acid, dexamethasone, or corticosterone had no affect on TGF-β-induced phosphorylation of p38 MAP kinase. On the contrary, we showed that the maximal concentration of vitamin D_3 (10 nM) plus that of TGF-β (5 ng/ml) on p38 MAP kinase phosphorylation was additive. Therefore, it is likely that vitamin D_3 plus TGF-β stimulates VEGF release in A10 cells via enhancement of p38 MAP kinase activation. SB203580, a p38 MAP kinase inhibitor [Cuenda et al., 1995], inhibited TGF-β-induced VEGF release in A10 cells in the absence or presence of vitamin D_3. SB203580 did not affect p38 MAP kinase phosphorylation induced by TGF-β plus vitamin D_3. SB203580 is not an inhibitor of the upstream kinase of p38 MAP kinase but is a direct inhibitor of p38 MAP kinase. Therefore, it is likely that p38 MAP kinase, at least in part, mediates TGF-β-induced VEGF release in A10 cells in the absence or presence of vitamin D_3.

Other members of the nuclear hormone receptor superfamily (e.g., progesterone, testosterone, estradiol, and T_3) are known to modulate blood pressure and vascular smooth muscle cell growth [Mizuma et al., 2001; Dubey et al., 2002]. However, these factors had no affect on TGF-β-induced VEGF release from A10 cells. It has been reported that deoxycorticosterone and pregnenolone have no effect on VEGF gene expression or release in vascular smooth muscle cells [Nauck et al., 1998]. Thus, it is likely that retinoic acid, vitamin D_3, or glucocorticoids regulate the vascular system, at least in part, by modulating the release of endogenous and TGF-β-induced VEGF from vascular smooth muscle cells.

A10 cells derived from fetal rat aorta express many characteristics of vascular smooth muscle cells such as the production of spontaneous action potentials and increase myokinase and creatine phosphokinase activities [Kimes and Brandt, 1976]. VEGF which is synthesized and released predominantly by vascular smooth muscle cells plays a central role in the regulation of angiogenesis in both physiologic and pathologic states, such as wound healing and tumorigenesis, through the induction of vascular endothelial cell proliferation, migration, and inhibition of apoptosis [Neufeld et al., 1999]. It is likely that the modulation by steroid hormones of TGF-β-induced VEGF synthesis in vascular smooth muscle cells plays a role in pathophysiologic angiogenesis. However, discrepancies between in vivo and in vitro findings may occur. Further investigations are necessary to elucidate these effects in vivo.

In conclusion, nuclear hormone receptor superfamily members, including retinoic acid, vitamin D_3, and glucocorticoids, affect TGF-β-stimulated VEGF release from aortic smooth muscle cells. The stimulatory effect of vitamin D_3 occurs, in part, via up-regulation of TGF-β-induced p38 MAP kinase activation.

REFERENCES

Auerbach W, Auerbach R. 1994. Angiogenesis inhibition: A review. Pharmacol Ther 63:265–311.

Brogi E, Wu T, Namiki A, Isner JM. 1994. Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. Circulation 90:649–652.

Carlberg C. 1995. Mechanisms of nuclear signalling by vitamin D_3: Interplay with retinoid and tyroid hormone signalling. Eur J Biochem 231:517–527.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364: 229–233.

Dubey RK, Oparil S, Imthurn B, Jackson EK. 2002. Sex hormones and hypertension. Cardiovasc Res 53:688–708.

Evans RM. 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889–895.

Gospodarowicz D, Abraham JA, Schilling J. 1989. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc Natl Acad Sci USA 86:7311–7315.

Hungerford JE, Little CD. 1999. Developmental biology of the vascular smooth muscle cell: Building a multilayered vessel wall. J Vasc Res 36:2–27.

Kato S. 2000. The function of vitamin D receptor in vitamin D action. J Biochem 127:717–722.

Kimes BW, Brandt BL. 1976. Characterization of two putative smooth muscle cell lines from rat thoracic aorta. Exp Cell Res 98:349–366.

Kojima S, Rifkin DB. 1993. Mechanism of retinoid-induced activation of latent transforming growth factor-β in bovine endothelial cells. J Cell Physiol 155:323–332.

Kornel L. 1993. The role of vascular steroid receptors in the control of vascular contractility and peripheral vascular resistance. J Steroid Biochem Mol Biol 45:195–203.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Mantell DJ, Owens PE, Bundred NJ, Mawer EB, Canfield AE. 2000. 1α,25-Dihydroxyvitamin D_3 inhibits angiogenesis in vitro and in vivo. Circ Res 87:214–220.

Massagué J. 1998. TGF-β signal transduction. Annu Rev Biochem 67:753–791.

Miano JM, Berk BC. 2000. Retinoids: Versatile biological response modifiers of vascular smooth muscle phenotype. Circ Res 87:355–362.

Miyazono K, ten Dijke P, Heldin CH. 2000. TGF-β signaling by Smad proteins. Adv Immunol 75:115–157.

Mizuma H, Murakami M, Mori M. 2001. Thyroid hormone activation in human vascular smooth muscle cells: Expression of type II iodothyronine deiodinase. Circ Res 88:313–318.

Nauck M, Karakiulakis G, Perruchoud AP, Papakonstantinou E, Roth M. 1998. Corticosteroids inhibit the expression of the vascular endothelial growth factor gene in human vascular smooth muscle cells. Eur J Pharmacol 341:309–315.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. 1999. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 13:9–22.

Neuville P, Bochaton-Piallat ML, Gabbiani G. 2000. Retinoids and arterial smooth muscle cells. Arterioscler Thromb Vasc Biol 20:1882–1888.

Pedram A, Razandi M, Hu RM, Levin ER. 1997. Vasoactive peptides modulate vascular endothelial cell growth factor production and endothelial cell proliferation and invasion. J Biol Chem 272:17097–17103.

Rebsamen MC, Sun J, Norman AW, Liao JK. 2002. 1α,25-Dihidroxyvitamin D_3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase. Circ Res 91:17–24.

Ross R. 1999. Atherosclerosis—an inflammatory disease. N Engl J Med 340:115–126.

Schulick AH, Taylor AJ, Zuo W, Qiu CB, Dong G, Woodward RN, Agah R, Roberts AB, Virmani R, Dichek DA. 1998. Overexpression of transforming growth factor β1 in arterial endothelium causes hyperplasia, apoptosis, and cartilaginous metaplasia. Proc Natl Acad Sci USA 95:6983–6988.

Seko Y, Seko Y, Takahashi N, Shibuya M, Yazaki Y. 1999. Pulsatile stretch stimulates vascular endothelial growth factor (VEGF) secretion by cultured rat cardiac myocytes. Biochem Biophys Res Commun 254:462–465.

Stavri GT, Zachary IC, Baskerville PA, Martin JF, Erusalimsky JD. 1995. Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells: Synergistic interaction with hypoxia. Circulation 92:11–14.

Tanabe K, Hirade K, Ishisaki A, Shu E, Suga H, Kitajima Y, Katagiri Y, Dohi S, Kozawa O. 2004. Possible involvement of p44/p42 MAP kinase in retinoic acid-stimulated vascular endothelial growth factor release in aortic smooth muscle cells. Aterosclerosis 175:245–251.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA. 1991. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266:11947–11954.

Yamamoto T, Kozawa O, Tanabe K, Akamatsu S, Matsuno H, Dohi S, Uematsu T. 2001. Involvement of p38 MAP kinase in TGF-β-stimulated VEGF synthesis in aortic smooth muscle cells. J Cell Biochem 82:591–598.

Yamamoto T, Kozawa O, Tanabe K, Akamatsu S, Matsuno H, Dohi S, Hirose H, Uematsu T. 2002. 1,25-Dihydroxyvitamin D_3 stimulates vascular endothelial growth factor release in aortic smooth muscle cells: Role of p38 mitogen-activated protein kinase. Arch Biochem Biophys 398:1–6.

Yoshizawa M, Miyazaki H, Kojima S. 1998. Retinoids potentiate transforming growth factor-β activity in bovine endothelial cells through up-regulating the expression of transforming growth factor-β receptors. J Cell Physiol 176:565–573.

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Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion

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Summary

The secretion and extracellular transport of Wnt protein are thought to be well-regulated processes. Wnt is known to be acylated with palmitic acid at a conserved cysteine residue (Cys77 in murine Wnt-3a), and this residue appears to be required for the control of extracellular transport. Here, we show that murine Wnt-3a is also acylated at a conserved serine residue (Ser209). Of note, we demonstrated that this residue is modified with a monounsaturated fatty acid, palmitoleic acid. Wnt-3a defective in acylation at Ser209 is not secreted from cells in culture or in *Xenopus* embryos, but it is retained in the endoplasmic reticulum (ER). Furthermore, Porcupine, a protein with structural similarities to membrane-bound O-acyltransferases, is required for Ser209-dependent acylation, as well as for Wnt-3a transport from the ER for secretion. These results strongly suggest that Wnt protein requires a particular lipid modification for proper intracellular transport during the secretory process.

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Introduction

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis, as well as in carcinogenesis (Logan and Nusse, 2004; Moon et al., 2004; Reya and Clevers, 2005). Most Wnt proteins transmit signals locally, presumably since their secretion and transport are under tight control. Although the molecular mechanism underlying their secretion and transport remains largely unknown, recent successes in identifying various molecules involved in these processes provide further clues. For instance, Wntless/Evi, a seven-pass membrane protein, plays an essential role in Wnt secretion (Banziger et al., 2006; Bartscherer et al., 2006), and a protein complex involved in intracellular membrane trafficking, called the retromer complex, participates in the long-range transport of Wnt protein (Coudreuse et al., 2006; Prasad and Clark, 2006). In addition, heparan sulfate-modified proteoglycans are involved in Wnt signaling, possibly by regulating the extracellular transport of Wnt (Hacker et al., 2005; Lin, 2004).

One important step regulating the extracellular transport of various secreted signal proteins, including Wnt, Hedgehog (Hh), and Spitz (*Drosophila* Transforming Growth Factor α), involves posttranslational modification with lipid moieties (Mann and Beachy, 2004; Miura et al., 2006; Nusse, 2003). A well-known example is cholesterol modification of the COOH terminus of the active form of Hh, which is generated by auto-proteolytic cleavage and is required for restricting the range of action of this protein (Lewis et al., 2001; Porter et al., 1996a, 1996b). In addition to cholesterol modification, a fatty acid modification, i.e., acylation, occurs with Hh, Wnt, and Spitz (Miura et al., 2006; Pepinsky et al., 1998; Willert et al., 2003). To date, at least three types of acylation are known to occur in eukaryotic cells: N-myristoylation, S-palmitoylation, and N-palmitoylation. N-myristoylation refers to the covalent modification with myristate at the N-glycine of proteins, S-palmitoylation refers to the reversible addition of fatty acids to cysteine residues through thioester linkages, and N-palmitoylation, first described for Hedgehog protein, involves modification at the N-terminal residues of proteins (Linder and Deschenes, 2003; Smotrys and Linder, 2004). In the case of Wnt, Nusse and coworkers reported that murine Wnt-3a is S-palmitoylated at a conserved cysteine residue at the 77th residue (Cys77) (Willert et al., 2003). A mutant form of mouse Wnt-3a, in which the palmitoylated Cys77 is substituted with alanine (C77A), shows a diminished ability to activate Wnt signaling, but is secreted normally into the culture medium (Willert et al., 2003). Thus, the authors proposed that palmitoylation of this cysteine residue may be required to produce an increased local concentration of Wnt on the plasma membrane. However, although their mass spectrometry analysis covered 85% of the primary amino acid sequence of Wnt-3a (Willert et al., 2003), there remains the possibility of additional acylation sites.

There is strong evidence to suggest that acylation is involved in the processing and intracellular trafficking

of Wnt prior to secretion. Genetic evidence suggests that Wnt-secreting cells require the action of specific genes, e.g., *porcupine* (*porc*) in *Drosophila* or its ortholog, *mom1*, in *C. elegans*, both of which encode proteins with structural similarities to those of a family of membrane-bound O-acyltransferases (MBOAT), which transfer acyl groups, such as a palmitoyl group, to substrates (**Hofmann, 2000**; **Kadowaki et al., 1996**; **Thorpe et al., 1997**; **van den Heuvel et al., 1993**). Porcupine (Porc) is localized at the endoplasmic reticulum (ER), and its overexpression in culture cells enhances the intracellular processing, for example, N-glycosylation, of Wingless (Wg: the *Drosophila* Wnt-1 ortholog) (**Tanaka et al., 2002**). In addition, treatment with a chemical inhibitor of acyltransferases produces defective intracellular trafficking of Wg (**Zhai et al., 2004**). Thus, *porc*-dependent acylation may regulate the processing and intracellular trafficking of Wnt, although acylation at Cys77 does not appear to be involved in these processes.

To resolve inconsistencies between studies examining the roles of Wnt acylation, and to better understand the biological significance and molecular mechanism of Wnt acylation, we carefully examined which amino acid residues are acylated, as well as the function of acylation. Our results indicate that another acylation with a monounsaturated fatty acid occurred at a conserved serine, and we suggest that Wnt-3a defective in acylation at this site does not exit the ER.

Results

Ser209 Is Required for Acylation of Wnt-3a, as Determined by Metabolic Labeling

We first examined acylation of Wnt-3a by metabolic labeling of cultured cells. In cultures of Wnt-3a-expressing mouse L cells (**Shibamoto et al., 1998**), specific labeling of secreted Wnt-3a protein with radiolabeled palmitic acid was observed, as previously reported. This labeling was detected after only 4 hr of incubation (**Figure 1A**), while labeling with radiolabeled cholesterol was not detected, even after 36 hr of incubation (data not shown). Palmitoyl moieties, when linked by thioester, but not oxyester, bonds, are known to be displaced from proteins by high concentrations of disulfide-reducing agents, such as 2-mercaptoethanol or dithiothreitol (**Bizzozero, 1995**). However, even though a previous report describes palmitoylation of Wnt-3a through a thioester linkage at Cys77 (**Willert et al., 2003**), we detected labeling even after incubation with a high concentration of a disulfide-reducing agent, i.e., 1.3 M 2-mercaptoethanol. We also observed that this labeling was resistant to neutral hydroxylamine (pH 7.0), which specifically cleaves thioester linkages (data not shown) (**Bizzozero, 1995**). These results suggest that acylation occurs at another site of Wnt-3a via an oxyester bond, in addition to the known acylation of Wnt-3a at Cys77 through a thioester linkage. Similarly, radiolabeled Wnt-5a was detected in cultures of Wnt-5a-expressing L cells under the same conditions, indicating that acylation commonly occurs among Wnt family members (**Figure 1B**). To confirm acylation of Wnt-3a at a site other than Cys77, we examined acylation of a mutant form of Wnt-3a, in which Cys77 was substituted by alanine (C77A). A significant reduction in acylation was not observed in C77A, indicating that another acylation site, besides Cys77, exists in Wnt-3a (**Figure 1A**).

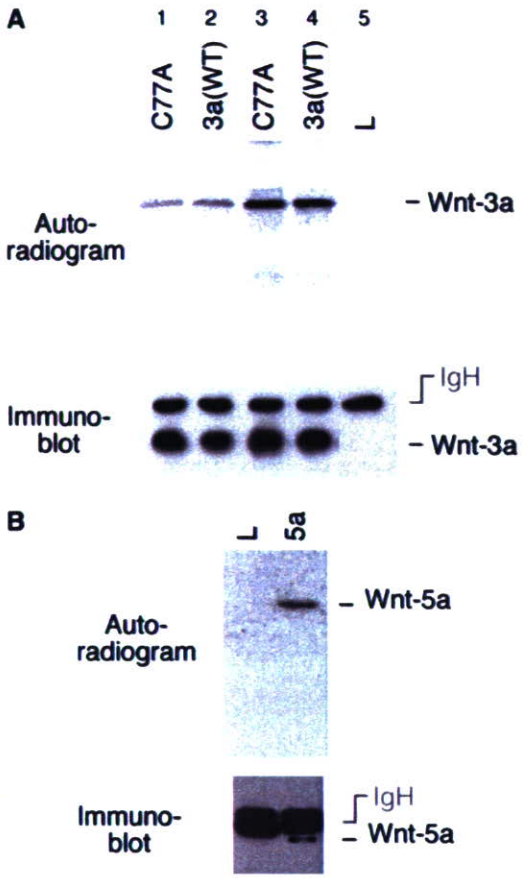

Figure 1. Acylation of Wnt-3a Protein Detected by Metabolic Labeling with Radiolabeled Palmitic Acid

(A) Acylation of wild-type and C77A forms of Wnt-3a proteins secreted from metabolically labeled cells was examined by autoradiography of anti-Wnt-3a immunoprecipitates (upper panel). Wild-type-producing (lanes 2 and 4), C77A-producing (lanes 1 and 3), and control (lane 5) L cells were incubated with ^{14}C-palmitic acid for 4 hr (lanes 1 and 2) or 36 hr (lanes 3–5). Immunoprecipitated Wnt-3a protein from culture supernatant was identified by immunoblotting with anti-Wnt-3a antibody (lower panel). Immunoglobulin heavy chains (IgH) reactive with the secondary antibody were also detected.

(B) Acylation of Wnt-5a protein secreted from Wnt-5a-producing L cells incubated with ^{3}H-palmitic acid for 36 hr was examined by immunoprecipitation by following the same procedure described in (A).

To map out the additional site of acylation within the amino acid sequence of Wnt-3a, we generated C-terminal-truncated forms of Wnt-3a protein and examined acylation (**Figure 2A**). Whereas Wnt-3a truncated at the 281st residue (ΔC-281) contained radiolabeled palmitic acid upon recovery from the cell lysate, as did the full-length protein (FL), Wnt-3a protein truncated at the 202nd residue (ΔC-202) did not contain the radiolabel (**Figure 2B**). Of note, in the region extending between the 203rd and 281st residues, numerous amino acid residues, including three serines and a threonine, were found to be highly conserved among members of the Wnt family (**Figure 2A**). Because serine and threonine may form oxyester linkages with acyl moieties, we next examined whether any of the conserved residues

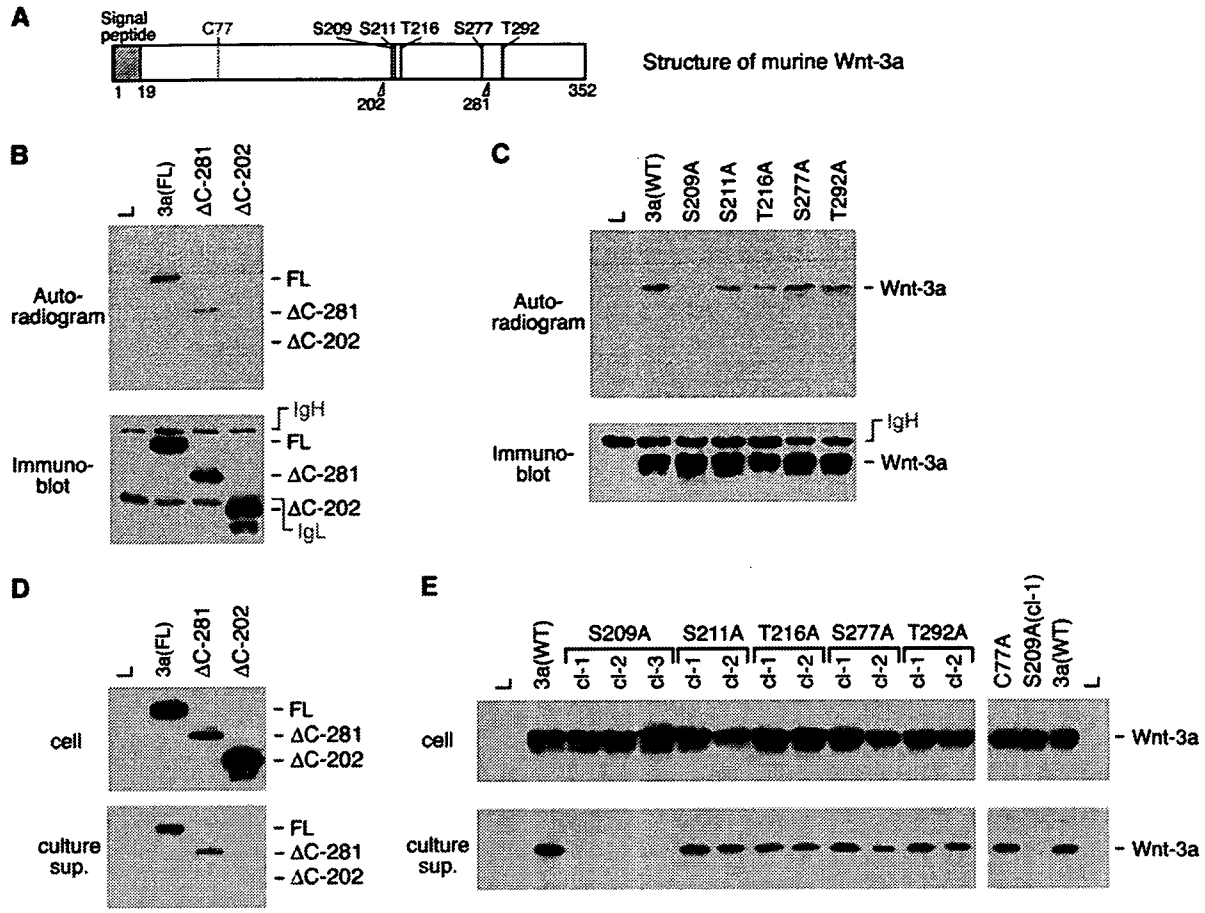

Figure 2. Serine 209 Is Required for the Acylation and Secretion of Wnt-3a in Cultured Cells

(A) Schematic representation of the sites truncated or mutated in this study.

(B) Acylation of truncated forms of Wnt-3a protein was examined by metabolic labeling by following the same procedure described in Figure 1A, except that samples were prepared from cell lysates. Control L cells and L cells expressing the HA-tagged full-length (FL) or one of two truncated (ΔC-281, ΔC-202) forms of Wnt-3a were incubated with ^{3}H-palmitic acid for 36 hr. All immunoprecipitates were obtained from cell lysates by using anti-HA antibody (upper panel). Immunoprecipitation of Wnt-3a protein was confirmed by using anti-HA antibody (lower panel). Coprecipitation of immunoglobulin heavy chains (IgH) and light chains (IgL) was identified.

(C) Acylation of point-mutated forms of Wnt-3a protein was examined as shown in (B) by using anti-Wnt-3a antibody. As controls, analyses of immunoprecipitates from L cells producing wild-type Wnt-3a (3a(WT)) and from control L cells (L) are also shown.

(D) Secretion of the two truncated (ΔC-281, ΔC-202) forms of Wnt-3a protein, as well as the HA-tagged full-length (FL) Wnt-3a protein, into the culture medium was examined. The amount of Wnt-3a protein within cells (upper panel) and released into the culture medium (lower panel) was examined by immunoblotting with anti-HA antibody.

(E) Secretion of the five point-mutated forms of Wnt-3a proteins was examined by using anti-Wnt-3a antibody as shown in (D). For each point-mutated form, more than two independent cell clones (cl) are shown. The representative clones of each form shown in (C) are numbered "cl-1." As controls, secretion of wild-type and C77A forms of Wnt-3a was also examined.

might be required for acylation to occur, by substituting each of them with alanine (S209A, S211A, T216A, and S277A). A threonine-to-alanine mutation outside of this region (T292A) was also included in the analysis as a control. It was remarkable that no acylation was detected for S209A, while acylation of the four other mutated Wnt-3a proteins (S211A, T216A, S277A, and T292A) matched that of wild-type Wnt-3a (Figure 2C). Thus, we concluded that Ser209 was required for detectable acylation of Wnt-3a under these assay conditions. We should also note that despite a previous report indicating that overexpression of *porc*, a putative membrane-bound O-acyltransferase (Hofmann, 2000), enhances Wg glycosylation (Tanaka et al., 2002), no obvious defect in N-glycosylation was detected in the S209A mutant when compared with wild-type Wnt-3a, in which N-glycosylation produces two products of different sizes, both of which are sensitive to tunicamycin treatment (Figure S1; see the Supplemental Data available with this article online).

Wnt-3a Is Modified with a Monounsaturated Fatty Acid at Ser209

Next we investigated whether acylation of Wnt-3a actually occurs at Ser209, as well as the structure of the attached moiety, by mass spectrometry (MS). FLAG-tagged Wnt-3a protein was purified from the conditioned media of the expressing cells (Figure S2), treated with trypsin, and then subjected to nano-flow reverse-phase liquid chromatography (LC) followed by MALDI-MS/MS. A number of peptides obtained after trypsin digestion, including 75% of the amino acid sequence

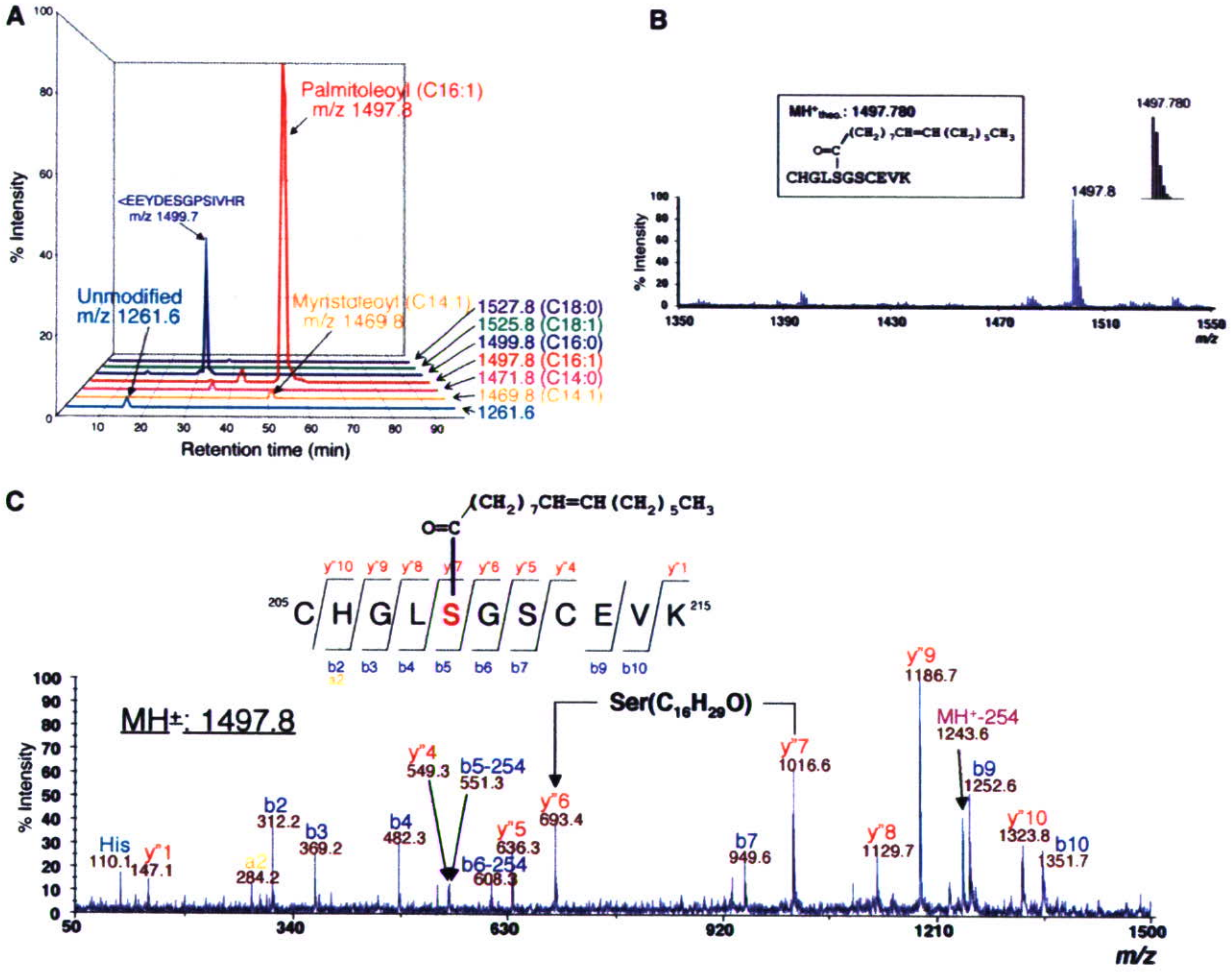

Figure 3. Lipid Modification of Wnt-3a Protein Probed by Nano-Flow LC/MALDI-MS and MS/MS

(A) The selected ion chromatogram reconstituted on the basis of m/z 1261.6, 1469.8, 1471.8, 1497.8, 1499.8, 1525.8, and 1527.8, corresponding to the respective theoretical MH^+ values of unmodified peptide and peptide modified with C14:1-, C14:0-, C16:1-, C16:0-, C18:1-, and C18:0-fatty acids. The ion intensities of each trace were normalized to that of the ion at m/z 1497.8. The peptide (m/z 1499.7) eluted at 32 min turned out to be a peptide (<EEYDESGPSIVHR, where <E is pyroglutamic acid) derived from γ-actin.

(B) The MALDI-MS spectrum of the peptide modified with C16:1-fatty acid. The $MH^+_{theo.}$ value and the theoretical isotopic ion distribution in the spectrum (inset) were calculated from the chemical structure indicated in the box.

(C) The MS/MS spectrum from the ion at m/z 1497.8. The spectrum was interpreted by the software *SeqMS* (see Experimental Procedures). The sequences from the N and C termini and the position of the modified residue were read out based on bm and y"l ions, respectively, where m and l denote the arbitrary positions counted from the N and C termini, which were produced by cleavage of peptide bonds during MS/MS. His, b-254 and MH^+254, respectively, in the spectrum denote the immonium ion of histidine and the fragment ions derived from β elimination of the lipid moiety ($C_{16}H_{29}O$) at Ser209 from the respective b or MH^+ ions.

of the secreted form of Wnt-3a, were recovered by using this procedure (Table S1). Unexpectedly, Ser209-containing peptides with the sequence CHGLS^{209}GSCEVK were not obtained at m/z 1499.8, corresponding to a peptide modified with palmitate (C16:0), nor at m/z values of 1471.8, 1525.8, and 1527.8, corresponding to peptides modified with myristate (C14:0), oleate (C18:1), and stearate (C18:0), respectively (Figure 3A). It should be noted that the peptide prominently observed at m/z 1499.7 turned out to be a trypsin digestion product of γ-actin (42 kDa) on MS/MS analysis. Rather, the Ser209-containing peptides were obtained at m/z 1497.8, 1469.8, and 1261.6, corresponding to the theoretical MH^+ values of the peptides modified with palmitoleic acid (C16:1), myristoleic acid (C14:1), and the unmodified peptide, respectively (Figure 3A). Among the three peptides detected, the one observed at m/z 1497.8 (corresponding to modification with C16:1) was the predominant molecular species (Figures 3A and 3B), although the relative amounts of each peptide were not explicitly determined. MS/MS analysis of the predominant peptide revealed the site of modification to be Ser209, based on the prominent fragment ions, y"6 and y"7, as well as b4 and b5 − 254 (Figure 3C). Differences in the masses of these ions could be assigned to Ser (323.2) modified with a C16:1-fatty acid or dehydro-Ala (69.0), respectively, the latter of which is a product of β elimination of a lipid moiety from modified Ser209 during the MS/MS measurement.

Further characterization of this prominent peptide (at m/z 1497.8) was carried out by accurate mass measurement, which indicated a molecular mass of 1496.776.

Thus, the accurate mass of the modification was calculated to be 236.217. This value suggests a unique elemental composition, $C_{16}H_{28}O_1$ (theoretical mass: 236.214), indicating modification with a monounsaturated C16-fatty acid (C16:1). It is also noteworthy that the isotopic ion distribution observed for the ion at m/z 1497.8 almost completely matched the theoretical one calculated for a peptide modified with a C16:1-fatty acid (see Figure 3B and the inset). In addition, this isotopic ion distribution was partially shifted when deuterium-labeled palmitic acid ($CD_3(CH_2)_{14}COOH$) was added into culture of Wnt-3a-expressing L cells (data not shown), indicating that the deuterium-labeled palmitic acid (C16:0), as well as the radiolabeled one in the experiments described above, was metabolically processed to C16:1-fatty acid, which then bound to the peptide. Finally, to elucidate the position of the double bond within the C16:1-fatty acid moiety, the olefinic double bond of the prominent peptide (m/z 1497.8) was subjected to oxidative cleavage. The original peptide was mostly degraded into its oxidative products via the loss of C_7H_{14} from the C16:1-fatty acid moiety (data not shown), suggesting that the double bond is located at Δ^9, which is identical to the position within biosynthesized palmitoleic acid (C16:1). Based on these lines of evidence, we conclude that Wnt-3a is modified with a monounsaturated fatty acid, palmitoleic acid (C16:1), at Ser209.

Ser209 Is Essential for Secretion of Wnt-3a

Next, we investigated whether Ser209-dependent acylation is involved in the secretion of Wnt-3a. We examined the secretion of the two C-terminal-truncated forms and five point-mutated forms of Wnt-3a proteins from L cells into their culture medium, as well as that of the wild-type and C77A forms of Wnt-3a. Interestingly, acylation was coupled with secretion for all Wnt-3a variants. Full-length (FL) and ΔC-281 forms of Wnt-3a demonstrated similar levels of secretion; however, secretion of the ΔC-202 form into the culture medium was not detected (Figure 2D). Furthermore, among the five point mutants, only S209A was not detected in the medium (Figure 2E).

Turnover of wild-type and S209A forms of Wnt-3a proteins was then examined during cycloheximide-mediated inhibition of de novo protein synthesis. S209A protein was stably retained in cells, while most wild-type Wnt-3a protein was secreted into the medium within 24 hr of treatment with cycloheximide (Figures 5A and 5B). Thus, the S209A mutation, preventing the palmitoleoyl modification, did not affect the stability of Wnt-3a protein, but rather blocked its secretion.

Porcupine Is Required for the Acylation and Secretion of Wnt-3a

The gene *porc*, encoding a putative O-acyltransferase, is thought to be required for the secretion and intracellular transport of Wg in *Drosophila* (Hofmann, 2000; Kadowaki et al., 1996; van den Heuvel et al., 1993; Zhai et al., 2004). Thus, we examined whether *porc* is required for detectable Wnt-3a acylation under the conditions of this study. In a number of independent L cell transfectants, in which expression levels of *porc* were stably reduced by expression of siRNA specific for mouse *porc*, reduced acylation of Wnt-3a was observed, reflecting

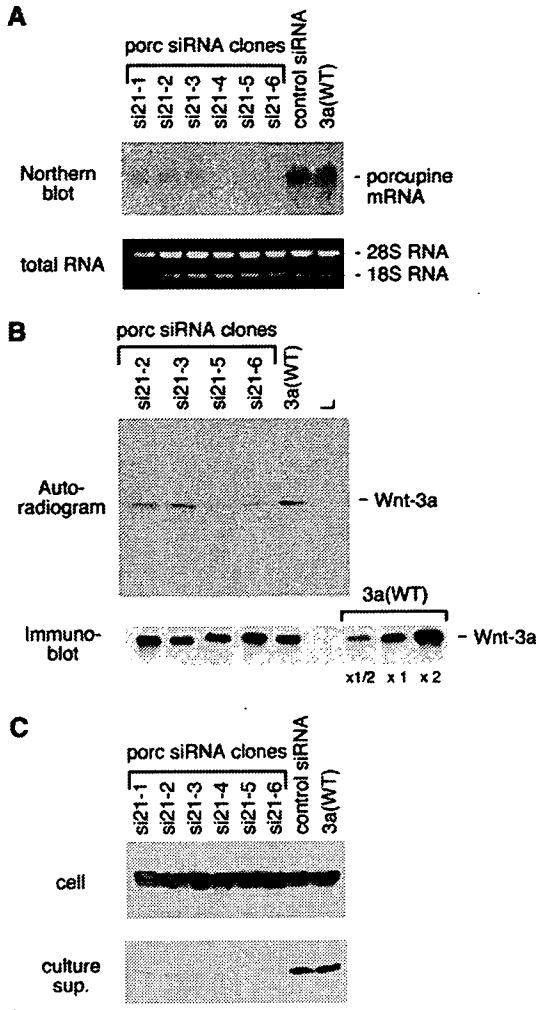

Figure 4. Porcupine Is Required for Palmitoleic Modification and Secretion of Wnt-3a

(A) Endogenous *porc* mRNA expression in clones expressing different levels of *porc*-specific siRNA (si21) is shown (upper panel). As controls, endogenous expression levels in cells expressing control siRNA and in parental Wnt-3a-expressing cells (3a(WT)) are also shown. Total RNAs loaded onto each lane are shown as well (lower panel).

(B) Acylation of Wnt-3a in *porc*-deficient clones, as well as in parental cells expressing Wnt-3a (3a(WT)), and control L cells (L) was analyzed as shown in Figure 2C. Standards to enable quantification of Wnt-3a were also loaded (right).

(C) Reduction in Wnt-3a protein secretion in *porc*-deficient cells was examined as shown in Figure 2E.

some residual *porc* expression, while the production and glycosylation of Wnt-3a appeared not to be perturbed (Figures 4A and 4B; data not shown). It is not certain whether the acylation remaining in *porc*-deficient transfectants was actually due to residual Porc activity. However, the possibility that the residual acylation was Cys77 palmitoylation, possibly detected even under this condition, can be excluded because levels of residual acylation were not reduced by substitution of Cys77 with alanine (Figure S3). Thus, *porc* is required for most of the acylation, which is also dependent on Ser209. Furthermore, as observed with S209A, Wnt-3a protein was retained in untreated *porc*-deficient cells and in

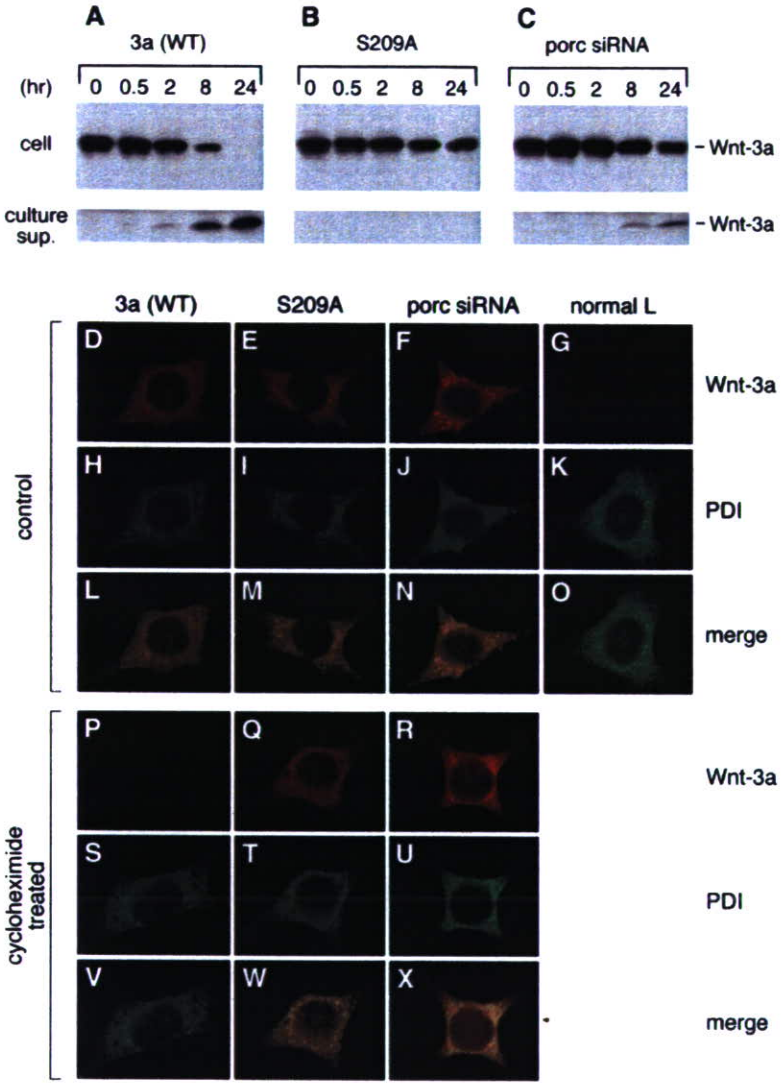

Figure 5. Defective Acylation Results in Preferential Retention of Wnt-3a Protein in the Endoplasmic Reticulum

(A–C) L cells expressing (A) wild-type Wnt-3a or (B) Wnt-3a (S209A-cl 1) as well as (C) wild-type Wnt-3a-expressing L cells defective in Porc activity (si21-6) were incubated with cyclohexymide. The amounts of Wnt-3a remaining within cells ([A]–[C], upper panel) and released into the culture supernatant ([A]–[C], lower panel) were examined by immunoblotting at the indicated time points after the addition of cyclohexymide.

(D–X) L cells expressing (D, H, L, P, S, and V) wild-type Wnt-3a or the (E, I, M, Q, T, and W) S209A mutant form, as well as (F, J, N, R, U, and X) wild-type Wnt-3a-expressing L cells defective in Porc activity and (G, K, and O) control L cells, were fixed 24 hr after incubation (D–O) without or (P–X) with cycloheximide. Cells were then stained with anti-Wnt-3a antibody (shown as red: [D]–[G] and [P]–[R]) or anti-PDI antibody, which is an ER marker (shown as green: [H]–[K] and [S]–[U]). (L–O and V–X) Merged pictures are also shown. The percentage of stained cells is shown in Table S2.

porc-deficient cells treated with cycloheximide (Figures 4C and 5C), although reduced levels of Wnt-3a secretion were detected, reflecting residual Porc activity. These results show that *porc* is required for secretion, as well as for Ser209-dependent acylation, of Wnt-3a from the cultured cells.

Wnt-3a with Defective Acylation Is Not Transferred from the ER

To determine the importance of acylation to Wnt-3a secretion, we examined the intracellular localization of Wnt protein with defective acylation (Figures 5D–5X; Table S2). Most Wnt-3a protein transcribed from the transfected gene is retained in the ER, probably due to limitations of protein trafficking from the ER (Figures 5D–5O). During cycloheximide-mediated inhibition of de novo protein synthesis, S209A protein was retained in the ER (Figures 5Q and 5W), whereas most newly synthesized wild-type Wnt-3a protein was secreted from cells under the same conditions (Figures 5P and 5V). Similarly, under the same conditions, most Wnt-3a protein was retained in the ER in *porc*-deficient cells (Figures 5R and 5X). Thus, Ser209-dependent acylation appears to be required for transit of Wnt-3a from the ER.

Ser209 Is Required for the Function and Appropriate Localization of Wnt-3a in *Xenopus* Embryos

To examine whether Ser209-dependent acylation would also be required for the function and secretion of Wnt-3a in vivo, we next injected S209A mRNA into *Xenopus* embryos (Figure 6). While the injection of wild-type Wnt-3a or S211A mRNAs into the ventral side of *Xenopus* eggs at the four (or two)-cell stage caused ectopic axis formation (Figures 6B and 6D; Table S3), as well as induction of the target genes *Siamois* and *Xnodal related-3* (Figure 6G), injection of S209A mRNA had no obvious effect, indicating that Ser209 is essential for the in vivo function of Wnt-3a (Figures 6C and 6G). Furthermore, whereas most wild-type Wnt-3a and S211A proteins were transported to the apical cell border, most S209A proteins were retained inside the expressing cells, probably in the ER (Figures 6I–6K). These results confirm that Ser209, which is required for acylation and secretion of Wnt-3a in cultured cells, is also essential for proper intracellular transport of Wnt-3a proteins in vivo.

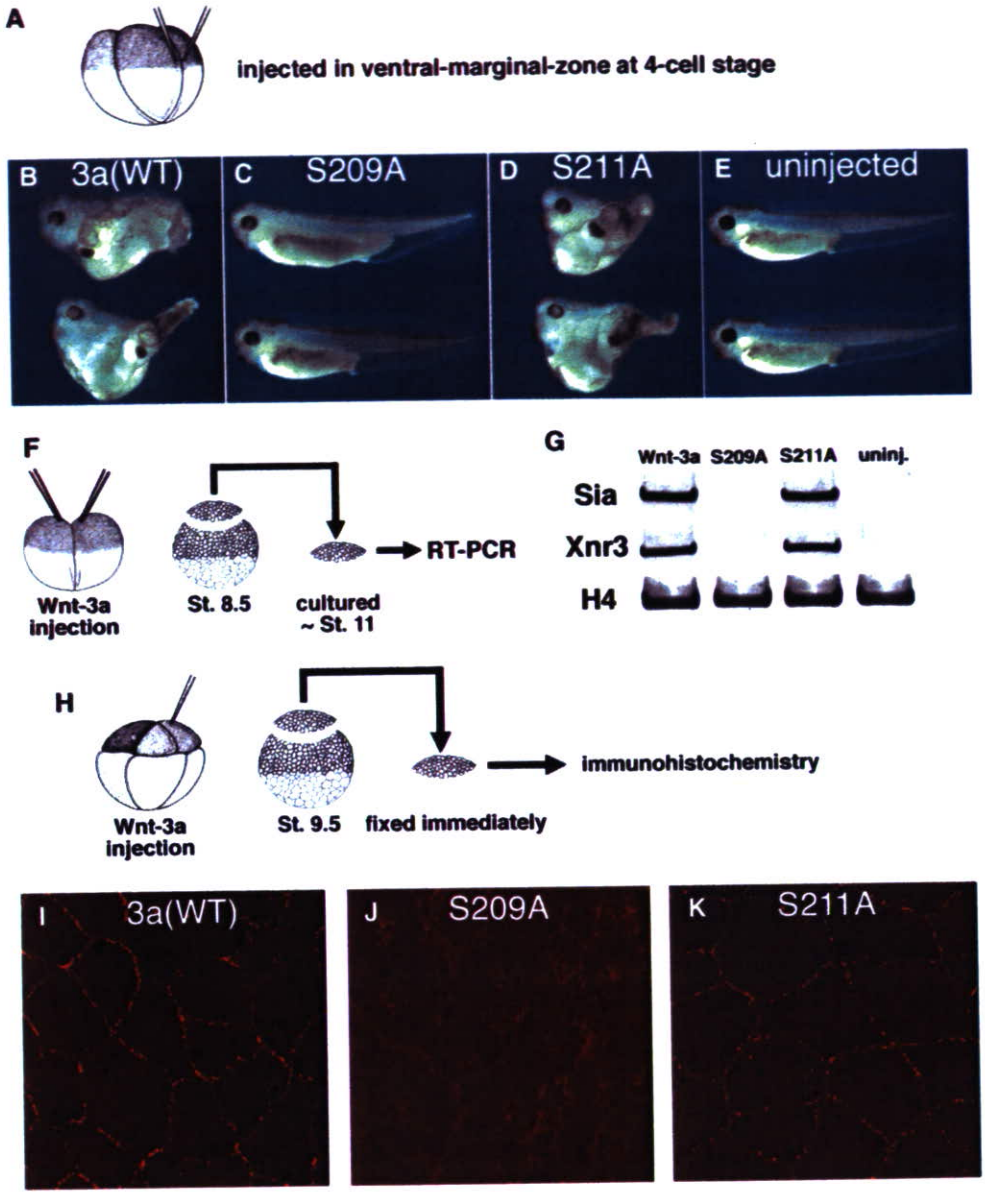

Figure 6. Acylation-Defective Wnt-3a Protein Is Largely Retained within the Cells of *Xenopus* Embryos

(A–K) (A), (F), and (H) indicate schematic representations of the experiments performed in (B)–(E), (G), and (I)–(K), respectively. (B–E) *Xenopus* embryos injected with mRNAs encoding (B) wild-type, (C) S209A, or (D) S211A forms of Wnt-3a, as well as (E) uninjected embryos, are shown. (G) Expression of *siamois* (Sia), *Xnodal related-3* (Xnr3), and *histone H4* (H4) in animal caps dissected from embryos injected with mRNAs encoding these forms of Wnt-3a, as well as in those from uninjected embryos, was analyzed by use of the reverse transcription-polymerase chain reaction. (I–K) Confocal images of animal caps dissected from the embryos injected with mRNAs encoding (I) wild-type, (J) S209A, or (K) S211A forms of Wnt-3a stained with anti-Wnt-3a antibody are shown.

Discussion

In this study, we demonstrated that Wnt-3a protein is acylated with an unsaturated fatty acid, palmitoleic acid (C16:1), at a conserved serine at the 209th residue, in addition to the previously reported Cys77 palmitoylation. We analyzed a number of mutant forms of Wnt-3a to demonstrate that Ser209 is required for appropriate trafficking of Wnt-3a protein from the ER during secretion, both in cultured cells and in embryos. In addition, Ser209-dependent acylation requires the function of Porc, a putative membrane-bound O-acyltransferase, in the ER. Thus, these results strongly suggest that Ser209-dependent acylation, catalyzed by Porc in the ER, is essential for transport of Wnt-3a from the ER during the secretion process.

Since acylation has been implicated in regulation of protein trafficking to intracellular organelles and particular domains of the plasma membrane (Smotrys and Linder, 2004; Huang and El-Husseini, 2005; Resh, 1999), Ser209-dependent acylation may be required for targeting of Wnt-3a proteins to specific organelles or membrane components required for secretion. Consistent with this model, an inhibitor of acyltransferase activity is known to inhibit the intracellular trafficking of Wg protein (Zhai et al., 2004). On the other hand, since

Figure 7. The Amino Acid Sequence Surrounding Ser209 Is Highly Conserved among Members of the Wnt Family

Aligned amino acid sequences surrounding Ser209 are shown for various forms of Wnt protein. Ser209 is shown in red, and other conserved residues are shown in blue. The consensus sequence among the members of the Wnt family is indicated at the bottom.

Wnt protein is transported between cells by lipoprotein particles in *Drosophila* wing imaginal discs (Greco et al., 2001; Panakova et al., 2005), an acylation-dependent trafficking system might be prerequisite for attachment of Wnt proteins to lipoprotein particles for secretion. Although it is not certain at present whether Wnt protein is secreted from cultured mammalian cells in association with lipoprotein particles, further characterization of the secreted Wnt protein might reveal whether acylation of Wnt protein has this proposed role in mammalian cell culture systems.

In addition, acylation may affect the function of several molecules involved in Wnt secretion, such as Wntless/Evi, a seven-pass membrane protein required for Wnt secretion in *Drosophila* and mammalian cultured cells (Banziger et al., 2006; Bartscherer et al., 2006), as well as the retromer complex, a protein complex involved in intracellular membrane trafficking and required for the long-range extracellular transport of Wnt protein in *C. elegans* (Coudreuse et al., 2006; Prasad and Clark, 2006). Because Wntless/Evi is primarily localized at the Golgi apparatus or plasma membrane, and since components of the retromer complex can be identified in *trans*-Golgi and trafficking vesicles, acylation-dependent trafficking of Wnt-3a from the ER appears to be prerequisite for Wntless/Evi and retromer complex function.

Another important aspect of this study pertains to the discovery of an anticipated lipid modification. This modification demonstrates several important characteristics, especially as related to the type of fatty acids involved. We demonstrated modification of Wnt-3a protein at Ser209 with monounsaturated fatty acids, such as palmitoleic acid (C16:1), but not with saturated fatty acids like palmitic acid (C16:0). Since the addition of deuterium-labeled palmitic acid (C16:0) into the cell culture medium resulted in a partial shift of the isotopic ion distribution of palmitoleic acid (C16:1) in mass spectrometry, Wnt-3a protein appeared to be modified with C16:1-fatty acid metabolically processed from palmitic acid (C16:0). Most cases of acylation with unsaturated fatty acids occur at sites where acylation with saturated fatty acids also occurs (DeMar et al., 1999; Dizhoor et al., 1992; Johnson et al., 1994; Kokame et al., 1992; Liang et al., 2001; Muszbek and Laposata, 1993; Neubert et al., 1992). In contrast, the results of our mass spectrometry analysis indicate a strong preference for modification with monounsaturated fatty acids at Ser209 of Wnt-3a. Given that cells store considerable amounts of saturated fatty acids as substrates for acyltransferases, the predominant modification with a palmitoleoyl (C16:1) moiety must be the result of preferential utilization of this unsaturated fatty acid as a substrate.

In addition to modification with a palmitoleoyl (C16:1) moiety, another interesting feature of Ser209 acylation involves the amino acid residue to which the acyl moiety is attached. In most cases of protein fatty acylation examined to date, including N-myristoylation, S-palmitoylation, and N-palmitoylation, a serine residue is not utilized for connecting to the acyl moieties, although there are a few exceptions, including attachment of oxyester-linked octanoate (C8:0) to serine in ghrelin, a growth-hormone-releasing peptide of 28 amino acids (Kojima et al., 1999; Smotrys and Linder, 2004). Here, we showed a different example of serine-linked O-acylation, where serine is used to bond a monounsaturated acyl moiety to a protein. Thus, Wnt-3a acylation demonstrates several unusual characteristics, suggesting that some specific machinery appears to be involved in this acylation.

One strong candidate for a player involved in this machinery is Porc. Since most Wnt acylation detected with the labeling assay of this study was dependent on Ser209 and was abolished in cells with markedly reduced Porc activity, *porc* appears to be required for acylation at Ser209. If this is the case, it will be important to elucidate the mechanism by which this enzyme utilizes unsaturated fatty acids and recognizes serine residues. Interestingly, additional members of the MBOAT family of membrane-bound O-acyltransferases, including acyl-CoA cholesterol acyltransferase (ACAT) and diacylglycerol acyltransferase (DGAT), utilize monounsaturated fatty acids as substrates (Cases et al., 1998; Seo et al., 2001). Thus, certain structural characteristics of this family may contribute to their preferential utilization of unsaturated fatty acids, as well as their role in O-acylation.

Another question pertains to the biological significance of acylation by unsaturated fatty acids. Although the functional significance of such acylation remains unclear, the bent structure produced by the double bond as a result of this process may influence the interaction of acylated proteins with lipid structures. Both in vivo and in vitro results indicate that acylation with unsaturated fatty acids results in displacement of proteins, including Fyn, annexinII, and Gai, from membrane domains within ordered lipid structures (Liang et al., 2001; Moffett et al., 2000; Zhao and Hardy, 2004). Thus, it seems unlikely that unsaturated fatty acylation enhances protein targeting into some ordered lipid structures, but rather that the folded structure might be advantageous for packaging fatty acid chains into the interior of small lipid particles. As such, we can speculate that palmitoleoyl (C16:1) modification may enable packaging of Wnt proteins into protein-lipid particles. An intracellular precursor of a lipoprotein particle might be a candidate for such a particle (Greco et al., 2001; Panakova et al., 2005).

Finally, it is of interest to note that the amino acid sequence surrounding Ser209 (C-K-C-H-G-(LIVMT)-S-G-S-C, where the bold S indicates Ser209) is highly conserved among members of the Wnt family, although the function of this motif remains unknown (Figure 7). Our results indicate that Ser209 in this conserved motif is essential for palmitoleoyl (C16:1) modification and secretion of Wnt-3a protein, suggesting that other Wnt members may likewise be modified by palmitoleoyl acid (C16:1), depending on the presence of this conserved motif. An attempt to reveal the role of this motif might provide clues regarding the molecular mechanism behind acylation.

Experimental Procedures

Cell Culture, Transfection, and Metabolic Labeling of Wnt Protein

L cells were cultured at 37°C in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 8.3% fetal calf serum and antibiotics. Stable transfectants expressing truncated or point-mutated forms of Wnt-3a were established as previously described (Shibamoto et al., 1998). Supernatants from cultures of Wnt-3a- or Wnt-5a-producing L cells were prepared as previously described (Shibamoto et al., 1998; Yamanaka et al., 2002). For inhibition of de novo protein synthesis, L cells were treated with 10 μg/ml cycloheximide for up to 24 hr; for that of Porc activity, a plasmid vector, pSilencer 3.0-H1 (Ambion), expressing siRNA specific for *porc* (si21: 5′-AAGTT GTCACAAGCTGGAACC-3′), was used to transfect Wnt-3a-expressing L cells. Stable transfectants with varying levels of defective *porc* expression were thus established and used in the experiments.

L cells secreting various forms of Wnt-3a (or Wnt-5a) and control L cells were inoculated into 35 mm dishes and were then incubated overnight at 37°C. Next, these cells were incubated for 36 hr in serum-free medium containing 0.37 MBq/ml [U-^{14}C] palmitic acid (30.4 GBq/mmol) or 14.8 MBq/ml [9,10-^{3}H] palmitic acid (2.2 TBq/mmol), or they were incubated in serum-free medium without labeled fatty acid for 20 hr and then with 0.37 MBq/ml [U-^{14}C] palmitic acid (30.4 GBq/mmol) for 4 hr. The culture supernatant or cell lysate was collected and immunoprecipitated with anti-Wnt-3a (or anti-Wnt-5a) antibody (R.T. and S.T., unpublished data) and protein G Sepharose beads. Immunoprecipitates mixed with sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2-mercaptoethanol at the final concentration of 1.3 M were incubated at 37°C for 1 hr, separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography with a Kodak BioMax TranScreen LE system or by image analysis with a BAS 2500 (Fuji Photo Film).

Antibodies, Immunoblotting, and Immunocytology

The preparation of monoclonal anti-Wnt-3a and anti-Wnt-5a antibodies is described elsewhere (R.T and S.T., unpublished data). These antibodies specifically recognize Wnt-3a or Wnt-5a proteins, respectively. Anti-protein disulfide isomerase rabbit polyclonal antibody (Calbiochem), anti-HA antibody (Covance), and anti-FLAG M2 affinity gel (Sigma), were purchased. Immunoblotting was performed according to a standard protocol. For immunocytological analysis, cells were fixed with 3% paraformaldehyde in PBS(−) for 10 min at room temperature, made permeable by treatment with cold 100% methanol for 10 min, and then incubated with anti-Wnt-3a antibody and anti-protein disulfide isomerase (anti-PDI, a rabbit polyclonal antibody) for 1 hr at room temperature. Thereafter, the cells were probed with CY3-conjugated anti-mouse IgG and Alexa 488-conjugated anti-rabbit secondary antibodies and were observed under a confocal microscope (Carl-Zeiss, LSM510).

Nano-Flow Liquid Chromatography

FLAG-tagged Wnt-3a protein was purified with anti-FLAG M2 antibody from the culture supernatant of Wnt-3a-expressing L cells in serum-free medium. Purified Wnt-3a protein was reduced with 0.2 M dithiothreitol, and cysteine alkylation was carried out with monoacrylamide. Wnt-3a protein was directly applied to a gel for 8.0% SDS-PAGE, after which the gel was stained with silver-staining reagents or Coomassie brilliant blue (CBB). The protein band at ~45 kDa was excised from a strip of the gel. The excised protein band that had been stained with silver-staining reagents was destained with 20 mM EDTA 2Na-50 mM NH_4HCO_3 in 30% aqueous acetonitrile and was then washed with 15 mM potassium hexacyanoferrate(III)-50 mM sodium thiosulfate. The band stained with CBB was destained with 100 mM NH_4HCO_3 in 60% aqueous acetonitrile. The gel was then subjected to in-gel digestion with trypsin (Promega).

The trypsin digest was extracted from the gel with 0.1% trifluoroacetic acid in 60% aqueous acetonitrile. The resultant solution was injected into an Ultimate nano-LC system (Dionex), where the digested peptides were first concentrated with a C18 trapping column (0.3 mm × 1 mm, Dionex, Idstein, Germany) at a flow rate of 30 μl/min, and then separated by using a C_{18}-Pepmap column (0.075 × 150 mm, Dionex). A linear gradient of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile) was used for the separation, and the peptides were eluted by increasing the concentration of solvent B from 5% to 80% over a period of 60 min at a flow rate of 200 nl/min. The effluent was monitored at 214 and 280 nm and was directly blotted at 1 min intervals onto the flat surface of a stainless steel plate (a MALDI sample plate) over a 96 min period. Thereafter, the matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid) was blotted manually onto each sample spot and then dried.

Matrix-Assisted Laser Desorption/Ionization, MALDI, Mass Spectrometry

Overall peptide identification was carried out by using a MALDI-TOF/TOF (4700 proteomics analyzer, Applied Biosystems, Framingham, MA), followed by a database search with Mascot ver. 2.0 (Matrix Science, Manchester, UK). Ions were generated by irradiating the sample area with a 200 Hz Nd:YAG laser operated at 355 nm. Calibration was performed by using MH^+ ions from a mixture of angiotensin I (m/z 1296.6), dynorphin (m/z 1604.0), ACTH (1-24) (m/z 2932.6), and β-endorphin (m/z 3463.8). The isotopic envelopes of the observed ions were closely compared with the theoretical envelopes generated by *Isotopica*, a software aid for calculating and assessing complex isotopic envelopes (http://coco.protein.osaka-u.ac.jp/Isotopica) (Fernandez-de-Cossio et al., 2004). For MS/MS, the precursor ions were accelerated at 8 kV in MS1 and were fragmented in a collision cell by using air as the collision gas. The resultant fragment ions, re-accelerated at 15 kV, were analyzed in MS2 equipped with a reflectron. MS/MS spectra were interpreted by *SeqMS*, a software aid for de novo sequencing by MS/MS (http://www.protein.osaka-u.ac.jp/rcsfp/profiling) (Fernandez-de-Cossio et al., 2000).

Xenopus Injection, Detection of Target Gene Expression, and Immunohistology

For morphological analysis, in vitro-synthesized wild-type, S209A, or S211A Wnt-3a mRNA sequences were injected into the ventral marginal zones of *Xenopus* eggs at the four-cell stage (Sokol et al., 1991). To examine the expression of *Siamois* and *Xnr3* (Brannon and Kimelman, 1996; McKendry et al., 1997), we injected the various Wnt-3a mRNA sequences at the two-cell stage, after which the animal caps of the injected embryos were dissected at stage 8.5, cultured to stage 11, and analyzed for gene expression by use of the reverse transcription-polymerase chain reaction. The following specific primers were used: forward, 5′-GATAACTGGCATTCCTGAGC-3′; reverse, 5′-ACAAGTCAGTGTGGTGATTC-3′ (23 cycles) for *siamois*; forward, 5′-CCATGTGAGCACCGTTCC-3′; reverse, 5′-GAGCAAACT CTTAATGTAG-3′ (18 cycles) for *Xnr3*; and forward, 5′-ATAACATC CAGGGCATCACC-3′; reverse, 5′-ACATCCATAGCGGTGACGGT-3′ (18 cycles) for *histone H4* as the internal input control. For immunohistological analysis, the various Wnt-3a mRNA sequences were injected into the blastomeres in the animal side of eight-cell-stage blastulas, and the explants from the injected embryos were fixed at stage 9.5 in MEMFA (0.1 M MOPS [pH 7.4], containing 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) for 1 hr at room temperature. After bleaching in 10% H_2O_2 and blocking with FCS-PBS (PBS with 15% fetal calf serum) for 20 min, the explants were incubated overnight at 4°C with anti-Wnt-3a antibody in FCS-PBS. They were then incubated for 2 hr at room temperature with rhodamine-conjugated

rabbit anti-mouse IgG. The washed explants were mounted and observed under a confocal microscope (Carl-Zeiss, LSM510).

Supplemental Data

Supplemental Data include three figures and three tables and are available at http://www.developmentalcell.com/cgi/content/full/11/6/791/DC1/.

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References

Banziger, C., Soldini, D., Schutt, C., Zipperlen, P., Hausmann, G., and Basler, K. (2006). Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. Cell *125*, 509–522.

Bartscherer, K., Pelte, N., Ingelfinger, D., and Boutros, M. (2006). Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. Cell *125*, 523–533.

Bizzozero, O.A. (1995). Chemical analysis of acylation sites and species. Methods Enzymol. *250*, 361–379.

Brannon, M., and Kimelman, D. (1996). Activation of Siamois by the Wnt pathway. Dev. Biol. *180*, 344–347.

Cases, S., Smith, S.J., Zheng, Y.W., Myers, H.M., Lear, S.R., Sande, E., Novak, S., Collins, C., Welch, C.B., Lusis, A.J., et al. (1998). Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. Proc. Natl. Acad. Sci. USA *95*, 13018–13023.

Coudreuse, D.Y., Roel, G., Betist, M.C., Destree, O., and Korswagen, H.C. (2006). Wnt gradient formation requires retromer function in Wnt-producing cells. Science *312*, 921–924.

DeMar, J.C., Jr., Rundle, D.R., Wensel, T.G., and Anderson, R.E. (1999). Heterogeneous N-terminal acylation of retinal proteins. Prog. Lipid Res. *38*, 49–90.

Dizhoor, A.M., Ericsson, L.H., Johnson, R.S., Kumar, S., Olshevskaya, E., Zozulya, S., Neubert, T.A., Stryer, L., Hurley, J.B., and Walsh, K.A. (1992). The NH2 terminus of retinal recoverin is acylated by a small family of fatty acids. J. Biol. Chem. *267*, 16033–16036.

Fernandez-de-Cossio, J., Gonzalez, J., Satomi, Y., Shima, T., Okumura, N., Besada, V., Betancourt, L., Padron, G., Shimonishi, Y., and Takao, T. (2000). Automated interpretation of low-energy collision-induced dissociation spectra by SeqMS, a software aid for de novo sequencing by tandem mass spectrometry. Electrophoresis *21*, 1694–1699.

Fernandez-de-Cossio, J., Gonzalez, L.J., Satomi, Y., Betancourt, L., Ramos, Y., Huerta, V., Amaro, A., Besada, V., Padron, G., Minamino, N., and Takao, T. (2004). Isotopica: a tool for the calculation and viewing of complex isotopic envelopes. Nucleic Acids Res. *32*, W674–W678.

Greco, V., Hannus, M., and Eaton, S. (2001). Argosomes: a potential vehicle for the spread of morphogens through epithelia. Cell *106*, 633–645.

Hacker, U., Nybakken, K., and Perrimon, N. (2005). Heparan sulphate proteoglycans: the sweet side of development. Nat. Rev. Mol. Cell Biol. *6*, 530–541.

Hofmann, K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling. Trends Biochem. Sci. *25*, 111–112.

Huang, K., and El-Husseini, A. (2005). Modulation of neuronal protein trafficking and function by palmitoylation. Curr. Opin. Neurobiol. *15*, 527–535.

Johnson, D.R., Bhatnagar, R.S., Knoll, L.J., and Gordon, J.I. (1994). Genetic and biochemical studies of protein N-myristoylation. Annu. Rev. Biochem. *63*, 869–914.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., and Perrimon, N. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. Genes Dev. *10*, 3116–3128.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature *402*, 656–660.

Kokame, K., Fukada, Y., Yoshizawa, T., Takao, T., and Shimonishi, Y. (1992). Lipid modification at the N terminus of photoreceptor G-protein α-subunit. Nature *359*, 749–752.

Lewis, P.M., Dunn, M.P., McMahon, J.A., Logan, M., Martin, J.F., St-Jacques, B., and McMahon, A.P. (2001). Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. Cell *105*, 599–612.

Liang, X., Nazarian, A., Erdjument-Bromage, H., Bornmann, W., Tempst, P., and Resh, M.D. (2001). Heterogeneous fatty acylation of Src family kinases with polyunsaturated fatty acids regulates raft localization and signal transduction. J. Biol. Chem. *276*, 30987–30994.

Lin, X. (2004). Functions of heparan sulfate proteoglycans in cell signaling during development. Development *131*, 6009–6021.

Linder, M.E., and Deschenes, R.J. (2003). New insights into the mechanisms of protein palmitoylation. Biochemistry *42*, 4311–4320.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.

Mann, R.K., and Beachy, P.A. (2004). Novel lipid modifications of secreted protein signals. Annu. Rev. Biochem. *73*, 891–923.

McKendry, R., Hsu, S.C., Harland, R.M., and Grosschedl, R. (1997). LEF-1/TCF proteins mediate wnt-inducible transcription from the *Xenopus* nodal-related 3 promoter. Dev. Biol. *192*, 420–431.

Miura, G.I., Buglino, J., Alvarado, D., Lemmon, M.A., Resh, M.D., and Treisman, J.E. (2006). Palmitoylation of the EGFR ligand Spitz by Rasp increases Spitz activity by restricting its diffusion. Dev. Cell *10*, 167–176.

Moffett, S., Brown, D.A., and Linder, M.E. (2000). Lipid-dependent targeting of G proteins into rafts. J. Biol. Chem. *275*, 2191–2198.

Moon, R.T., Kohn, A.D., De Ferrari, G.V., and Kaykas, A. (2004). WNT and β-catenin signalling: diseases and therapies. Nat. Rev. Genet. *5*, 691–701.

Muszbek, L., and Laposata, M. (1993). Myristoylation of proteins in platelets occurs predominantly through thioester linkages. J. Biol. Chem. *268*, 8251–8255.

Neubert, T.A., Johnson, R.S., Hurley, J.B., and Walsh, K.A. (1992). The rod transducin α subunit amino terminus is heterogeneously fatty acylated. J. Biol. Chem. *267*, 18274–18277.

Nusse, R. (2003). Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface. Development *130*, 5297–5305.

Panakova, D., Sprong, H., Marois, E., Thiele, C., and Eaton, S. (2005). Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature *435*, 58–65.

Pepinsky, R.B., Zeng, C., Wen, D., Rayhorn, P., Baker, D.P., Williams, K.P., Bixler, S.A., Ambrose, C.M., Garber, E.A., Miatkowski, K., et al. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. J. Biol. Chem. *273*, 14037–14045.

Porter, J.A., Ekker, S.C., Park, W.J., von Kessler, D.P., Young, K.E., Chen, C.H., Ma, Y., Woods, A.S., Cotter, R.J., Koonin, E.V., and Beachy, P.A. (1996a). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. Cell *86*, 21–34.

Porter, J.A., Young, K.E., and Beachy, P.A. (1996b). Cholesterol modification of hedgehog signaling proteins in animal development. Science *274*, 255–259.

Prasad, B.C., and Clark, S.G. (2006). Wnt signaling establishes anteroposterior neuronal polarity and requires retromer in *C. elegans*. Development *133*, 1757–1766.

Resh, M.D. (1999). Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim. Biophys. Acta. *1451*, 1–16.

Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843–850.

Seo, T., Oelkers, P.M., Giattina, M.R., Worgall, T.S., Sturley, S.L., and Deckelbaum, R.J. (2001). Differential modulation of ACAT1 and ACAT2 transcription and activity by long chain free fatty acids in cultured cells. Biochemistry *40*, 4756–4762.

Shibamoto, S., Higano, K., Takada, R., Ito, F., Takeichi, M., and Takada, S. (1998). Cytoskeletal reorganization by soluble Wnt-3a protein signalling. Genes Cells *3*, 659–670.

Smotrys, J.E., and Linder, M.E. (2004). Palmitoylation of intracellular signaling proteins: regulation and function. Annu. Rev. Biochem. *73*, 559–587.

Sokol, S., Christian, J.L., Moon, R.T., and Melton, D.A. (1991). Injected Wnt RNA induces a complete body axis in *Xenopus* embryos. Cell *67*, 741–752.

Tanaka, K., Kitagawa, Y., and Kadowaki, T. (2002). *Drosophila* segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum. J. Biol. Chem. *277*, 12816–12823.

Thorpe, C.J., Schlesinger, A., Carter, J.C., and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. Cell *90*, 695–705.

van den Heuvel, M., Harryman-Samos, C., Klingensmith, J., Perrimon, N., and Nusse, R. (1993). Mutations in the segment polarity genes wingless and porcupine impair secretion of the wingless protein. EMBO J. *12*, 5293–5302.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., 3rd, and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448–452.

Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S., and Nishida, E. (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. EMBO Rep. *3*, 69–75.

Zhai, L., Chaturvedi, D., and Cumberledge, S. (2004). *Drosophila* wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine. J. Biol. Chem. *279*, 33220–33227.

Zhao, H., and Hardy, R.W. (2004). Long-chain saturated fatty acids induce annexin II translocation to detergent-resistant membranes. Biochem. J. *381*, 463–469.

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ORIGINAL ARTICLE

Suppression of tumorigenicity, but not anchorage independence, of human cancer cells by new candidate tumor suppressor gene CapG

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Previously, we isolated a series of cell lines from a human diploid fibroblast lineage as a model for multistep tumorigenesis in humans. After passaging a single LT-transfected fibroblast clone, differently progressed cell lines were obtained, including immortalized, anchorage-independent and tumorigenic cell lines. In the present paper, we analysed the gene expression profiles of these model cell lines, and observed that expression of the CapG protein was lost in the tumorigenic cell line. To examine the possibility that loss of CapG protein expression was required for tumorigenic progression, we transfected CapG cDNA into the tumorigenic cell line and tested for tumor-forming ability in nude mice. Results showed that ectopic expression of CapG suppressed tumorigenicity, but not growth in soft agar or liquid medium. We also found that certain cancer cell lines including stomach cancer, lung cancer and melanoma had also lost CapG expression. One such cancer cell line AZ521 also became non-tumorigenic after the introduction of CapG cDNA. Moreover, we showed that CapG expression was repressed in small-cell lung cancer tissues. Together, our findings indicated that CapG is a new tumor suppressor gene involved in the tumorigenic progression of certain cancers.
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Keywords: CapG; tumor suppressor gene; multistep tumorigenesis; gastric cancer; lung cancer

Introduction

Carcinogenesis is thought to proceed in a stepwise fashion with the accumulation of multiple genetic abnormalities, such as activation of proto-oncogenes and inactivation of tumor suppressor genes (Hahn and Weinberg, 2002). In the case of colorectal cancer, for example, sequential alterations of a specific set of genes, *APC*, *K-ras* and *p53*, can account for each clinical stage of carcinogenesis (Rajogopalan *et al.*, 2003). However, the direct correlation between such genetic abnormalities and defined clinical stages and the molecular mechanism of progression through the different clinical stages remain poorly understood.

To clarify the mechanisms of multistep carcinogenesis, we decided that it would be useful to study a series of differently transformed cell lines derived from a single line of normal cells and then analyse the differences in gene expression between these cell lines. To this end, we previously isolated a series of variously transformed cell lines (retinoblastoma (RB) cell lineage) from human skin fibroblasts (RB) from a patient with hereditary RB (Oka *et al.*, 1999). Whereas RB cells had the normal diploid set of 46 chromosomes, one copy of chromosome 13 contained a large deletion spanning the region from q14 to q22. We introduced early genes of SV40 into RB cells and obtained several mortal clones with extended lifespan (RBSV). After repeated passages of a single RBSV cell clone, we succeeded in isolating immortalized (RBI), anchorage-independent (RBS) and tumorigenic (RBT) cell lines. Such model cell lines of multistep tumorigenesis are very rare, and to our knowledge no other similar cell lines have been previously published.

In the present study, we searched for differences in gene expression between these model cell lines and found that CapG protein expression was completely absent only in the most progressed line, the tumorigenic RBT cells. When introduced into RBT cells, the *CapG* gene suppressed RBT tumorigenicity in nude mice, but did not affect RBT colony formation in soft agar. This effect of CapG was not limited to our model cell lines. Although the *CapG* gene is ubiquitously expressed in normal tissues, expression was frequently lost in human cancer cell lines and tissues including the tumorigenic gastric cancer cell line AZ521. This cell line was also converted to a non-tumorigenic state by ectopic expression of CapG protein. These results suggested that the *CapG* functioned as a tumor suppressor gene and was

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involved in tumorigenic conversion in various human cancers.

Results

CapG expression was lost at the tumorigenic stage of the RB cell lineage

As previously reported (Oka *et al.*, 1999), we isolated a set of human cell lines (RB cell lineage) as a model of multistep carcinogenesis. These cell lines were derived from a single human diploid fibroblast strain (RB) that was transformed by the introduction of SV40 early genes (RBSV) and resultant lines selected for progression to the immortalized (RBI), anchorage-independent (RBS) or tumorigenic (RBT) stages (Figure 1a). To investigate for altered gene expression between the RB cell lineage stages, we performed Western blot analysis using various antibodies. When one polyclonal antibody (HD-2) was used, a protein band with apparent molecular weight of 45 kDa was found to be absent only from the RBT tumorigenic cell line (Supplementary Figure 1a). This antibody was made by us to analyse another cancer-related protein, but the antibody obtained recognized several unknown protein bands probably because of contamination of other proteins in the antigen preparation for immunization. During analyses using this antibody, we found loss of the 45 kDa protein in some cell lines including the RBT cell line and strong expression of this protein in other cell lines (Supplementary Figure 1b). We next determined a protein spot corresponding to the 45 kDa protein on two-dimensional gel (Supplementary Figure 2a). Mass spectrometric analysis identified this protein as CapG (Supplementary Figure 2b). We then cloned CapG cDNA, expressed and purified glutathione-S-transferase-fused CapG protein from *Escherichia coli* and generated polyclonal anti-CapG antibody (Supplementary Figure 3). Using this antibody, we confirmed that RBT cells showed no CapG protein expression (Figure 1b). To understand the mechanism by which CapG protein expression was inhibited in the RBT cell line, we performed Southern and Northern blot hybridizations. As shown in Figure 1c and d, whereas CapG mRNA was not detected in RBT cells, no gross structural alterations of the *CapG* gene were observed in any of the RB cell lines. This suggested that CapG expression was repressed at the transcriptional level at the stage of progression from anchorage independence to tumorigenicity in the RB cell lineage.

Ectopic CapG expression suppressed RBT cell tumorigenicity

The finding that loss of CapG expression was associated with progression from the anchorage-independent to the tumorigenic stage of the RB cell lineage led us to hypothesize that the CapG protein played a role in tumor suppression. To test this possibility, we introduced CapG cDNA into RBT cells by infection with a recombinant retroviral vector, and isolated cell clones expressing CapG protein. Levels of CapG protein in these clones were largely comparable to those observed

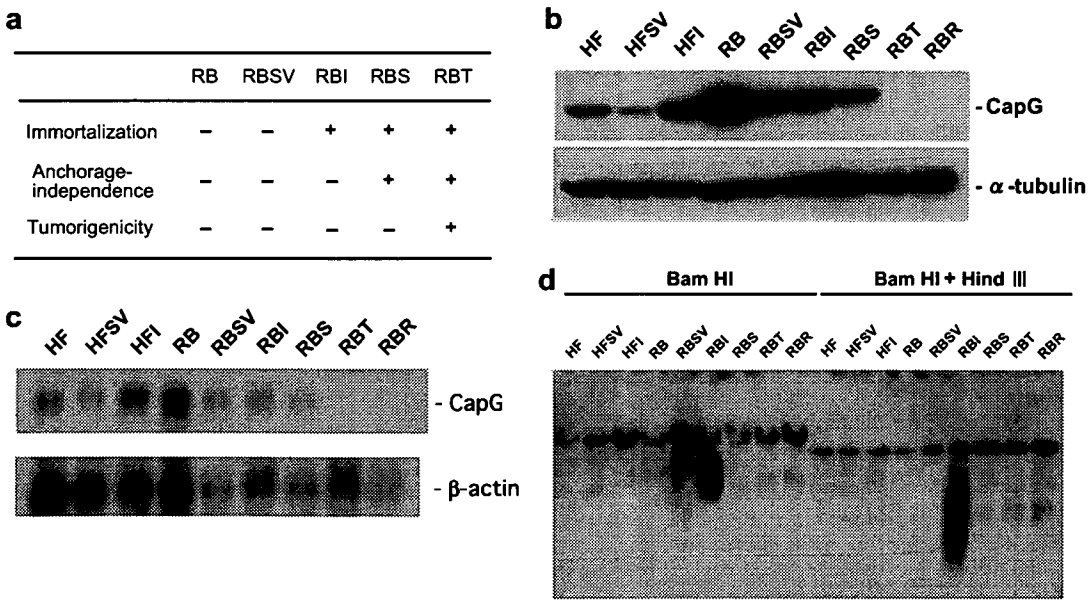
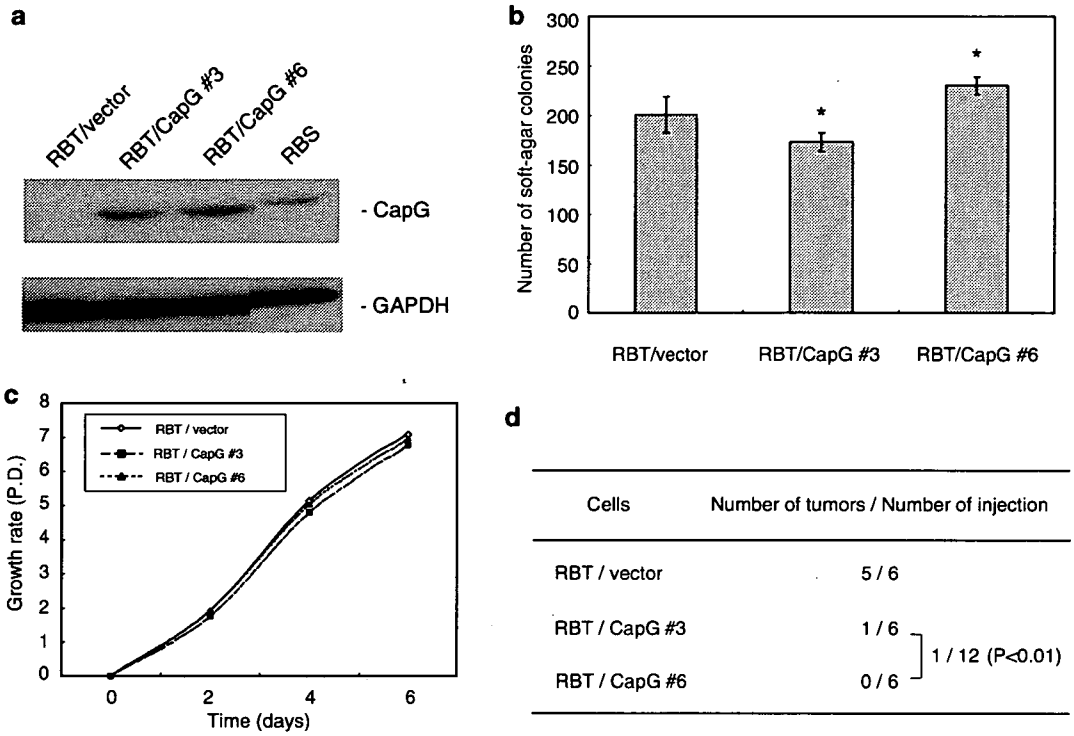


Figure 1 Loss of CapG expression in a tumorigenic cell line of the RB cell lineage. (**a**) Multistep transformation of the RB cell lineage. (**b**) CapG protein expression in the RB cell lineage detected by Western blot analysis. α-Tubulin was used as a loading control. RBR is a cell line reconstituted from an RBT tumor from a nude mouse. HF: human diploid fibroblasts; HFSV: HF cell clone transfected with SV40 early genes; HFI: immortalized HF cell line obtained from HFSV. (**c**) CapG mRNA expression in the RB cell lineage analysed by Northern blot. The RNA blot was hybridized with ^{32}P-labeled CapG cDNA (1.2 kb). β-Actin was used as a loading control. (**d**) No structural alteration of the *CapG* gene were observed in the RB cell lineage cell lines. Genomic DNA was analysed by Southern blot hybridization using ^{32}P-labeled CapG cDNA as a probe.



Cells	Number of tumors / Number of injection	
RBT / vector	5 / 6	
RBT / CapG #3	1 / 6	1 / 12 (P<0.01)
RBT / CapG #6	0 / 6	

Figure 2 Tumorigenicity and growth properties of the RBT cell line infected with a CapG retroviral construct. (**a**) CapG protein expression as determined by Western blot. RBT/vector and RBT/CapG are mock- and CapG retrovirus-infected RBT cell clones, respectively. RBS is an anchorage-independent but non-tumorigenic cell line of the RB cell lineage. GAPDH was used as a loading control. (**b**) Anchorage-independent growth of RBT cell clones expressing CapG protein. Ten thousand cells were plated in 0.35% soft agar medium, and colonies larger than 0.125 mm counted 2 weeks later. The experiment was performed in triplicate. Error bars represent standard deviation from mean value. Asterisk shows 'nonsignificance' ($P > 0.05$). (**c**) Growth of RBT cell clones expressing CapG protein. Twenty-five thousand cells were seeded into liquid medium in 24-well culture plates. At the times indicated, cells in triplicate wells were trypsinized and counted using a hemocytometer. (**d**) Tumorigenicity in nude mice of RBT cell clones expressing CapG protein. Ten million cells were injected subcutaneously into nude mice. Data represent number of mice with tumors at 7 weeks after injection/number of mice injected.

in RBS cells, which grow anchorage independently but do not form tumors in nude mice (Figures 1a and 2a, and data not shown). The growth rate in liquid media and colony-forming ability in soft agar of RBT cell clones expressing CapG protein were similar to those of parental non-infected RBT cells (Figure 2b and c), which indicated that proliferation and anchorage-independent growth were not affected by CapG expression. However, when injected subcutaneously into nude mice, the tumor-forming capacity of CapG-expressing RBT cells was greatly reduced compared to non-infected RBT cells and RBT cells infected with a control retroviral vector (Figure 2d). These observations indicated that *CapG* gene suppressed RBT tumorigenicity, and that tumor suppression was independent of growth in liquid culture or soft agar.

Lack of CapG expression in various human cancer cell lines

Although ectopic CapG expression suppressed RBT cell tumorigenesis, the RB cell lineage is a series of artificially transformed cell lines rather than actual cancer cell lines. Therefore, we examined whether CapG expression was altered in human cancer cells. As shown in Figure 3a, CapG mRNA was ubiquitously expressed in normal human tissues, although expression levels were not uniform. However, some cancer cell lines, especially those derived from human stomach cancer (1/3), cutaneous melanoma (1/3), small-cell lung cancer (3/5) and lung adenocarcinoma (1/5) had completely lost CapG expression (Figure 3b). Downregulated or reduced CapG protein expression was also observed at a high frequency in various lung cancers (Figure 3b). These results suggested that loss of CapG expression may contribute to the development of human cancers.

CapG suppressed the tumorigenicity of stomach cancer cell line AZ521

To examine whether ectopic CapG expression could also suppress the tumorigenicity of an established human cancer cell line, we introduced CapG cDNA into the human stomach cancer cell line AZ521 that lacked CapG protein expression as shown in Figure 3 and isolated three cell clones that expressed CapG protein (Figure 4a). These CapG-expressing AZ521 clones exhibited similar growth rates and colony-forming ability compared to control AZ521 cells (Figure 4b and c), which again suggested that CapG protein

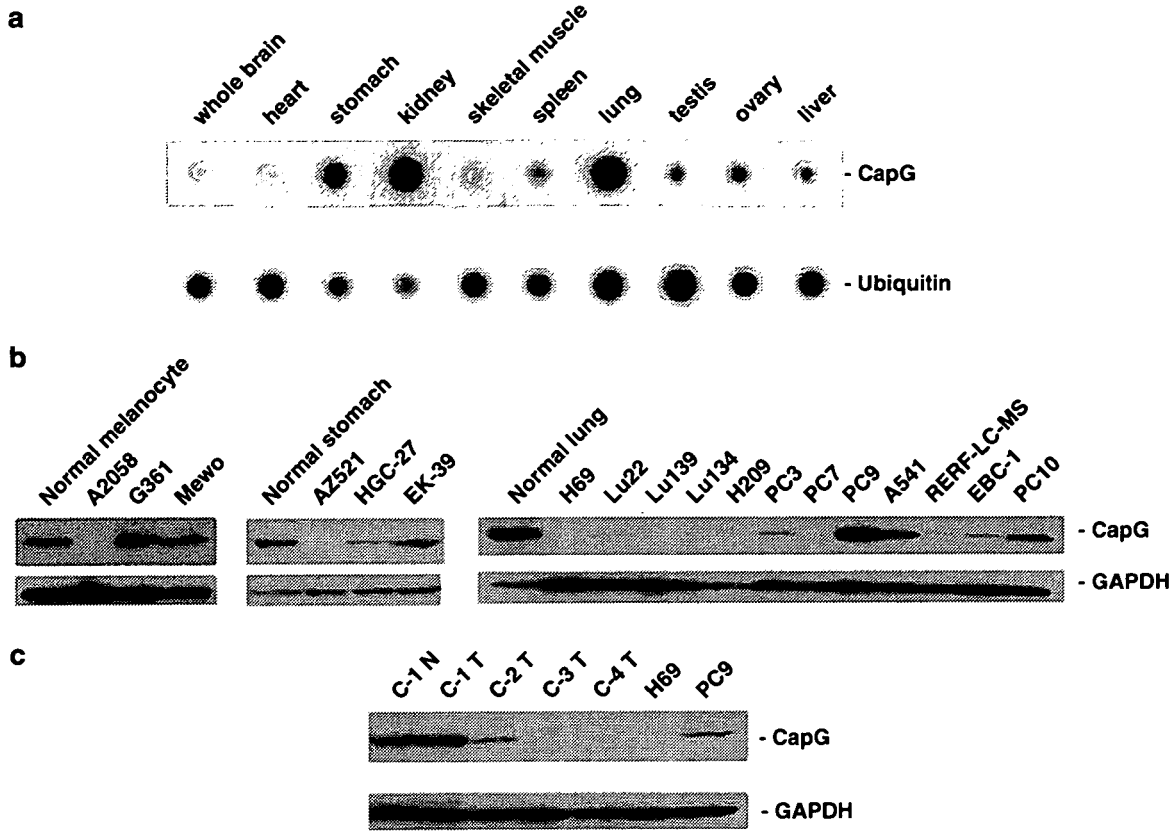


Figure 3 CapG expression in human normal tissues, cancer cell lines and primary tumors. (**a**) Expression of CapG mRNA in normal human tissues. Human RNA blot (Clonthech) was subjected to Northern blot hybridization with ^{32}P-labeled CapG cDNA. Several major tissues were selected from the original autoradiogram (Supplementary Figure 4). Ubiquitin cDNA supplied by the manufacturer was used as an internal control. (**b**) Expression of CapG protein in human melanoma (A2058, G361, MeWo), stomach cancer (AZ521, HGC-27, EK-39), small-cell lung cancer (H69, Lu22, Lu139, Lu134, H209), lung adenocarcinoma (PC3, PC9, PC9, A541, RERF-LC-MS) and lung squamous-cell carcinoma (EBC-1, PC10) cell lines. Expression levels were determined by Western blot. GAPDH was used as an internal control. (**c**) Expression of CapG protein in human small-cell lung cancer tissues. Lysates from four tumor tissues (C-1T to C-4T) and one normal lung tissue (C-1N) were subjected to Western blot. GAPDH was used as a loading control.

expression did not affect cell growth in liquid media or soft agar. However, CapG-expressing AZ521 clones did not produce tumors in nude mice (Figure 4d), which suggested that CapG expression suppressed the tumorigenicity of the human cancer cells in nude mice.

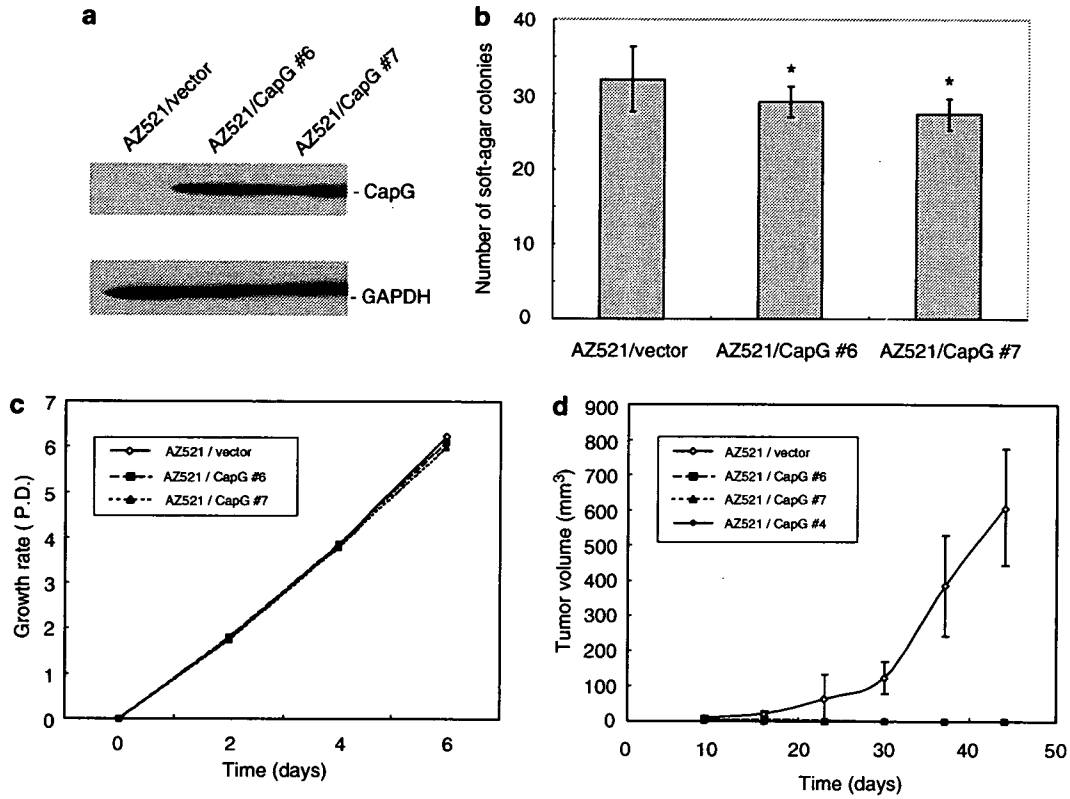
Loss of CapG protein expression was also observed in primary tumors

As most cancer cell lines are cultured *in vitro* over long periods of time, it can be argued that cancer cell lines do not necessarily accurately reflect primary tumor cells. To examine whether primary tumors also showed loss of CapG protein expression, we performed Western blot analysis using human tumor tissues isolated from cases of stomach cancer (four cases), small-cell lung cancer (four cases) and melanoma (two cases). The results showed that although downregulation of CapG protein was not observed in the stomach cancer and melanoma samples, CapG protein levels were greatly reduced in three of the four small-cell lung cancer samples (Figure 3c). Although some CapG protein was still present, tumor tissues can also contain normal cells such as stromal cells or infiltrating lymphocytes that would express normal levels of CapG. Therefore, our results strongly suggested that the development of certain human primary cancers could also involve the loss of the *CapG* gene function.

Taken together, our findings indicated that CapG may act as a tumor suppressor protein involved in tumorigenicity in nude mice but not in cell growth in liquid media or soft agar.

Effect of ectopic CapG expression on microfilament organization and cell motility

Dynamic reorganization of the actin cytoskeleton is an underlying factor in the tumor-related processes of invasion and metastasis. Because the CapG protein is known to possess actin-modulating activity (Silacci *et al.*, 2004), it is possible that an alteration of microfilaments contributes to the acquisition or loss of tumorigenicity. Therefore, microfilament organization was examined in the RB cell lineage. Although the expression level of actin protein was comparable in all of the RB cell lines regardless of the presence or absence of



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Cells	Number of tumors / Number of injection	
AZ521 / vector	3 / 3	
AZ521 / CapG #4	0 / 3	0 / 2 (P<0.01)
AZ521 / CapG #6	0 / 3	
AZ521 / CapG #7	0 / 3	

Figure 4 Suppression of tumorigenicity of a stomach cancer cell line AZ521 infected with CapG retroviral vector. (**a**) CapG protein expression. Cell lysates from AZ521 cell clones infected with control or CapG retroviral constructs were subjected to Western blotting with GAPDH used as a loading control. (**b**) Anchorage-independent growth of AZ521 cells expressing CapG protein. Ten thousand cells were plated in 0.35% soft agar medium, and the number of colonies larger than 0.125 mm counted 2 weeks later. The experiment was performed with triplicate samples. The data represent the mean value and standard deviation. Asterisk shows 'nonsignificance' ($P > 0.05$). (**c**) Growth of AZ521 cells expressing the CapG protein. Twenty-five thousand cells were seeded into liquid medium in 24-well culture plates. At the times indicated, cells in triplicate wells were trypsinized and counted using a hemocytometer. (**d**) Suppression of tumor formation by ectopic CapG expression. AZ521 cell clones (2.5×10^6) were injected subcutaneously into nude mice, and the size of tumors that appeared measured every week. Data represent the mean value ($n = 3$ each) and standard deviation. (**e**) Tumorigenicity of AZ521 cell clones expressing CapG protein. Data were obtained from the experiment (**d**), and represent number of mice with tumors at 7 weeks after injection/number of mice injected.

CapG (Figure 5a), actin filament organization was greatly altered with malignant progression of the RB cell lineage (Figure 5b). Microfilaments were well organized and stretched long in diploid RB cells, and were maintained, though much finer and shorter, even in the anchorage-independent cell line RBS (Figure 5b). In contrast, the actin stress fibers seemed to disappear in the tumorigenic RBT cell line, but then reassembled to form a thick bundle at the perinuclear region (Figure 5b). These findings led us to hypothesize that the tumorigenicity of the RBT cell line might be caused, at least in part, by alteration of microfilament organization. One caveat, however, is that the ectopic CapG expression that resulted in the suppression of tumorigenicity in RBT cells did not affect formation of the actin bundle (Figure 5b).

We next examined the effect of ectopic CapG expression on cell motility that is believed to have some relation to actin cytoskeleton. The same RBT transfectants that were used for the tumorigenicity test and microfilament staining were subjected to a migration assay. As shown in Figure 5c, ectopic expression of CapG enhanced migration activity of the RBT cell line. This finding implies that cell motility and tumorigenicity may be controlled by different functions of the CapG protein.

These results suggest that the actin-modulation function of CapG may not be involved in the

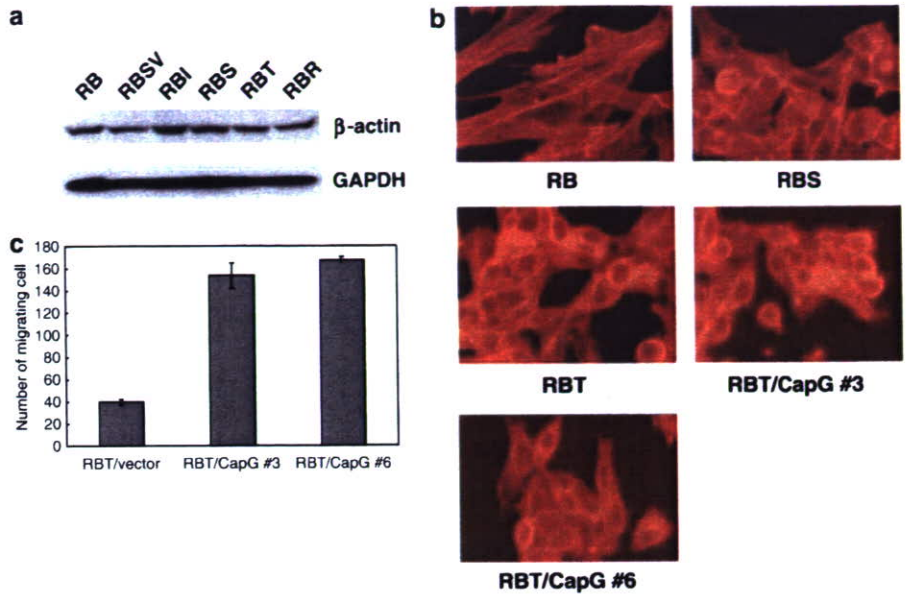

Figure 5 Effect of ectopic CapG expression on microfilament organization and cell motility. (**a**) Expression level of β-actin protein. Total cell lysates of the RB cell lines were subjected to Western blot analysis with anti-β-actin and anti-GAPDH antibodies. (**b**) Cytoskeleton organization of the RB cell lines. The RB cell lines were fixed with 2% paraformaldehyde, and stained with BODIPY 558/568 phalloidin (Molecular Probes, Eugene, OR, USA). (**c**) Enhanced migration of the RBT cells by overexpression of CapG protein. The RBT cell line was transfected with CapG expression vector or empty vector, and stably transfected clones were isolated. These cell clones (4×10^4 cells) were subjected to cell migration assay. Data represent mean value ($n = 3$ each) and standard deviation, and are significant ($P < 0.01$).

suppression of tumorigenicity. Clarification of the mechanism of tumor suppression by CapG will necessitate the isolation and characterization of CapG-interacting proteins.

Discussion

In this paper, we analysed the RB cell lineage, a human model of multistep tumorigenesis, to identify genes involved in malignant progression. As the different RB cell lines were derived from a single line of human diploid fibroblasts, it is likely that they shared a uniform genetic background with alterations limited to the expression of genes associated with malignant progression. Therefore, the RB cell lineage represented an effective tool for the identification of oncogenes or tumor suppressor genes responsible for each step of malignant progression. The cell lineage was not transfected with exogenous genes for malignant progression from immortalized RBI cell lines, such that the malignant progression observed in this cell lineage was likely to be due, at least in part, to the inactivation of endogenous tumor suppressor genes (Oka *et al*., 1999). In this way, the process of malignant progression in the RB cell lineage may mimic the process that occurs in naturally occurring cancer. Therefore, identification of genes with altered expression in response to malignant progression using the RB cell lineage may be a useful tool to help elucidate the mechanisms of the multistep tumorigenic process.

By comparing gene expression profiles of the RB cell lineage, we found that CapG expression was lost at the transcriptional level at the stage of progression from the non-tumorigenic to the tumorigenic state. Similar downregulation of CapG expression was also observed in various human cancer cell lines and cancer tissues. These findings allowed us to hypothesize that the CapG protein may be involved in the tumorigenic progression of cancer cells as a tumor suppressor gene. To examine this possibility, we introduced CapG cDNA into the tumorigenic line of the RB cell lineage, RBT, as well as the gastric cancer cell line AZ521, both of which showed no endogenous CapG protein expression. We then tested the ability of these cell lines to form tumors in nude mice and to form colonies in soft agar. Our results indicated that ectopic *CapG* gene expression suppressed tumorigenicity, but did not affect the anchorage-independent growth of these cell lines.

CapG, also known as gCap39, Mbh1 or MCP, is a 348-amino-acid protein that is ubiquitously expressed in normal tissues, being particularly abundant in macrophages (Yu *et al*., 1990; Prendergast and Ziff, 1991; Dabiri *et al*., 1992). CapG is a member of the gelsolin family of actin filament modulating proteins that also includes gelsolin, villin, adseverin, advellin, supervillin and flightless I (Silacci *et al*., 2004). However, CapG has features that distinguish it from other gelsolin family proteins. CapG has only three repeated gelsolin-like domains, in contrast to the usual six domains present in other gelsolin family proteins, and it lacks the actin-severing activity exhibited by the other family members. Another unique characteristic of CapG is its subcellular

localization. Whereas CapG localizes to both the cytoplasm and nucleus, the other gelsolin family members are present only in the cytoplasm. Therefore, CapG may have a function in addition to actin or cytoskeleton modulation. Indeed, it has been reported that CapG represses transcriptional activation (De Corte *et al.*, 2004), although it was not clear which gene was trans-repressed by CapG.

Among these CapG properties, the cytoskeleton-modulating function may be dispensable for the suppression of tumorigenicity, as ectopic CapG expression in the tumorigenic RBT cell line did not affect cytoskeletal appearance (Figure 5b) but did suppress tumorigenicity. CapG expression and nuclear localization patterns were also unchanged between the non-tumorigenic cell line RBS and the non-tumorigenic transfectants, RBT/CapG cells and AZ521/CapG cells, which expressed ectopic CapG protein (Supplementary Figure 5). This persistent expression suggests that tumor suppression does not result from abnormal expression or localization of CapG protein.

On the other hand, CapG reportedly possesses an oncogenic function involved in the control of cell migration or invasion. When cells were transfected with CapG expression vector, they acquired stimulated migration/invasion activity (Pellieux *et al.*, 2003; De Corte *et al.*, 2004). Enhanced migration of RBT cells was also observed in this study after ectopic expression of CapG (Figure 5c). This latter experiment utilized the same transfected cell clones that were examined for tumorigenicity (Figures 2d and 5c). Consequently, the indication is that CapG overexpression leads to the simultaneous suppression of tumorigenicity and activation of migration/invasion. Thus, the implication is that activated migration/invasion does not hamper tumor suppression. In this regard, it is noteworthy that CapG protein overexpression in ocular melanoma and glioblastoma has been reported (Van Ginkel *et al.*, 1998; Lal *et al.*, 1999). Although the effect of the overexpression on tumorigenesis has not been clarified, it may be interesting to examine whether these cancers contain cells with *CapG* gene mutations.

Given that CapG function can be activated via the Ras/MAPK signaling pathway (De Corte *et al.*, 2004), it is possible that there may be a relationship between loss of CapG expression and activation of the Ras/MAPK signaling pathway. It is well known that the *K-Ras*, *N-Ras* and *BRAF* genes are frequently mutated and activated in melanoma and stomach cancer (Davies *et al.*, 2002; Rajagopalan *et al.*, 2002). Furthermore, our results showed that some melanoma and stomach cancer cell lines had lost CapG protein expression, which suggests that Ras/MAPK mutations may affect CapG protein expression levels. Therefore, we examined the melanoma and stomach cancer cell lines used in this paper for the presence of K-Ras, N-Ras and BRAF mutations by RT–PCR and sequencing. However, we found no relationship between mutation of these genes and CapG protein levels (data not shown). Further study is necessary to clarify the mechanism of tumor suppression by CapG.

Gelsolin, the prototype gelsolin family protein, also exhibits tumor suppressor activity in certain human cancer cell lines. Downregulation of gelsolin has been observed at a high frequency in various cancer cell lines, and ectopic expression of gelsolin suppressed tumorigenicity of bladder and lung cancer cell lines (Tanaka *et al.*, 1995; Sagawa *et al.*, 2003). However, in these cases, gelsolin suppressed not only tumorigenicity but also anchorage-independent cell growth, which suggests that the mechanisms of tumor suppression by CapG and gelsolin are different.

In this study, based on RB cell lineage cell lines, we attempted to investigate the mechanisms that underlie multistep malignant progression and identified a candidate tumor suppressor gene, *CapG*. Loss of CapG protein and mRNA expression was observed at the progression from the non-tumorigenic to tumorigenic state, and ectopic CapG expression in tumorigenic RBT cells resulted in the inhibition of tumorigenicity, but not anchorage-independent cell growth. CapG protein levels in the RB cell lineage coincided well with the activity of the *CapG* gene, which indicated that our cell lines were a useful model to clarify the mechanisms of malignant progression. Although the molecular mechanism of CapG-induced tumor suppression remains unclear, we expect our model cell lines will contribute greatly to the understanding of tumorigenesis.

Materials and methods

Cell culture

RB cell lines were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as described previously (Oka *et al.*, 1999). The gastric cancer cell line AZ521 was cultured in minimal essential medium (MEM) supplemented with 10% FBS. Cancer cell lines were obtained from the Japanese Cancer Research Resources Bank, Health Science Research Resources Bank or Riken Cell Bank.

Western blot analysis

Cells were lyzed with RIPA buffer (0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride and 0.25 TIU/ml aprotinin). The cell lysates were run on 5–20% SDS–polyacrylamide gel and electroblotted onto polyvinylidene difluoride membrane. The membranes were incubated successively with rabbit polyclonal anti-CapG (TransGenic, Kumamoto, Japan), mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon International, Temecula, CA, USA) or α-tubulin (Sigma, St Louis, MO, USA) antibody and then with horseradish peroxidase-conjugated anti-rabbit or mouse IgG antibody (Cell Signaling Technology, Beverly, MA, USA). Protein bands were detected using enhanced chemiluminescence reagent (Amersham, Piscataway, NJ, USA).

Southern blot hybridization

DNA was digested with restriction enzymes, subjected to 1% agarose gel electrophoresis and transferred onto nylon