

Fig. 3 Voltage-dependency of ATP-induced currents in mock-, wild-type UCH L1- and C90S UCH L1-transfected PC12 Tet-Off cells. **A:** The voltage protocol shown in the upper panel was applied before and during application of 1 mM ATP at the time indicated by (i) and (ii) in the lower panel. **(b)** Cumulated current traces obtained in wild type UCH L1-transfected cells before (i) and during (ii) application of ATP.

The subtracted current traces [(ii)-(i)] show the ATP-induced currents. **(c)** The current-voltage relationships of ATP-induced currents. The amplitudes of subtracted currents [(ii)-(i)] in (b) at the end of 50 ms pulses were plotted against the pulse potentials in mock- (○), wild-type (●) and C90S UCH L1-transfected cells (△).

Then the amplitudes of ATP-induced currents at different voltages were obtained by subtracting the one before application of ATP from the one during application of ATP, and were plotted as in Fig. 3(c). In consideration of desensitization, the current-voltage relationships were obtained by applying voltage steps in opposite direction, i.e. from +50 to -100 mV, but there was almost no change (data not shown). The reversal potential was about 0 mV, suggesting that these currents were due to non-specific cationic channels.

ATP-induced inward currents were concentration dependent and the sensitivity of ATP was not significantly changed by overexpression of either wild- or C90S UCH L1. Each EC_{50} was 34 μ M, 40 μ M and 62 μ M and each Hill coefficient (n_H) was 1.38, 1.48 and 1.34 in mock-, wild-type and C90S UCH L1-transfected cells, respectively (Fig. 4).

Effects of wild type and C90S UCH L1 on mono-ubiquitin expression

It was reported that absence of UCH L1 reduced the mono-ubiquitin level in mouse brain and UCH L1 overexpression increased the level of mono-ubiquitin by alteration of ubiquitin metabolism in cultured cells. Therefore, UCH L1-mediated increases in ubiquitin levels are a function of UCH L1 affinity for ubiquitin rather than hydrolase activity (Osaka *et al.* 2003). To clarify the effect of UCH L1 on ubiquitin levels in PC12 Tet-Off cells, ubiquitin was visualized using confocal immunofluorescence microscopy (Fig. 5). Wild-type UCH L1-transfected cells showed stronger immunoreactivity for ubiquitin compared with those

in mock-transfected cells or non-transfected cells in the same field. Increased ubiquitin immunoreactivity was also evident in C90S UCH L1-transfected cells. These results were consistent with the previous report that ubiquitin were up-regulated by UCH L1 (Osaka *et al.* 2003).

Effects of Kinase inhibitors on ATP-induced currents in UCH L1-transfected cells

The mechanism by which ATP-induced currents were augmented in UCH L1-transfected cells was investigated. It was reported that in *Aplysia* UCH activated PKA as a result of the degradation of regulatory subunit of PKA, which contributed the long-term potentiation (Hegde *et al.* 1997). Therefore, the possibility of the involvement of the activated PKA was tested by using H-89, a PKA inhibitor. After obtaining a large ATP-induced currents in the UCH L1-transfected cells, 10 μ M H-89 was applied for 10 min. The amplitude of ATP-induced currents in the presence of H-89 was $48.1 \pm 3.51\%$ ($n = 8$) compared to the first ATP-induced current in the same cell (control without H-89; $71.2 \pm 5.6\%$ ($n = 7$)) (Fig. 6a). Also, it was reported that the intracellular carboxyl terminus of P2X receptor contains several consensus phosphorylation sites for PKC as well as PKA, suggesting that the function of the P2X receptor could be regulated by protein phosphorylation (Chow and Wang 1998). Hence, the possibility of the involvement of the activated PKC was tested by using chelerythrine, a PKC inhibitor. Application of 5 μ M chelerythrine for 10 min had no effect on the ATP-induced inward current in the UCH L1-transfected cell. The relative amplitude of second

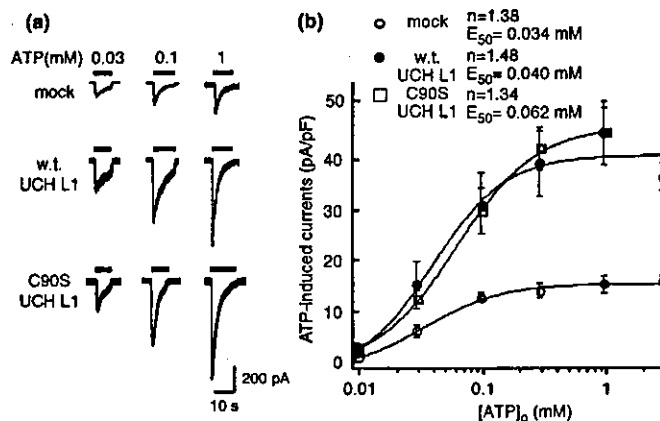


Fig. 4 Concentration-dependent curve of ATP-induced currents in mock-, wild-type and UCH L1-transfected PC12 Tet-Off cells. (a) Inward membrane currents induced by 0.03, 0.1 and 1 mM ATP at the holding potential of -70 mV in mock-, wild-type and C90S UCH L1-transfected PC12 Tet-Off cells. (b) The peak inward current induced by ATP at the holding potential of -70 mV was plotted against the

ATP concentration between 0.01 and 3 mM in mock (○), wild-type (●) and C90S UCH L1 (□)-transfected PC12 Tet-Off cells. Each point represents the mean of five or six cells and the bar shows \pm SEM. The curve shows the least squares fit, where n_H = Hill coefficient and EC_{50} = the half maximum concentration.

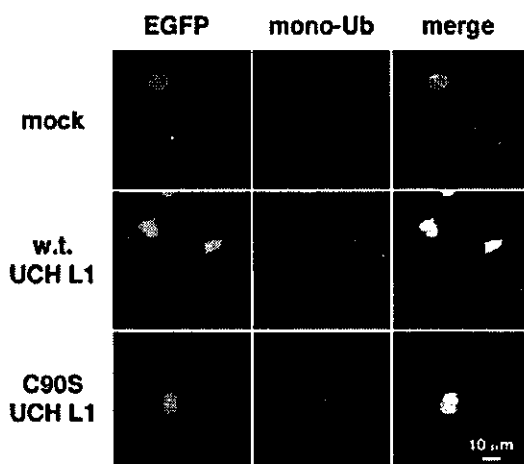


Fig. 5 Effects of wild-type and C90S UCH L1 on mono-ubiquitin expression. Confocal image of PC12 Tet-Off cells transfected pBI-EGFP-mock, wild-type (wt) UCH L1 and C90S UCH L1 with Lipofectamine 2000 were double stained with mono-ubiquitin (red) and GFP (green).

ATP-induced inward currents in the presence of chelerythrine was $71.9 \pm 4.2\%$ ($n = 7$) (control without chelerythrine; $71.2 \pm 5.6\%$ ($n = 7$)) (Fig. 6a). Furthermore, the possibility of the involvement of CaMKII was also tested by using KN-93, a CaMKII inhibitor. Application of $10 \mu\text{M}$ KN-93 for 20 min significantly reduced the ATP-induced inward current in the UCH L1-transfected cell ($69.9 \pm 6.7\%$ ($n = 5$); control, $90.2 \pm 3.5\%$ ($n = 5$)) (Fig. 6b).

In UCH L1-transfected PC12 Tet-Off cells, increased ATP-induced currents were not completely reversed by H-89 or KN-93, suggesting that both PKA and CaMKII contributed

independently. Hence, the combination of PKA and CaMKII was tested to see whether coapplication of H-89 and KN-93 inhibited the effect of UCH L1 additively. Coapplication of $10 \mu\text{M}$ KN-93 and $10 \mu\text{M}$ H-89 attenuated ATP-induced currents more strongly than that with single kinase inhibitor. The relative amplitude of ATP-induced inward currents was $45.8 \pm 2.7\%$ ($n = 7$) (control without inhibitors; $90.2 \pm 3.5\%$ ($n = 5$)) (Fig. 6b).

In PC12 cells and hippocampal neurons, it was reported that activation of PKA caused activation of extracellular signal-regulated kinase (ERK), subsequent phosphorylation of Ca^{2+} -stimulated cAMP response element binding protein (CREB) and stimulated transcription. Such signal transduction was predicted to contribute to long-term potentiation (Impey *et al.* 1998). Likewise, the augmentation of ATP response in UCH L1-transfected cell might be due to the stimulation of transcription that increased the number of P2X receptors. To test this possibility, we examined whether mitogen-activated protein kinase (MAPK) including ERK was activated following the activation of PKA in PC12 Tet-Off cells. The result was that even after application of cells with $5 \mu\text{M}$ PD98059, one of the MAPK kinase inhibitors, for 4 days, ATP-induced currents in UCH L1-transfected cells were not affected. The amplitude of ATP-induced inward currents after the application of PD98059 was 53.3 ± 3.5 pA/pF ($n = 2$) (control without PD98059; 50.8 ± 5.2 pA/pF ($n = 8$)) (Fig. 6c).

Expression of DARPP-32 in PC12 Tet-Off cells

In rat striatum, positive feedback mechanism of dopamine signaling via PKA was reported. Activation of PKA causes phosphorylation at threonine 34 (Thr-34) of dopamine and cAMP-regulated phosphoprotein with molecular weight of

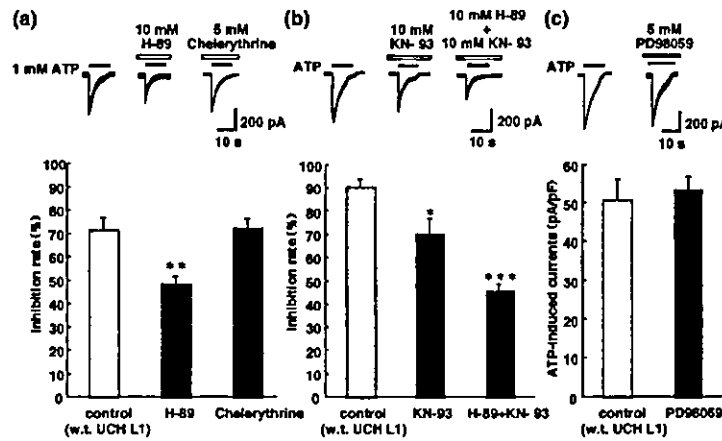


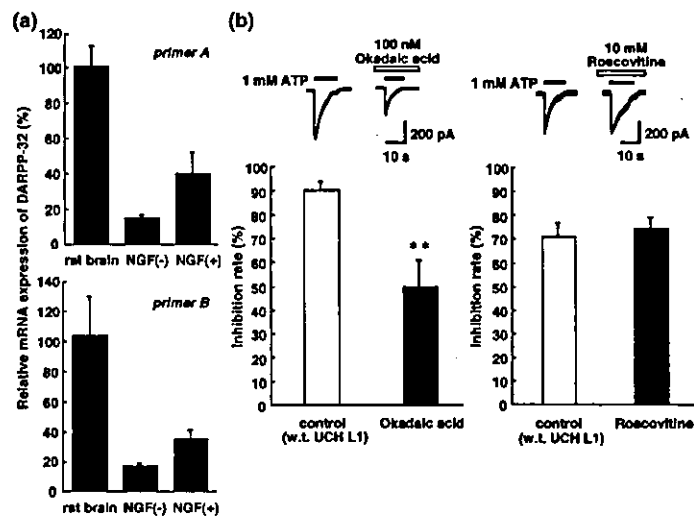
Fig. 6 Inhibition of ATP-induced currents by kinase inhibitors in wild type UCH L1-transfected PC12 Tet-Off cells. (a) ATP-induced currents were attenuated by pre-application of 10 μ M H-89, a PKA inhibitor, but not by 5 μ M chelerythrine, a PKC inhibitor, for 10 min. (b) ATP-induced currents were attenuated by pre-application of 10 μ M KN-93, a CaMKII

inhibitor, for 20 min. ATP-induced currents were further attenuated by copreapplication of 10 μ M KN-93 and 10 μ M H-89. C: ATP-induced currents were not affected by application of 5 μ M PD98059, a MAPKK inhibitor, for four days. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

about 32 000 (DARPP-32), which reduces PP1 activity and consequently inhibits dephosphorylation of various substrates in the cell. On the other hand, activation of PKA stimulates PP2A activity and suppresses the phosphorylation at Thr-75 of DARPP-32. Since phosphorylation of DARPP-32 at Thr-75 has negative feedback regulation on PKA activity, dephosphorylation at Thr-75 reduces the inhibition of PKA activity (Nishi *et al.* 2000). To examine whether the similar mechanism exists in PC12 Tet-Off cells, we analyzed the expression of DARPP-32 first. With RT-PCR method using two kinds of primer sets specific to DARPP-32 (primer set A and B), the relative expression levels of DARPP-32 in rat whole brain and PC12 Tet-Off cells under non-differen-

tiated and differentiated conditions were compared. As shown in Fig. 7(a), DARPP-32 was expressed in PC12 Tet-Off cells and the expression level tended to increase after differentiation of the cells with NGF. The possibility of the involvement of DARPP-32 and its phosphorylation was further tested using okadaic acid, a PP1 and PP2A inhibitor. The relative amplitudes of ATP-induced inward currents after the application of 100 nM okadaic acid for 20 min was significantly inhibited to $49.9 \pm 11.1\%$ ($n = 5$) (control without okadaic acid; $90.2 \pm 3.5\%$ ($n = 5$)), presumably due to the inhibition of PP2A and subsequent dephosphorylation of Thr-75, leading the release of negative feedback on PKA (Fig. 7b). Since phosphorylation of Thr-75 was also

Fig. 7 Quantitative RT-PCR of DARPP-32 and effects of okadaic acid and roscovitine on ATP-induced currents in wild-type UCH L1-transfected PC12 Tet-Off cells. (a) The expression level of DARPP-32 mRNA was normalized to that of rat brain β -actin mRNA. Using two kinds of primer (a and b), PC12 Tet-Off cells were shown to express DARPP-32 whose level was increased by differentiation of the cells with NGF. B: ATP-induced currents were attenuated by preapplication of 100 nM okadaic acid, a PP1 and PP2 inhibitor, for 20 min. (c) ATP-induced currents were not affected by pre-application 10 μ M roscovitine, a CDK5 inhibitor, for 10 min ** $p < 0.01$.



mediated by cyclin-dependent kinase (CDK), the effect of a CDK inhibitor was tested to see if ATP-induced currents were more enhanced. However, application of 10 μ M roscovitine for 10 min did not have significant effect ($74.4 \pm 5.1\%$ ($n = 4$); control; $71.2 \pm 5.6\%$ ($n = 7$)) (Fig. 7c).

ATP-induced currents in mock-transfected cells

We concluded that the increase of ATP-induced inward currents in UCH L1-transfected cells was partly attributed to the activation of PKA. Hence, we tested whether ATP-induced currents in the cells not transfected with UCH L1 were increased by the application of forskolin, an adenylate cyclase activator that increases intracellular level of cAMP (Conn *et al.* 1989). The ATP-induced currents in mock-transfected cells were significantly increased after the application of 10 μ M forskolin for 10 min ($109.2 \pm 2.2\%$ ($n = 5$); control without forskolin; $87.4 \pm 3.8\%$ ($n = 5$)) (Fig. 8a). It was confirmed that application of inactive analogue of forskolin, 10 μ M 1,9-dideoxyforskolin, did not have such effect ($79.2 \pm 2.2\%$ ($n = 5$); control; $87.4 \pm 3.8\%$ ($n = 5$)) (Fig. 8a). Next, the effects of kinase inhibitors on ATP-induced currents were tested in mock-transfected cells. In mock-transfected cells, application of 10 μ M H-89 for 10 min or 10 μ M roscovitine for 10 min had no effect on the ATP-induced inward current (H-89, $84.3 \pm 3.0\%$ ($n = 4$); roscovitine, $93.4 \pm 5.0\%$ ($n = 4$); control; $87.4 \pm 3.8\%$ ($n = 5$)) (Fig. 8a). On the other hand, ATP-induced inward currents were significantly increased after application of 100 nM okadaic acid for 20 min ($62.5 \pm 6.7\%$ ($n = 5$)). However, application of 10 μ M KN-93 had no effect ($111.3 \pm 7.1\%$ ($n = 3$); control; $79.0 \pm 3.8\%$ ($n = 5$)) (Fig. 8b).

ATP-induced currents in C90S UCH L1-transfected cells

Since ATP-induced currents in and C90S UCH L1-transfected PC12 Tet-Off cells were significantly potentiated as well, the same pharmacological analyses were done to see whether

or not the mechanism was the same as in wild-type UCH L1-transfected cells. Application of 10 μ M H-89 for 10 min significantly reduced ATP-induced currents in C90S UCH L1-transfected cells ($61.9 \pm 2.0\%$ ($n = 8$); control without H-89, $78.4 \pm 3.3\%$ ($n = 4$)) (Fig. 9a). Also, applications of 10 μ M KN-93 or 100 nM okadaic acid for 20 min significantly reduced ATP-induced inward currents in C90S UCH L1-transfected cells (KN-93, $58.0 \pm 3.7\%$ ($n = 5$); okadaic acid, $65.1 \pm 4.9\%$ ($n = 6$); control; $88.0 \pm 3.4\%$ ($n = 5$)) (Fig. 9b). Furthermore, ATP-induced currents were more attenuated by copreapplication of 10 μ M KN-93 and 10 μ M H-89 ($44.2 \pm 3.7\%$ ($n = 6$); control; $88.0 \pm 3.4\%$ ($n = 5$)) (Fig. 9b).

Discussion

To analyze the functional role of UCH L1 in the central nervous system (CNS), it is important to know whether UCH L1 has any effects on ion channels and receptors that are the basic elements of neurotransmission. There are many ways to analyze them, but one of the most simple but efficient ways to start is to analyze the effects of exogenous UCH L1 in neuronal cultured cell line. To confirm that cells express the transfected protein, we used CHO-AA8-Lucl cells because of the higher transfection efficacy and not having endogenous UCH L1 (Fig. 1). For functional analyses of UCH L1 in nervous system, we used PC12 Tet-Off cells. The engineered PC12 cells are constructed to have higher transfection efficiency than wild-type PC12 cells which are one of the popular neuronal cell line (unpublished data). Among neurotransmitter receptors in PC12 cells, we analyzed ATP-activated receptors that are widely distributed in the brain and involved in various biological activities including neurosecretion. In PC12 cells, P2X₂ and P2X₄ receptors (Hur *et al.* 2001) with lower level of P2X₆ (unpublished data) are expressed and ATP-induced inward currents were well characterized (Nakazawa *et al.* 1994). We recorded

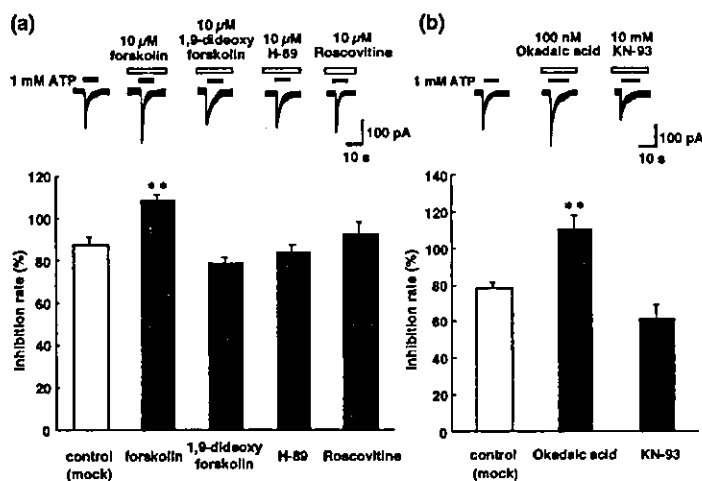


Fig. 8 ATP-induced currents in mock-transfected PC12 Tet-Off cells. (a) ATP-induced currents were augmented by preapplication of 10 μ M forskolin for 10 min, but were not affected by 10 μ M H-89 and 10 μ M roscovitine. (b) ATP-induced currents were augmented by 100 nM okadaic acid, but were not affected by 10 μ M KN-93. ** $p < 0.01$.

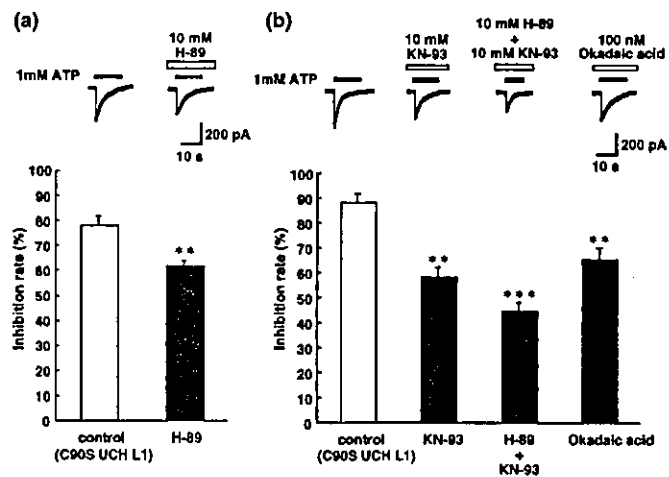


Fig. 9 ATP-induced currents in C90S UCH L1-transfected PC12 Tet-Off cells were also dependent on PKA and CaMKII. (a) ATP-induced currents were attenuated by preapplication of 10 μ M H-89 for 10 min. (b) ATP-induced currents were attenuated by preapplication of 10 μ M KN-93 and 100 nM okadaic acid for 20 min. ATP-induced currents were further attenuated by copreapplication of 10 μ M H-89 and 10 μ M KN-93. ** $p < 0.01$, *** $p < 0.001$.

ATP-induced inward currents due to the activation of P2X receptor channels at the holding potential of -70 mV under the conventional whole-cell patch clamp configuration. To analyze the effects of overexpression of UCH L1 and for pharmacological characterization, high concentration of ATP (1 mM) was used to see the effects on the maximum response to ATP. As to the possibility to analyze other receptor channels expressed in PC12 cells, nicotinic acetylcholine receptor (nAChR) was likely to be analyzed. However, no currents were recorded using ACh or nicotine (not shown), probably because nAChR required longer time to be expressed after differentiation (Fukukawa *et al.* 1992).

The amplitude of ATP-induced inward currents was significantly greater in both wild-type and mutant (C90S) UCH L1-transfected PC12 Tet-Off cells (Fig. 2a). We found that the potentiation of ATP-induced currents was due to the activation of PKA and CaMKII (Fig. 6). Though activation of PKA by homologous UCH was reported in *Aplysia* (Hegde *et al.* 1997), the mechanism of PKA activation in PC12 cells would be different from the one in *Aplysia*. In *Aplysia*, UCH enhances the degradation of the regulatory subunit of PKA and consequently activate PKA. However, in our study, the mutant (C90S) UCH L1, which lacks hydrolase activity, also potentiated ATP-induced currents, which was also attenuated by H-89 and KN-93 (Figs 2 and 9). Therefore, function of UCH L1 other than hydrolase activity should play a fundamental role. Based on the previous reports that UCH L1 has multifunction, one of the plausible reason would be the ability to increase free ubiquitin by both wild-type and C90S mutant UCH L1. It was reported that C90S UCH L1 lacks hydrolase activity, but retains ubiquitin binding affinity and increases free ubiquitin level in SH-SY5Y cells (Osaka *et al.* 2003). Actually, immunostaining of mono-ubiquitin was stronger in the cytoplasm of wild-type and C90S UCH L1-transfected PC12 Tet-Off cells (Fig. 5). The mechanism how increased level of mono-ubiquitin activated PKA and

CaMKII were not yet known and should be investigated further.

The mechanism how activated PKA and CaMKII potentiate ATP-induced currents also remains to be investigated. One possible mechanism would be phosphorylation of P2X receptors by these protein kinases, though it was reported that activation of PKA reduced the magnitude of the ATP-activated current in P2X₂-transfected HEK293 cells (Chow and Wang 1998). The potentiation of ATP-induced currents by PKA in our study might be similar to the one observed for Ca²⁺ channels (Kamp and Hell 2000). Likewise, in mock-transfected cells, the amplitude of ATP-induced currents was significantly increased by forskolin, an adenylate cyclase activator that increases intracellular levels of cAMP and activates PKA (Fig. 8). Therefore, it was suggested that at least an activation of PKA contributed to the potentiation of ATP-induced currents. As for the involvement of CaMKII, it has been recently reported that CaMKII potentiates ATP responses by promoting trafficking of P2X receptors (Xu and Huang 2004), suggesting that phosphorylation of P2X receptors were not the sole mechanism of the potentiation of ATP-induced currents. Furthermore, since increased ATP-induced currents were not completely reduced by H-89, KN-93 nor copapplication of H-89 and KN-93 in wild-type or C90S UCH L1-transfected cells (Figs 6 and 9), it was also suggested that activation of PKA and CaMKII was not the sole mechanism of the potentiation of ATP-induced currents but there may be other components, too.

Another possible mechanism how PKA potentiates ATP-induced currents would be an increase in number of P2X receptors. In *Aplysia*, homologous UCH activates PKA and consequently activates MAPK and subsequent transcription (Hegde *et al.* 1997). Such signal transduction is predicted to contribute to a long-term potentiation (Impey *et al.* 1998). If the similar signaling exists in mammalian cells, the number of P2X receptors could increase during the differentiation

after transfection of UCH L1 or C90S UCH L1. However, it was unlikely because even after incubation with MAPK inhibitor during differentiation, the augmented ATP-induced currents were still observed in wild-type (Fig. 6c) or C90S UCH L1-transfected cells (not shown).

There may be indirect effects of phosphorylation by PKA on P2X receptors. In rat striatum, it has been suggested that there are positive and negative feedback system of DARPP-32 via activation of PKA and CDK5, respectively (Nishi *et al.* 2000). To test whether the similar mechanism exists in PC12 Tet-Off cells, we first analyzed the expression of DARPP-32 with RT-PCR. DARPP-32 was expressed in PC12 Tet-Off cells and we found that the expression level of DARPP-32 was increased after differentiation of the cells with NGF (Fig. 7). Thus, the possibility of the involvement of DARPP-32 in the P2X receptor activation was tested with using okadaic acid, a PP1 and PP2A inhibitor, and roscovitine, a CDK5 inhibitor. Roscovitine had no effects on the ATP-induced currents in mock- and UCH L1-transfected PC12 Tet-Off cells, suggesting that CDK5 did not play an important role in the regulation of P2X receptor via DARPP-32 in PC12 Tet-Off cells. On the other hand, the ATP-induced inward currents were attenuated by okadaic acid in wild-type or C90S UCH L1-transfected PC12 Tet-Off cells (Figs 7b and 9b), but increased in mock-transfected PC12 Tet-Off cells (Fig. 8b). Based on these results, we assume the followings; (1) In wild-type and C90S UCH L1-transfected cells, activation of PKA by wild-type or C90S UCH L1 stimulates PP2A activity and dephosphorylates DARPP-32 at Thr-75. Since phosphorylation of DARPP-32 at Thr-75 inhibits PKA activity, inhibition of PP2A by okadaic acid accelerates phosphorylation at Thr-75, which in turn has a negative feedback effect on PKA activity and their substrates. On the other hand, activation of PKA by wild-type or C90S UCH L1 causes phosphorylation at Thr-34 of DARPP-32, which in turn reduces PP1 activity. If PP1 activity is

already low enough, okadaic acid does not have significant effect on dephosphorylation of P2X receptors and presumably other proteins by PP1 (Fig. 10, *right*). (2) In mock-transfected PC12 Tet-Off cells, PKA activity is supposed to be low, because H-89 did not have significant effect and forskolin augmented the ATP-induced currents (Fig. 8a). Under this condition, PP1 activity might be prominent, which dephosphorylates various substrates including P2X receptors. Without activation of PKA, less phosphorylation at Thr-34 and less activation of PP2A, which in turn cause more phosphorylation at Thr-75 (Fig. 10, *left*). Therefore inhibition of mainly PP1 by okadaic acid could prevent the dephosphorylation of P2X receptor, increasing ATP-induced currents. (3) In both UCH L1- and mock-transfected PC12 Tet-Off cells, phosphorylation of DARPP-32 at Thr-75 by CDK5 might be negligible and CDK5 signaling did not have significant effect, unlike in neostriatal neurons (Nishi *et al.* 2000; Bibb *et al.* 2001).

As a conclusion, our present observation indicates that UCH L1 potentiates ATP responses due to activation of P2X receptors by up-regulation of ubiquitin level, activation of PKA and CaMKII, and regulation of DARPP-32. As UCH L1 has multifunction and is known to be transported over long distances via slow axonal transport to synapses (Bizzi *et al.* 1991), UCH L1 is supposed to have various effects on neuronal function. Our finding shows the first evidence that there is a relationship between UCH L1 and neurotransmitter receptor, suggesting that UCH L1 may play an important role in synaptic activity. The question whether UCH L1 can affect other neurotransmitter receptors such as GABA and glutamate receptors should be investigated further. On the contrary, ubiquitin reduction and the consequent inadequate ubiquitination of proteins may trigger accumulation of proteins that should undergo ubiquitin-dependent degradation (Wang *et al.* 2004). The question whether lack of UCH L1 interfere the functional role of neurotransmitter receptors should be

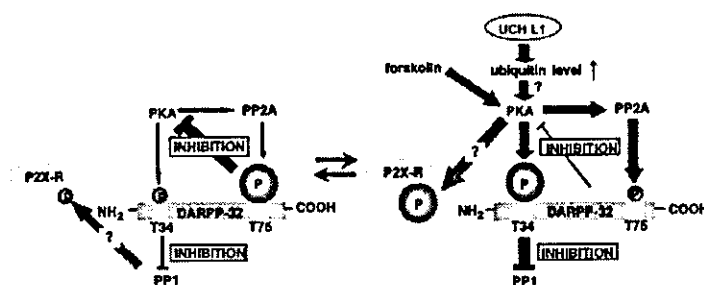


Fig. 10 Predicted PKA signaling via DARPP-32 in mock- and wild-type or C90S UCH L1-transfected PC12 Tet-Off cells. *Left*: In mock-transfected cells, corresponding to basal condition, phosphorylation of DARPP-32 at Thr-75 has negative feedback effect on PKA. Down-regulation of PKA also results in less phosphorylation of DARPP-32 at Thr-34 and therefore less inhibition of PP1. Down-regulated PKA and PP1 are supposed to reduce the phosphorylation of P2X receptors.

Right: In wild-type or C90S UCH L1-transfected cells, PKA is reported to activate PP2A, which dephosphorylates DARPP-32 at Thr-75, subsequently removing the negative feedback on PKA. Activation of PKA also results in the increased phosphorylation of DARPP-32 at Thr-34, which in turn inhibits PP1. Activation of PKA and inhibition of PP1 are supposed to increase the phosphorylation of P2X receptors.

investigated next. These studies may help to understand how dysfunction of UCH L1 causes neurodegeneration.

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Influence of assembly of siRNA elements into RNA-induced silencing complex by fork-siRNA duplex carrying nucleotide mismatches at the 3'- or 5'-end of the sense-stranded siRNA element

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Abstract

RNA interference (RNAi) is a powerful method for suppressing the expression of a gene of interest, and can be induced by 21–25 nucleotide small interfering RNA (siRNA) duplexes homologous to the silenced gene, which function as sequence-specific RNAi mediators in RNA-induced silencing complexes (RISCs). In the previous study, it was shown that fork-siRNA duplexes, whose sense-stranded siRNA elements carried a few nucleotide mismatches at the 3'-ends against the antisense-stranded siRNA elements, could enhance RNAi activity more than conventional siRNA duplexes in cultured mammalian cells. In this study, we further characterized fork-siRNA duplexes using reporter plasmids carrying target sequences complementary to the sense- or antisense-stranded siRNA elements in the untranslated region of *Renilla* luciferase. The data presented here suggest that nucleotide mismatches at either the 3'- or 5'-end of the sense-stranded siRNA elements in fork-siRNA duplexes could influence assembly of not only the antisense-stranded siRNA elements but also the sense-stranded elements into RISCs. In addition, we further suggest the possibility that there could be a positional effect of siRNA duplex on RNAi activity.

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RNA interference (RNAi) is the process of a sequence-specific post-transcriptional gene silencing triggered by double-stranded RNAs (dsRNAs) homologous to the silenced gene (reviewed in [1–4]). DsRNAs introduced or generated in cells are subjected to digestion with an RNase III enzyme, Dicer, into 21–25 nucleotide (nt) RNA duplexes [5–8], and the resultant RNA duplexes, referred to as small interfering RNA (siRNA) duplexes, can be associated with the RNA-induced silencing complexes (RISCs) and function as sequence-specific RNAi mediators in the complexes [5,7]. In terms of rapid and potent induction of

RNAi by exogenous dsRNAs, RNAi has become a powerful reverse genetic tool for suppressing the expression of a gene of interest in various species including mammals.

In mammals, direct introduction of chemically synthesized 21–25 nt siRNA duplexes into cells is often used for induction of RNAi [9–12], although different siRNAs induce different levels of RNAi activities [10,13]. In previous studies, where the effect of various types of synthetic siRNAs on the induction of mammalian RNAi was tested, an improvement of the siRNA duplexes for enhancing RNAi activity was found [14]. The improved siRNA duplexes, named 'fork-siRNA duplexes,' possess mismatched sequences at their termini due to introduction of base substitutions into the

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3'-ends of the sense-stranded siRNA elements: one of the ends of the fork-siRNA duplex, on the 3'-end of the sense-stranded siRNA element, remains open (unannealed). In addition to the properties of fork-siRNA duplex, recent studies suggested that siRNA duplexes conferring a strong RNAi activity could be characterized by a low base-stability due to AU-rich sequences around the 3'-ends of the sense-stranded siRNA elements, i.e., the functional siRNA duplexes have the asymmetrical features of the AU (or GC) content in their sequences [14–16]. Based on these observations, a model was proposed: the ease of unwinding from one of the ends of the siRNA duplex could determine the orientation of the duplex, and the siRNA element unwound from the 5'-end could be determined and function as a sequence-specific RNAi mediator in RISC [14].

In order to further characterize fork-siRNA duplexes, we report herein the construction of reporter plasmids carrying target sequences complementary to the sense- and antisense-stranded siRNA elements, and examination of the levels of gene silencing depending upon the sense- and antisense-stranded siRNA elements functioning as RNAi mediators in RISCs. The data presented here suggested that the structural features of fork-siRNA duplexes could influence incorporation of their siRNA elements into RISCs.

Materials and methods

Preparation of oligonucleotides. RNA and DNA synthetic oligonucleotides were obtained from PROLIGO and INVITROGEN, respectively. For preparation of duplexes, sense- and antisense-stranded oligonucleotides (20 μ M each) were mixed in an annealing buffer (30 μ M Hepes, pH 7.4, 100 μ M potassium acetate, and 2 μ M magnesium acetate), heat-denatured at 90 $^{\circ}$ C for 3 min, and annealed at 37 $^{\circ}$ C overnight. The siRNA duplexes used in this study were as follows: the La21-conv. siRNA duplex (a conventional siRNA duplex), La21-3'm2 siRNA duplex (a fork-siRNA duplex carrying two-base mismatches at the 3'-end of the sense-stranded siRNA element), La21-5'm2 siRNA duplex (a fork-siRNA duplex carrying two-base mismatches at the 5'-end of the sense-stranded siRNA element), and La21-ss19 siRNA duplex (a siRNA duplex containing 19 nt sense-stranded siRNA element) (Fig. 1B) [14]. Note that these La21 siRNA duplexes possess the same antisense-stranded siRNA elements. Non-silencing siRNA duplex (Qiagen) was used as a negative control.

Construction of reporter plasmids. In order to insert target sequence complementary to the sense- or antisense-stranded La21 siRNA element [10] against *Photinus* luciferase in the 3' untranslated region (UTR) of the *Renilla* luciferase transcripts, the phRL-TK plasmid (Promega) carrying the *Renilla* luciferase gene was digested with *Xba*I and *Not*I, and subjected to ligation with synthetic oligonucleotide duplexes carrying the La21 siRNA target sequences. The sequences of the synthesized oligonucleotides were as follows:

sLa21Fw; 5'-CTAGCATGCAACCGCTGGAGAGCAACTGCA-3'
 asLa21Fw; 5'-GGCCTGCAGTTGCTCTCCAGCGGTTGCATG-3'
 sLa21Rv; 5'-CTAGCATGCAGCAGTTGCTCTCCAGCGGTA-3'
 asLa21Rv; 5'-GGCTACCGCTGGAGAGCAACTGCTGCATG-3'

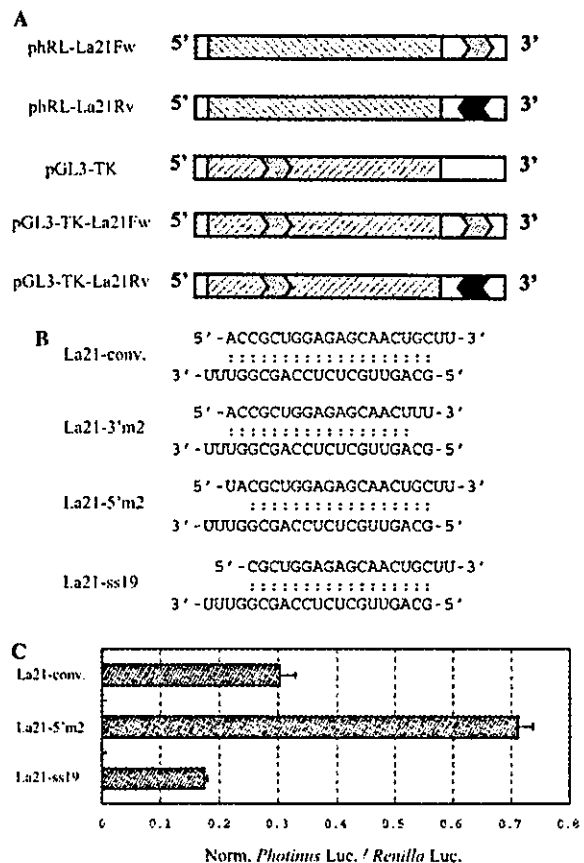


Fig. 1. Schematic drawing of the luciferase transcripts derived from plasmids constructed in this study (A), used synthetic siRNA duplexes (B), and RNAi activities induced by the siRNA duplexes (C). (A) The names of constructed plasmids are indicated. Hatched and open boxes represent the luciferase coding and untranslated regions, respectively. Gray and solid (black) regions indicate target sequences complementary to the antisense- and sense-stranded La21 siRNA elements, respectively. (B) The names of synthetic La21 siRNA duplexes are indicated. Upper and lower sequences in the duplexes represent the sense- and antisense-stranded siRNA elements, respectively. (C) Gene silencing of the *Photinus* luciferase gene. RNAi induction was carried out as described previously [14]. Indicated La21 siRNA duplexes together with pGL3-control and phRL-TK plasmids carrying *Photinus* and *Renilla* luciferase genes, respectively, were transfected into HeLa cells. Twenty-four hours after transfection, cell lysate was prepared and the level of luciferase activity was examined. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are shown: the ratios of luciferase activity determined in the presence of the La21 siRNA duplexes were normalized to the ratio obtained for a control in the presence of a non-silencing siRNA duplex (Qiagen). Data are averages of at least three independent determinations. Error bars represent standard deviations.

The resultant plasmids possessing the target sequences for the antisense- and sense-stranded La21 siRNA elements were named 'phRL-La21Fw' and 'phRL-La21Rv,' respectively.

We further constructed reporter plasmids carrying two target sites of the La21 siRNA duplex. The *Hind*III-*Xba*I fragment encoding the *Photinus* luciferase gene was isolated from the pGL3-control plasmid

(Promega), and substituted for the *Hind*III–*Xba*I regions carrying *Renilla* luciferase in the pHRL-La21Fw and pHRL-La21Rv plasmids. The resultant plasmids derived from the pHRL-La21Fw and pHRL-La21Rv plasmids were named 'pGL3-TK-La21Fw' and 'pGL3-TK-La21Rv,' respectively, and possessed two La21 siRNA duplex target sites, one in the *Photinus* luciferase coding region and the other in the 3' UTR. We also constructed 'pGL3-TK' plasmid by substitution of *Photinus* luciferase for *Renilla* luciferase in the pHRL-TK plasmid using the same procedure described above.

Cell culture, transfection, and luciferase and β -galactosidase assays. HeLa cells were grown as described previously [10]. The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and seeded into 24-well culture plates (approximately 0.5×10^5 cells/well). Cotransfection of synthetic siRNA duplexes with reporter plasmids was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions, and to each well, 0.24 μ g siRNA duplexes, 0.05 μ g pHRL-La21Fw or pHRL-La21Rv plasmid, and 0.1 μ g pSV- β -galactosidase control vector (Promega) as a control were applied. Twenty-four hours after transfection, cell lysate was prepared and the expression levels of luciferase and β -galactosidase were examined by a Dual-Luciferase reporter assay system (Promega) and a Beta-Glo assay system (Promega), respectively, according to the manufacturer's instructions. In the case of transfection with pGL3-TK, pGL3-Tk-La21Fw or pGL3-TK-La21Rv, 0.24 μ g siRNA duplexes, 0.1 μ g of any one of the pGL3-TK, pGL3-Tk-La21Fw, and pGL3-Tk-La21Rv plasmids, and 0.05 μ g of the pHRL-TK plasmid as a control were applied into HeLa cells. Twenty-four hours after transfection, a Dual-Luciferase reporter assay was conducted.

Results and discussion

Influence of assembly of siRNA elements into RISCs by fork-siRNA duplexes

In the previous study, it was shown that fork-siRNA duplexes carrying nucleotide mismatches at the 3'-ends of the sense-stranded siRNA elements could enhance RNAi activity more than conventional siRNA duplexes [14]. This suggests the possibility of greater occurrence of assembly of the antisense-stranded siRNA elements rather than the sense-stranded elements into RISCs in fork-siRNA duplexes over that in conventional duplexes. Accordingly, we attempted to examine whether fork-siRNA duplexes could influence incorporation of their siRNA elements into RISCs. To address this, we constructed two reporter plasmids, pHRL-La21Rv and pHRL-La21Fw, carrying the target sequences for the sense- and antisense-stranded La21 siRNA elements, respectively, in the 3' untranslated region (UTR) of *Renilla* luciferase (Fig. 1A). This is because the previous study showed that the La21-conv., La21-3'm2, and La21-5'm2 siRNA duplexes (Fig. 1B) could confer different levels of RNAi activities, although they possessed the same antisense-stranded siRNA element [14]. In addition to the previous results, the result with a newly designed siRNA duplex, the La21-ss19 siRNA duplex (Figs. 1B and C), also supported the idea that the forked terminus of siRNA duplex could influence RNAi activity. Accordingly, we decided to use the sequences of the

sense- and antisense-stranded La21 siRNA elements as targets in this study. Using the pHRL-La21Fw and -La21Rv plasmids, and a series of the La21 siRNA duplexes, the levels of gene silencing depending upon the sense- and antisense-stranded La21 siRNA elements were investigated.

First we examined if the sense-stranded siRNA elements, like the antisense-stranded siRNA elements, could have potential for functioning as sequence-specific RNAi mediators in RISCs. To see this, the La21-conv. siRNA duplex together with pHRL-La21Rv, pHRL-La21Fw or pHRL-TK and pSV- β -galactosidase control vector as a control were cotransfected into HeLa cells, and the levels of the expression of *Renilla* luciferase were examined. As a result, significant suppression of the expression of *Renilla* luciferase was detectable in the presence of either pHRL-La21Rv or pHRL-La21Fw, whereas little or no suppression was seen in the presence of pHRL-TK as a negative control (Fig. 2). Therefore, these results strongly suggest that either the sense- or antisense-stranded La21 siRNA element can be incorporated into RISC and function as a sequence-specific RNAi mediator in the complex.

We next examined the RNAi activities directed by the sense- and antisense-stranded siRNA elements derived from the La21-3'm2 and La21-5'm2 siRNA duplexes (fork-siRNA duplexes) as well as the La21-conv. siRNA duplex. As shown in Fig. 3A, when the pHRL-La21Fw plasmid was used, ~86%, 95%, and 72% gene silencing mediated by the antisense-stranded La21 siRNA elements derived from the La21-conv., La21-3'm2, and

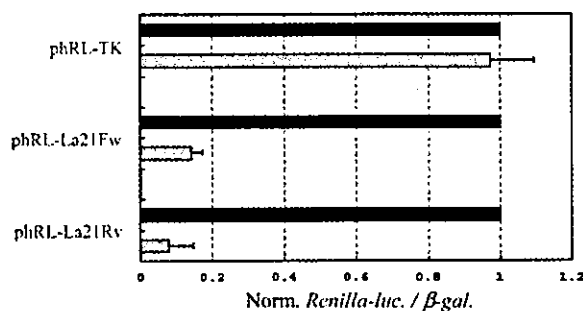


Fig. 2. Gene silencing of exogenous reporter gene with conventional siRNA duplexes. The conventional La21 (La21-conv.) siRNA duplex against the *Photinus* luciferase gene together with pHRL-TK, pHRL-La21Fw, or pHRL-La21Rv plasmid carrying the *Renilla* luciferase reporter gene, and pSV- β -galactosidase control vector as a control were cotransfected into HeLa cells. Twenty-four hours after transfection, cell lysate was prepared, and the levels of the luciferase and β -galactosidase activities were examined. Ratios of normalized target (*Renilla*) luciferase activity to control β -galactosidase activity are shown; the ratios of luciferase activity determined in the presence of the La21-conv. siRNA duplex (gray bars) are normalized to the ratio obtained for a control in the presence of a non-silencing siRNA duplex (Qiagen) (solid bars). Data are averages of at least four independent experiments. Error bars represent standard deviations.

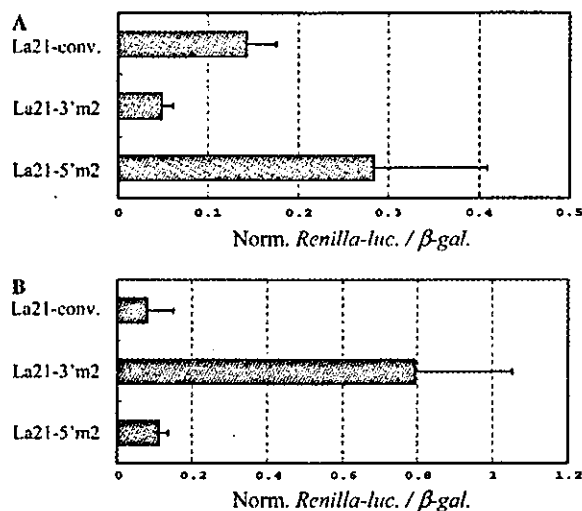


Fig. 3. Silencing of the expression of exogenous reporter gene with various types of siRNA duplexes. The conventional La21 (La21-conv.) or fork-La21 (La21-3'm2 and La21-5'm2) siRNA duplexes together with pHRL-La21Fw (A) or pHRL-La21Rv (B) reporter plasmid and pSV- β -galactosidase control vector as a control were cotransfected into HeLa cells, and the expression levels of luciferase and β -galactosidase were examined as in Fig. 2. Ratios of normalized target (*Renilla*) luciferase activity to control β -galactosidase activity are shown as in Fig. 2. Data are averages of at least four independent determinations. Error bars indicate standard deviations.

La21-5'm2 siRNA duplexes, respectively, was observed. Although the levels of the gene silencing with pHRL-La21Fw as a reporter plasmid increased further than those with the pGL3-control plasmid carrying *Photinus* luciferase in the previous study [14] (further discussion below), the effects of mismatches at the 3'- and 5'-ends of the sense-stranded elements in the La21 fork-siRNA duplexes on RNAi activity appeared to remain unchanged in the experiments using either pHRL-La21Fw or pGL3-control.

When the pHRL-La21Rv plasmid was used, i.e., when the levels of the RNAi activity directed by the sense-stranded La21 siRNA elements were examined, significant differences in the level of RNAi activity among the La21 siRNA duplexes used were observed: while ~92% and 89% suppression of the expression of *Renilla* luciferase was detectable in the presence of the La21-conv., and La21-5'm2 siRNA duplexes, respectively, the gene silencing mediated by the sense-stranded element derived from the La21-3'm2 duplex appeared to confer ~20% inhibition of the expression of *Renilla* luciferase (Fig. 3B), suggesting that the degree of assembly of the sense-stranded siRNA element into RISC in the La21-3'm2 siRNA duplex could be much lower than those in the La21-conv., and La21-5'm2 siRNA duplexes. Taking all the data together, these observations suggest that nucleotide mismatches at the ends of fork-siRNA duplexes can influence assembly of not only

the antisense-stranded siRNA elements but also the sense-stranded siRNA elements into RISCs.

The previous *in vitro* RNAi reaction with *Drosophila* embryo lysate has demonstrated that single nucleotide mismatch around the termini of siRNA duplex can affect target-RNA cleavages directed by the sense- and antisense-stranded siRNA elements [15]. The results of our present study using cultured human cells consistently agree with those in the previous study. Therefore, it appears that the effect of low base-pairing stabilities due to either AU-rich or nucleotide mismatches around the termini of siRNA duplexes on RNAi activity is likely common among various species. In addition, such low base-pairing stability could contribute to ready unwinding of the duplex from that end by a possible helicase activity in RISCs.

Another important point to note in this study is that the sense-stranded siRNA elements have potential for functioning as sequence-specific RNAi mediators in RISCs. As previously suggested [14], this indicates that off-target gene silencing mediated by the sense-stranded siRNA elements could occur in RNAi induction by siRNA duplexes. Our present data also indicated a possible avoidance of such off-target gene silencing: fork-siRNA duplexes carrying nucleotide mismatches at the 3'-end of the sense-stranded elements could reduce such off-target silencing. Therefore, fork-siRNA duplexes may provide us with not only an increase in RNAi activity but also decrease in off-target gene silencing directed by the sense-stranded siRNA elements.

Positional effect of siRNA target site on RNAi activity

The results shown in Fig. 3A led us to the possibility that the position of an siRNA target site on a silenced gene transcript could influence its RNAi activity, i.e., there could be a positional effect of the siRNA target site on RNAi activity. To examine this possibility, we constructed two reporter plasmids carrying *Photinus* luciferase, pGL3-TK-La21Rv and pGL3-TK-La21Fw, whose 3' UTRs contained the target sequences complementary to the sense- and antisense-stranded La21 siRNA elements, respectively (Fig. 1A). Thus, the resultant *Photinus* luciferase transcripts derived from pGL3-TK-La21Fw and pGL3-TK-La21Rv possess two target sites: one site complementary to the antisense-stranded La21 siRNA element is in the luciferase coding region, and the other complementary to the sense- or antisense-stranded siRNA element is in its 3' UTR.

The La21-conv., La21-3'm2, or La21-5'm2 siRNA duplexes together with the pGL3-TK (carrying one target site in the luciferase coding region), pGL3-TK-La21Rv or pGL3-TK-La21Fw plasmid (Fig. 1A) and the pHRL-TK plasmid as a control were cotransfected into HeLa cells, and the levels of RNAi activity were examined by a dual-luciferase assay. When the

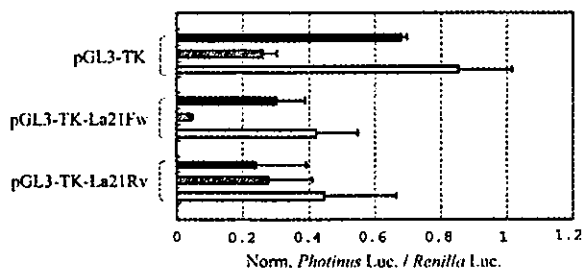


Fig. 4. Gene silencing of *Photinus* luciferase carrying two target sites of the La21 siRNA duplex. The La21-conv., La21-3'm2 or La21-5'm2 siRNA duplex together with pGL3-TK, pGL3-TK-La21Fw or pGL3-TK-La21Rv reporter plasmid carrying *Photinus* luciferase and phRL-TK plasmid carrying *Renilla* luciferase as a control were cotransfected into HeLa cells, and the expression levels of luciferase were examined as in Fig. 1. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are shown as in Fig. 1. Solid, gray, and open bars indicate the data in the presence of the La21-conv., La21-3'm2, and La21-5'm2 siRNA duplexes, respectively. Data are averages of at least three independent determinations. Error bars represent standard deviations.

pGL3-TK plasmid was used, results similar to those in the previous study using the pGL3-control plasmid encoding *Photinus* luciferase driven by the SV40 promoter [14] were observed (Fig. 4). When the pGL3-TK-La21Fw and pGL3-TK-La21Rv plasmids were used, the levels of RNAi activity, other than those in the presence of the pGL3-TK-La21Rv plasmid and the La21-3'm2 siRNA duplex, appeared to increase more greatly in both the pGL3-TK-La21Fw and pGL3-TK-La21Rv plasmids than in the pGL3-TK plasmid used, suggesting that two target sites of the La21 siRNA duplex on the silenced *Photinus* luciferase transcript probably contributed to the enhancement of RNAi activity. As for the RNAi activity in the presence of pGL3-TK-La21Rv and the La21-3'm2 siRNA duplex, it may be that the target site in the luciferase coding region, not in the 3' UTR, is only recognizable for active RISCs, since a rather weak RNAi activity mediated by the sense-stranded siRNA element in the La21-3'm2 siRNA duplex was detected in the presence of phRL-La21Rv (Fig. 3B). This may account for the lack of significant difference in the level of RNAi activity between pGL3-TK and pGL3-TK-La21Rv in the presence of the La21-3'm2 siRNA duplex.

It should be noted that the La21-5'm2 siRNA duplex was able to confer ~60% suppression of the expression of *Photinus* luciferase in pGL3-TK-La21Fw, although the duplex was able to induce ~15% inhibition of the *Photinus* luciferase expression in pGL3-TK. Since the *Photinus* luciferase transcripts derived from pGL3-TK-La21Fw carry two identical target sites complementary to the antisense-stranded La21 siRNA element, and since the target site in the luciferase coding region appeared not to contribute much to gene silencing when

using the La21-5'm2 siRNA duplex (Fig. 4), it is conceivable that the target site in the 3' UTR could be more sensitive to cleavage by RISCs than that in the luciferase coding region on the *Photinus* luciferase transcripts derived from pGL3-TK-La21Fw in the presence of the La21-5'm2 siRNA duplex. These observations thus suggest a possible positional effect of target site of siRNA duplex on RNAi activity.

Finally, we add that a difference in RNAi activity between phRL-La21Rv (Fig. 3) and pGL3-TK-La21Rv in the presence of the La21-5'm2 siRNA duplex was observed, although the *Renilla* and *Photinus* luciferase transcripts derived from phRL-La21Rv and pGL3-TK-La21Rv, respectively, which carried the same target sites complementary to the sense-stranded La21 siRNA element in their 3' UTRs, could be subjected to gene silencing chiefly mediated by the sense-stranded siRNA element. The difference might be attributable to possibly different stabilities between the *Renilla* and *Photinus* luciferase gene products in cells. To further evaluate such a possible difference and also the positional effect of siRNA target site on RNAi activity, more extensive studies need to be carried out.

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RNAi induction and activation in mammalian muscle cells where *Dicer* and *eIF2C1* translation initiation factors are barely expressed

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Abstract

Dicer plays an important role in the course of RNA interference (RNAi), i.e., it digests long double-stranded RNAs into 21–25 nucleotide small-interfering RNA (siRNA) duplexes functioning as sequence-specific RNAi mediators. In this study, we investigated the expression levels of *Dicer* and *eIF2C1~4*, which, like *Dicer*, appear to participate in mammalian RNAi, in various mouse tissues. Results indicate that the levels of *eIF2C1~4* as well as *Dicer* are lower in skeletal muscle and heart than in other tissues. To see if RNAi could occur under such a condition with low levels of expression of *Dicer* and *eIF2C1~4*, we examined RNAi activity in mouse skeletal muscle fibers. The results indicate that RNAi can be induced by synthetic siRNA duplexes in muscle fibers. We further examined RNAi activity during myogenic differentiation of mouse C2C12 cells. The data indicate that although the expression levels of *Dicer* and *eIF2C1~4* decrease during the differentiation, RNAi can be induced in the cells. Altogether, the data presented here suggest that muscle cells retain the ability to induce RNAi, although *Dicer* and *eIF2C1~4* appear to be barely expressed in them.

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RNA interference (RNAi) is the process of a sequence-specific post-transcriptional gene silencing triggered by double-stranded RNAs (dsRNAs) homologous to the silenced genes. This intriguing gene silencing has been found in various species including flies, worms, protozoa, vertebrates, and higher plants (reviewed in [1–4]). DsRNAs introduced or generated in cells are digested by an RNase III enzyme, *Dicer*, into 21–25 nucleotide (nt) RNA duplexes [5–8] and the resultant duplexes, referred to as small-interfering RNA (siRNA) duplexes, function as essential sequence-specific RNAi mediators in the RNA-induced silencing complexes (RISCs) [5,7]. Thus, *Dicer* appears to play an important role in the process of RNAi induction.

In mammalian cells except for a part of undifferentiated cells [9–12], long dsRNAs (>30 bp) can trigger a rapid and non-specific RNA degradation involving the sequence-non-specific RNase, RNase L [13], and a rapid translation inhibition involving the interferon-inducible, dsRNA-activated protein kinase, PKR, instead of induction of RNAi [14]. In contrast, chemically synthesised siRNA duplexes can induce the sequence-specific RNAi activity in mammalian cells without triggering the rapid and non-specific RNA degradation and translation inhibition [15]. Together, it is likely that RNAi activity induced by the long dsRNAs could be masked by those rapid responses to the long dsRNAs in most of mammalian cells.

It may be of interest to examine the role of *Dicer* in differentiated mammalian cells possessing the rapid responses to long dsRNAs. Mammalian *dicer* has been identified and found to be a large multi-domain

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polypeptide (~215kDa) characterised by containing a putative DEXH/DEAH RNA helicase/ATPase domain, a PAZ domain, two RNase domains, and a dsRNA-binding domain [16–20]. The expression of *Dicer* appears to be ubiquitous, but the level of its expression varies among tissues. Of the tissues examined previously, skeletal muscle appeared to express *Dicer* at a low level, i.e., the *Dicer* transcript appeared to be barely detectable at least using RT-PCR [16,17].

In this study, we investigated not only RNAi activity but also the expression levels of *Dicer* and *eIF2C1~4*, which, like *Dicer*, appear to participate in mammalian RNAi [21,22], in mouse skeletal muscle fibers, and muscle cells that differentiated from mouse C2C12 cells. The results indicate that RNAi can be induced by synthetic siRNA duplexes in those cells although the expression levels of *Dicer* and *eIF2C1~4* are lower than those in other tissues and undifferentiated C2C12 cells.

Materials and methods

Preparation and culture of muscle fibers isolated from extensor digitorum longus in mice. Isolation of muscle fibers from mice was carried

out as described previously [23]. Briefly, extensor digitorum longus (EDL) was isolated from mice (ICR mouse strain), treated with 0.5% type 1 collagenase (Washington biochemical) in Dulbecco's modified Eagle's medium (DMEM) (Sigma), and incubated at 37°C for 90 min. After incubation, the EDL was dissociated into single muscle fibers by gently pipetting, and dissociated single fibers were plated on matrigel-coated 24-well culture plates (approximately 100 fibers/well). The muscle fibers were cultured at 37°C in DMEM supplemented with 10% horse serum (Invitrogen) in a 5% CO₂-humidified chamber. Two–three hours after starting culture, transfection was carried out.

Cell culture. C2C12 cells were grown at 37°C in DMEM supplemented with 15% fetal calf serum (Sigma), 100 U/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen) in a 5% CO₂-humidified chamber. For induction of myogenic differentiation, cells were cultured at 37°C in DMEM supplemented with 5% horse serum (Invitrogen) in a 5% CO₂-humidified chamber [24]. The medium was changed everyday.

Synthetic oligonucleotides. RNA and DNA synthetic oligonucleotides were obtained from PROLIGO and SIGMAGENOSIS, respectively. The La2 siRNA duplex described previously was used in this study, and preparation of RNA duplexes was performed as described previously [25].

Transfection and luciferase assay. Reporter plasmids and siRNA duplexes were cotransfected into isolated single muscle fibers and undifferentiated and differentiated C2C12 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. When undifferentiated C2C12 cells were used, the day before transfection, the cells were trypsinised, diluted with the fresh medium without

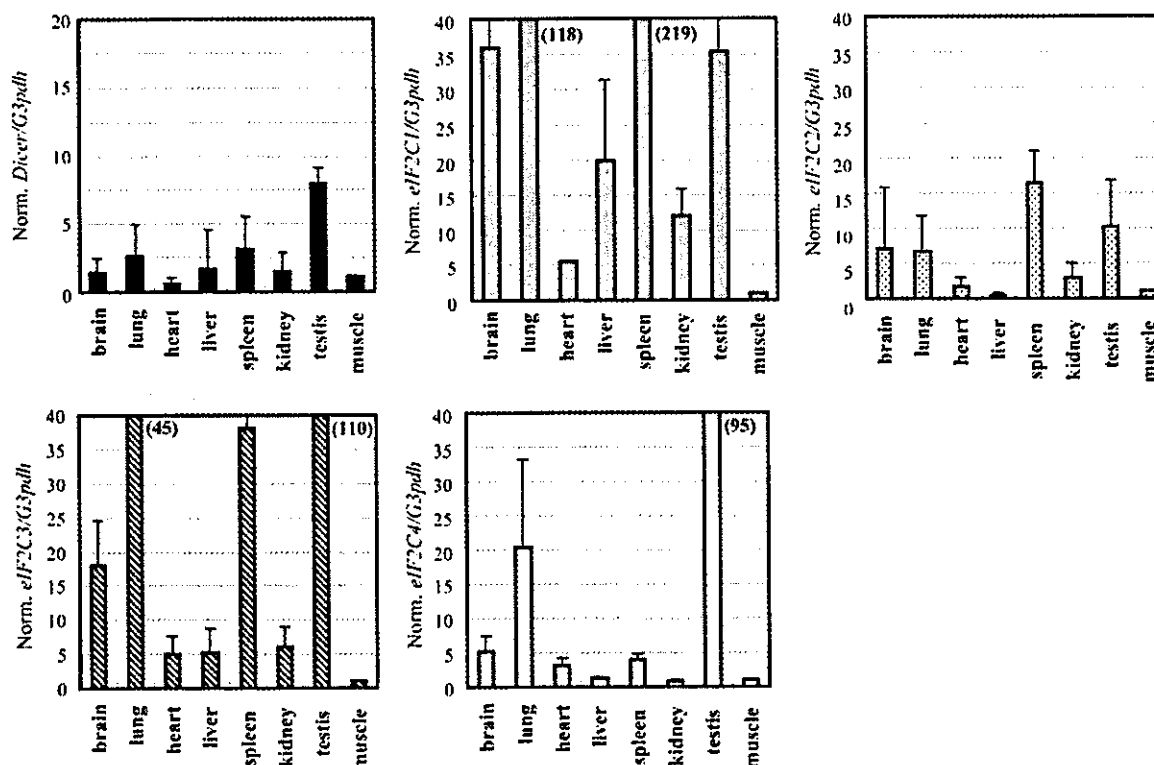


Fig. 1. Expression profiles of *Dicer* and *eIF2C1~4* in various mouse tissues. Total RNA was extracted from indicated tissues and subjected to cDNA synthesis with oligo(dT) primer and a reverse transcriptase. The expression levels of *Dicer* and *eIF2C1~4* were examined by means of a real-time PCR using the synthesised cDNAs as templates. The expression levels of the genes are normalised to that of the *G3pdh* gene examined as a control, and plotted when the expression level of either *Dicer* or *eIF2C1~4* in skeletal muscle is given as 1. Figures in parentheses indicate the averaged expression levels which are over the plotted areas. Data are averages of three independent experiments. Error bars represent standard deviations.

antibiotics, and seeded into 24-well culture plates (approximately 5×10^4 cells/well). Before the transfection, the culture medium was replaced with 0.5 ml OPTI-MEM I (Invitrogen), and to each well, 0.25 μ g pGL3-control plasmid (Promega), 0.05 μ g pRL-SV40 plasmid (Promega), and 0.2 μ g siRNAs were applied. After 4-h incubation, 0.5 ml of the fresh culture medium without antibiotics was added, and further incubation at 37°C was carried out. In the case of transfection into the isolated muscle fibers, the transfection mixture was directly applied into wells, and further incubation at 37°C was carried out. When a short-hairpin expression plasmid, pRNA-U6.1/Neo/siRNA (GenScript), was used instead of synthetic siRNAs, 0.1 μ g pGL3-control and 0.05 μ g pRL-TK (Promega) together with various amounts of pRNA-U6.1/Neo/siRNA were introduced into C2C12 cells. The expression of luciferase was examined using a Dual-Luciferase reporter assay system (Promega) according to the directions provided by the manufacturer.

RT-PCR. Total RNA was extracted from the cultured cells and various mouse tissues using Trizol reagent (Invitrogen). Reverse-transcription (RT) for synthesizing the first-strand cDNAs was carried out using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, and the resultant cDNAs were examined by real-time PCR using the ABI PRISM 7000 sequence detection system (Applied Biosystems) with a SYBER Green PCR Master Mix or a TaqMan Universal PCR Master Mix together with Assays-on-Demand Gene Expression products (Applied Biosystems) according to the manufacturer's instructions. For plotting a standard curve, the 1, 5, 25, 125, and 625-fold diluted brain cDNA samples, which were prepared from a brain tissue (total RNA) and designated as standards, were used in every real-time PCR. Expression levels of the genes examined were normalised to that of the control *G3pdh* gene. The PCR primers used in the real-time PCR were as follows:

G3pdh-F; 5'-TCTTCACCACCATGGAGAAG-3'
G3pdh-R; 5'-TCATGGATGACCTTGGCCAG-3'
Dicer-F; 5'-GCAGGCTTTTACACACGCCT-3'
Dicer-R; 5'-GGGTCTTCATAAAGGTGCTT-3'
eIF2C2-F; 5'-AGATGAAGAGGAAGTACCGT-3'
eIF2C2-R; 5'-CAGAACCAGCTTGTGCCTGT-3'

The Assays-on-Demand Gene Expression products used (the Assay ID numbers) were as follows:

eIF2C1; Mm00462977m1, *eIF2C3*; Mm00462959m1, *eIF2C4*; Mm00462659m1.

5-Bromodeoxyuridine incorporation assay. Cells were metabolically labeled in the culture medium containing 10 μ M of 5-bromodeoxyuridine (BrdU) (Sigma) for 20 h, and rinsed with phosphate-buffered saline solution (PBS) followed by fixation with 70% ethanol containing 0.5 M HCl at -20°C for 1 h. The resultant cells were incubated with anti-BrdU antibody (Oxford biotechnology) at 4°C overnight. The BrdU-antibody complexes were visualised with Alexa488 conjugated secondary antibody (Invitrogen) and examined using a ZEISS (Axiovert) microscope.

Results and discussion

Expression profiles of *Dicer* and *eIF2C1~4* in various mouse tissues

Previous studies suggested that *Dicer* and *eIF2C* translation initiation factors (*eIF2C1~4*) homologous to the *Ago* genes in *Drosophila* [26,27] contributed to mammalian RNAi [21,22]. *Dicer* appears to be expressed ubiquitously, but its expression level varies among tissues [16,17]. Since little is known about the expression levels of *eIF2C1~4* among tissues, we first

examined the levels of expression of *eIF2C1~4* and *Dicer* in various tissues. Total RNA was extracted from mouse tissues and subjected to cDNA synthesis with oligo(dT) primer and reverse transcriptase. The resultant cDNAs were examined by a real-time PCR. The results are shown in Fig. 1. The expression level of *Dicer* in either skeletal muscle or heart appears to be lower than those in other tissues, which agrees with the previous observations [16,17]. It should be noted that the expression levels of *eIF2C1~4* in either skeletal muscle or heart, like the expression profile of *Dicer*, are also significantly lower than those in the other tissues examined. Consequently, the observations suggest that

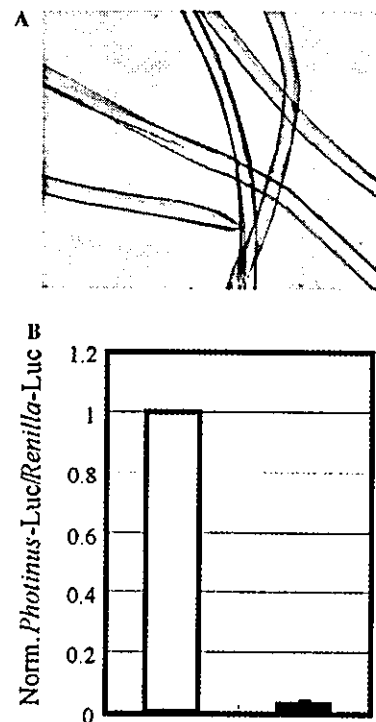


Fig. 2. RNAi induction by synthetic siRNA duplexes in muscle fibers prepared from mouse extensor digitorum longus. (A) Photograph of isolated muscle fibers. Isolation of muscle fibers from mouse extensor digitorum longus was carried out as described in Materials and methods. (B) RNAi activity in isolated muscle fibers. The La2 siRNA duplex against the *Photinus* luciferase gene [25] or a non-silencing siRNA duplex (Qiagen) together with pGL3-control and pRL-SV40 plasmids carrying *Photinus* and *Renilla* luciferase reporter genes, respectively, were cotransfected into the isolated muscle fibers. Twenty-four hours after transfection, cell lysate was prepared and dual luciferase assay was carried out. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of the La2 siRNA duplex are normalised to the ratios obtained in the presence of the non-silencing siRNA duplex. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

skeletal and cardiac muscle cells express either *Dicer* or *eIF2C1~4* at a low level.

RNAi activity in muscle fibers isolated from mouse extensor digitorum longus

The observations described above raised the question whether RNAi could occur in muscle, i.e., whether RNAi could be induced under a condition with a low level of expression of either *Dicer* or *eIF2C1~4*. In order to address the question, we isolated mouse muscle fibers from extensor digitorum longus of ICR mice (Fig. 2A), and introduced synthetic 21-nt siRNA duplex targeting the exogenous reporter gene, *Photinus luciferase*, together with a pGL3-control plasmid carrying the *Photinus luciferase* gene and a pRL-SV40 plasmid carrying the *Renilla luciferase* gene as a control into the isolated muscle fibers. For realizing an efficient RNAi

induction, we used the La2 siRNA duplex having the potential for inducing a strong RNAi activity in cultured mammalian cells [25]. As shown in Fig. 2B, the results indicate that the La2 siRNA duplex can induce a strong gene silencing of the *Photinus luciferase* gene in the muscle fibers. This result suggests that RNAi can be induced by synthetic siRNA duplexes in skeletal muscle which barely expresses either *Dicer* or *eIF2C1~4*.

RNAi activity during myogenic differentiation of mouse C2C12 cells

To further examine the properties of RNAi in muscle cells and during myogenic differentiation, we investigated RNAi activity in C2C12 cells, a mouse myoblast cell line, which can be induced by changing culture conditions (detailed in Materials and methods) to differentiate into contractile myotubes [24]. First, we

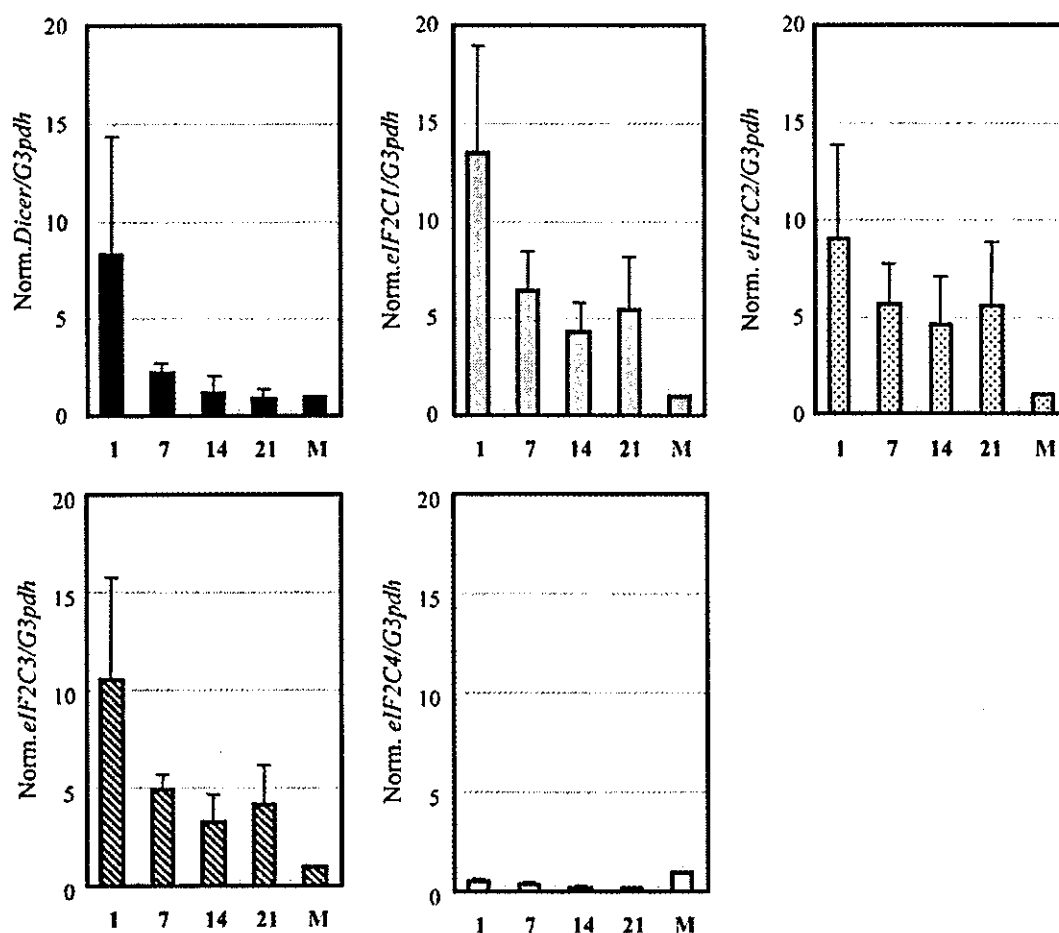


Fig. 3. Expression profiles of *Dicer* and *eIF2C1~4* during myogenic differentiation of mouse C2C12 cells. Total RNA was extracted from C2C12 cells at various days (indicated) after induction of myogenic differentiation of the cells (day 1 indicates undifferentiated C2C12 cells), and subjected to RT-PCR to examine the expression levels of *Dicer* and *eIF2C1~4* as in Fig. 1. The expression levels of the genes are normalised and plotted as in Fig. 1. M indicates skeletal muscle. Data are averages of three independent experiments. Error bars represent standard deviations.

examined the expression profiles of *Dicer* and *eIF2C1~4* during the myogenic differentiation of C2C12 cells and compared them with those of skeletal muscle examined above. As shown in Fig. 3, the expression profiles reveal that the level of expression of either *Dicer* or *eIF2C1~3* is gradually decreased during the myogenic differentiation of C2C12 cells, and that the *eIF2C4* gene is expressed at a low level in either C2C12 myoblast or myotube.

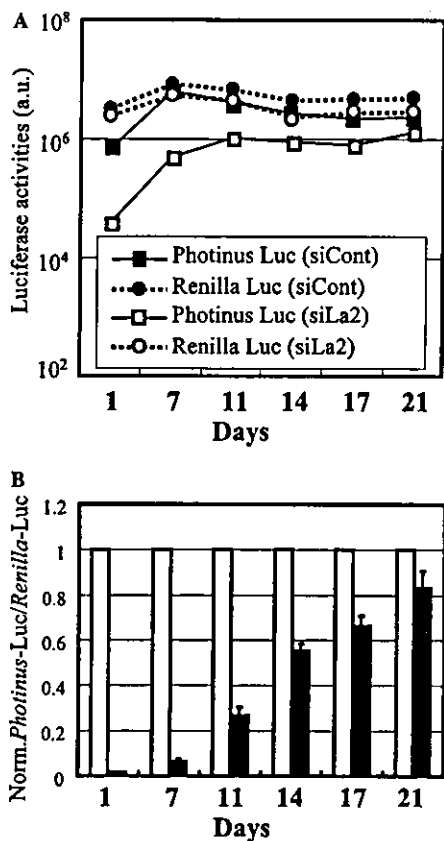


Fig. 4. Persistence of RNAi activity during myogenic differentiation of mouse C2C12 cells. The La2 siRNA duplex or a non-silencing siRNA duplex (Qiagen) together with pGL3-control and pRL-SV40 plasmids were cotransfected into C2C12 cells as in Fig. 2. Before transfection, the culture medium (DMEM containing 15% fetal calf serum) was replaced with DMEM containing 5% horse serum for induction of the myogenic differentiation of C2C12 cells. RNAi activity was examined 24 h after transfection (day 1), and thereafter examined at various days (indicated) up to 3 weeks after the transfection. (A) Absolute *Photinus* and *Renilla* luciferase expressions. The expression levels are plotted in arbitrary luminescence units (a.u.). (B) Ratios of normalised target to control luciferase. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 2. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

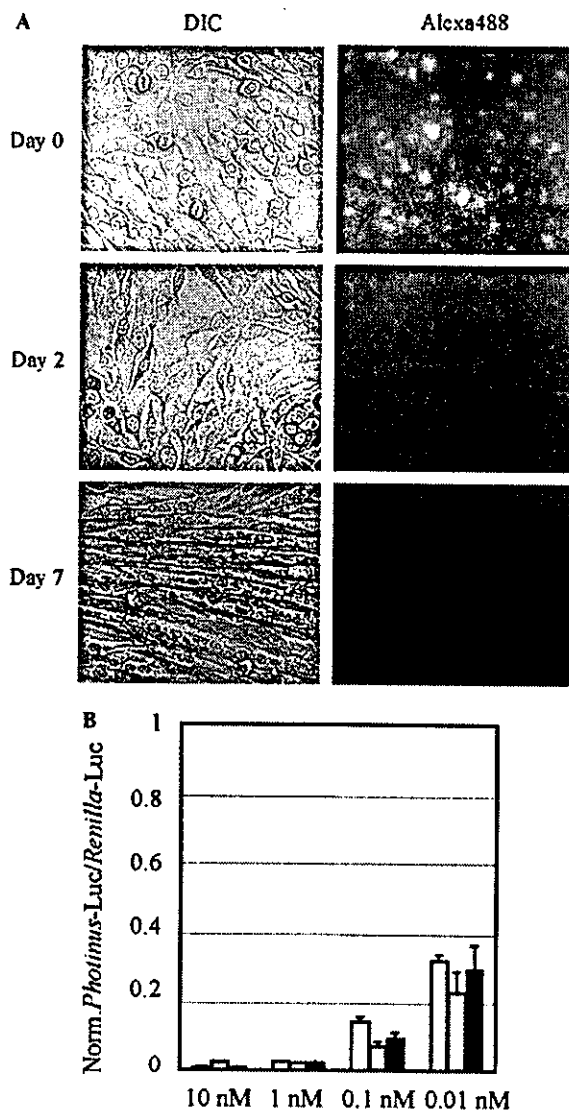


Fig. 5. Cell-cycle arrest and RNAi activity during myogenic differentiation of C2C12 cells. Myogenic differentiation of C2C12 cells was induced by changing the culture medium from DMEM containing 15% fetal calf serum to DMEM containing 5% horse serum. (A) Cell-cycle arrested C2C12 cells. Metabolically labeling of the cells with BrdU was carried out at indicated days after the differentiation. Day 0 indicates undifferentiated C2C12 cells. BrdU incorporated into the cells was visualised with an anti-BrdU antibody and an Alexa488 conjugated secondary antibody. The cells were examined by a fluorescent microscope. Left (DIC, differential interference contrast) and right (Alexa488, fluorescence image) panels are identical in visual field. (B) RNAi activity during the differentiation. The reporter plasmids carrying the *Photinus* and *Renilla* luciferase genes were cotransfected with a decreasing amount of the La2 siRNA or non-silencing siRNA duplexes (Qiagen), from 10 to 0.01 nM, into either undifferentiated or differentiated C2C12 cells. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 2. Open, dotted, and solid bars indicate the data in C2C12 cells that differentiated for 0 (undifferentiated), 2, and 7 days, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

Next we examined RNAi activity during the myogenic differentiation of C2C12 cells. The La2 siRNA duplex together with pGL3-control and pRL-SV40 plasmids was cotransfected into undifferentiated C2C12 cells, and simultaneously myogenic differentiation of the cells was carried out by changing culture medium as described above (see Materials and methods). As a result, a strong RNAi activity was detected by day 7 after RNAi induction (Fig. 4), when morphological changes of C2C12 cells into myotubes appeared to be completed (Fig. 5A); thereafter, the cells gradually lost the RNAi activity and lost most of the activity by day 21 after the induction (Fig. 4).

Because proliferating mammalian cells gradually lose RNAi activity with an increase in the number of cell divisions [12,28,29], we investigated whether cell division occurred in C2C12 cells during the differentiation by means of a BrdU incorporation assay. As shown in Fig. 5A, while the incorporation of BrdU into nuclei

could be observed in undifferentiated C2C12 cells, few or no BrdU-positive cells were detectable at day 2 and 7 after induction of the differentiation. In addition, from the data of Fig. 5B, the nature of RNAi activity during the differentiation appears to remain unchanged. Consequently, these observations suggest that C2C12 cells differentiated over 2 days are probably cell-cycle arrested cells, and thus that the decrease in RNAi activity during the myogenic differentiation of C2C12 cells is not caused by cell division.

We further examined RNAi activities in C2C12 myotubes that differentiated for 14 and 21 days. The results indicate that RNAi activities induced by synthetic siRNA duplexes are detectable in those differentiated C2C12 myotubes (Fig. 6), although the transfection efficiency of siRNA and plasmid DNA into the cells seemed to become lower as the culture was long. Taking all the data together, it is conceivable that the decrease in RNAi activity during the myogenic

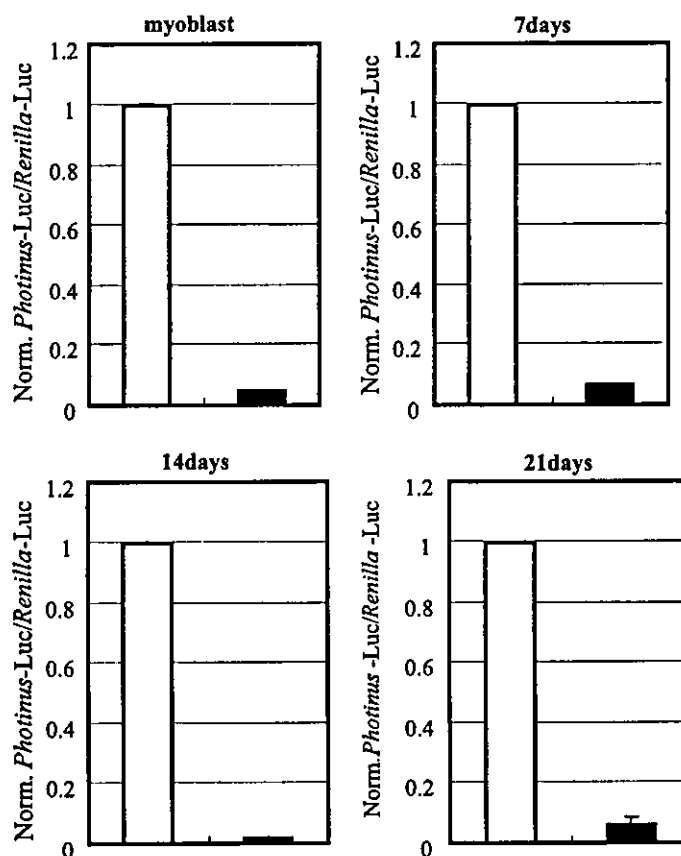


Fig. 6. RNAi induction after myogenic differentiation of C2C12 cells. Myogenic differentiation of C2C12 cells was performed as in Fig. 5. RNAi induction was carried out as in Fig. 2 at indicated days after induction of the myogenic differentiation, and each RNAi activity was examined 24 h after RNAi induction. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 2. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.