Immunocytometry System) at 4°C and washed with 2% calf serum-containing PBS. Labeled B cells were cultured in RPMI with 10% fetal calf serum (FCS) in the presence of LysoTracer Red DND-99 or Alexa 594-conjugated transferrin (Invitrogen) at 37°C. Cells were washed in an acidic solution to remove uninternalized Abs, fixed, and examined with a LEICA DMIRE2 confocal laser scanning microscope.

Analysis of stability of surface MHC II
Splenic B cells were incubated with Sulfo-NHS-biotin (2 mg/ml) (Pierce) in PBS for 2 min on ice and chased for the indicated times. At the end of the chase periods, the protein samples were extracted with 0.1% SDS-containing PBS, and biotinylated proteins were precipitated with streptavidin–agarose (Pierce). Precipitated biotinylated samples were analyzed with KL295 anti-I-A β chain Ab.

Bone marrow transfer
Sca-1$^+$ BM cells from C57BL/6 mice were purified with the MACS system (Miltenyi Biotec) and cultured at a density of 1×10^6 cells/ml in RPMI with 10% FCS, 10 ng/ml IL-7, and 100 ng/ml stem cell factor (PeproTech). At days 1 and 2, cells were infected with the I-A β-chain expressing retrovirus. Four days after infection, hCD8$^+$ cells were purified with the MACS system (Miltenyi Biotec) and transferred intravenously into irradiated (8.5 Gy) C57BL/6 mice. Eight weeks after transfer, examinations were performed.

Antigen presentation assay
Splenic B cells were incubated with 10 μM I-Eα peptide 52–68 for 20 h, and incubated B cells were stained with Y-Ae mAb that specifically recognizes the complexes of I-A^b MHC II presenting I-Eα peptide 52–68. Splenic B cells incubated with I-Eα peptides (3×10^5) were fixed with 0.5% paraformaldehyde for 10 min at room temperature, mixed with 20.6 T-T hybridoma (5×10^4) in 200 μl of a 96-well plate, and cultured overnight. IL-2 production from 20.6 T-T hybridoma was determined by enzyme-linked immunosorbent assay (BD).

Internalization assay
Internalization of MHC II was analyzed by cell-surface biotinylation assay. Splenic B cells were subjected to biotinylation on ice with the reversible membrane-impermeable derivative of biotin, sulfo-NHS-S-S-biotin (1.5 mg/ml) (Pierce), in PBS. Biotinylated B cells were incubated at 37°C for 30 min, and the remaining cell-surface biotin was cleaved by reducing its disulfide linkage with glutathione cleavage buffer. The internalized I-A β chain molecules were precipitated with streptavidin–agarose (Pierce) and analyzed with KL295 anti-I-A β chain Ab.

Cell preparation and reagents
Splenic B cells, T cells, macrophages, and DCs were purified from the spleens of C57BL/6 mice using the MACS system (Miltenyi Biotec). The fraction shown as 'other' indicates the cell fraction that contained no B cells, T cells, Mϕ or DCs. Among the splenic B cells, the T1, T2, T3, FO, and MZ fractions were collected based on the expression levels of B220, AA4.1. CD23 and were detected IgM using a FACS Vantage SE high-speed sorter (BD Immunocytometry System). HL3 CD11c Ab for DCs, 145-2C11 CD3 Ab for T cells, RA3-6B2 B220 Ab for B cells, and CI:A3-1 F4/80 for Mϕ were used for the MACS system. RA8-6B2 B220, AA4.1 C1qRp, R6-60.2 IgM, and B3B4 CD23 Abs were used for FACS Vantage. Immature DCs were prepared by culturing BM cells obtained from each mouse with GM-CSF (20 ng/ml) (Pepro Tech) for 7 days. Seven days after cultivation, immature DCs were purified using CD11c beads (Miltenyi Biotec).

Statistics
Data from enzyme-linked immunosorbent assay and real-time RT–PCR were analyzed with the Student's *t* test. Values with $P<0.05$ were considered significant.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein tyrosine kinase Syk

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Hepatitis C virus (HCV) is the major causative agent of hepatocellular carcinoma. However, the precise mechanism underlying the carcinogenesis is yet to be elucidated. It has recently been reported that Syk, a non-receptor protein tyrosine kinase, functions as a potent tumour suppressor in human breast carcinoma. This study first examined the possible effect of HCV infection on expression of Syk *in vivo*. Immunohistochemical analysis revealed that endogenous Syk, which otherwise was expressed diffusely in the cytoplasm of normal hepatocytes, was localized near the cell membrane with a patchy pattern in HCV-infected hepatocytes. The possible interaction between HCV proteins and Syk in human hepatoma-derived Huh-7 cells was then examined. Immunoprecipitation analysis revealed that NS5A interacted strongly with Syk. Deletion-mutation analysis revealed that an N-terminal portion of NS5A (aa 1–175) was involved in the physical interaction with Syk. An *in vitro* kinase assay demonstrated that NS5A inhibited the enzymic activity of Syk and that, in addition to the N-terminal 175 residues, a central portion of NS5A (aa 237–302) was required for inhibition of Syk. Moreover, Syk-mediated phosphorylation of phospholipase C-γ1 was downregulated by NS5A. An interaction of NS5A with Syk was also detected in Huh-7.5 cells harbouring an HCV RNA replicon or infected with HCV. In conclusion, these results demonstrated that NS5A interacts with Syk resulting in negative regulation of its kinase activity. The results indicate that that NS5A may be involved in the carcinogenesis of hepatocytes through the suppression of Syk kinase activities.

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INTRODUCTION

Hepatitis C virus (HCV) is the major aetiological agent of viral hepatitis worldwide after hepatitis A and B viruses (Choo *et al.*, 1989), with about 170 million people being infected. The majority of HCV-infected individuals develop chronic infection, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC). HCV is a member of the family *Flaviviridae* and its genome consists of a single-stranded, positive-sense RNA of approximately 9600 nt, which encodes a polyprotein precursor of about 3010 aa. Currently, clinical HCV isolates are classified into six genotypes and more than 60 subtypes (Doi *et al.*, 1996; Mellor *et al.*, 1995; Robertson *et al.*, 1998). The polyprotein is cleaved by signal peptidase, signal peptide peptidase and two virally encoded proteases to generate at least ten mature proteins: core, envelope glycoprotein 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Okamoto *et al.*, 2004; Reed & Rice, 2000).

HCV NS5A is part of the replication complex that catalyses replication of the viral genome. NS5A takes two forms, p56 and p58, with different degrees of phosphorylation, which may play distinct roles in the virus replication cycle (Evans

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