

# The Palmitoylation of Metastasis Suppressor KAI1/CD82 Is Important for Its Motility- and Invasiveness-Inhibitory Activity

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## ABSTRACT

The cancer metastasis suppressor protein KAI1/CD82 is a member of the tetraspanin superfamily. Recent studies have demonstrated that tetraspanins are palmitoylated and that palmitoylation contributes to the organization of tetraspanin webs or tetraspanin-enriched microdomains. However, the effect of palmitoylation on tetraspanin-mediated cellular functions remains obscure. In this study, we found that tetraspanin KAI1/CD82 was palmitoylated when expressed in PC3 metastatic prostate cancer cells and that palmitoylation involved all of the cytoplasmic cysteine residues proximal to the plasma membrane. Notably, the palmitoylation-deficient KAI1/CD82 mutant largely reversed the wild-type KAI1/CD82's inhibitory effects on migration and invasion of PC3 cells. Also, palmitoylation regulates the subcellular distribution of KAI1/CD82 and its association with other tetraspanins, suggesting that the localized interaction of KAI1/CD82 with tetraspanin webs or tetraspanin-enriched microdomains is important for KAI1/CD82's motility-inhibitory activity. Moreover, we found that KAI1/CD82 palmitoylation affected motility-related subcellular events such as lamellipodia formation and actin cytoskeleton organization and that the alteration of these processes likely contributes to KAI1/CD82's inhibition of motility. Finally, the reversal of cell motility seen in the palmitoylation-deficient KAI1/CD82 mutant correlates with regaining of p130<sup>CAS</sup>-CrkII coupling, a signaling step important for KAI1/CD82's activity. Taken together, our results indicate that palmitoylation is crucial for the functional integrity of tetraspanin KAI1/CD82 during the suppression of cancer cell migration and invasion.

## INTRODUCTION

Cancer metastasis suppressor KAI1/CD82 is pathologically as well as clinically connected to the progression, invasion, and metastasis of various malignancies, including cancers of the prostate, breast, cervix, colon, and skin (1–5). The gene expression of KAI1/CD82 is frequently down-regulated or lost in poorly differentiated cancers or at the advanced clinical stage of cancers (6–10). KAI1/CD82 expression in a tumor correlates with a better prognosis of cancer patients (11–13). Inhibition of cancer cell migration, invasion, and suppression of cancer metastasis are the well-recognized biological characteristics of KAI1/CD82, although KAI1/CD82 has been occasionally reported to regulate cell survival (14, 15). That KAI1/CD82 inhibits cancer cell motility by attenuating the biological activities of its associated proteins has emerged as a putative mechanism by which KAI1/CD82 functions as a metastasis suppressor (16, 17).<sup>3</sup> However, the structural elements in KAI1/CD82 required for this attenuation are not well understood. Identification of these elements or biochemical features holds the key to fully understand the metastasis suppressive activity of KAI1/CD82.

As a tetraspanin, KAI1/CD82 is a component of tetraspanin webs

(18) or tetraspanin-enriched microdomains (19, 20) in which tetraspanins are associated with one another as well as with nontetraspanin transmembrane proteins such as integrins, growth factors, and growth factor receptors (20–23). Associations between protein components within the tetraspanin web likely include both protein-protein and protein-lipid-protein interactions (21, 24, 25). Participation of KAI1/CD82 in tetraspanin webs may affect the organization of the tetraspanin webs and subsequently the functional status of growth factors, their receptors, and cell adhesion molecules in the webs. Formation of the tetraspanin web is apparently determined by the intrinsic biochemical features of tetraspanins. The goal of this study is to assess the role of KAI1/CD82's biochemical features or structural elements such as palmitoylation in KAI1/CD82-mediated inhibition of cell motility.

Palmitoylation is a posttranslational acylation process in which saturated fatty acids, predominantly palmitate, are covalently linked to, in most cases, the cysteine residues in proteins. Palmitoylation typically occurs on membrane-proximal cysteine residues of the membrane-spanning and -anchored proteins such as G-protein-coupled receptors and Src kinases (26, 27). Palmitoylation contributes to membrane targeting of proteins, protein trafficking, localization of proteins into organized membrane microdomains such as lipid rafts, and regulation of functional activities of proteins (26, 28, 29).

Early studies showed that tetraspanins were palmitoylated (30–32). Recently, analyses on the palmitoylation of CD9 and CD151 demonstrated that tetraspanin palmitoylation contributes to the organization of tetraspanin webs or tetraspanin-enriched microdomains (19, 33, 34). However, the effect of tetraspanin palmitoylation on cellular function remains to be established. In this study, using PC3 human metastatic prostate cancer cells as the experimental model, we found that palmitoylation of KAI1/CD82 is necessary for KAI1/CD82's inhibitory effect on cell migration and invasion.

## MATERIALS AND METHODS

**Antibodies and Extracellular Matrix Proteins.** The monoclonal antibodies (mAbs) against human tetraspanins used in this study were CD82 mAbs M104 (35), BL-2 (Diacclone SAS, Besancon, France), 6D7 (36), 50F11 (BD PharMingen, San Diego, CA), CD9 mAb Du-all (Sigma, St. Louis, MO) and mAb7 (kindly provided by Dr. Lisa Jennings, University of Tennessee, Memphis, TN), and CD81 mAb M38 (35). The p130<sup>CAS</sup> and CrkII mAbs were purchased from BD Transduction Laboratory (Lexington, KY). A mouse IgG2 (clone MOPC 141; Sigma) was used as a negative control antibody in flow cytometry. The secondary antibodies were horseradish peroxidase-conjugated goat antimouse IgG (Sigma) and FITC-conjugated goat-antimouse IgG antibody (Biosource International, Camarillo, CA). The extracellular matrix proteins used in this study were human plasma fibronectin (Invitrogen, San Diego, CA), mouse laminin 1 (Invitrogen), and rat tail collagen type I (BD Biosciences, Bedford, MA).

**Cell Culture and Transfectants.** Prostate cancer cell line PC3 was obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FCS, 100 units/mL penicillin, and 100 µg/mL streptomycin. The full-length KAI1/CD82 wild-type cDNA was originally obtained from Dr. Christopher Class (German Cancer Research Center, Heidelberg, Germany). As previously described (37), the C<sup>5</sup>A, C<sup>7</sup>4A, C<sup>83</sup>A, C<sup>251</sup>,<sup>253</sup> A, and C<sup>5</sup>,<sup>74</sup>,<sup>83</sup>,<sup>251</sup>,<sup>253</sup> A [or “full monty” (FM)] mutants of KAI1/CD82 were generated by PCR-based site-directed mutagenesis using KAI1/CD82

Received 5/4/04; revised 7/7/04; accepted 8/11/04.

**Grant support:** NIH Grant CA-96991 and the United States Army Grant PC030970 (X. Zhang).

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wild-type cDNA as the template and pCR3.1-uni plasmid (Invitrogen) as the expression vector. The introduced mutations were confirmed by DNA sequencing. The Mock, KAI1/CD82 wild-type, and KAI1/CD82 mutants were established as described previously (38). Briefly, plasmid DNA were transfected into PC3 cells via Lipofectamine 2000 (Invitrogen) and selected under G418 (Invitrogen) at a concentration of 1 mg/mL. Hundreds of G418-resistant clones were pooled, and the KAI1/CD82-positive ones were collected by flow cytometric cell sorting. The pooled Mock or KAI1/CD82-positive clones constituted the stable transfectants used in all subsequent experiments.

**Flow Cytometry.** Cells were harvested with 2 mmol/L EDTA/PBS, washed once with PBS, treated with DMEM supplemented with 5% goat serum at 4°C for 10 to 15 minutes, and then incubated with a primary mAb such as CD82 mAb M104, CD81 mAb M38, or a mouse IgG2 at 4°C for 1 hour. After removing unbound primary mAbs with two washes, cells were additionally labeled with FITC-conjugated goat-antimouse IgG at 4°C for 30 minutes. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

**Fluorescent Microscopy.** As described in earlier studies (39), circular glass coverslips (Fisher, Pittsburgh, PA) were coated with the extracellular matrix proteins fibronectin (10  $\mu\text{g}/\text{mL}$ ) or laminin 1 (10  $\mu\text{g}/\text{mL}$ ) at 4°C overnight and then blocked with 0.1% heat-inactivated BSA at 37°C for 45 min. Cells were harvested in 2 mmol/L EDTA/PBS, washed once in PBS, and plated on the extracellular matrix-coated coverslips at 37°C for 3 hours. Cells were then fixed with 3% paraformaldehyde at room temperature for 15 minutes and permeabilized with 0.1% Brij 98 in PBS at room temperature for 2 minutes. Nonspecific binding sites were blocked with 20% goat serum in PBS at room temperature for 1 hour. Primary mAbs were diluted at a final concentration of  $\sim 1 \mu\text{g}/\text{mL}$  in 20% goat serum/PBS and incubated with cells at room temperature for 1 hour followed by three washes with PBS. Cells were then labeled with FITC-conjugated goat antimouse IgG at room temperature for 1 hour, followed by four washes with PBS. For F-actin staining, cells were incubated with Texas Red-conjugated  $\alpha$ -phalloidin at room temperature for 30 minutes. Finally, the coverslips were mounted on glass slides in FluoroSave reagent (Calbiochem, Carlsbad, CA). Digital images were captured using a Zeiss Axiophot fluorescent microscope at a magnification of  $\times 63$ .

**Cell Labeling, Immunoprecipitation, Immunoblot, and Western Blotting.** For metabolic labeling with [ $^3\text{H}$ ]palmitate, PC3 cells stably transfected with wild-type or various mutant KAI1/CD82 cDNAs were incubated with 300  $\mu\text{Ci}/\text{mL}$  [9,10- $^3\text{H}$ ]palmitic acid (Perkin-Elmer Life Sciences, Boston, MA) in DMEM containing 2% fetal bovine serum at 37°C for 3 hours. Cells were lysed in a 1% NP40 lysis buffer containing 1% NP40 (Sigma), 25 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L  $\text{MgCl}_2$ , 1 mmol/L phenylmethylsulfonyl fluoride, 10  $\mu\text{g}/\text{mL}$  aprotinin, 10  $\mu\text{g}/\text{mL}$  leupeptin, 2 mmol/L sodium orthovanadate, and 2 mmol/L sodium fluoride. Insoluble material was removed by centrifugation at 14,000  $\times g$  for 15 minutes, and lysates were precleared by incubation at 4°C for 6 hours with protein A- and G-Sepharose beads (PharmaciaAmersham Biotech, Uppsala, Sweden). Then the mAb-preabsorbed protein A- and G-Sepharose beads were incubated with cell lysate overnight at 4°C. Immune complexes were collected by centrifugation followed by four washes in the immunoprecipitation buffer. Immune complexes were eluted from the beads with Laemmli sample buffer and then analyzed by SDS-PAGE (12% acrylamide) under nonreducing conditions. The gels were treated with autoradiography enhancement reagent (Perkin-Elmer Life Sciences) according to the manufacturer's protocol. The gels were dried and exposed to BioMax MR film (Kodak, Rochester, NY) at  $-80^\circ\text{C}$  for 14 days.

For cell surface biotinylation, PC3 transfectants were labeled with 100  $\mu\text{g}/\text{mL}$  EZlink sulfo-NHS-LC biotin (Pierce, Rockford, IL) in PBS at room temperature for 1 hour followed by three washes with PBS. The biotinylated cells were lysed in 1% Brij 97 lysis buffer, and cell lysates were immunoprecipitated as described above. Immunoprecipitated proteins resolved by SDS-PAGE were transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and incubated with horseradish peroxidase-conjugated extravidin (Sigma). Blots were visualized by chemiluminescence (Perkin-Elmer Life Sciences).

Immunoprecipitation and immunoblotting were performed as described in our earlier studies (40). Briefly, cells were lysed with 1% NP40/0.2% SDS or 1% Brij97 lysis buffer. Lysates were immunoprecipitated as described above, and the precipitates were then separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and sequentially blotted with a primary

antibody and a horseradish peroxidase-conjugated antimouse or antirabbit IgG (Sigma) secondary Ab, followed by chemiluminescence detection. For Western blotting, lysates were directly separated by SDS-PAGE followed by the blotting procedures as indicated above.

**Cell Migration Assay.** Migration assays were performed in Transwell membrane filter inserts in 24-well tissue culture plates (BD Labware, Bedford, MA) as described previously (41). The Transwell filters were 6.5 mm in diameter, and the pore size for polycarbonate membranes is 8  $\mu\text{m}$ . Filters were spotted with fibronectin diluted in 10 mmol/L  $\text{NaHCO}_3$  or laminin 1 diluted in PBS on the lower surface of the Transwell inserts at 4°C overnight and then blocked with 0.1% heat-inactivated BSA at 37°C for 30 minutes. Cells were detached at 90% confluence with 2 mmol/L EDTA/PBS, washed once in PBS, and resuspended in serum-free DMEM containing 0.1% BSA. A 300- $\mu\text{L}$  cell suspension was added to inserts at a density of  $3 \times 10^4$  cells/insert. DMEM containing 1% FCS was added to the lower wells. Migration was allowed to proceed at 37°C for 3 hours. Cells that did not migrate through the filters were removed using cotton swabs, and cells that migrated through the inserts were fixed and stained with Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL). The number of migrated cells per insert was counted under a light microscope at magnification  $\times 40$ . Data from several independent experiments were pooled and analyzed using a two-tailed, Student's *t* test.

**Cell Invasion Assay.** Cell invasiveness was assayed basically as described by Albini et al. (42). Type I collagen was coated onto the upper surface of Transwell inserts (100  $\mu\text{L}$  per insert) and solidified by placing the inserts into a chamber saturated with ammonia vapors for 3 minutes. The inserts were then washed  $3 \times 30$  minutes with PBS and incubated with serum-free DMEM at 37°C overnight to equilibrate the pH of the collagen gel to 7. The undersides of the inserts were coated with 10  $\mu\text{g}/\text{mL}$  fibronectin at 37°C for 3 hours and blocked with 0.1% heat-inactivated BSA at 37°C for 1 hour. The inserts were then transferred into a 24-well plate containing 10% FCS-DMEM. Each insert was filled with 500  $\mu\text{L}$  of serum-free DMEM containing  $7.5 \times 10^4$  cells. The plates were incubated in a 37°C incubator for 48 hours. After incubation, the collagen layer in the inserts was carefully removed using a cotton swab. The undersides of the inserts were fixed and stained using Diff-Quick. The number of invaded cells per insert was counted under a light microscope. Data from several independent experiments were pooled and analyzed using two-tailed, Student's *t* test.

## RESULTS

**KAI1/CD82 Is Palmitoylated at Multiple Sites.** Previous studies have shown that KAI1/CD82 is palmitoylated (19, 33, 34). To confirm this observation, we isolated KAI1/CD82 proteins via immunoprecipitation using [ $^3\text{H}$ ]palmitate-labeled, KAI1/CD82-transfected/expressed PC3 human metastatic prostate adenocarcinoma cells (43). As shown in Fig. 1A, KAI1/CD82 was labeled by [ $^3\text{H}$ ]palmitate. The higher and lower molecular weight forms of KAI1/CD82 likely represent heavily glycosylated and less glycosylated KAI1/CD82 proteins, respectively (35). Consistent with previously reports (19, 33, 34, 44), tetraspanin CD81 is heavily labeled with palmitate, and it was used here as a positive control. Paxillin was not palmitoylated as expected and served as a negative control in this experiment.

For transmembrane proteins, palmitoylation usually occurs on membrane-proximal intracellular cysteine residues (26, 27). Palmitoylation of tetraspanins typically involves multiple cysteine residues adjacent to the predicted borders between the cytoplasmic and transmembrane domains (19, 33, 34, 44). CD82 contains five cysteine residues near the interface of membrane and cytosol: C<sup>5</sup>, C<sup>74</sup>, C<sup>83</sup>, C<sup>251</sup>, and C<sup>253</sup> (Fig. 1B). To map palmitoylation sites in KAI1/CD82, five cysteine $\rightarrow$ alanine mutants were generated as schematically represented in Fig. 1C. In the mutant called FM, all five cysteine residues were replaced simultaneously by alanines (Fig. 1C).

All KAI1/CD82 mutants were individually transfected into PC3 cells, which barely express endogenous KAI1/CD82 (see following), and each individual stable transfectant was established by collecting and pooling hundreds of multiple KAI1/CD82-expressing clones with

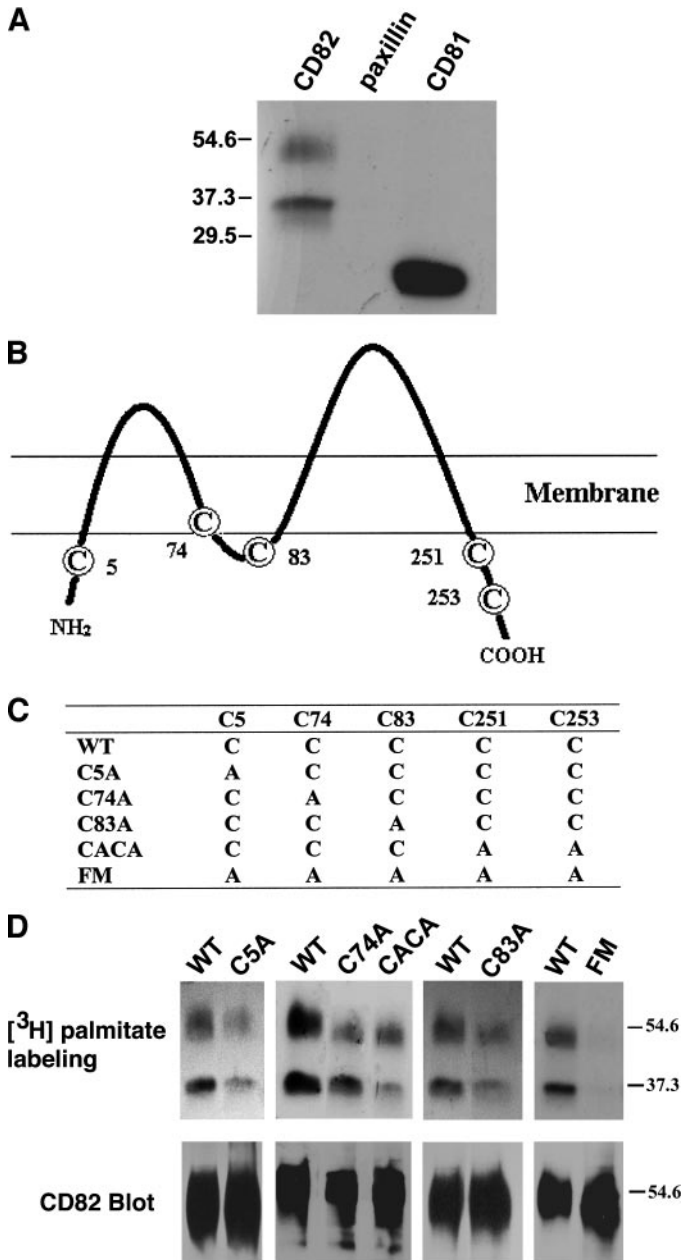


Fig. 1. KAI1/CD82 is palmitoylated at multiple sites. **A**, metabolic labeling of KAI1/CD82 with [ $^3$ H]palmitate. PC3-KAI1/CD82 wild-type-transfectant cells were radiolabeled with [ $^3$ H]palmitate and lysed in 1% NP40. KAI1/CD82, CD81, and paxillin were immunoprecipitated using their specific mAbs. The immunoprecipitates were resolved using nonreducing SDS-PAGE and labeled proteins were visualized by autoradiography. Molecular masses are given in kDa. **B**, Candidate sites of KAI1/CD82 palmitoylation are schematically represented. Shown are the four transmembrane domains, two extracellular loops, two cytoplasmic tails, and one intracellular loop of KAI1/CD82. The cysteine residues proximal to the interface of plasma membrane and cytoplasm are depicted on the KAI1/CD82 backbone. **C**, site-directed mutagenesis of KAI1/CD82. Various C $\rightarrow$ A (cysteine $\rightarrow$ alanine) mutations in KAI1/CD82 are schematically indicated. **D**, mapping the palmitoylation sites of KAI1/CD82. PC3 cells were transfected with individual KAI1/CD82 constructs and metabolically labeled with [ $^3$ H]palmitate. Cells were lysed and immunoprecipitations were performed with KAI1/CD82 mAbs. Half of the immunoprecipitate was resolved by SDS-PAGE and the extent of [ $^3$ H]palmitate labeling was determined by autoradiography. The other half of the immunoprecipitate was analyzed by KAI1/CD82 immunoblotting after SDS-PAGE separation and served as a loading control to demonstrate that equal amounts of KAI1/CD82 were immunoprecipitated under the indicated experimental conditions.

a flow cytometric cell sorting technique as described in our earlier studies (38, 45). The effects of these cysteine mutations on KAI1/CD82 palmitoylation were assessed using [ $^3$ H]palmitate-labeling of PC3 transfectants followed by immunoprecipitation of KAI1/CD82.

The results are shown in Fig. 1D. Only substitution of all five cysteines (mutant FM) leads to the full abolition of palmitoylation in KAI1/CD82 (Fig. 1D). In contrast, the palmitoylation levels of other cysteine mutants such as C $^5$ A, C $^{74}$ A, C $^{83}$ A, and C $^{251, 253}$ A were decreased  $\sim$ 61,  $\sim$ 53,  $\sim$ 62, and  $\sim$ 76%, respectively, relative to wild-type KAI1/CD82's palmitoylation level from separate experiments (Fig. 1D). The incorporation of [ $^3$ H]palmitate into KAI1/CD82 was quantitated by densitometric analysis using NIH Image version 1.62 software. Interestingly, different cysteine residues appear to be differentially involved in the palmitoylation of higher and lower molecular weight KAI1/CD82 forms. For example, C $^{74}$  is more involved in the palmitoylation of the higher molecular weight KAI1/CD82 form, whereas the C $^{251}$  and C $^{253}$  residues seem to be more important for the palmitoylation of the lower molecular weight form (Fig. 1D). Together, we concluded that mutations of any single cysteine residue were not sufficient to abolish KAI1/CD82 palmitoylation. The complete removal of KAI1/CD82 palmitoylation required the mutation of all five C $^5$ , C $^{74}$ , C $^{83}$ , C $^{251}$ , and C $^{253}$  residues. Thus, KAI1/CD82 could be palmitoylated at all C $^5$ , C $^{74}$ , C $^{83}$ , C $^{251}$ , and C $^{253}$  residues that are proximal to the membrane either simultaneously or in a temporaspatial manner. The bottom panel of Fig. 1D showed equal amounts of KAI1/CD82 proteins were used for palmitate labeling analyses. The KAI1/CD82 proteins were detected in immunoblots with its mAb, which only recognizes the higher molecular weight, heavily glycosylated form of KAI1/CD82 in immunoblots (35).

**Palmitoylation-Deficient KAI1/CD82 Failed to Inhibit Cell Migration and Invasion.** It has been well-established that KAI1/CD82 inhibits cancer cell migration, invasiveness, and metastasis. To investigate whether palmitoylation is necessary for KAI1/CD82-mediated inhibition of cellular motility, we analyzed cell migration and invasion using the PC3-KAI1/CD82 palmitoylation-deficient mutant (FM) along with PC3-Mock and -KAI1/CD82 wild-type transfectants. The cell surface expression of KAI1/CD82 was negligible in Mock transfectant and approximately two times higher in the PC3-KAI1/CD82 wild-type transfectant (mean fluorescence intensity: 68) than that in the PC3-KAI1/CD82 palmitoylation-deficient (FM) transfectant (mean fluorescence intensity: 37; Fig. 2A). Tetraspanin CD81 is a ubiquitously expressed tetraspanin protein that physically associates with KAI1/CD82 (refs. 18, 22, also see following). CD81 expression was equivalent among the PC3 transfectants (Fig. 2A), indicating that expression of KAI1/CD82 did not alter the cell surface expression of this associated tetraspanin. Other proteins such as integrin  $\alpha_5\beta_1$  were also equivalently expressed on the cell surfaces of PC3 transfectants (data not shown). However, the total cellular level of KAI1/CD82 proteins, examined by immunoblotting the total cell lysate, were equivalent between PC3-wild type and -FM KAI1/CD82 transfectants (Fig. 2B). In Fig. 2B,  $\beta$ -tubulin was used as an internal control to demonstrate the equal loading of cell lysate. It is worth noting that palmitoylation-deficient KAI1/CD82 proteins are still recognized by various KAI1/CD82 mAbs against different antigen epitopes of KAI1/CD82 such as M104, 50F11, 4F9, 6D7, and BL-2 in flow cytometry, immunofluorescence, immunoblotting, or immunoprecipitation (Figs. 1, 2, and 3). This result suggests that the tertiary structure of KAI1/CD82, at least the extracellular domains of KAI1/CD82, remain globally sound after the removal of acylates.

In the directional cell migration toward fetal calf sera on fibronectin or laminin substrate, PC3-KAI1/CD82 wild-type cell migration was markedly less compared with that of PC3-Mock cells (Fig. 2C). This result is consistent with previous studies using other cancer cell lines (4, 10, 14, 38, 41). The palmitoylation-deficient KAI1/CD82 or the FM mutant, however, largely reversed KAI1/CD82-mediated inhibition of PC3 cell migration on both fibronectin and laminin (Fig. 2C). This reversal in cell migration has been reproducibly seen in multiple

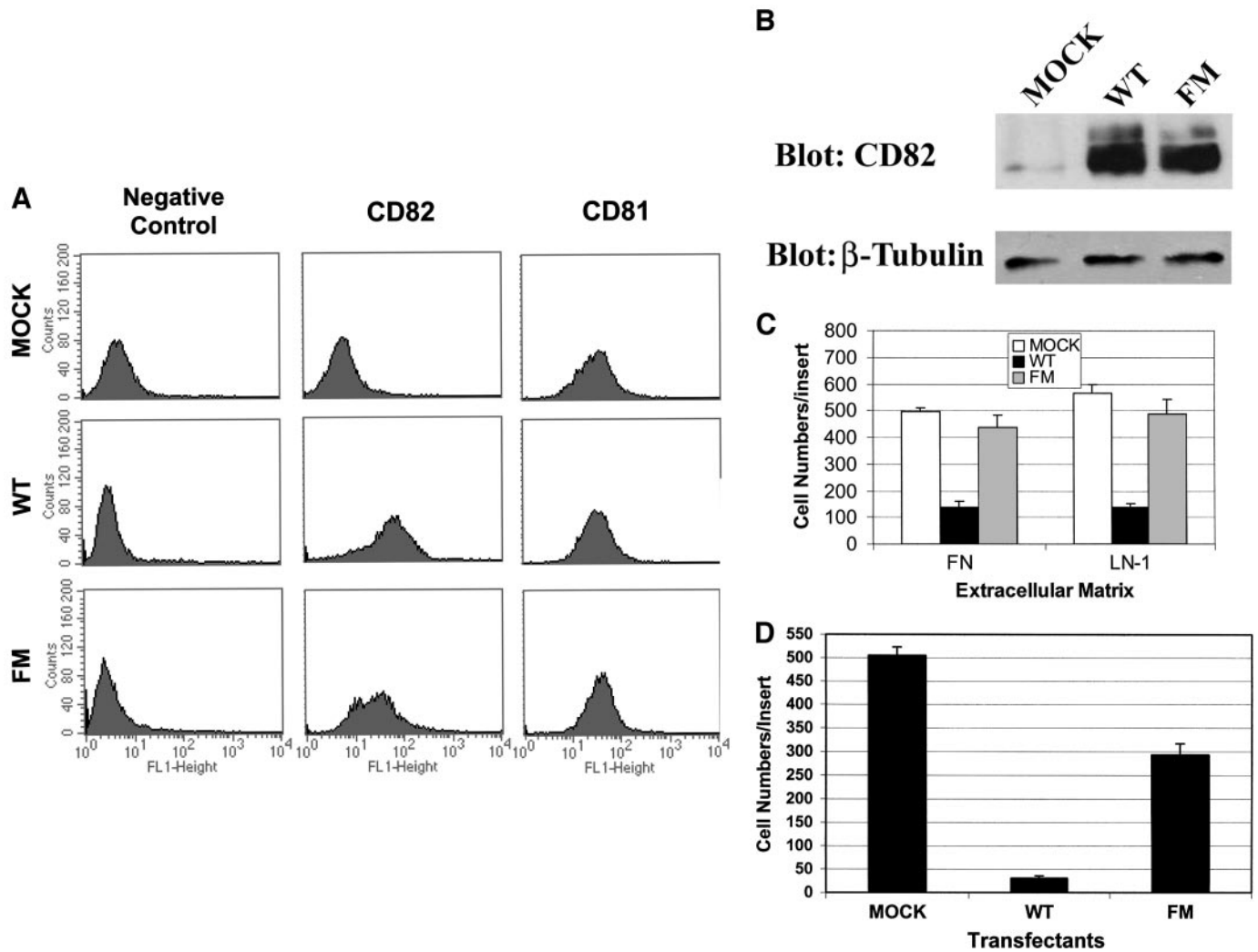


Fig. 2. Palmitoylation-deficient KAI1/CD82 reverses KAI1/CD82-mediated suppression of cell migration and invasion. **A**, cell surface expression of tetraspanins in PC3 transfectants. PC3 stable transfectants were established as described in Materials and Methods. The transfectant cells were detached, incubated with a negative control mAb (mouse IgG2b), human CD81 mAb M38, or human KAI1/CD82 mAb M104, and then stained with FITC-coupled goat antimouse antibodies. Stained cells were then analyzed by flow cytometry. The mean fluorescence intensity of KAI1/CD82 staining in PC3-Mock, -wild type (WT), and -FM transfectants were 1, 68, and 37, respectively. **B**, total KAI1/CD82 level in PC3 transfectants. PC3-Mock, -KAI1/CD82 -WT, -KAI1/CD82 FM transfectant cells were lysed with radioimmunoprecipitation assay buffer, and the lysates were Western blotted with KAI1/CD82 and  $\beta$ -tubulin mAbs. **C**, Cell migration of the PC3 transfectants was determined using Transwell inserts coated with fibronectin (FN; 10  $\mu$ g/mL) or laminin 1 (LN1; 10  $\mu$ g/mL). Cells that migrated onto the lower surface were fixed, stained, and counted. Results were obtained from multiple experiments and represented as the number of cells that migrated through per insert. Each bar represents the mean  $\pm$  SD,  $P < 0.01$ , between the WT and Mock, as well as between the WT and FM mutant on both FN and LN1. There was no difference,  $P > 0.05$ , between Mock and FM on both FN and on LN1. **D**, Cell invasion of the PC3 transfectants was determined using the Transwell inserts coated with collagen gel (11 mg/mL). Cells that invaded through collagen gel onto the lower surface were fixed, stained, and counted. Results were obtained from multiple experiments and represented as the number of invaded cells per insert. Each bar represents the mean  $\pm$  SD,  $P < 0.01$ , between the WT and Mock, the WT and FM mutant, and Mock and FM.

experiments using different batches of PC3 stable transfectants. Meanwhile, we examined the effect of KAI1/CD82 palmitoylation on cell invasion, a migratory process through three-dimensional matrices. Consistent with the results obtained from cell migration experiments, cell invasion through collagen type I gel was remarkably suppressed in KAI1/CD82 wild-type transfectant (Fig. 2D). This suppression was substantially reversed by the KAI1/CD82 FM mutant (Fig. 2D). The reversal of FM in invasion ( $\sim 60\%$  of Mock) was not as complete as that in cell migration ( $\sim 85$  to  $90\%$  of Mock). However, FM was  $\sim 10$  times more invasive than the wild type, as compared with that FM was  $\sim 3$  to 4 times more migratory than wild type. Thus, our result indicates that palmitoylation is needed for the motility-suppressive activity of KAI1/CD82 in PC3 cells.

**Palmitoylation Regulates KAI1/CD82-Tetraspanin-Enriched Microdomain Association, Subcellular Distribution of KAI1/CD82, and Motility-Related Cellular Events.** Palmitoylation of tetraspanins CD9 and CD151 plays an important role in tetraspanin-

tetraspanin interactions and the organization of tetraspanin-enriched microdomains (19, 33, 34). To further investigate why KAI1/CD82 palmitoylation is needed for its motility-inhibitory activity, we analyzed the effect of KAI1/CD82 palmitoylation on the association of KAI1/CD82 with tetraspanins CD9 and CD81. We found that KAI1/CD82-CD9 and -CD81 associations are substantially decreased in PC3-KAI1/CD82 FM-transfectant cells compared with those in PC3-KAI1/CD82 wild-type cells (Fig. 3A). These results are consistent with previous observations made with CD9 and CD151 (19, 33, 34). We then demonstrated that total cellular CD9 and CD81 levels remained equivalent among Mock, wild-type, and FM transfectants, with the control of  $\beta$ -tubulin (Fig. 3B). Therefore, the decreased levels of CD9 and CD81 observed in KAI1/CD82 immunoprecipitates were due to diminished association of CD9 and CD81 with KAI1/CD82.

Because fatty acylation regulates cellular distribution of proteins (27), we examined the subcellular localization of wild-type and palmitoylation-deficient KAI1/CD82 in PC3 cells using immunofluores-

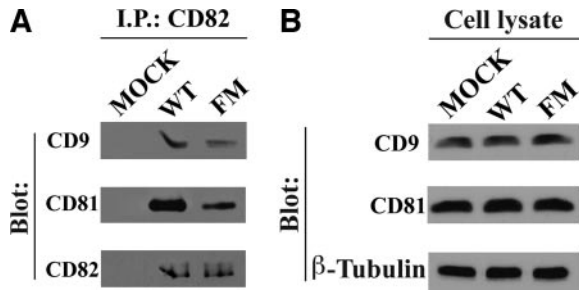


Fig. 3. Palmitoylation of KAI1/CD82 regulates the KAI1/CD82-tetraspanin associations. **A.** PC3-Mock, -KAI1/CD82 wild-type, and -KAI1/CD82 FM transfectant cells were lysed in 1% Brij 97, and the lysates were immunoprecipitated with CD82 mAb BL-2. The precipitates were resolved by nonreducing SDS-PAGE. After transfer to nitrocellulose, the membrane was blotted with CD9 mAb MAB7, CD81 mAb M38, and CD82 mAb BL-2, respectively, and the detected proteins were visualized by chemiluminescence. **B.** CD9 and CD81 protein levels in PC3 transfectants were examined by Western blotting using CD9 mAb MAB7 and CD81 mAb M38, respectively.  $\beta$ -Tubulin in the same cell lysates was also measured to use as an internal loading control.

cence. When cells were spread on fibronectin- or laminin-coated plates, wild-type KAI1/CD82 proteins were localized to the cell periphery or plasma membrane as well as intracellular vesicles (Fig. 4A). The palmitoylation-deficient KAI1/CD82 proteins were also distributed in both compartments but significantly more KAI1/CD82 molecules were detected in larger intracellular vesicles in PC3-KAI1/CD82 FM cells (Fig. 4A). These data are consistent with the decreased cell surface expression of KAI1/CD82 FM as detected by FACS analyses (Fig. 2A). These intracellular vesicles belong to the endosome/lysosome compartment.<sup>4</sup> Laser scanning confocal microscopic analysis also indicated less distribution on the cell surface and more in intracellular vesicles of KAI1/CD82 FM mutant, as compared with wild type (data not shown). Next, we analyzed the subcellular distribution of CD81 in PC3 transfectants to discern if KAI1/CD82 palmitoylation also regulates the localization of its associated tetraspanins such as CD81, a prominent member of tetraspanin-enriched microdomains. On the fibronectin- and laminin-coated plates, CD81 was also found at the cell periphery and in intracellular vesicles (Fig. 4B). The distributions of CD81 were not significantly altered among Mock-, wild-type-, and FM-transfectant cells, except that more CD81 was found in intracellular vesicles in PC3-KAI1/CD82 wild-type cells on the laminin-coated substratum (Fig. 4B). Meanwhile, CD81 was detected in the nucleus. The reason that CD81 was stained inside the nucleus is unclear and requires additional examination.

A striking difference in cellular morphology among PC3 transfectants was observed when cells were spread on extracellular matrices such as fibronectin or laminin (Fig. 4). PC3-Mock cells displayed a rounded, fan-like, or cobblestone-like morphology with continuous, well-developed lamellipodia that are especially evident on laminin. However, PC3-KAI1/CD82 wild-type cells showed an elongated, polarized, or bipolar morphology. Lamellipodia in KAI1/CD82 wild-type cells are polarized and relatively much less developed. Notably, cellular morphology of PC3-KAI1/CD82 FM cells is completely reverted back to that of Mock cells. The same results were also obtained under phase contrast microscopy (data not shown). Taken together, these data suggest that palmitoylation is responsible for the unique and rigid cellular morphology induced by KAI1/CD82.

The remarkable difference in cellular morphology resulting from KAI1/CD82 palmitoylation makes us question if palmitoylation also regulates cytoskeleton organization, which ultimately determines cell shape. In F-actin-fluorescent staining, the palmitoylation-deficient KAI1/CD82 mutant reversed wild-type KAI1/CD82-induced alter-

ation of actin cytoskeleton, namely the disruption of the cortical actin network (Fig. 4C). The cortical actin network is a circular meshwork-like structure located in the cellular peripheral area and is important for cell motility. Both Mock and FM cells developed well-organized cortical actin networks on either fibronectin or laminin. In KAI1/CD82 wild-type cells, this structure was absent. Thus, KAI1/CD82 palmitoylation regulates actin cytoskeleton organization and is important for the formation of the cortical actin network.

**Palmitoylation Is Required for the Uncoupling of p130<sup>CAS</sup>-CrkII Complex by KAI1/CD82.** Our previous study demonstrated that in Du145 metastatic prostate cancer cells, KAI1/CD82 downregulates the formation of p130<sup>CAS</sup>-Crk complex (38), a critical signaling event leading to cell motility (46). Here, we assessed whether the palmitoylation of KAI1/CD82 is involved in the diminished p130<sup>CAS</sup>-CrkII coupling resulting from the KAI1/CD82 expression. The formation of p130<sup>CAS</sup>-CrkII complexes was significantly decreased in PC3-KAI1/CD82 wild-type cells as compared with those seen in Mock cells (Fig. 5). The reduction of p130<sup>CAS</sup>-CrkII complexes was ~2 to 3-fold among different experiments, as determined by densitometric analysis (data not shown). Importantly, p130<sup>CAS</sup>-CrkII coupling in palmitoylation-deficient KAI1/CD82-transfectant cells was recovered to the level observed in Mock cells (Fig. 5), indicating that the formation of p130<sup>CAS</sup>-CrkII complex was no longer affected by KAI1/CD82 upon the removal of palmitate.

## DISCUSSION

**Palmitoylation Is Needed for the Optimal Inhibition of Cell Motility and Invasiveness via KAI1/CD82.** Herein we have demonstrated that metastasis suppressor KAI1/CD82 is palmitoylated in PC3 cells and that palmitoylation involves all cysteine residues of KAI1/CD82 near the boundary of its transmembrane and cytoplasmic domains, which includes Cys<sup>5</sup>, Cys<sup>74</sup>, Cys<sup>83</sup>, Cys<sup>251</sup>, and Cys<sup>253</sup>. The involvement of multiple cysteine residues in palmitoylation has been seen in other tetraspanins such as CD9 and CD151 (19, 33, 34), but the biochemical relevance remains unknown. Multiple palmitoylation in a tetraspanin molecule may assist putative interactions between its transmembrane domains and may also facilitate the sorting of tetraspanins into acylated tetraspanin webs or tetraspanin-enriched microdomains. Although all five cysteines were mutated, the KAI1/CD82 palmitoylation-deficient mutant still retained different antigen epitopes and showed no alterations in molecular size and in glycosylation, suggesting that the palmitoylation-deficient mutant still remains globally sound in tertiary structure.

Most importantly, we found that the palmitoylation-deficient KAI1/CD82 mutant largely reversed wild-type KAI1/CD82-inhibited migration on fibronectin and laminin, as well as invasion through collagen gel in PC3 metastatic prostate cancer cells. Although previous studies indicated that palmitoylation of tetraspanins such as CD9 and CD151 plays an important role in assembly of tetraspanin-enriched microdomains and contributes to integrin-dependent signaling and cellular morphology (19, 33, 34), whether palmitoylation is required for the tetraspanin-regulated or -mediated cellular functions such as cell migration and cellular fusion remained unanswered. Our study demonstrated for the first time that the palmitoylation of KAI1/CD82 is necessary for its biological activity, *i.e.*, the inhibition of cancer cell migration and invasion. The optimal suppressive activity of KAI1/CD82 on cell migration, as well as invasion, was observed when KAI1/CD82 was palmitoylated. Because the suppressive activity of KAI1/CD82 on metastasis primarily results from KAI1/CD82-induced deficiency in motility and invasiveness (1, 4, 10), KAI1/CD82 palmitoylation is also likely to play an essential role in KAI1/

<sup>4</sup> B. Zhou and X. Zhang, unpublished data.

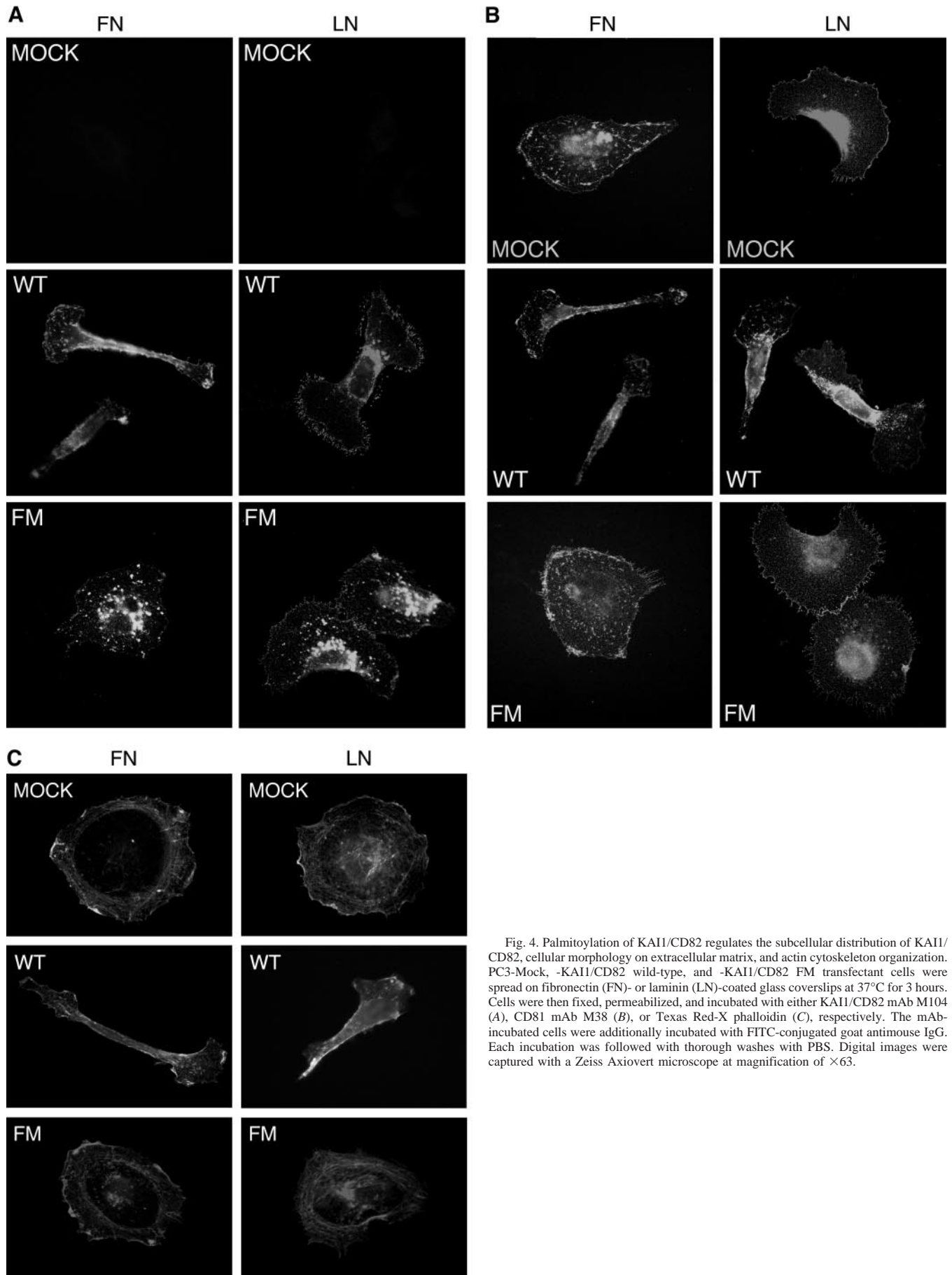


Fig. 4. Palmitoylation of KAI1/CD82 regulates the subcellular distribution of KAI1/CD82, cellular morphology on extracellular matrix, and actin cytoskeleton organization. PC3-Mock, -KAI1/CD82 wild-type, and -KAI1/CD82 FM transfectant cells were spread on fibronectin (FN)- or laminin (LN)-coated glass coverslips at 37°C for 3 hours. Cells were then fixed, permeabilized, and incubated with either KAI1/CD82 mAb M104 (A), CD81 mAb M38 (B), or Texas Red-X phalloidin (C), respectively. The mAb-incubated cells were additionally incubated with FITC-conjugated goat antimouse IgG. Each incubation was followed with thorough washes with PBS. Digital images were captured with a Zeiss Axiovert microscope at magnification of  $\times 63$ .

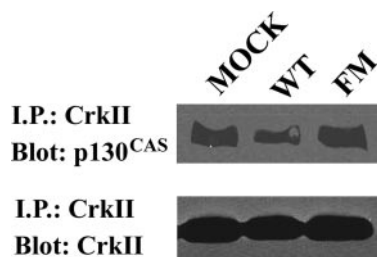


Fig. 5. Palmitoylation is involved in KAI1/CD82-induced down-regulation of p130<sup>CAS</sup>-CrkII coupling. PC3-Mock, -KAI1/CD82 wild-type, and -KAI1/CD82 FM transfectant cells were lysed using 1% NP40 and 0.2% SDS. Lysates were immunoprecipitated with CrkII mAb, and the precipitates were resolved by SDS-PAGE. After electric transference, the membrane was immunoblotted with p130<sup>CAS</sup> mAb. After stripping, the membrane was reblotted with CrkII mAb. CrkII signals serve as loading controls to demonstrate equal loading.

CD82-mediated suppression of cancer metastasis *in vivo*. This issue will be addressed in future studies.

**Palmitoylation Regulates the Cellular and Molecular Events Critical for Cell Motility.** The palmitoylation of KAI1/CD82 is involved in several motility-related cellular events that are affected by KAI1/CD82. These cellular events include overall cellular morphology, lamellipodia formation, and cortical actin organization. The KAI1/CD82 wild-type transfectant displayed a rigid cellular morphology. Some cells looked in a bipolar shape and others polarized with elongated retraction tails. These morphologic features suggest a defect in cell motility. In general, cellular polarization initiates cell motility (47). However, in some KAI1/CD82 wild-type-expressing PC3 cells, the polarized morphology is derived from the deficient retraction of elongated cellular extensions.<sup>5</sup> The palmitoylation-deficient KAI1/CD82 and Mock cells exhibit a fan-like or an asymmetrically rounded morphology with extensive lamellipodia, which are especially evident when plated on laminin. This morphology reflects active motile actions. In term of lamellipodia, they contain intrusive plasma membrane ruffling that leads to cell migration (47, 48). During the formation of lamellipodia, cortical actin fibers assemble into branched or meshwork-like structures, which also promotes cell motility (49). Palmitoylation is apparently required for KAI1/CD82-dependent disruption of lamellipodia formation and cortical actin network organization, both of which contribute to KAI1/CD82's motility-inhibitory activity.<sup>6</sup> Although palmitoylation of tetraspanin CD151 has been reported to regulate cellular morphology (34), our study further demonstrates that tetraspanin palmitoylation also contributes to lamellipodia formation and cortical actin organization.

Our earlier study showed that KAI1/CD82 inhibits Du145 prostate cancer cell migration through p130<sup>CAS</sup>-CrkII coupling (38). Consistent with this observation, the palmitoylation-deficient KAI1/CD82 does not diminish p130<sup>CAS</sup>-CrkII coupling, and the reversal of inhibited cell motility via palmitoylation-deficient KAI1/CD82 correlates with the recovery of p130<sup>CAS</sup>-CrkII coupling in this mutant. Thus, palmitoylation of KAI1/CD82 also appears to participate in the regulation of signaling originated by its associated integrins, which is consistent with what was observed with CD151 (19). Because the Rho small GTPases Cdc42 and Rac determine the formation of lamellipodia and cortical actin meshwork and Rac transmits p130<sup>CAS</sup>-CrkII signaling to the actin cytoskeleton (48), KAI1/CD82 palmitoylation is expected to regulate Rho small GTPase activities. These signaling events are the subject of our ongoing studies.

**Why Palmitoylation Is Important for KAI1/CD82-Mediated Suppression of Motility and Invasiveness.** Palmitoylation often plays a role in directing signaling proteins such as Gα and Src family

kinases to caveolae and/or cholesterol/sphingolipid-enriched lipid rafts (26, 29). Indeed, tetraspanins can be detected in lipid rafts (50–52), and tetraspanin CD9 has been reported to directly bind to cholesterol (25). Therefore, besides partitioning into tetraspanin webs or tetraspanin-enriched microdomains, KAI1/CD82 may also be directed by palmitoylation into cholesterol/sphingolipid-enriched lipid rafts. Thus, KAI1/CD82 may inhibit cell motility by modulating the functional activities of signaling proteins localized within lipid rafts. However, palmitoylation of other tetraspanins such as CD9 and CD151 did not alter their partition into lipid rafts (19, 33, 34). Although the localization of KAI1/CD82 into the lipid rafts has not yet been tested, we extrapolate that, as with CD9 and CD151, KAI1/CD82 palmitoylation would not alter its partition into lipid rafts. Therefore, the functional consequence observed in palmitoylation-deficient KAI1/CD82 unlikely results from an alteration in the direct partitioning of KAI1/CD82 into cholesterol/sphingolipid-enriched lipid rafts.

The common and, also very likely, immediate consequence of the removal of palmitates is the deterioration or disruption of tetraspanin-enriched microdomains or tetraspanin webs (18, 33, 34). The associations between KAI1/CD82 and other tetraspanins such as CD9 and CD81 are indeed disrupted in PC3 cells expressing palmitoylation-deficient KAI1/CD82, in agreement with what was seen with CD9 and CD151 (19, 33, 34). In this regard, KAI1/CD82-mediated inhibition of cell migration is likely determined by the association between KAI1/CD82 and tetraspanin-enriched microdomain. However, the disruption of KAI1/CD82-tetraspanin-enriched microdomain (CD9 and CD81) association was only partial after the removal of palmitates in KAI1/CD82. If the KAI1/CD82-tetraspanin-enriched microdomain interaction is fully responsible for KAI1/CD82 activity, it is not immediately clear why a nearly complete reversal of KAI1/CD82-suppressed cell migration could result from partially decreased KAI1/CD82-tetraspanin-enriched microdomain interactions. A 60% overturn in invasion, however, correlates well with the incomplete disruption of KAI1/CD82 and tetraspanin-enriched microdomain association. Nevertheless, the KAI1/CD82-tetraspanin-enriched microdomain association emerged as an important clue to the mechanism of KAI1/CD82 through the analysis of KAI1/CD82 palmitoylation in this study.

Notably, palmitoylation contributes to the intracellular distribution of KAI1/CD82. Palmitoylation-deficient KAI1/CD82 proteins are preferentially localized in intracellular endosome/lysosome vesicles *versus* plasma membrane. Thus, palmitoylation appears to promote KAI1/CD82 localization to the cell surface. Other studies have also indicated that palmitoylation promotes the cell surface expression of membrane-spanning proteins such as CCR5, thyrotropin receptor, vasopressin V<sub>2</sub> receptor, and adenosine A1 receptor (53–58). Although palmitoylation-deficient KAI1/CD82 *per se* may associate with tetraspanin-enriched microdomain less efficiently than wild-type KAI1/CD82, less surface expression of KAI1/CD82 could also result in the decreased association of KAI1/CD82 with CD9 and CD81, which are mainly localized on the cell surface. Because palmitoylation is a dynamic process, many palmitoylated proteins can be de-palmitoylated, and their functions are regulated by the turnover of palmitoylation (29). For example, palmitoylation and de-palmitoylation regulate the conformation and activity of Gα protein (29). For tetraspanins, palmitoylation is also a regulated process. A recent study showed that the palmitoylation of tetraspanin CD81 was inhibited under oxidative treatment (44). Combining the fact that de-palmitoylation catalyzed by thioesterase PPT1 occurs in lysosome (59), the KAI1/CD82 proteins present in endosome/lysosome vesicles under physiologic conditions may represent the un- or de-palmitoylated KAI1/CD82. In other words, palmitoylation may prevent KAI1/CD82

<sup>5</sup> Unpublished data.

<sup>6</sup> Unpublished data.

degradation in lysosomes by promoting its cell surface expression. Thus, palmitoylation and de-palmitoylation likely contribute to the regulation of KAI1/CD82 trafficking to and/or from the plasma membrane. In migrating cells, vesicle trafficking assists the migratory process, and the fusion of intracellular vesicles at the leading edge directly leads to front extensions of the migrating cell (60). Also, endocytosis and vesicle trafficking potentiate or attenuate the activities of various growth factors and signaling molecules that regulate cell motility (61, 62). Indeed, KAI1/CD82 accelerates the endocytosis of growth factor receptor (16) and integrin.<sup>3</sup> Therefore, the regulation of KAI1/CD82 trafficking could be the mechanistic role that palmitoylation plays in cell migration and invasion.

Taken together, we have shown that palmitoylation of KAI1/CD82 is critical for its motility- and invasiveness-suppressive activity. Palmitoylation is needed for KAI1/CD82-dependent disruption of the cellular and molecular events that are important for cell movement, including lamellipodia formation, cortical actin organization, and p130<sup>CAS</sup>-CrkII coupling. Mechanistically, palmitoylation regulates the distribution (therefore trafficking) of KAI1/CD82 between the plasma membrane and intracellular vesicles and the participation of KAI1/CD82 in tetraspanin-enriched microdomain, which are both likely to be pivotal for the motility-inhibitory activity of KAI1/CD82.

## ACKNOWLEDGMENTS

We thank Drs. Lisa Jennings, Dan Baker, and Svetozar Grgurevich for constructive suggestions and critical review of the article. We also thank all colleagues who provided mAbs used in this study.

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