Qiagen (Venlo, The Netherlands) and designated ATP7A siRNA4 and ATP7A siRNA6, respectively. For gene silencing, a specific sense strand 5′-GCAGCUUGUAGUAUUGAA ATT-3′ was used for ATP7A siRNA4, and an antisense strand 5′-UUUCAAUACUACAAGCUGCTA-3′ was also used. For ATP7A siRNA6, a specific sense strand 5′-GCGUA GCUCCAGAGGUUUATT-3′ was used, and an antisense strand 5′-UAAACCUCUGGAGCUACGCAG-3′ was also used. Cells were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Selective silencing of ATP7A was confirmed by Western blot analysis.

In vivo model of cisplatin resistance

All animal experiments were conducted in accordance with the Institutional Ethical Guidelines for Animal Experimentation of our National Institute of Biomedical Innovation (Osaka, Japan). Four-week-old, female Institute of Cancer Research (ICR) nu/nu mice were obtained from Charles River Japan (Yokohama, Japan). For subcutaneous xenograft experiments, 2.5×10^6 HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were suspended in 100 μl of 1/1 (v/v) phosphate-buffered saline (PBS)/Matrigel (Becton Dickinson, Bedford, MA) and injected subcutaneously into the flanks of the ICR nu/nu mice ($n = 5$ per group). One week after xenograft establishment, tumors measured ~100 mm^3. Mice were then randomly divided into two groups and administered cisplatin (3 mg/kg) or PBS i.p. twice weekly for 4 weeks. Tumor volumes were determined twice weekly by measuring length (L), width (W) and depth (D). Tumor volume was calculated using the formula: tumor volume (mm^3) = $W \times L \times D$. At 56 days after tumor implantation, tumors were removed and weighed.

Quantification of intracellular platinum accumulation

Cisplatin accumulation in cells was analyzed according to a previously established method, with certain minor modifications. In brief, 6×10^6 cells (HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells) were seeded into two 150-mm tissue culture dishes and incubated for 24 hr. The cells were then exposed to 1 mM cisplatin for 60 min at 37°C and then washed twice with PBS. After 3 hr of incubation in cisplatin-free D-MEM medium (supplemented with 10% FBS), whole extracts were prepared and the concentration of intracellular platinum was determined using an Agilent 7500ce inductively coupled plasma mass spectrometer (ICP-MS; Agilent, Santa Clara, CA). The absolute concentration of platinum in each sample was determined from a calibration curve prepared with a platinum standard solution.

Preparation of crude membrane fractions

To investigate the localization of Anx A4, crude membrane fractions (CMFs) of cells treated in various ways were prepared. Cells were divided into three groups: those that received no treatment, those pretreated with 10 μM cisplatin for 4 hr and those pretreated with 50 μM carboplatin for 4 hr. CMF were prepared as described elsewhere,[30] with modifications. Prepared proteins were investigated using Western blot analysis. Additional information can be found in Supporting Information Material and Methods.

Biotinylation of HEC1 cell membrane surface proteins after cisplatin or carboplatin exposure

To investigate the localization of ATP7A after exposure to platinum drugs, treated or mock-treated HEC1 cells were surface-biotinylated and the presence of ATP7A was investigated by Western blot analysis. Additional information can be found in Supporting Information Material and Methods.

Immunofluorescence for ATP7A and Anx A4

Immunofluorescence staining was performed 2 days after cells had been seeded on cover slips. Before staining, cells in the treatment groups were pretreated with 10 μM cisplatin or 50 μM carboplatin for 4 hr. Cells were then analyzed for localization of Anx A4 and ATP7A. Additional information can be found in Supporting Information Material and Methods.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's analysis to evaluate the significance of differences. In all analyses, $p < 0.05$ was considered to be statistically significant.

Results

Expression of Anx A4 in endometrial carcinoma cell lines

To investigate Anx A4 expression in nine common endometrial carcinoma cell lines, Western blot analyses were performed. Expression of Anx A4 was strongest in SNGM cells compared with the other eight cell lines (Fig. 1*a*). Thus, enhanced expression of Anx A4 was confirmed in this endometrial carcinoma cell line.

Anx A4 and platinum resistance in HEC1 cell lines

From control HEC1 cells (low Anx A4 expression levels), four stable lines of Anx A4-overexpressing cells (HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells) and one line of empty vector transfected cells (HEC1-CV cells) were established. Overexpression of Anx A4 was confirmed using Western blot analysis and was compared with CCC cell lines (OVTOKO and OVISE) used as positive controls (Fig. 1*b*). Significantly higher IC_{50} values for cisplatin were observed in HEC1-A25 (32.1 μM, $p < 0.01$), HEC1-A43 (23.8 μM, $p < 0.01$), HEC1-A63 (34.9 μM, $p < 0.01$) and HEC1-A77 cells (17.3 μM, $p < 0.01$) compared with HEC1 (9.8 μM) and HEC1-CV cells (8.4 μM) (Fig. 1*c*). Similarly, IC_{50} values for carboplatin were significantly increased in HEC1-A25 (194.6 μM, $p < 0.01$), HEC1-A43 (153.3 μM, $p < 0.01$), HEC1-A63 (371.5 μM, $p < 0.01$) and HEC1-A77 cells (158.1 μM, $p < 0.01$) compared with HEC1 (59.1 μM) and HEC1-CV cells (60.9 μM) (Fig. 1*c*). Thus, Anx A4 overexpression conferred platinum resistance in HEC1 cell lines.

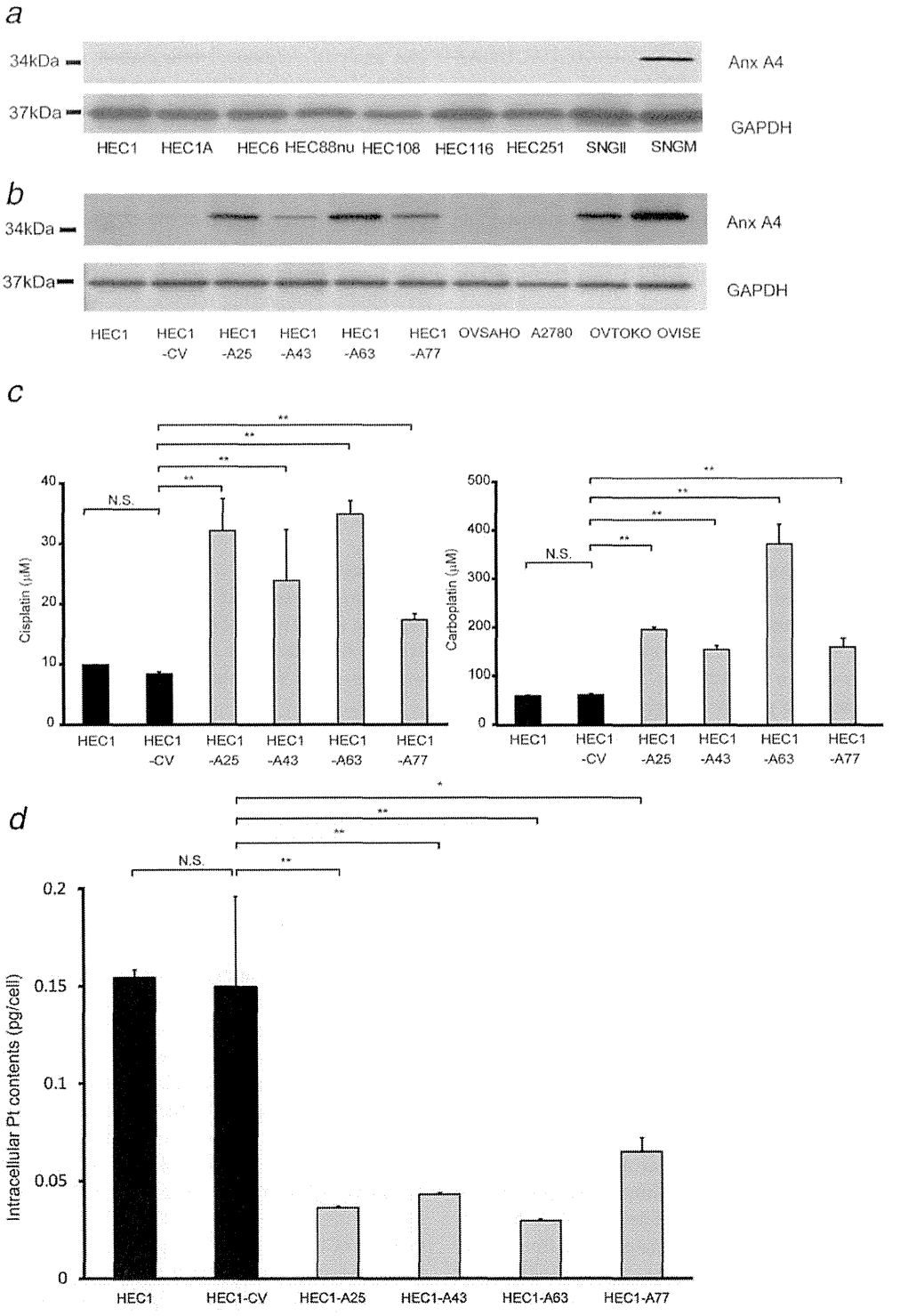

Figure 1. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vitro*. (*a*) Western blot analysis of nine endometrial carcinoma cell lines. Anx A4 was expressed in one cell line. (*b*) Establishment of an Anx A4-stably-expressing HEC1 cell line by transfection with the pcDNA3.1-Anx A4 expression plasmid into a HEC1 cell line with low Anx A4 expression levels. Enforced expression of Anx A4 was confirmed by Western blot analysis. (*c*) The IC_{50} sensitivity to cisplatin or carboplatin was investigated in HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells. (*d*) Intracellular platinum accumulation was investigated after treatment with 1 mM cisplatin for 60 min and further incubation with cisplatin-free medium for 180 min and was determined by ICP-MS analysis.

Intracellular platinum accumulation in Anx A4-overexpressing cells

To elucidate the mechanism underlying platinum resistance induced by Anx A4, intracellular platinum accumulation of HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells after cisplatin exposure was analyzed. Significantly less platinum had accumulated in HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells compared with HEC1 and HEC1-CV cells (0.036 pg/cell, $p < 0.01$; 0.04 pg/cell, $p < 0.01$; 0.03 pg/cell, $p < 0.01$; 0.065 pg/cell, $p < 0.05$ and 0.154 and 0.150 pg/cell, respectively) (Fig. 1*d*). Thus, intracellular platinum accumulation was decreased in Anx A4-overexpressing cells.

Anx A4-overexpressing cells and cisplatin in xenograft models

To determine the involvement of Anx A4 in platinum resistance *in vivo*, HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were subcutaneously injected into nude mice. After the tumor xenograft had been established, cisplatin or PBS was given twice a week for 1 month. On Day 56, average tumor volumes were $11,496 \pm 950$ mm^3 in PBS-treated HEC1-CV control mice and $3,554 \pm 872$ mm^3 in cisplatin-treated HEC1-CV controls. A significant antitumor effect of cisplatin was therefore observed in HEC1-CV-xenografted mice compared with the PBS-treated group. The parent HEC1 and HEC1-CV xenografts responded similarly to cisplatin (Fig. 2*a*; $p < 0.01$).

In HEC1-A63-xenografted mice, the average tumor volume on Day 56 was $8,245 \pm 160$ mm^3 in the PBS-treated group and only slightly less ($7,078 \pm 257$ mm^3) in the cisplatin-treated group (Fig. 2*a*; $p = 0.42$). A similar response to cisplatin was observed in the HEC1-A63 and HEC1-A77 xenografts. On Day 56, no significant differences in tumor weight were found in HEC1-A63-xenografted mice between the PBS treatment (4.66 ± 0.42 g) and the cisplatin treatment groups (4.43 ± 0.16 g) (Fig. 2*b*). Similar results were observed in HEC1-A77 xenograft models. In contrast, a significant decrease in tumor weight was observed in HEC1-CV-xenografted mice between the PBS mock treatment (5.95 ± 1.16 g) and the cisplatin treatment groups (3.20 ± 0.76 g; $p < 0.05$) (Fig. 2*b*). Similar results were observed for the HEC1 and HEC1-CV xenografts. No significant differences in tumor weight in the PBS treatment group were observed among HEC1-CV-xenografted (5.95 ± 1.16 g), HEC1-xenografted (7.48 ± 0.34 g), HEC1-A63-xenografted (4.66 ± 0.42 g) and HEC1-A77-xenografted mice (4.82 ± 1.08 g) (Fig. 2*b*). These results indicated that overexpression of Anx A4 in HEC1 endometrial carcinoma cell lines conferred significant platinum resistance to the cells as tumors growing *in vivo*.

Translocation of Anx A4 and ATP7A after platinum exposure

In our study, platinum transporters were the focus of an investigation of the molecular mechanisms of chemoresistance induced by Anx A4. In previous research, intracellular platinum levels were decreased after enhanced expression of Anx A4, and ATP7A and ATP7B are well known as efflux transporters of platinum drugs.[27,28,31] However, the relationship of Anx A4 with ATP7A and ATP7B has not been previously examined. The results of our study demonstrated no change in expression of ATP7A at the protein levels owing to enforced overexpression of Anx A4 (Fig. 3*a*) and no ATP7B expression in HEC1 cells (data not shown). Therefore, the effects of Anx A4 expression on ATP7B in these cells were not investigated.

Because Anx A4 is normally localized to the cytoplasm, we theorized that exposure to platinum drugs may induce translocation of Anx A4 to the cellular membrane, resulting in an increase in chemoresistance owing to the influence of ATP7A. To investigate the possibility of induced translocation of Anx A4 and ATP7A by platinum drugs, CMFs were prepared. By Western blot analysis, Anx A4 expression in CMF of HEC1 and HEC1-CV cells before and after treatment with cisplatin or carboplatin was barely detectable because of its low endogenous expression in these cells (Fig. 3*b*). In contrast, Anx A4 expression was increased in CMF of HEC1-A63 cells and HEC1-A77 cells treated with cisplatin and carboplatin compared with untreated cells (Fig. 3*b*). Biotinylation-based cell surface membrane protein enrichment revealed a marked increase in biotinylation of ATP7A after exposure to cisplatin or carboplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (Fig. 3*c*). In the biotinylated samples, no Anx A4 expression was detected on the cell surface, although it had been previously detected in the cell CMF (data not shown). These results suggested that exposure to cisplatin or carboplatin induced massive translocation of Anx A4 to CMF, including the inner surface of the cell membrane (inaccessible to biotinylation). Before exposure of the cell to cisplatin or carboplatin, ATP7A was not expressed in biotinylated samples but after exposure, strong ATP7A expression was detected. These results suggested that exposure to cisplatin or carboplatin induced massive translocation of ATP7A to the outer surface of the cell (accessible to biotinylation).

Anx A4 and ATP7A localization

By immunofluorescence analysis, Anx A4 was localized in the perinuclear and cytoplasmic regions of untreated cells, whereas ATP7A was localized mainly in the perinuclear and cytoplasmic regions and slightly less in the cellular membrane in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (Figs. 4*a*–4*d*). After 4-hr exposure to cisplatin or carboplatin, Anx A4 and ATP7A were found to be colocalized to the cellular membrane in HEC1-A63 cells (Fig. 4*c*). Similar findings were observed in HEC1-A77 cells (Fig. 4*d*). Because of the low expression of Anx A4 in HEC1 and HEC1-CV cells, no Anx A4 was detected in the cellular membranes in these cells (Figs. 4*a* and 4*b*). Thus, the results of the immunofluorescence analysis were in accordance with those of both Western blot analysis of CMF preparations and biotinylation

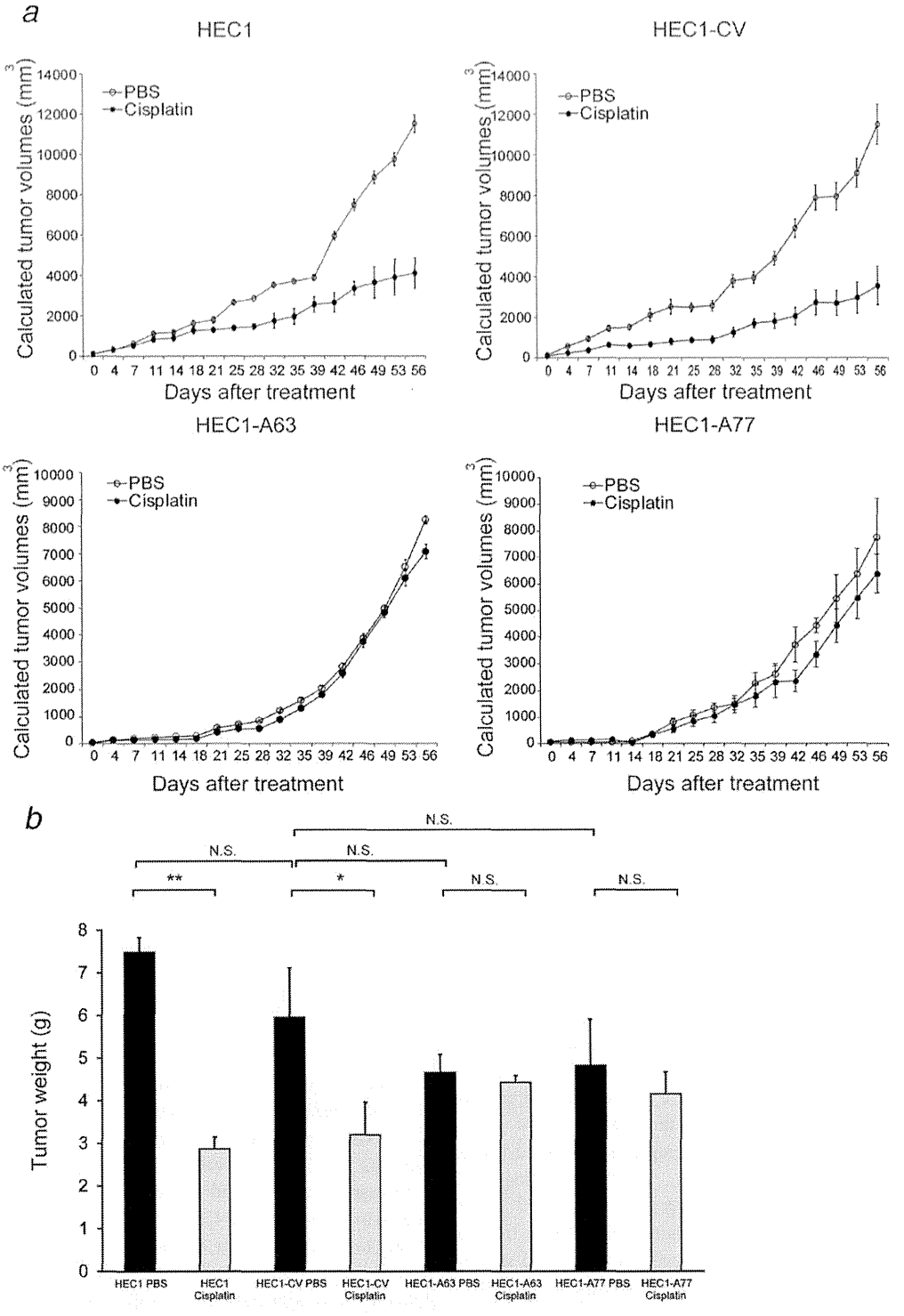

Figure 2. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vivo*. Analysis of Anx A4 as a platinum-resistant protein *in vivo*. (*a*) To determine the resistance of Anx A4-stably-expressing HEC1 cells to platinum *in vivo*, parent HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were subcutaneously injected into nude mice ($n = 5$ per group). After tumor xenografts were established, cisplatin (3 mg/kg) or PBS was administered i.p. twice weekly for 1 month. Figure shows the average (points) for five animals ± SD (bars). (*b*) Fifty-six days after implantation, tumors were removed and weighed. Values shown are the means (±SD) of five mice. NS: not significant (*$p <$ 0.05; **$p < 0.01$; one-way ANOVA, followed by Dunnett's analysis).

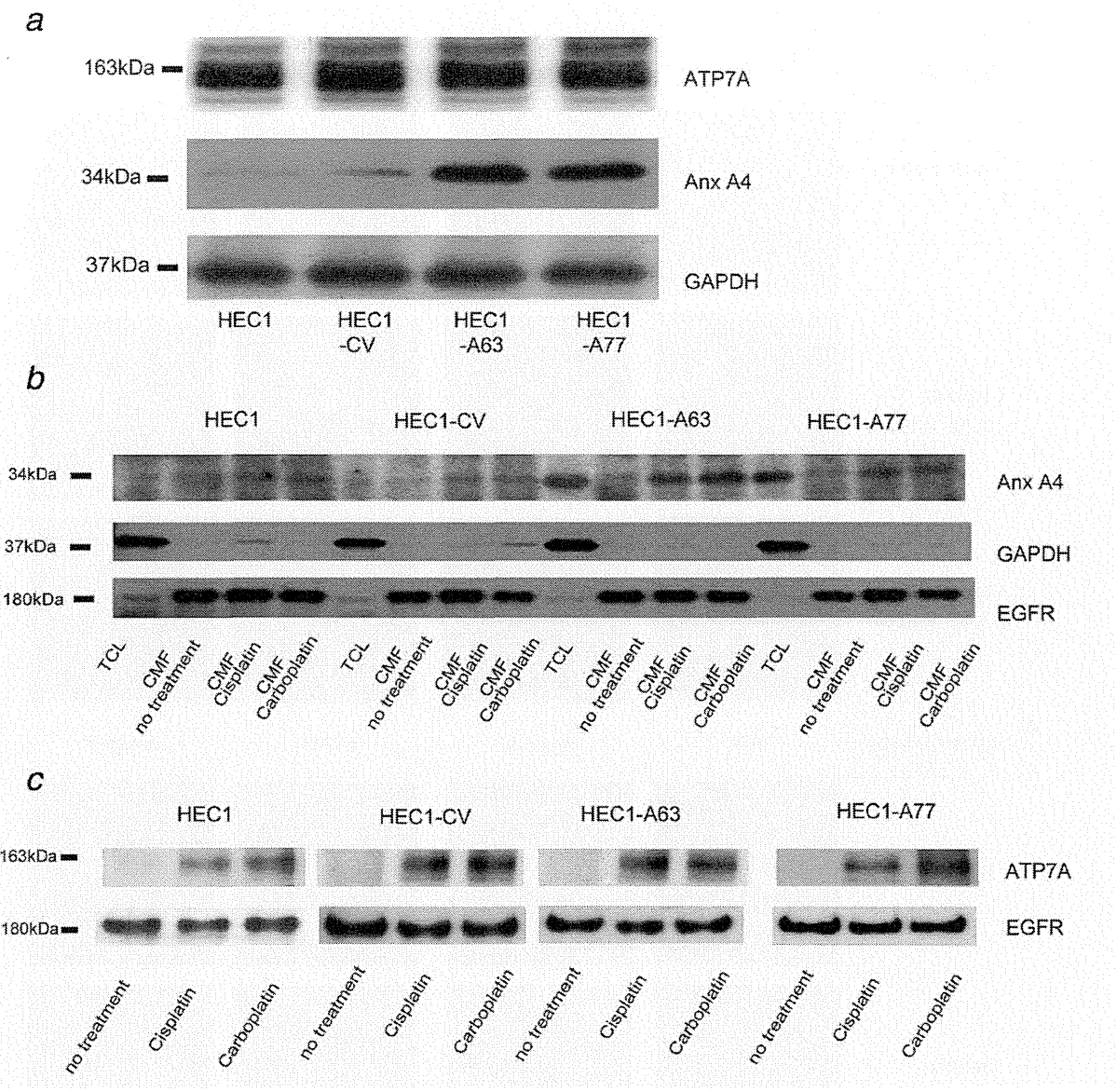

Figure 3. Localization of Anx A4 and ATP7A was investigated using Western blot analysis. The localization of Anx A4 and ATP7A was investigated using two techniques: orthogonal crude membrane fractions and biotinylation of cell surface proteins. (*a*) No significant change in expression levels of ATP7A was observed in HEC1, HEC1-CV, HEC1-A63 or HEC1-A77 cells. (*b*) In both HEC1-A63 and HEC1-A77 cells (but not in HEC1 and HEC1-CV cells), the drug-induced translocation of Anx A4 into the crude membrane fraction was shown by Western blot analysis after exposure to 10 μM cisplatin or 50 μM carboplatin for 4 hr. TCL: total cell lysate. Epidermal growth factor receptor was used as the control for cell surface protein labeling. (*c*) In HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells, translocation of ATP7A to the cell surface was shown by Western blot analysis. Cells were treated with 25 μM cisplatin or 150 μM carboplatin for 4 hr, and cell surface proteins were biotinylated with 500 μM sulfo-NHS-SS-biotin. Biotinylated surface proteins were enriched with UltraLink Immobilized Neutroavidin (Thermo Fisher Scientific, Waltham, MA) and analyzed by Western blot analysis using anti-ATP7A. Levels of epidermal growth factor receptor, a surface protein, are shown as loading controls.

assays (Figs. 3*b* and 3*c*). Anx A4 and ATP7A were localized in the cytoplasm before cisplatin or carboplatin exposure; Anx A4 and ATP7A were then translocated to the cellular membrane after cisplatin or carboplatin exposure. Thus, Anx A4 and ATP7A are colocalized to the cellular membrane in platinum-treated HEC1-A63 and HEC1-A77 cells but not in HEC1 and HEC1-CV cells.

Effect of ATP7A expression on resistance to platinum drugs

The mechanism of platinum resistance conferred by Anx A4 overexpression was explored further by suppression of ATP7A expression using siRNA. The suppression of ATP7A was confirmed using Western blot analysis (Fig. 5*a*). Anx A4 expression was unchanged by silencing ATP7A (Fig. 5*a*). The

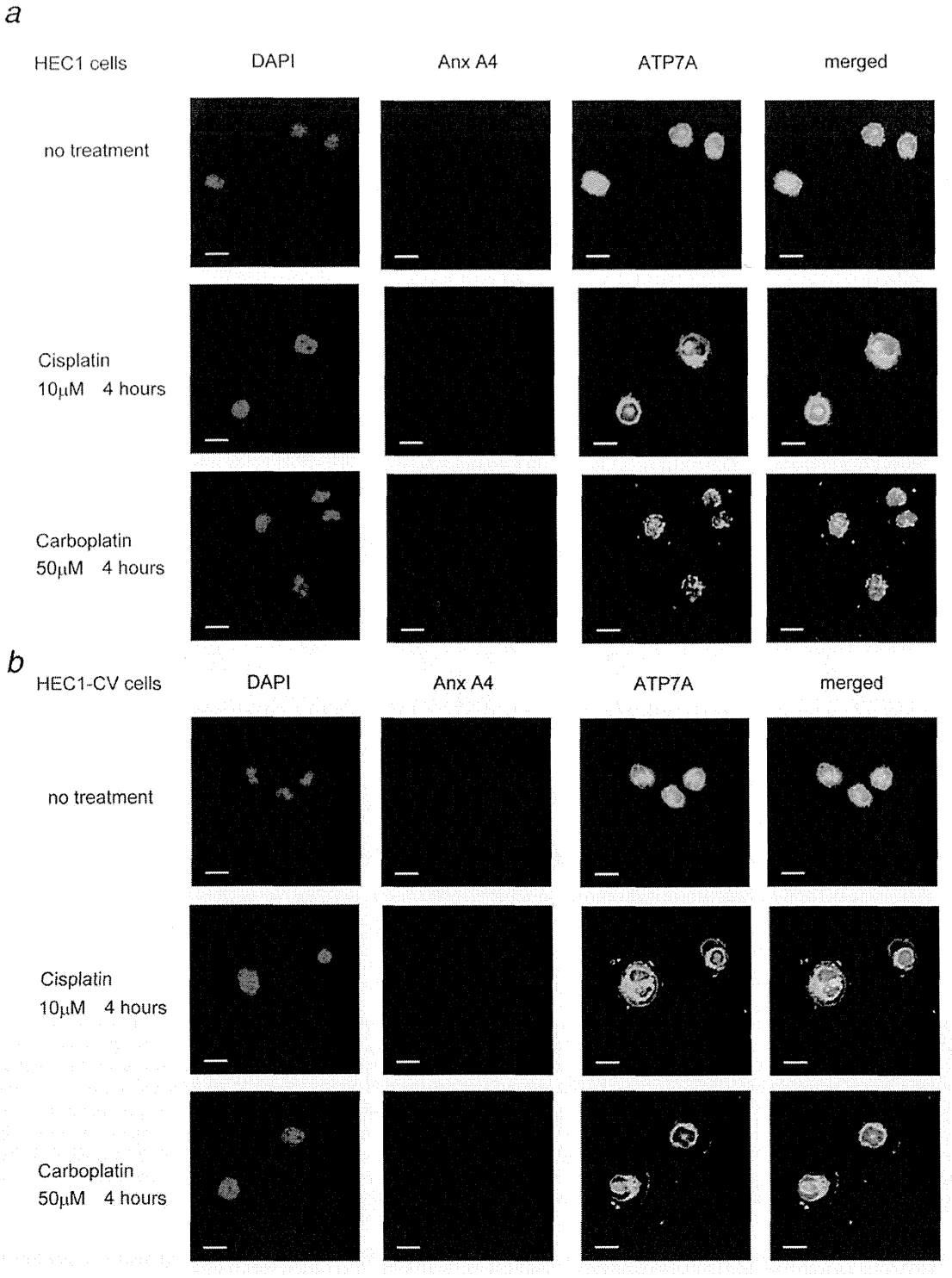

Figure 4. Immunofluorescence staining for ATP7A and Anx A4. HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were divided into three groups: the no treatment, cisplatin exposure and carboplatin exposure groups. (*a*) HEC1 cells, (*b*) HEC1-CV cells, (*c*) HEC1-A63 cells and (*d*) HEC1-A77 cells. Cells were incubated with anti-Anx A4 antibody (red) or anti-ATP7A antibody (green). Nuclei were stained with DAPI (blue). In the no treatment group for each cell, Anx A4 was localized in perinuclear and cytoplasmic regions and ATP7A was strongly localized in perinuclear regions. In HEC1 and HEC1-CV cells, after exposure to cisplatin or carboplatin, ATP7A was relocalized in the cellular membrane, although some ATP7A remained in the cytoplasm; however, no change in location of Anx A4 was observed. In HEC1-A63 and HEC1-A77 cells, Anx A4 and ATP7A were newly colocalized in the cellular membrane as well as remaining in the cytoplasm. In a comparison of HEC1 and HEC1-CV cells with HEC1-A63 and HEC1-A77 cells, expression of Anx A4 in HEC1-A63 and HEC1-A77 cells was stronger in the cytoplasm and cellular membrane. Scale bar = 30 μm.

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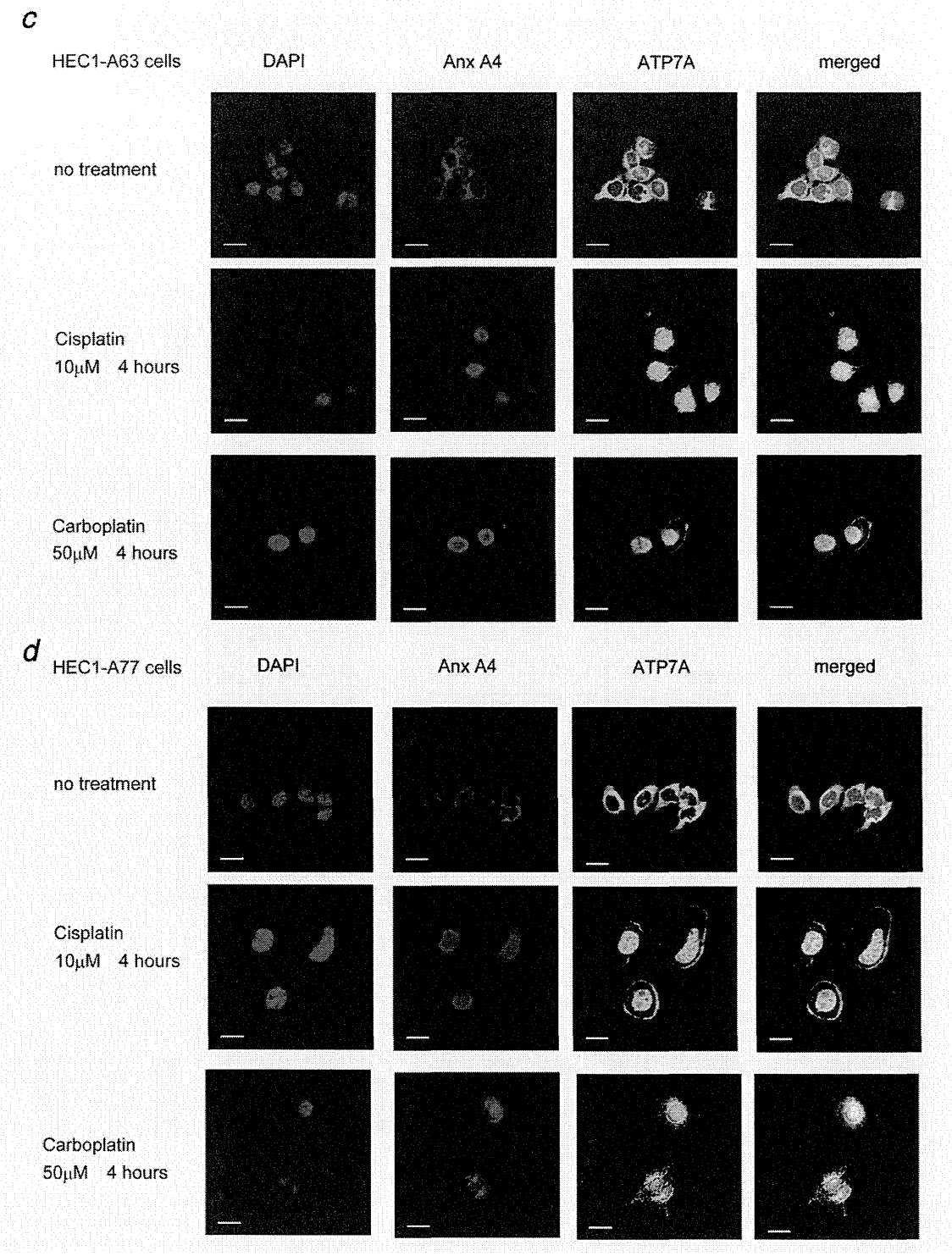

Figure 4. (Continued)

control and commercial siRNAs against ATP7A were transfected and the IC_{50} values of cisplatin and carboplatin were determined for each cell line. The IC_{50} value for cisplatin was significantly lower for the two kinds of ATP7A-silenced HEC1-A63 cells (ATP7A siRNA4, $IC_{50} = 11.0$ μM, $p < 0.01$; ATP7A siRNA6, $IC_{50} = 11.2$ μM, $p < 0.01$) compared with

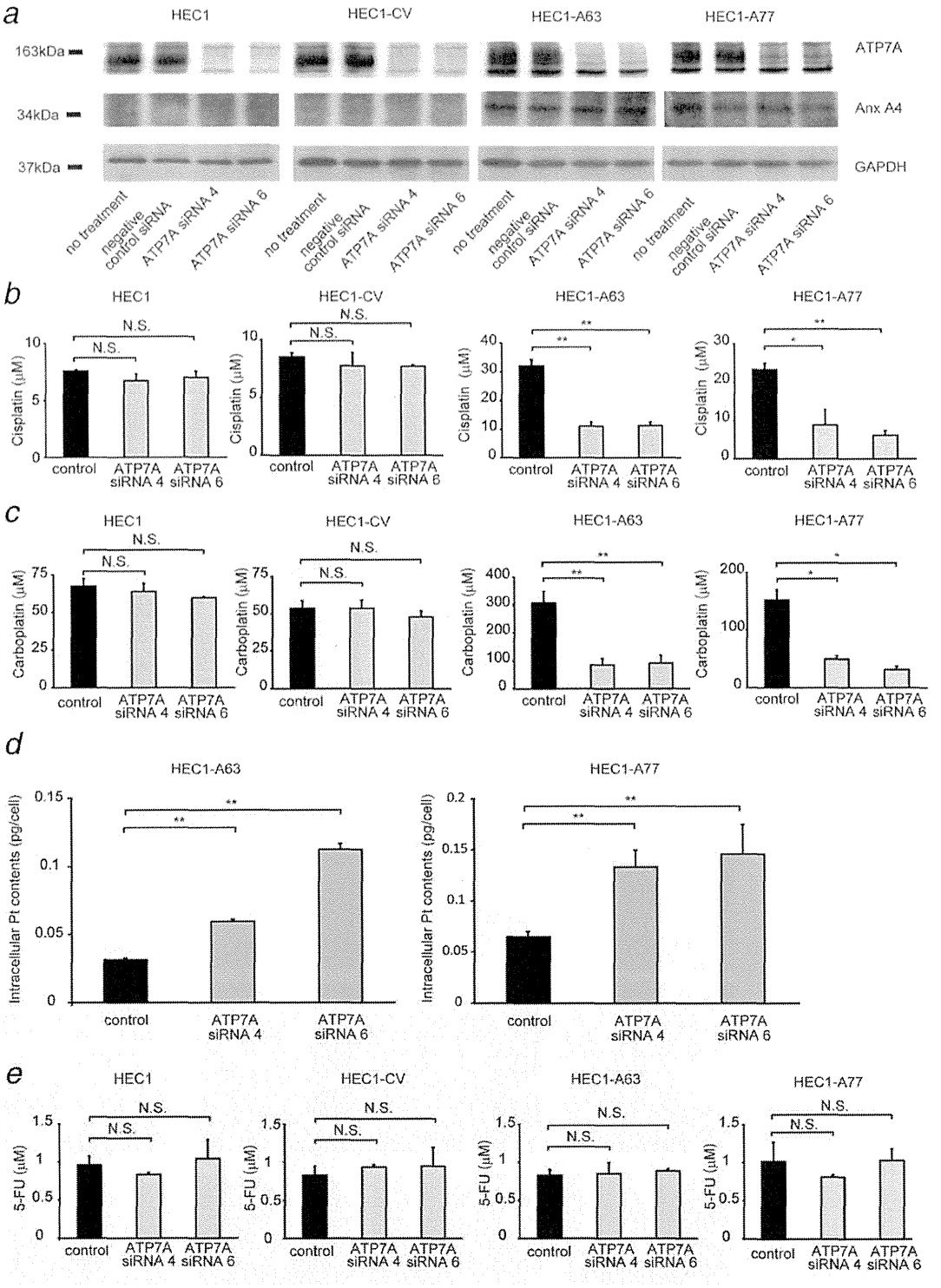

Figure 5. Knockdown of ATP7A expression improves platinum chemosensitivity in Anx A4-overexpressing cells. (*a*) Knockdown expression of ATP7A by siRNA in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells by Western blot analysis. (*b*) IC_{50} values are shown for cisplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells transfected with negative control siRNA and two types of siRNA targeting ATP7A. A significant decrease in IC_{50} value for cisplatin was observed for the two types of ATP7A-silenced HEC1-A63 and HEC1-A77 cells but not for the HEC1 and HEC1-CV cells. (*c*) IC_{50} values are shown for carboplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells transfected with negative control siRNA and two kinds of siRNA targeting ATP7A. A significant decrease in IC_{50} value for carboplatin was observed for the two types of ATP7A-silenced HEC1-A63 and HEC1-A77 cells but not for the HEC1 and HEC1-CV cells. (*d*) Intracellular platinum content after treatment with 1 mM cisplatin for 60 min and further incubation with cisplatin-free medium for 180 min in D-MEM medium in HEC1-A63 cells and HEC1-A77 cells transfected with negative control siRNA and ATP7A-targeting siRNA, as determined by ICP-MS analysis. Significantly higher intracellular platinum accumulation was observed in HEC1-A63 cells and HEC1-A77 ATP7A-silencing cells than in control siRNA-transfected HEC1-A63 cells and HEC1-A77 cells. (*e*) No significant differences in IC_{50} values for 5-FU were noted between HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells. Similar results were observed in ATP7A-silenced cell lines for HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (*$p < 0.05$; **$p < 0.01$; one-way ANOVA followed by Dunnett's analysis).

the HEC1-A63 control siRNA-transfected cells (IC_{50} = 32.2 μM) (Fig. 5*b*).

In addition to cisplatin, improved chemosensitivity associated with ATP7A silencing was observed with carboplatin. Significantly lower IC_{50} values for carboplatin were observed in both types of ATP7A-silenced HEC1-A63 cells (siRNA4, IC_{50} = 85.9 μM, $p < 0.01$; siRNA6, IC_{50} = 92.8 μM, $p < 0.01$) compared with the HEC1-A63 control siRNA-transfected cells (IC_{50} = 300.7 μM) (Fig. 5*c*). Similar results were found for HEC1-A77 ATP7A-silenced cells, where a significantly lower IC_{50} value for cisplatin was observed (siRNA4, IC_{50} = 8.9 μM, $p < 0.05$; siRNA6, IC_{50} = 6.2 μM, $p < 0.01$) compared with that for HEC1-A77 control siRNA-transfected cells (IC_{50} = 23.3 μM). IC_{50} values for carboplatin were also significantly lower for the two kinds of ATP7A-silenced HEC1-A77 cells (siRNA4, IC_{50} = 49.8 μM, $p < 0.05$; siRNA6, IC_{50} = 31.9 μM, $p < 0.05$) compared with the HEC1-A77 control siRNA-transfected cells (IC_{50} = 152.1 μM, $p < 0.01$) (Fig. 5*c*). In contrast, siRNA treatments targeting ATP7A were ineffective in HEC1 and HEC1-CV cells treated with cisplatin or carboplatin (Figs. 5*b* and 5*c*). Intracellular platinum accumulation after cisplatin exposure was significantly increased in HEC1-A63 cells treated with ATP7A siRNA (0.060 pg/cell, $p < 0.01$ to 0.113 pg/cell, $p < 0.01$) compared with control siRNA-transfected cells (0.030 pg/cell) (Fig. 5*d*). Similarly, a significant increase in intracellular platinum accumulation was observed in HEC1-A77 cells treated with ATP7A siRNA (0.133 pg/cell, $p < 0.01$ to 0.146 pg/cell, $p < 0.01$) compared with control siRNA-transfected cells (0.065 pg/cell) (Fig. 5*d*).

To investigate the relationship between resistance to drugs other than platinum drugs and Anx A4 or ATP7A expression, IC_{50} values for 5-FU were determined for each cell line. No significant change in IC_{50} values for 5-FU was observed in HEC1 (IC_{50} = 0.96 μM), HEC1-CV (IC_{50} = 1.00 μM), HEC1-A63 (IC_{50} = 0.83 μM) or HEC1-A77 cells (IC50 = 1.01 μM) (Fig. 5*e*). Similar results were observed in the ATP7A-silenced cell lines for HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells as well as in ATP7A-silenced cell lines (Fig. 5*e*). These results suggested that platinum resistance induced by enforced expression of Anx A4 was mainly dependent on the platinum transporter ATP7A and that expression of Anx A4 and ATP7A was unrelated to resistance to 5-FU.

Discussion

In our study, overexpression of Anx A4 in HEC1 cells decreased cell sensitivity to platinum drugs *in vitro*. Increased drug efflux was the mechanism underlying this change. In addition, an association between Anx A4 and platinum resistance was demonstrated for the first time *in vivo*. The mechanism of Anx A4-induced drug efflux may prove to be a promising therapeutic target because blockage of that mechanism may improve the prognosis of patients with Anx A4-associated platinum-resistant tumors.

Anx A4 itself is not a drug transporter, but it does bind to phospholipids in a Ca^{2+}-dependent manner and self-associates onto phospholipid membrane surfaces, causing membrane aggregation.[12,14–17] Thus, we assumed an indirect mediating effect of Anx A4 on drug efflux through an association between an unidentified drug transporter and Anx A4. Recently, MRP2 (an ABC ATPase-like multidrug-resistant protein) and ATP7A and ATP7B (two P-type Cu-transporting ATPases) were identified as platinum efflux transporters strongly associated with platinum resistance.[32,33] In an analysis of clinical gynecological samples, expression of MRP2 failed to predict tumor response to chemotherapy and did not correlate with overall survival.[34–36] In contrast, poor survival rates were associated with overexpression of ATP7A in patients with ovarian cancer.[27] Similarly, a correlation was found between ATP7B overexpression in endometrial carcinomas and an unfavorable clinical outcome in patients treated with cisplatin-based chemotherapy.[37] Therefore, we focused on the platinum transporters ATP7A and ATP7B and investigated their relationships with expression of Anx A4. In normal, unchallenged cells, ATP7A and ATP7B are localized in the Golgi apparatus and are involved in copper homeostasis, using ATP hydrolysis to transport copper ions across cellular membranes. They function in both the export of excess copper and its delivery to copper-dependent enzymes. ATP7A and ATP7B are also known to be efflux transporters of platinum drugs.[8,27,28,31,38,39] In one study, only a slight increase in expression of transfected ATP7A was seen in a human ovarian cancer cell line; however, that small increase was sufficient to confer significant resistance to cisplatin or carboplatin.[40] In a similar study in another human cisplatin-resistant ovarian cancer cell line, silencing of ATP7B by siRNA transfection resulted in a 2.5-fold decrease in cisplatin IC_{50} levels and a significant increase in DNA-platinum adduct formation.[41] Preparing CMF of treated cells facilitated the localization of Anx A4 expression in cells before and after exposure to platinum drugs. The abundance of AnxA4 in the membrane fraction along with the translocation to the membrane was increased. Using the orthogonal method of cell surface protein labeling to monitor proteins appearing on the cell surface, biotinylated ATP7A was increased after cisplatin or carboplatin exposure both in HEC1 and HEC1-CV cells (cells expressing low levels of Anx A4) and HEC1-A63 and HEC1-A77 cells (cells overexpressing Anx A4). Taken together, these results suggest that platinum drug exposure causes relocalization of Anx A4 expression to the membrane fraction and relocalization of ATP7A transporters (to a minimum) to the external surface of the cellular membrane. Unfortunately, no similar analysis of ATP7B was possible because it is not expressed in HEC1 cells (data not shown). However, in cells that express both ATP7A and ATP7B proteins, other immunofluorescence studies have shown similar changes in localization of both proteins after cisplatin exposure.[42] After cisplatin or carboplatin exposure in HEC1-A63 and HEC1-A77 Anx

A4-overexpressing cells, immunofluorescence showed that Anx A4 expression was relocated from the perinuclear and cytoplasmic Golgi regions to the cellular membrane. This relocalization was not observed in HEC1 and HEC1-CV cells, in which overexpression of Anx A4 does not occur.

ATP7A also relocates from the perinuclear and cytoplasmic regions to the cellular membrane after cisplatin or carboplatin exposure. However, this occurs both in HEC1 and HEC1-CV cells (cells expressing low levels of Anx A4) and HEC1-A63 and HEC1-A77 cells (cells overexpressing Anx A4). Although no direct interaction between ATP7A and Anx A4 was detected by coimmunoprecipitation analysis (data not shown), immunofluorescence analysis showed colocalization of ATP7A and Anx A4 at least within the cellular membrane in Anx A4-overexpressing cells. These results suggested that Anx A4 is not required for ATP7A translocation and that ATP7A translocation is unrelated to expression of Anx A4.

Translocation of Anx A4 to plasma membranes is reportedly mediated by an increase in intracellular free Ca^{2+}, which is increased by exposure to platinum drugs.[43,44] In addition to the translocation of ATP7A and Anx A4 to the plasma membrane, our results also showed translocation of ATP7A to the nucleus in HEC1 and HEC1-CV cells. Translocation to the nucleus and colocalization of both ATP7A and Anx A4 were also observed in HEC1-A63 and HEC1-A77 cells after exposure to cisplatin or carboplatin in the immunofluorescence staining analysis in our study (Fig. 4). Anx A4 translocates to the nucleus after etoposide treatment and suppresses NF-κB transcriptional activity, which induces expression of Bax, a proapoptotic Bcl-2 family protein.[18] In addition, a correlation has been reported between nuclear staining of Anx A4 and poor survival in patients with ovarian cancer.[45] However, the role of ATP7A in the nucleus and its relationship with NF-κB transcriptional activity has not been investigated. Further investigation is needed to elucidate the role of nuclear colocalization of Anx A4 and ATP7A in platinum resistance.

In our study, translational silencing of ATP7A in HEC1 and HEC1-CV (Anx A4-nonexpressing cells) and HEC1-A63 and HEC1-A77 cells (Anx A4-overexpressing cells) was performed. Western blot analysis demonstrated no detectable changes in protein expression of Anx A4 when ATP7A was silenced in any of these four cell lines.

In HEC1 and control HEC1-CV cells (low Anx A4 expression levels), IC_{50} values for cisplatin or carboplatin cells after the knockdown of ATP7A expression caused no improvement in the sensitivity of these cells to cisplatin or carboplatin. Similar results were observed in a previous study in which no improvement in sensitivity to cisplatin resulted from silencing of ATP7A in platinum-resistant or -sensitive ovarian cancer cell lines.[41] However, Mangala *et al.* reported improved sensitivity to cisplatin in both platinum-resistant ovarian cancer cells and parental cells expressing ATP7B as a result of silencing of ATP7B expression.[41] An important discovery related to ATP7A was communicated in our study: in cells overexpressing both Anx A4 and ATP7A, silencing of ATP7A significantly improved sensitivity to cisplatin and carboplatin, thus restoring them to sensitivity levels comparable to those of HEC1 and HEC1-CV cells. These results were supported by a quantitative analysis of the accumulation of intracellular platinum, demonstrating that siRNA silencing of ATP7A in Anx A4-overexpressing HEC1-A63 and HEC1-A77 cells resulted in greater intracellular platinum accumulation than HEC1-A63 and HEC1-A77 cells transfected with a control siRNA. On the other hand, the analysis of IC_{50} values for 5-FU showed no relationship between overexpression of Anx A4 and resistance to 5-FU. In addition, no improvement in sensitivity to 5-FU was observed as a result of ATP7A silencing. These results suggested a specific relationship of Anx A4 with ATP7A and resistance to platinum drugs but with to nonplatinum drugs such as 5-FU. Differences in efficacy and improvement in drug sensitivity of ATP7A silencing were observed between cell lines (HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells). These variations may be related to the colocalization of Anx A4 and ATP7A in the cellular membrane after cisplatin or carboplatin exposure. Colocalization of Anx A4 and ATP7A after exposure to platinum drugs was specific to changes in Anx A4-overexpressing cells, which are probably related to drug efflux. These results suggest that in conjunction with higher Anx A4 expression levels, ATP7A had a positive effect on efflux of platinum drugs, resulting in significantly increased platinum resistance. Because overexpression of Anx A4 had no effect on ATP7A expression and because no direct interaction between ATP7A and Anx A4 was detected in the coimmunoprecipitation analysis, Anx A4 seems to promote ATP7A activity in a manner which is currently unexplained.

In addition to the effects of Anx A4 on drug resistance in ovarian cancer, similar findings have been reported for other overexpressed members of the Annexin family such as Annexin A3 (Anx A3).[46,47] Intracellular platinum concentrations of cisplatin and levels of platinum DNA binding in that study were significantly lower in Anx A3-overexpressing cells than in control cells, suggesting a more general involvement of the Annexin family in platinum resistance.[46] From the results of these related reports and those of our study, we conclude that the Annexin family may potentially enhance the activity of numerous drug transporters. Identifying these enhancement mechanisms may be extremely useful for developing additional therapeutic targets for drug-resistant tumors.

In summary, our study demonstrated that enhanced expression of Anx A4 induces chemoresistance by promoting platinum drug efflux *via* ATP7A. These findings suggested that Anx A4 is a potential therapeutic target for chemosensitization, particularly in tumors with higher expression of both Anx A4 and ATP7A. Thus, our study provides a clear example of applied genotoxicology. However, platinum resistance induced by overexpression of Anx A4 may occur as a

result of multiple processes, including regulation of apoptosis and efflux of platinum drugs. Thus, other unknown chemoresistant mechanisms may be induced by overexpression of Anx A4. Because overexpression of Anx A4 has been reported in several other types of clinically important cancers, such as rectal, renal, lung and pancreatic cancer,[19–23] targeting Anx A4 may lead to the development of an effective therapy for overcoming chemoresistance in more types of cancer.

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Varicella-Zoster Virus ORF49 Functions in the Efficient Production of Progeny Virus through Its Interaction with Essential Tegument Protein ORF44

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Varicella-Zoster Virus ORF49 Functions in the Efficient Production of Progeny Virus through Its Interaction with Essential Tegument Protein ORF44

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The ORF49 tegument protein of varicella-zoster virus (VZV) is one of the core gene products that is conserved among herpesvirus family members. Although ORF49 is known to be a cell-tropic factor, its detailed functions remain elusive. ORF44 is another core gene product reported to be essential, although its characterization and detailed functional analysis have not been reported. These two core gene products form a complex in other herpesviruses beyond the host species and herpesvirus subfamilies. Here, we show that complex formation between ORF44 and ORF49 is conserved in VZV. We serendipitously found that binding is eliminated by an amino acid substitution at position 129 (phenylalanine 129), and four amino acids in the carboxyl-terminal half of the acidic cluster in ORF49 (i.e., aspartate-phenylalanine-aspartate-glutamate from positions 41 to 44 [41DFDE44]) were identified as its binding motif. Alanine substitutions in each domain rendered the ORF44F129A mutation lethal for VZV, similar to deletion of the entire ORF44. The phenotype of the ORF49-41AAAA44 mutation was comparable to that of the ORF49-defective virus, including small-plaque formation, impaired growth, and low infectious virus production. These results suggest that the interaction between ORF44 and ORF49 is essential for their role in VZV infection and that ORF49 is required for the efficient production of infectious progeny virus mediated by the conserved interaction between the two proteins.

Varicella-zoster virus (VZV) is a member of the human alphaherpesvirus subfamily and the etiologic agent of two diseases: varicella is the result of primary infection with VZV, and herpes-zoster is caused by reactivation of the virus from the latent state (1). VZV shares many features, especially a tropism for epithelial and neural tissues, with other human alphaherpesvirus members, including herpes simplex viruses 1 and 2 (HSV-1 and -2, respectively), and with the nonhuman alphaherpesviruses. However, VZV spreads only via cell-to-cell infection in culture and is more akin to the betaherpesviruses (i.e., human herpesviruses 6 and 7) in its apparent T-cell-tropism (1).

The VZV genome is approximately 125 kb and contains at least 70 unique open reading frames (ORFs), and it is the smallest genome in terms of length and gene set among human herpesviruses (1–3). Of the 70 identified ORFs, 44 are core genes that are conserved among all human herpesvirus subfamilies (4). Recent genome-wide mutagenesis analysis showed that 34 ORFs among the core genes are essential for virus reconstitution in cell culture, whereas deletion of seven ORFs results in viral growth defects, and three ORFs are dispensable in cell culture or skin organ culture (5). Eight core genes encode tegument proteins, which are the structural components of the virion and are located between the nucleocapsid and the envelope.

VZV ORF49 encodes a nonessential tegument protein that functions as a cell-tropic factor in cell culture via an unknown mechanism (6). VZV ORF49 is the homolog of HSV-1 UL11 and human cytomegalovirus (HCMV) UL99, which are among the most extensively studied tegument protein-encoding genes. The UL11 and UL99 gene products, pUL11 and pp28, function in secondary envelopment (7–9), but they have different roles in the viral life cycle. HSV-1 UL11 is not essential for the viral life cycle; however, the UL11 deletion mutant forms small plaques, and the final titers are reduced to 80 to 95% of wild-type levels (10). In contrast, HCMV UL99 is an essential gene, and pp28-deficient mutants show extremely impaired growth in normal fibroblasts and produce no detectable infectious progeny (9). However, this mutant spreads from cell to cell via an unknown mechanism (11).

Several recent reports, beginning with one on HSV-1 UL16, which is a core gene within the intron of a conserved herpesvirus spliced gene, (12), showed that interactions between pUL11 and pUL16 homologs were conserved beyond the host species and herpesvirus subfamilies (13–15). HSV pUL16 localizes to the nucleus and the cytoplasm of infected cells and functions in virus entry and in nuclear and cytoplasmic egress (16–19); pUL16 homologs may function in secondary envelopment, as reviewed in reference 20. As described for UL11 homologs, whether UL16 homologs are required for the viral life cycle differs among viruses (13, 15, 21–25). In a genome-wide mutagenesis analysis, deletion of the entire gene region from the viral genome of VZV ORF44, the UL16 homolog, showed that it is an essential gene by loss-of-function analysis in the MeWo cell line (5), although this was not

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confirmed by a revertant virus generated for gain-of-function analysis.

The LI motif and the conserved acidic cluster of pUL11 are essential for its interaction with pUL16 of HSV-1, whereas the critical sequences in pUL16 have not been determined because it is highly sensitive to deletions. Its short N-terminal 75-amino-acid (aa) fragment was recently shown to include the pUL11 binding site, and its C-terminal region functions as the binding regulatory domain (26), although this has not been confirmed in the context of HSV-1-infection. HCMV pUL94 directs pp28 to the assembly compartment, where it plays a role in secondary envelopment. Amino acids 37 to 39, near the acidic cluster of pp28, and one of the conserved cysteine residues of pUL94 are involved in binding in the context of infection (24, 27). In VZV, potential ORF49 protein (ORF49p)-binding proteins, including the pUL16 homolog ORF44 protein (ORF44p), were identified by global screening using the yeast two-hybrid system (28, 29), although these interactions have not been confirmed, even by coexpression experiments in mammalian cells.

In our previous study on VZV ORF49 (6), ORF49p was identified as one of the cell-tropic factors for VZV lytic infection in cell culture. However, the precise function of ORF49 in cells in which the ORF49-defective virus showed impaired growth was not elucidated. To address this issue, we established a complete *trans*-complementation system for ORF49 and identified ORF44p as its binding partner in the context of infection. In the present study, we aimed to reveal the precise role of ORF49p by using this system and by analyzing the conserved mechanism of interaction between these proteins and its role in VZV infection.

MATERIALS AND METHODS

Cells and viruses. The melanoma cell line MeWo was propagated in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Ueno, Tokyo) supplemented with 8% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 0.6 mg/ml L-sodium glutamate, and 0.02 mg/ml gentamicin sulfate (Nacalai Tesque, Kyoto, Japan) (DMEM complete). MeWo cells stably expressing Cre recombinase, designated MeWo-Cre cells, were maintained in DMEM complete supplemented with 500 μg/ml G418 (Nacalai Tesque) (30). MeWoORF49 cells stably expressing ORF49 were generated as follows: MeWo cells were transfected with CAG/ORF49 (described below) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, selected, and propagated in DMEM complete supplemented with 1.5 μg/ml puromycin (Invitrogen). Recombinant viruses derived from the parental VZV strain Oka (pOka), rpOka, rpOkaΔ44Rev, rpOkaORF44F129ARev, rpOkaORF44T128A, rpOkaORF44K130A, rpOkaORF49M1L, rpOkaORF49M1LRev, rpOka-ORF49-41AAAA44, and rpOkaORF49-41AAAA44Rev were maintained in DMEM complete supplemented with 3% FBS.

Cell-free virus was prepared as described previously with slight modifications (6). At 48 h postinfection (hpi) by cell-to-cell spread, cells were harvested with a cell scraper (Iwaki, Tokyo, Japan), spun at 800 × *g* for 5 min at 4°C, and suspended in SGP buffer (phosphate-buffered saline [PBS] containing 0.1% L-sodium glutamate and 7% sucrose). The suspended cells were treated with an ultrasonic disruptor (UD-201; Tomy Seiko, Tokyo, Japan) at 1.5° for 30 s on ice and spun at 800 × *g* for 5 min at 4°C, and the supernatant was stored at −80°C until use. The purified viral particles were prepared as described in reference 6. Briefly, the cell-free virus solutions were subjected to Histodenz (Sigma-Aldrich) gradient purification (5 to 50% in PBS) by ultracentrifugation at 50,200 × *g* for 2 h at 4°C in a P40ST rotor (CP80WX; Hitachi Koki, Hitachinaka, Japan). Aliquots of the peak particle-containing fractions were subjected to ultracentrifugation at 52,600 × *g* for 2 h at 4°C in a P28S rotor (CP80WX; Hitachi Koki), and the pellets were stored at −80°C for further analyses.

Plasmids. The pGEX/ORF44, pGEX/ORF44A, and pGEX/ORF44P plasmids were generated to express the full length (corresponding to aa 2 to 363), anterior half (aa 2 to 200), and posterior half (aa 181 to 363) of the ORF44 protein in *Escherichia coli*. The primer pairs ORF44ecoF4 (5′-ACCGAATTCGAATTACAACGCATATTTCCG-3′) and ORF44salR (5′-ACCGTCGACCTAGGTGGTTGTAGG-3′) for ORF44, ORF44ecoF4 and ORF44salR600 (5′-ACCGTCGACTAAATTAGGTTCCATAGCC-3′) for ORF44A, and ORF44ecoF541 (5′-ACCGAATTCGGAGTGTGGTGGTCAGACG-3′) and ORF44salR for ORF44P were used to amplify each indicated region of the ORF44 gene from the rpOka cDNA. The PCR products were inserted in frame into the pGEX6P-1 bacterial expression vector (GE Healthcare Bio-Sciences, Piscataway, NJ) via the EcoRI and SalI sites (underlined). The same procedure was used to construct pGEX/ORF61. The DNA fragment (positions 406 to 744) of ORF61 was cloned into pGEX6P-1 via the BamHI and SalI sites (underlined). The primer pair was ORF61bamF406 (5′-ACCGGATCCGGGCCCCTTCAATCGTCGG-3′) and ORF61salR744stop (5′-ACCGTCGACCTAGAATCTCGCGTTTCCCTC-3′). The eukaryotic ORF44 expression plasmid CAG/ORF44 or CAG/FLAGORF44, which was N-terminally tagged with FLAG (DYKDDDDK), was generated as follows: the entire ORF44 gene was amplified by PCR with the primers ORF44-25kpnF (5′-ACCGGTACCAATCCGCTAGACTG-3′) or ORF44FLAGkpnF4 (5′-ACCGGTACCGCCACCATGgactacaaagacgatgacgacaagGAATTACAACGCATATTTCCG-3′) and ORF44salR, and the PCR fragment was cut by KpnI and SalI (underlined) and cloned into pCAGGS-MCS-neo via the KpnI and XhoI sites. The FLAG tag coding sequence within the primer for ORF44FLAGkpnF4 is shown in lowercase letters. The ORF49 expression plasmid CAG/ORF49 was generated as follows: the entire ORF49 gene was amplified by PCR with the primers ORF49-24ecoF (5′-ACCGAATTCTTACATCAGCATTGCG−3′) and ORF49bamR (5′-ACCGGATCCTTAACATTTTGCGCATTTGG-3′), and the PCR fragment was cut by EcoRI and BamHI (underlined) and cloned into pCAGGS-MCS-puro via the EcoRI and BglII sites. The pCAGGS plasmid was kindly provided by Jun-ichi Miyazaki (Osaka University, Japan) (31). A Cre recombinase-expressing plasmid, pCX-Cre-neo, was previously generated (30) from pCX-Cre, which was a generous gift from Masaru Okabe (Osaka University, Japan).

Construction of mutant ORF44 and ORF49 expression plasmids. The ORF44 mutant plasmids pGEX/ORF44F129A, CAG/ORF44T128A, CAG/ORF44F129A, CAG/ORF44K130A, CAG/FLAGORF44I121stop, CAG/FLAGORF44P136stop, and CAG/FLAGORF44F129AP136stop and the ORF49 mutant plasmid CAG/ORF49-41AAAA44 were generated with a QuikChange Lightning multisite-directed mutagenesis kit (Agilent Technologies, La Jolla, CA) according to the manufacturer's recommendations, using the primers listed in Table 1 based on pGEX/ORF44, CAG/ORF44, CAG/FLAGORF44, and CAG/ORF49, respectively. The ORF49 C-terminal deletion mutant plasmids CAG/ORF49N40, CAG/ORF49N44, and CAG/ORF49N48 were generated using the following primer pairs: ORF49-24ecoF (5′-ACCGAATTCTTACATCAGCATTGCG-3′) as the forward primer for all the deletion mutants and ORF49mycxhoR120 (5′-ACCCTCGAGcagatcctcttctgagatgagtttttgttcAAAGTCTTCAAAGAACTCTG-3′), ORF49mycxhoR132 (5′-ACCCTCGAGcagatcctcttctgagatgagtttttgttcCTCATCAAAGTCAAAGTCTT-3′), or ORF49mycxhoR144 (5′-ACCCTCGAGcagatcctcttctgagatgag tttttgttcCTCTGTTACATTCTCATCAAAGTC-3′) as the reverse primer for CAG/ORF49N40, CAG/ORF49N44, or CAG/ORF49N48. All of the reverse primers contained a c-myc tag, indicated by lowercase letters, and the PCR products were cloned into pCAGGS-MCS-puro via the EcoRI and XhoI sites (underlined).

Antibodies. To produce a mouse monoclonal antibody (MAb) against ORF44, a glutathione *S*-transferase (GST)-ORF44A recombinant protein was expressed in *E. coli* BL21 transformed with pGEX/ORF44A, purified, and used to immunize mice; hybridoma clones producing the anti-ORF44 MAb were established as described previously (32). To produce polyclonal anti-ORF61 Abs, a GST-ORF61 fusion protein was purified from *E. coli* BL21 transformed with pGEX-ORF61 and used to immunize a rabbit

TABLE 1 Primers used for ORF44 or ORF49 mutations

Primer	Sequence[a]	Amino acid substitution(s)
ORF44 361at-ta	5′-TAT CCG GTT GAA AAC TAA GAC CAT GTT TTT GGA-3′	Ile 121 to stop (TAA)
ORF44 382a-g	5′-CAT GTT TTT GGA GCA GCG TTT AAG AAC CC-3′	Thr 128 to Ala
ORF44 385tt-gc	5′-TT TTT GGA GCA ACG GCT AAG AAC CCG ATC G-3′	Phe 129 to Ala
ORF44 388aa-gc	5′-TTT GGA GCA ACG TTT GCG AAC CCG ATC GCG-3′	Lys 130 to Ala
ORF44 406ccc-taa	5′-AAC CCG ATC GCG TAC TAA CTT CCA ACA TCT ATT-3′	Pro 136 to stop (TAA)
ORF49 M1L	5′-ATT GCG GTC ATT GCG TTG GGA CAA TCT TCA-3′	Met 1 to Leu
ORF49 41AAAA44	5′-TTT GAA GAC TTT GCC GCT GCT GCG AAT GTA ACA GAG-3′	Asp-Phe-Asp-Glu (41–44) to Ala-Ala-Ala-Ala

[a] Nucleotides that differ from those of the wild type are underlined.

(Sigma Genosys, Hokkaido, Japan). The anti-ORF61 Ab was purified with GST-conjugated normal human serum (NHS)-activated Sepharose and GST-ORF61-conjugated NHS-activated Sepharose. Rabbit polyclonal anti-ORF49 and anti-gB-C Abs were described previously (6). The mouse anti-glycoprotein E (anti-gE) (clone 9) Ab was described in reference 33. Mouse anti-glycoprotein H (gH) (VgIII-3) was obtained as described previously (33). Sheep anti-*trans*-Golgi network (anti-TGN46) Ab (AHP500G; AbD Serotec, Oxford, United Kingdom), anti-α-tubulin Ab (B-5-1-2; Sigma-Aldrich), and goat anti-GST Ab (GE Healthcare Bio-Sciences) were commercially available. Alexa Fluor 488-labeled donkey anti-mouse immunoglobulin G (IgG), Alexa Fluor 594-labeled donkey anti-rabbit IgG, and Alexa Fluor 647-labeled donkey anti-sheep IgG (Invitrogen) were used as secondary Abs, and Hoechst 33342 (Sigma-Aldrich) was used for nuclear staining in confocal microscopic analyses. ECL enhanced chemiluminescence anti-mouse or anti-rabbit IgG horseradish peroxidase-linked whole antibodies from donkey (GE Healthcare Bio-Sciences) were used as secondary Abs in immunoblotting.

Mutagenesis of viral genomes in *E. coli*. The mutant bacterial artificial chromosomes (BACs) pOka-BACORF49M1L, containing a methionine-to-leucine substitution at residue Met-1, and pOka-BACORF49-41AAAA44, containing a tetra-alanine substitution at residues 41-Asp-Phe-Asp-Glu-44, were generated by *recA*-mediated allelic replacement in pOka-BAC-harboring DH10B transformed with pST76A-SR/pOkaORF49M1L and pST76A-SR/pOkaORF49-41AAAA44, respectively, which were derived from pST76A-SR/pOkaORF50 (including nucleotide positions 84361 to 89970 of pOka) (30), using the primers listed in Table 1 and a QuikChange Lightning multisite-directed mutagenesis kit. The revertant BACs pOka-BACORF49M1LRev and pOkaBACORF49-41AAAA44Rev were generated by recA-mediated allelic replacement using pST76A-SRORF50 transformed into DH10B cells harboring pOka-BACORF49M1L and pOka-BACORF49-41AAAA44, respectively.

To generate pOka-BACΔ44, in which the nucleotides (nt) 1 to 800 of the ORF44 gene were replaced with an FRT (*flp* recombinase recognition target) sequence, a linear fragment was amplified by PCR using the primer pair ORF44FRTfKMF0 (5′-TTAAACCCACAAGTACC CGGGCGGCAATCCGCTAGACTGTTTTTCTGCTCGAAGTTCCTA TTCTCTAGAAAGTATAGGAACTTCAGCAAGCGAACCGGAAT TGC-3′) and ORF44FRTrKMR800 (5′-TCCCGCTGACCGGCCTTT CTCCACATACACGGAGCCCAACACACACAACCGAAGTTCCTAT ACTTTCTAGAGAATAGGAACTTCCTTTTTCAATTCAGAAGA ACTC-3′) (FRT is underlined) using pCR2.1-TOPO as the template (Invitrogen). The amplified fragment was then transformed into DH10B harboring pOka-BAC (34) with pGETrec (a kind gift from Panayiotis A. Ioannou, The Murdoch Institute for Research into Birth Defects, Royal Children's Hospital, Melbourne, Australia) (35); *recE/T* recombination for pOka-BACΔ44KMr and excision of the kanamycin resistance gene from pOka-BACΔ44KMr by the *flp*/FRT system using pCP20 (a kind gift from Wilfried Wackernagel, Universität Oldenburg, Germany) (36), resulting in pOka-BACΔ44, were carried out as described previously (6).

To construct pOka-BACΔ44Rev, the revertant BAC genome against pOka-BACΔ44, *recA*-mediated mutagenesis was performed as described previously (30), using the shuttle plasmid pST76A-SR/pOkaORF44. For pST76A-SR/pOkaORF44, a 3.2-kbp fragment of viral DNA corresponding to nt 79230 to 82453 of pOka was amplified from the pOka-BAC genome using the primer pair ORF43F1201 (5′-ACCGCTGCGTGTATA AATGCCCGGGTTGAC-3′) and ORF42/45F (5′-ACCATGTCATTGAT AATGTTTGG-3′), cloned into pCR2.1-TOPO, sequenced, cut with EcoRI, blunted, and cloned into the plasmid pST76A-SR (kindly provided by Ulrich H. Koszinowski, Max von Pettenkofer Institut, Ludwig-Maximilians-Universität München, Munich, Germany) (37), which was cut with KpnI and blunted.

The shuttle plasmids for the ORF44 point mutant BACs, pST76A-SRORF44T128A (with an alanine substitution for threonine at residue Thr-128), pST76A-SR/pOkaORF44F129A (with an alanine substitution for phenylalanine at residue Phe-129), and pST76A-SR/pOkaORF44K130A (with an alanine substitution for lysine at residue Lys-130), were generated from pST76A-SR/pOkaORF44 with a QuikChange Lightning multisite-directed mutagenesis kit using the primers listed in Table 1. Each mutated shuttle plasmid was transformed into DH10B harboring pOka-BACΔ44KMr, and *recA*-mediated allelic replacement was performed as described elsewhere (30) to generate pOka-BACORF44T128A, pOka-BACORF44F129A, and pOka-BACORF44K130A. The revertant BAC for the ORF44F129A mutant BAC, pOka-BACORF44F129ARev, was generated by *recA*-mediated allelic replacement using pST76A-SRORF44 transformed into pOka-BACORF44F129A-harboring DH10B.

All of the purified BACs were digested with BamHI or EcoRI to ensure that the expected DNA fragments were present, and the whole region for allelic replacement was sequenced to ensure that unexpected deletions or substitutions were not present.

Reconstitution of recombinant viruses and excision of the BAC cassette. MeWo cells or MeWoORF49 cells for rpOkaORF49M1L and rpOkaORF49-41AAAA44 seeded in one well of a 12-well plate were transfected with 3 μg of BAC DNA using Lipofectamine 2000 or X-treamGene HP (Roche Applied Science, Basel, Switzerland). After typical cytopathic effects (CPE) were seen in cells expressing green fluorescent protein (GFP), cell-free virus was prepared as described above and used to infect MeWo-Cre cells or pCX-Cre-neo-transfected MeWoORF49 cells for rpOkaORF49M1L and rpOkaORF49-41AAAA44 to excise the BAC cassette.

Immunoblotting, immunoprecipitation, and immunofluorescence. Immunoblotting, immunoprecipitation, and immunofluorescence were performed as described previously (30, 32) with slight modifications. The proteins in immunoblots were visualized by Chemi-Lumi One Super (Nacalai Tesque) in combination with LAS4000mini (GE Healthcare Bio-Sciences). Radioimmunoprecipitation assay (RIPA) lysis buffer (0.01 M Tris-HCl [pH 7.4], 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) was used for cell lysis, and the supernatants obtained by ultracentrifugation at 216,900 × *g* for 1 h at 4°C in a P50A3 rotor (CP80WX; Hitachi Koki) and precleared with protein G Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences) were used for immunoblotting and immunoprecipitation. Immunofluorescence images were captured and analyzed by an FV1000D confocal microscope (Olympus, Tokyo, Japan).

***In vitro* binding assay.** To analyze the interaction of GST-ORF44 with ORF49, GST-ORF44, GST-ORF44F129A, or GST-ORF44P recombinant protein was expressed in and purified using BugBuster master mix (Merck Millipore, Darmstadt, Germany) from *E. coli* BL21 transformed with pGEX/ORF44, pGEX/ORF44F129A, or pGEX/ORF44P as described above. ORF49p was expressed in MeWo cells by transfection of CAG/ORF49 using Lipofectamine 2000 and solubilized as described above. GST-ORF44 recombinant protein was bound to glutathione Sepharose 4B (GE Healthcare Bio-Sciences) overnight at 4°C, washed with PBS three times, pelleted, and reacted with soluble ORF49p overnight at 4°C. The bead–GST-ORF44 recombinant protein–ORF49p complex was washed with RIPA lysis buffer three times, pelleted, suspended in SDS-PAGE sample buffer, boiled, and subjected to SDS-PAGE and immunoblotting as described above.

Protein identification by MS. MeWo cells were infected with pOka by cell-to-cell infection and lysed with RIPA lysis buffer as described above. The cell lysates from pOka- or mock-infected MeWo cells were precleared with protein G Sepharose and subjected to immunoprecipitation with anti-ORF49 Ab cross-linked protein G Sepharose. The immunoprecipitates were separated by SDS-PAGE and stained with a SilverQuest silver staining kit (Invitrogen). Protein bands were excised from the gel and digested with trypsin (sequencing grade; Promega, Madison, WI) according to published procedures (38). Nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) analyses were performed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a nano-electrospray ionization (nano-ESI) source (AMR, Tokyo, Japan) and coupled to a Paradigm MG4 pump (Michrom Bioresources, Auburn, CA) and autosampler (HTC PAL; CTC Analytics, Zwingen, Switzerland). A spray voltage of 1,800 V was applied. The peptide mixture was separated on a Magic C_{18} AQ column (100 μm by 150 mm, 3.0-μm particle size, 300 Å; Michrom Bioresources) with a flow rate of 500 nl/min. The linear gradient was as follows: 5% to 45% B in 30 min, 45% to 95% B in 0.1 min, 95% B for 2 min, and finally 5% B (solvent A = 0.1% formic acid in 2% acetonitrile, and B = 0.1% formic acid in 90% acetonitrile). Intact peptides were detected in the Orbitrap at a resolution of 60,000. For the LC-MS/MS analysis, six precursor ions were selected for subsequent MS/MS scans in a data-dependent acquisition mode following each full scan (*m/z*, 350 to 1,500). A lock mass function was used for the LTQ-Orbitrap to obtain constant mass accuracy during the gradient analysis. Peptides and proteins were identified by automated database searches using Proteome Discoverer v.1.1 (Thermo Fisher Scientific, Waltham, MA) against human entries or all entries of the Swiss-Prot protein database (version 3.26) with a precursor mass tolerance of 10 parts per million (ppm), a fragment ion mass tolerance of 0.8 Da, and strict trypsin specificity, allowing for up to two missed cleavages. Cysteine carbamidomethylation was set as a fixed modification, and methionine oxidation was allowed as a variable modification.

Plaque size and infectious-center assays for growth kinetics. To analyze the growth kinetics of the recombinant viruses, infectious-center assays were performed as described previously (6) with slight modifications. Briefly, 5×10^5 MeWo or MeWoORF49 cells were seeded on one well of a 12-well plate and inoculated with 50 PFU of cell-free virus per well. For the plaque size measurement, the infected cells were cultured for 7 days. For the infectious-center assay, infected cells were harvested at 24-h intervals and then titrated on newly prepared cells. The cells were fixed in 30% methanol and 70% acetone and stained with an anti-gE MAb (clone 9) and secondary ECL anti-mouse IgG horseradish peroxidase-linked whole antibody (GE Healthcare Bio-Sciences). The stain was developed with 3,3′,5,5′-tetramethylbenzidine-H (TMB-H) peroxidase substrate (Moss, Inc., Pasadena, MD). Images of the plaques were captured and traced, and the number of plaques was counted, or the plaque area was measured using ImageJ (http://rsbweb.nih.gov/ij/).

RESULTS

ORF49 functions in the efficient production of infectious progeny virus. To examine the mechanism of action of ORF49, we performed loss-of-function and gain-of-function analyses by generating an ORF49-defective virus, rpOkaORF49M1L, and its revertant virus, rpOkaORF49M1LRev, from the pOka-BACORF49M1L and pOka-BACORF49M1LRev genomes, respectively (Fig. 1A and C). In addition, the MeWoORF49 cell line was established to express ORF49 constitutively in MeWo cells in which the previous ORF49-defective virus, rpOkaΔ49, specifically showed an impaired growth phenotype (6), and gain-of-function analysis was performed by ORF49 *trans*-complementation assay.

None of the assayed viral proteins, including glycoprotein H (gH), ORF61 protein (ORF61p), ORF44p, and ORF49p, were detected in MeWo cells (Fig. 2A, lane 1). In MeWoORF49 cells, ORF49p was the only protein detected among the tested viral proteins (Fig. 2A, lane 2), and its expression level was comparable to that of ORF49p in rpOka-infected MeWo cells (Fig. 2A, lane 3). In rpOkaORF49M1L-infected cells, ORF49p was not detected in the infected MeWo cells (Fig. 2A, lane 4) or in the viral particles (Fig. 2C, lane 2), although gH and ORF61p were clearly expressed in infected cells (Fig. 2A, lane 4), as was gH in the viral particles (Fig. 2C, lane 2). The rpOkaORF49M1LRev line expressed all of the viral proteins tested (Fig. 2A, lane 5), similar to rpOka-infected MeWo cells (Fig. 2A, lane 3).

To confirm the ORF49-defective phenotype and perform gain-of function analysis, plaque formation was analyzed on MeWo and MeWoORF49 cells (Fig. 3A). Plaque sizes were similar between rpOka-infected MeWo and MeWoORF49 cells (Fig. 3A, lanes 1 and 2), indicating that the exogenous expression of ORF49 had neither positive nor negative effects on normal VZV infection. Similar to our previous results using rpOkaΔ49, rpOkaORF49M1L formed significantly smaller plaques on MeWo cells (Fig. 3A, lane 3) than on MeWoORF49 cells, and this reduction was recovered in the revertant virus infection in MeWo cells (Fig. 3A, lane 5) and completely rescued by the exogenous expression of ORF49 in MeWoORF49 cells (Fig. 3A, lane 4). Consistent with the plaque formation assay results, rpOkaORF49M1L propagated on MeWo cells showed slower growth than rpOka or rpOkaORF49M1LRev on MeWo or MeWoORF49 cells in an infectious-center assay, and the growth impairment of rpOkaORF49M1L was completely rescued by exogenous ORF49p in MeWoORF49 cells (Fig. 3B).

During the preparation of cell-free viruses, another strikingly different phenotype of the ORF49 defect was observed. As summarized in Table 2, the titer of cell-free virus or plaque size formed by the cell-free virus infection of rpOka was almost the same whether the virus was propagated on MeWo or MeWoORF49 cells or titrated on MeWo or MeWoORF49 cells, again indicating that the exogenous ORF49p had no effect on normal VZV infection. When rpOkaORF49M1L was propagated on MeWo cells, the titer of cell-free virus was 3 to 5% of that observed with propagation of MeWoORF49 cells, and the plaque size depended on the kind of cells used for titration but not on the kind used for propagation. In addition, the rpOkaORF49M1L particles isolated from MeWoORF49 cells contained abundant ORF49p (Fig. 2C, lane 3) and produced almost the same titer of cell-free virus as the parental virus but formed significantly smaller plaques on MeWo cells (Table 2). This gain-of-function analysis performed using the ORF49 *trans*-complementation system suggested that the incom-

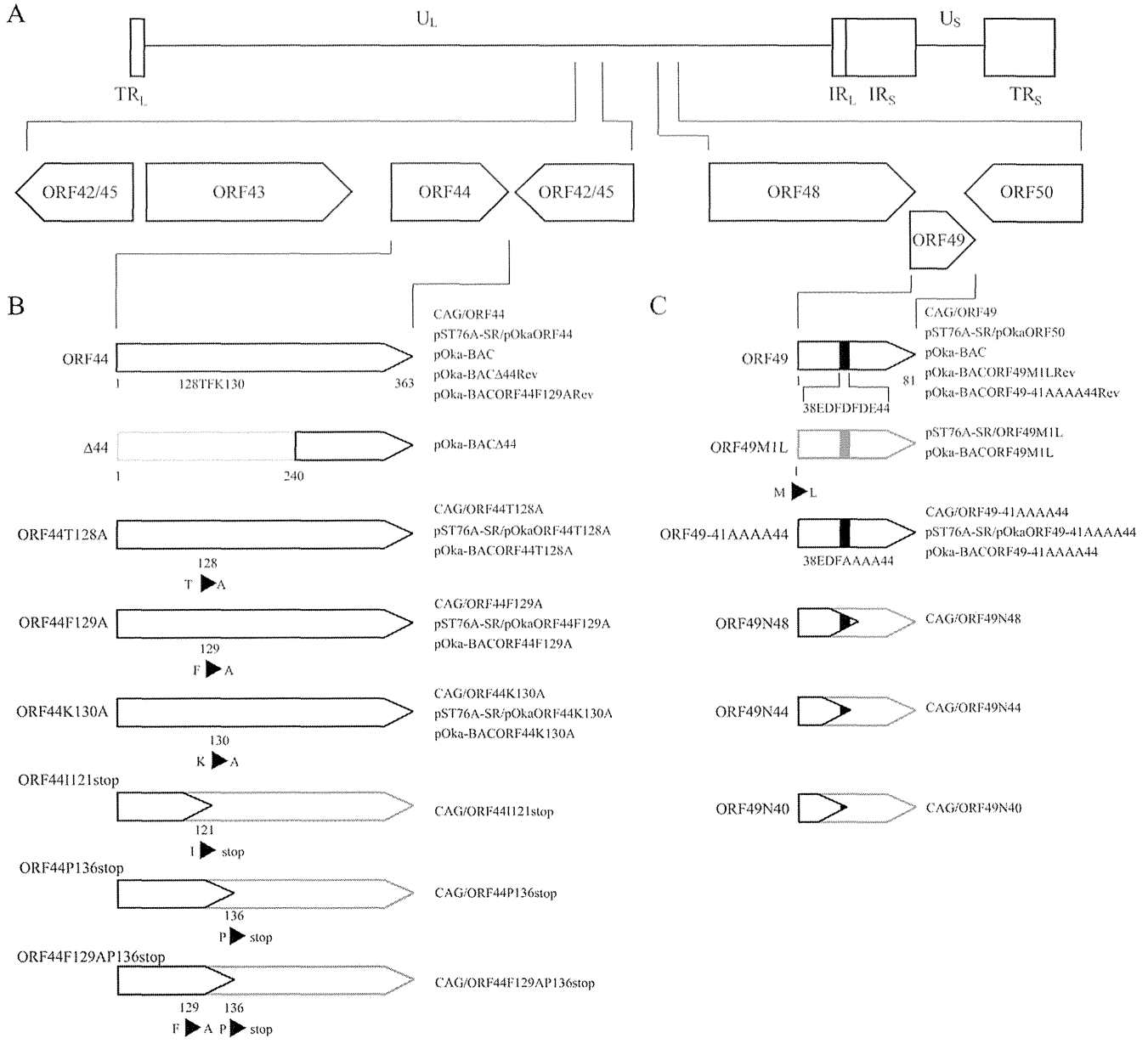

FIG 1 Schematics showing the plasmids and recombinant BAC genomes for ORF44 and ORF49. (A) Location of the ORF44 and ORF49 genes in the unique long region (U_L) segment of the genome of VZV strain pOka; terminal repeats (TR), unique short region (U_S), and internal repeats (IR) are indicated. (B) The wild type, deleted Δ44 region, and amino acid substitutions in ORF44p are shown. (C) The wild type, amino acid substitutions at the first methionine or the carboxyl-terminal half of the acidic cluster from amino acid positions 41 to 44, and the carboxyl-terminal truncations within ORF49p are shown. The acidic cluster is indicated as a black box. (B and C) Unexpressed regions of the mutant proteins are shown in gray outlined shapes. The names of relevant BACs, shuttle plasmids, and mammalian expression plasmids containing mutations are shown on the right.

ing ORF49p from the viral particles into the cells was not functional in any step and revealed that *de novo* ORF49p functioned in the production of infectious progeny viruses required for efficient propagation.

Furthermore, to examine the role of ORF49 in the production of infectious progeny viruses in detail, we redesigned our study on ORF49 to investigate its function by analyzing its binding partners.

Identification of the ORF44 protein as the binding partner for ORF49. ORF49p was immunoprecipitated from pOka- or mock-infected MeWo cells using an anti-ORF49 antibody (Ab), and the coimmunoprecipitating proteins were separated in a denaturing gel and visualized by silver staining (Fig. 4A). An approximately 36-kDa band was coimmunoprecipitated with the 13-kDa band corresponding to the ORF49p in pOka-infected MeWo cell lysates (Fig. 4A, lane 1). This 36-kDa band was identified as the ORF44 protein (ORF44p) of VZV by LC-MS/MS analysis (24.3% coverage of 363 amino acids).

ORF44p was specifically detected as a 36-kDa band in all recombinant VZV-infected MeWo cells, including rpOkaORF49M1L, by

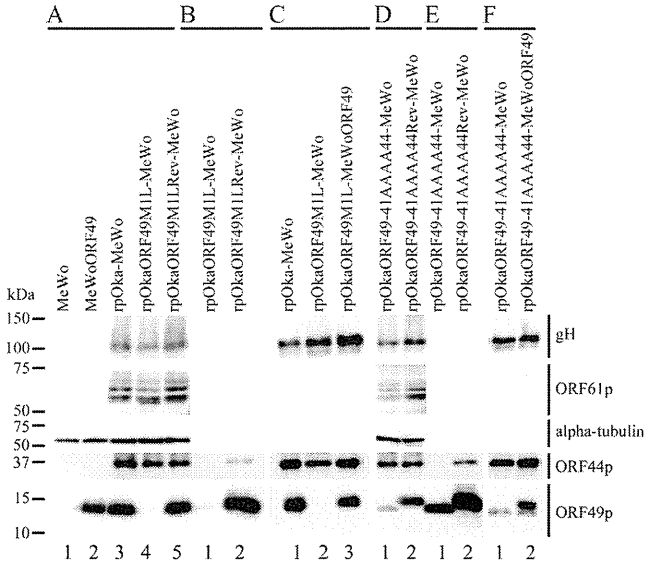

FIG 2 Expression and interaction of viral proteins during ORF49 mutant virus infection. (A) Proteins expressed in mock-infected MeWo cells (lane 1), mock-infected MeWoORF49 cells (lane 2), rpOka-infected MeWo cells (lane 3), rpOkaORF49M1L-infected MeWo cells (lane 4), and rpOkaORF49M1LRev-infected MeWo cells (lane 5) were visualized with Abs against gH, ORF61p, α-tubulin, ORF44p, and ORF49p. (B) The interaction between ORF44p and ORF49p was analyzed in rpOkaORF49M1L-infected cells (lane 1) and rpOkaORF49M1LRev-infected cells (lane 2). Immunoprecipitates obtained with an anti-ORF49 Ab from each type of virus-infected cells were electrophoretically separated and visualized using anti-ORF44 and anti-ORF49 Abs. (C) The viral proteins incorporated into virions from rpOka-infected MeWo cells (lane 1), rpOkaORF49M1L-infected MeWo cells (lane 2), and rpOkaORF49M1L-infected MeWoORF49 cells (lane 3) were visualized using Abs against gH, ORF61p, ORF44p, and ORF49p. (D) Proteins expressed in rpOkaORF49-41AAAA44-infected MeWo cells (lane 1) and rpOkaORF49-41AAAA44Rev-infected MeWo cells (lane 2) were visualized using Abs against gH, ORF61p, α-tubulin, ORF44p, and ORF49p. (E) The interaction between ORF44p and ORF49p was analyzed in rpOkaORF49-41AAAA44-infected cells (lane 1) and rpOkaORF49-41AAAA44Rev-infected cells (lane 2). Immunoprecipitates obtained using an anti-ORF49 Ab from each type of virus-infected cells were electrophoretically separated and visualized with anti-ORF44 and anti-ORF49 Abs. (F) The viral proteins incorporated into virions from rpOkaORF49-41AAAA44-infected MeWo cells (lane 1), and rpOka-ORF49-41AAAA44-infected MeWoORF49 cells (lane 2) were visualized with Abs against gH, ORF61p, ORF44p, and ORF49p.

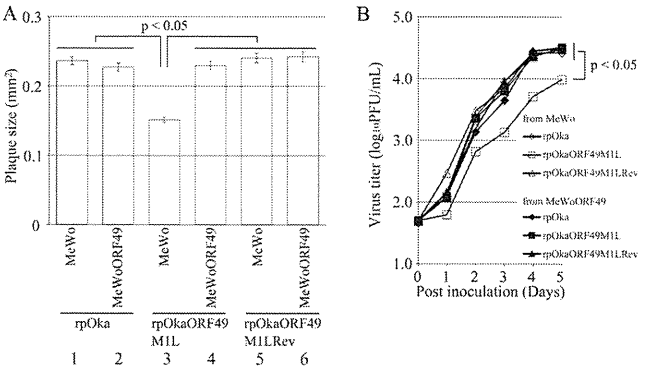

FIG 3 Growth properties of the ORF49M1L mutant virus in MeWo and MeWoORF49 cells. (A) Comparison of plaque sizes among recombinant viruses. MeWo cells or MeWoORF49 cells were infected with rpOka, rpOkaORF49M1L, or rpOkaORF49M1LRev (50 PFU/well) and cultured for 7 days. The infected cells were then stained with an anti-gE Ab, and the plaques were traced and measured by ImageJ software. Plaque size is shown with the standard error of the mean. Statistical significance was determined by Student's *t* test. (B) Growth kinetics of recombinant viruses in MeWo and MeWoORF49 cells. MeWo or MeWoORF49 cells were infected with rpOka, rpOkaORF49M1L, or rpOkaORF49M1LRev (50 PFU/well), harvested at the indicated times, serially diluted, added to newly prepared MeWo cells, and cultured for 5 days. The plaques were stained with an anti-gE Ab and counted. Each point represents the mean titer for two wells of one experiment. The experiments were performed twice independently. Statistical significance was determined by Student's *t* test.

immunoblotting with an anti-ORF44 Ab (Fig. 2A, lanes 3, 4, and 5). ORF44p was coimmunoprecipitated with ORF49p only in cells infected with the wild-type virus, rpOka, or rpOkaORF49M1LRev (data not shown) (Fig. 2B, lane 2) but not in the absence of ORF49p, as seen in rpOkaORF49M1L infection (Fig. 2B, lane 1). Thus, the interaction of ORF44p and ORF49p was specific and conserved in VZV.

ORF44 is essential for viral growth in cell culture. Because ORF49 is completely dispensable for viral reconstitution and propagation in MRC-5 cells, and the interaction between ORF44p and ORF49p was confirmed, we predicted that the ORF44 deletion mutant would be viable at least in MRC-5 cells, despite the fact that loss-of-function analysis showed that it is essential in MeWo cells (5). The ORF44 deletion mutant virus could not be reconstituted from pOka-BACΔ44 (Fig. 1B) in either MeWo cells or MRC-5 cells; however, the revertant virus of pOka-BACΔ44 reconstituted from the pOka-BACΔ44Rev genome (Fig. 1B) showed almost the same plaque size and growth as the parental virus, rpOka, in MeWo cells (data not shown). These findings confirmed that ORF44 is essential for VZV growth in cell culture even in MRC-5 cells.

ORF44p binds to and depends on ORF49p for its accumulation on the TGN in coexpressing cells and infection. When ORF44p was expressed alone by CAG/ORF44 transfection, it was dispersed throughout the cytoplasm and did not localize to the TGN (Fig. 5A). When ORF49p was expressed alone, it was predominantly localized to the juxtanuclear region with TGN46 (Fig. 5B), as reported previously (6). In cells coexpressing ORF44 and ORF49, ORF44p accumulated on the TGN with ORF49p (Fig. 5C), suggesting that the complex formation between ORF44p and ORF49p required no other viral factors and that it functioned in the accumulation of the ORF44p on the TGN. The expression of and interaction

TABLE 2 Comparison of cell-free virus titer and plaque formation

Virus	Cells for: Propagation	Titration	Titer (PFU/ml)[a]	Mean (SE) plaque size, mm²[b]
rpOka	MeWo	MeWo	2.3×10^3	0.232 (0.00891)
	MeWo	MeWoORF49	4.1×10^3	0.225 (0.00854)
	MeWoORF49	MeWo	4.0×10^3	0.232 (0.00911)
	MeWoORF49	MeWoORF49	1.3×10^3	0.206 (0.00828)
rpOkaORF49M1L	MeWo	MeWo	1.5×10^2	0.161 (0.00601)
	MeWo	MeWoORF49	1.7×10^2	0.235 (0.01319)
	MeWoORF49	MeWo	4.0×10^3	0.147 (0.00534)
	MeWoORF49	MeWoORF49	6.3×10^3	0.228 (0.01474)
rpOkaORF49-41AAAA44	MeWo	MeWo	1.2×10^2	0.141 (0.00726)
	MeWo	MeWoORF49	1.3×10^2	0.212 (0.00669)
	MeWoORF49	MeWo	2.1×10^3	0.149 (0.00796)
	MeWoORF49	MeWoORF49	4.7×10^3	0.227 (0.00740)

[a] Titers of cell free viruses are shown from one experiment performed in duplicate.
[b] Plaque sizes are shown as means (standard errors [SE]) from one experiment performed in duplicate.

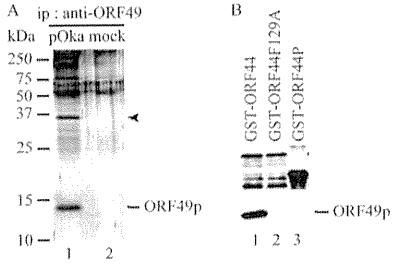

FIG 4 Identification of ORF44p as the binding partner of ORF49p by proteomic analysis and their *in vitro* binding assay. (A) pOka-infected MeWo cells expanded by cell-to-cell spread with full CPE at 2 to 3 days postinfection were lysed with RIPA buffer, and the binding molecules were coimmunoprecipitated with ORF49p using an anti-ORF49 Ab (lane 1). Mock-infected MeWo cells were used as a negative control (lane 2). The immunoprecipitates (ip) were electrophoretically separated and visualized by silver staining. (B) ORF49p expressed in and purified from MeWo cells was incubated with purified GST-ORF44 (lane 1), GST-ORF44F129A (lane 2), or GST-ORF44P (lane 3). Bound proteins were electrophoretically separated and visualized by anti-GST Abs (upper panel) and anti-ORF49 Abs (lower panel).

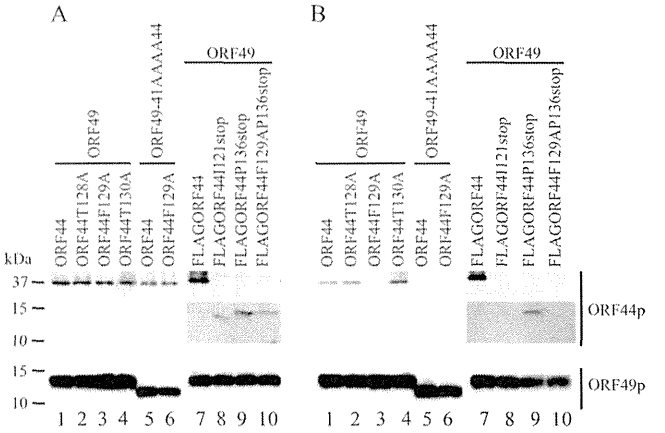

FIG 6 Expression and interaction of ORF44p and ORF49p in cotransfected cells. MeWo cells were cotransfected with CAG/ORF49 (lanes 1 to 4 and 7 to 10) or CAG/ORF49-41AAAA44 (lanes 5 and 6) and CAG/ORF44 (lanes 1 and 5), CAG/ORF44T128A (lane 2), CAG/ORF44F129A (lanes 3 and 6), CAG/ORF44K130A (lane 4), CAG/FLAGORF44 (lane 7), CAG/FLAGORF44I121stop (lane 8), CAG/FLAGORF44P136stop (lane 9), or CAG/FLAGORF44F129AP136stop (lane 10) (A and B). Protein expression was visualized with anti-ORF44p and anti-ORF49p antibodies (A), and proteins immunoprecipitated by anti-ORF49 Ab from cotransfected cells were electrophoretically separated and visualized using anti-ORF44 and anti-ORF49 Abs (B).

between ORF44p and ORF49p were confirmed by immunoblotting with the corresponding Abs and immunoprecipitation with anti-ORF49 Ab followed by immunoblotting with each Ab (Fig. 6A, lane 1, and B, lane 1, respectively). In rpOkaORF49M1LRev-infected MeWo cells, in spite of the broadly diffuse pattern seen for ORF44p, it appeared to accumulate on the TGN with ORF49p (Fig. 7B), as was seen in coexpressing cells (Fig. 5C), and this pattern was also observed in cells infected with rpOka (data not shown). In rpOkaORF49M1L-infected cells, ORF44p was dispersed as in cells expressing ORF44 alone and was not accumulated on the TGN (Fig. 7A), again indicating that the accumulation of ORF44p on the TGN depended on ORF49p and required no other viral factors.

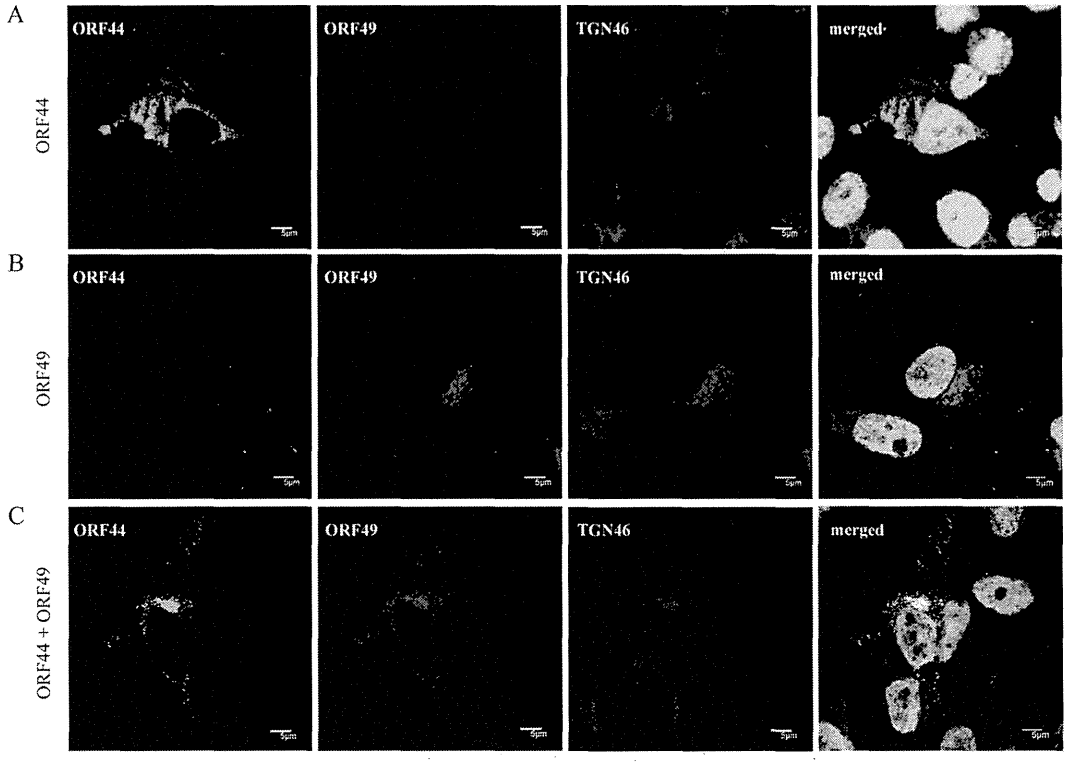

FIG 5 Localization of ORF44p and ORF49p in transiently transfected MeWo cells. MeWo cells were transfected with CAG/ORF44 (A) or CAG/ORF49 (B) or cotransfected with CAG/ORF44 and CAG/ORF49 (C). Cells were fixed at 48 h posttransfection and triple labeled for ORF44p (green), ORF49p (red), and TGN46 (blue). Nuclei were stained with Hoechst 33342 (cyan). Scale bars, 5 μm.