Metastasis-suppressor KAI1/CD82 induces homotypic aggregation of human prostate cancer cells through Src-dependent pathway

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Accepted 10 January 2003

Abbreviations: SFK, Src family kinases; TM4SF, transmembrane 4 superfamily

Abstract

To investigate the functional role of KAI1/CD82, a metastasis suppressor for human prostate cancer, in the regulation of homotypic cell adhesion, we transfected *KAI1* cDNA into DU 145 human prostate cancer cells and established stable transfectant clones with high KAI1/CD82 expression. The *KAI1* transfectant cells exhibited significantly increased homotypic cell aggregation in comparison with the control transfectant cells. This aggregation of the *KAI1* transfectants was further enhanced upon exposure to anti-CD82 antibody, suggesting that KAI1/CD82 may be involved in the intracellular signaling for the cell adhesion. Among several signal pathway inhibitors tested, PP1, an inhibitor of Src family kinases, significantly suppressed homotypic aggregation of the *KAI1* transfectant cells. Ligation of KAI1/CD82 with anti-CD82 antibody increased endogenous Src kinase activity of the *KAI1* transfectant cells. When different types of *src* expression constructs were retransfected into the *KAI1*-transfected DU 145 cells, kinase-negative mutant *src* transfectant cells exhibited much lower homotypic aggregation than the mock cells transfected with an empty vector. Moreover, homotypic aggregation of the mutant *src* transfectant cells

was not enhanced by KAI1/CD82 ligation with anti-CD82 antibody. These results suggest that Src mediates the intracellular signaling pathway of KAI1/CD82 for the induction of homotypic adhesion of human prostate cancer cells.

Keywords: cell adhesion; metastasis suppressor proteins; neoplasm metastasis; prostatic neoplasms; Src-family kinases

Introduction

The *KAI1* gene is a metastasis-suppressor for many types of human cancers including prostate cancer (Dong *et al.*, 1995; Adachi *et al.*, 1996; Dong *et al.*, 1996; Guo *et al.*, 1996; Ueda *et al.*, 1996; Yu *et al.*, 1997; Yang *et al.*, 2000; Yang *et al.*, 2001). The *KAI1* mRNA expression was reduced in human metastatic prostate cancer cell lines when compared to normal prostate tissue (Dong *et al.*, 1995; Ueda *et al.*, 1996; White *et al.*, 1998). Overexpression of the *KAI1* gene suppressed metastatic ability of rat AT6.1 prostate cancer cells (Dong *et al.*, 1995). The *KAI1* gene encodes an integral membrane protein CD82, which consists of four transmembrane domains and one large extracellular domain, indicating that it is a member of transmembrane 4 superfamily (TM4SF). The TM4SF has at least 15 members, including CD9, CD63, CD81, CD82 and CD151 (for reviews, Maecker *et al.*, 1997; Bienstock and Barrett, 2001), and some of them have been suggested to be involved in the regulation of cellular activities related to metastasis (Atkinson *et al.*, 1984; Ikeyama *et al.*, 1993; Miyake *et al.*, 1996; Domanico *et al.*, 1997).

Although the physiological role of KAI1/CD82 is not fully understood, it has been suggested that KAI1/CD82 plays putative roles in the regulation of intercellular adhesion. C33 antigen, which is identical to KAI1/CD82, has been known as a cell surface molecule to be involved in cell adhesion in the process of HTLV-1-induced syncytium formation in human T cells (Fukudome *et al.*, 1992). Similar to other members of TM4SF, KAI1/CD82 is physically associated with other TM4SF members, integrins, and other surface molecules including CD4, CD8, CD19, CD21 and MHC class I and class II in lymphocytes, most of which are strongly involved in cell adhesion process

during lymphocytes activation (Imai and Yoshie, 1993; Angelisova *et al.*, 1994; Mannion *et al.*, 1996; Rubinstein *et al.*, 1996; Lagaudriere-Gesbert *et al.*, 1997; Shibagaki *et al.*, 1999). Besides immune cells, KAI1/CD82 was shown to increase homotypic aggregation of epithelial cells including melanoma and colon cancer cells (Takaoka *et al.*, 1998a; 1998b). Since a reduction in the ability of tumor cells to adhere to each other facilitates their detachment from the primary tumor mass and invasion of the surrounding tissue, the suppressing role of KAI1/CD82 for cancer invasion and metastasis may be explained or correlated with its modulation of homotypic cell adhesion property. However, it is not known at present how KAI1/CD82 participates in the homotypic cell adhesion of epithelial cells.

As an effort to understand the mechanism(s) underlying the regulatory role of KAI1/CD82 in homotypic cell adhesion, we examined the regulation mode of homotypic cell adhesion by KAI1/CD82 in *KAI1*-transfected DU 145 human prostate adenocarcinoma cells. Our present data suggest that KAI1/CD82 signaling pathway induces homotypic adhesion of human prostate cancer cells through Src tyrosine kinase.

Materials and Methods

Cell culture and transfection

DU 145 human prostate adenocarcinoma cells were grown in RPMI 1640 (Gibco-BRL, Grand Island, NY) with 10% FBS at 37°C, 5% CO₂. For production of *KAI1*-transfected cells, DU 145 cells were transfected with pCMV-neo/*KAI1*, which was kindly provided by Dr. J. Carl Barrett (NIEHS, Research Triangle Park, NC), using lipofectAMINE (Gibco-BRL) according to the manufacturer's instructions. pcDNA3 vector (Invitrogen, Carlsbad, CA) only was also transfected as a control. Transfectant clones were isolated by growth in RPMI 1640 containing 10% FBS and 400 µg/ml G418 (Gibco-BRL). Stable high *KAI1* expressing clones were identified by Western blotting using anti-CD82 monoclonal antibody (mAb). In second-transfection experiments, the cDNA expression construct encoding c-Src, v-Src, or dominant-negative mutant Src-lacking kinase activity was cotransfected with pTK/Hyg (Invitrogen) into a DU 145 transfectant clone with high *KAI1* expression, DU 10-16. Src transfectants were selected with 400 µg/ml G418 and 200 µg/ml hygromycin (Gibco-BRL), and colonies were selected for Src protein expression by Western blotting.

Western blotting analysis

Cultured cells were lysed in TNEN buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 2 mM EDTA, 1%

NP-40, 1 mM PMSF, 10 µg/ml aprotinin, 20 µg/ml leupeptin and 2 mM benzimidazole) on ice for 10 min. After centrifugation at 15,000 *g* for 10 min, the supernatants were collected and quantified using Bradford assay. The proteins were mixed with SDS-sample buffer, boiled for 5 min, and then separated by 10% SDS-PAGE. After transferring to Immobilon-P (Millipore, Bedford, MA) membrane, proteins were probed with 2 µg/ml anti-CD82 mAb (BD PharMingen, San Diego, CA). Membranes were washed and probed with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, St. Louis, Mo), washed, and developed with ECL reagent (Amersham, Piscataway, NJ).

Cell aggregation assay

Adherent cells were rendered single cell suspension by trypsinization followed by incubation with 5 mM EDTA in PBS lacking Ca²⁺ and Mg²⁺ at 37°C for 5 min and by 7 gentle passes through a 22-gauge needle. After cells were washed with Puck's saline (5 mM KCl, 140 mM NaCl, 8 mM NaHCO₃, pH 7.4), suspensions of single cells (1 × 10⁵ cells/ml of Puck's saline) were seeded into each well of a 24-well culture plate, and incubated in 5% CO₂ at 37°C with agitation at 70-80 rpm using an orbital shaker. Photographs were taken at every 10 min after incubation under a microscope on 3 predetermined fields, and both the total cell number (A) and the number of cells remaining as single cells (B) were counted. The results were expressed as the percentage of cells that formed aggregates as follows: (A-B)/A × 100 (%). For KAI1/CD82 ligation, the *KAI1* transfectant cells were pretreated with anti-CD82 mAb for 3 h and then with goat anti-mouse IgG (γ chain-specific) for 4 h before single cell suspensions were made for aggregation assays.

Src kinase assay

Anti-Src mAb immune complex kinase assay was performed as described (Bolen *et al.*, 1987). Briefly, after treatment of the cells with either anti-CD82 mAb or mouse IgG for 2 h and then with goat anti-mouse IgG for 1 h, total cellular lysates were harvested in RIPA buffer supplemented with protease inhibitors and sodium orthovanadate (1 mM). Cellular proteins (200 µg) were incubated with anti-v-Src mAb (Oncogene Science; 10 µg) and immunoprecipitated using protein A-agarose (Sigma). Immunoprecipitates were washed twice in cold lysis buffer and once in kinase buffer and added to an *in vitro* kinase reaction mixture consisting of 50 mM PIPES (pH 7.0), 10 mM MnCl₂, 10 mM DTT, 10 µM ATP, 2 µg of acid-denatured enolase, and 10 µCi of [³²P]ATP (NEN). The reaction was incubated at 30°C for 30 min and then

stopped by boiling in SDS-sample buffer. After electrophoresis on a 10% polyacrylamide gel, the radioactive proteins were visualized by autoradiography. Src protein in immunoprecipitate was detected by Western blotting using 5 μ g/ml anti-v-Src mAb (Oncogene Science, Cambridge, MA).

Results

KAI1/CD82 promotes homotypic adhesion of prostate cancer cells

To examine the functional effects of *KAI1* expression on the metastatic phenotypes of prostate cancer cells,

Figure 1. Homotypic adhesion of the *KAI1*-transfected DU 145 human prostate carcinoma cells. (A) *KAI1*/CD82 protein levels in the stable clones of *KAI1*-transfected DU 145 cells were analyzed by Western blotting using anti-CD82 mAb. (B) Each transfectant clone (1×10^5 cells/ml) was pretreated with either anti-CD82 mAb or mouse IgG for 3 h and then with goat anti-mouse IgG for 4 h. After disrupting cell aggregates by pipetting, the cells were incubated in the Puck's saline with orbital shaking. The degree of aggregate was examined under microscope, and the result was expressed as a percent of total cell numbers forming aggregates at 30 min after incubation. Data represent the mean $\pm$ SEM of triplicate determinations. Asterisks indicate that the differences are statistically significant (**P*, ***P* < 0.01, Student's *t*-test).

we transfected *KAI1* expression construct into DU 145 human metastatic prostate adenocarcinoma cells devoid of *KAI1* expression. Several *KAI1* transfectant clones stably overproduced *KAI1*/CD82 protein (Figure 1A). When cell aggregation assay was performed, the *KAI1* transfectant clones exhibited much higher homotypic cell adhesiveness than the control transfectant, indicating that *KAI1*/CD82 expression induces homotypic adhesion of DU 145 prostate cancer cells (Figure 1B). Since many transmembrane proteins have known to be activated by oligomerization, we next treated the cells with mouse anti-CD82 mAb followed by goat anti-mouse IgG. As a result, cross-linking of *KAI1*/CD82 proteins on the cell surface with anti-CD82 mAb significantly increased homotypic cell adhesion of the *KAI1* transfectant clones, whereas no effect of anti-CD82 mAb treatment on cell aggregation was observed in the control transfectant devoid of *KAI1*/CD82 expression (Figure 1B). These results suggest that *KAI1*/CD82 promotes homotypic cell adhesion of prostate cancer cells by triggering intracellular signal involved in homotypic cell adhesion process.

A Src kinase inhibitor, PP1 suppresses homotypic cell adhesion of the *KAI1* transfectant

As an effort to explore signaling pathway(s) taken downstream of *KAI1*/CD82 for the induction of homotypic cell adhesion, we examined homotypic adhesion of the *KAI1* transfectant cells in the presence of several inhibitors for signal transduction mediators in a

Figure 2. Homotypic adhesion of the *KAI1* transfectant cells in the presence of inhibitors for signal transduction mediators. A *KAI1* transfectant clone, DU 10-16 was pretreated with GF109203X (2 μ M), U-73122 (5 μ M), wortmannin (0.1 μ M), U0126 (5 μ M) or PP1 (1 μ M) for 30 min, and then assayed for homotypic cell aggregation as in Figure 1. Data represent the mean $\pm$ SEM of triplicate determinations. Asterisk indicates that the difference is statistically significant (**P* < 0.01, Student's *t*-test).

Figure 3. Effect of an inhibitor of Src family kinases, PP1 on homotypic adhesion of the *KAI1* transfectant cells with cross-linked CD82. A *KAI1* transfectant clone, DU 10-16 was pretreated with anti-CD82 mAb for cross-linking of KAI1/CD82 proteins on cell surface and then with 0.5 μ M PP1 for 30 min, and cell aggregation assay was performed for the indicated periods. Data represent the mean $\pm$ SEM of triplicate determinations. Asterisk indicates that the differences is statistically significant (* $P < 0.01$, Student's *t*-test).

several fold higher, but not lethal, dose than a dose of IC_{50} : GF109203X, a protein kinase C inhibitor; U73122, a phospholipase C inhibitor; wortmanin, a phosphatidylinositol 3-kinase inhibitor; U0126, an extracellular signal-regulated kinase (MEK) inhibitor; PP1, an inhibitor of Src family kinases (SFKs). None of the inhibitors affected homotypic adhesion of the control transfectant cells. However, the inhibitor of SFKs, PP1 suppressed homotypic adhesion of the *KAI1* transfectant cells to a level at the control transfectant cells, whereas the other inhibitors tested did not exhibit the inhibition effect (Figure 2). In addition, the positive effect of KAI1/CD82 ligation with anti-CD82 mAb on homotypic cell adhesion was partially blocked when the *KAI1* transfectant cells were pretreated with PP1 (Figure 3). Because the inhibition effect of PP1 was not exerted at concentrations lower than 250 nM, it seems likely that Src kinase, the only kinase showing IC_{50} higher than 100 nM for PP1 among SFKs, is functionally involved in signaling pathway(s) of KAI1/CD82 for the induction of homotypic adhesion of human prostate cancer cells.

Activation of KAI1/CD82 increases cellular Src kinase activity

To clarify if a signal triggered by activated KAI1/CD82 indeed increases Src kinase activity of prostate cells,

Figure 4. Src kinase activity of the *KAI1* transfectant cells after cross-linking of KAI1/CD82 with anti-CD82 mAb. Lysates of the cells pretreated with either anti-CD82 mAb or mouse IgG were immunoprecipitated with anti-Src mAb. (A) Immune complex kinase assay was carried out for c-Src with acid-denatured enolase as the exogenous substrate. (B) Western blot showing Src protein level in immunoprecipitates used for immune complex kinase assay.

we examined endogenous Src kinase activity of *KAI1* transfectant cells after cross-linking of KAI1/CD82 with anti-CD82 mAb. As shown in Figure 4, Src kinase activity of the *KAI1* transfectant cells was significantly increased by anti-CD82 mAb treatment, while no effect of anti-CD82 mAb treatment on Src kinase activity was observed in the control transfectant cells devoid of KAI1 expression. Since cellular Src protein levels of the *KAI1* transfectants were similar to that of the control transfectant, an increase in Src kinase activity by anti-CD82 mAb treatment results from activation of KAI1/CD82 in the *KAI1* transfectant cells.

Overexpression of mutant Src protein lacking kinase activity inhibits homotypic adhesion of the *KAI1* transfectant cells

To confirm the signal-mediating role of Src kinase in the KAI1/CD82 signaling pathway(s) to induce homotypic adhesion of prostate cancer cells, we generated stable *src* transfectant clones after retransfection of the *KAI1*-transfected DU 145 cells with an expression construct encoding c-Src, v-Src or dominant-negative (DN) mutant Src lacking kinase activity. Src protein levels in those transfectant clones were far greater than an endogenous Src protein level (Figure 5A). We next analyzed homotypic adhesion of the DU 145 cells double transfected with *KAI1* and different types of *src*. The overexpression of c-*src* and v-*src* does not significantly affect homotypic adhesion of the *KAI1* transfectant cells (Figure 5B). However, the DN mutant *src*-transfected cells displayed 1/4 level of homotypic adhesion of the mock transfectant cells having endogeneous *src* expression only, indicating that overproduction of kinase-negative Src mutant protein interferes the promoting effect of KAI1/CD82 on homotypic adhesion of DU 145 cells. In addition, ho-

Figure 5. Homotypic adhesion of the *KAI1* transfectant cells after retransfection with the different types of *src*. (A) Src expression levels of the *src* transfectant clones were examined by Western blot analysis using anti-Src mAb. (B) Three different types of *src*-transfected DU 145 clones that had been pretransfected with *KAI1* were assayed for their homotypic cell aggregation as in Figure 1. Data represent the mean $\pm$ SEM of triplicate determinations. Asterisks indicate that the differences are statistically significant (**P*, ***P* < 0.01, Student's *t*-test).

motypic adhesion of the *KAI1* transfectant cells over-expressing DN mutant *src* was not increased by ligation of *KAI1*/CD82 with anti-CD82 mAb. This result strongly suggests that *KAI1*/CD82 induces homotypic adhesion of human prostate cancer cells through Src kinase-dependent pathway.

Discussion

Several studies have shown that *KAI1*/CD82 is involved in a variety of signal transduction pathways.

In monocytes, cross-linking of CD82 with anti-CD82 mAb induces cytoplasmic calcium fluxes and phosphorylations (Lebel-Binay *et al.*, 1995a). Treatment of human T cells with anti-CD82 mAb leads to cytokine production (IL-2 and IFN- γ) and modulates cellular proliferation by delivering co-stimulatory signals upon TCR/CD3 activation pathway (Lebel-Binay *et al.*, 1995b). In addition, ligation of CD82 with anti-CD82 mAb synergizes with and stabilizes anti-CD3 mAb-induced tyrosine phosphorylation, along with cytoskeletal rearrangements (Lagaudrière-Gesbert *et al.*, 1998). This CD82 signal also enhances both homotypic and heterotypic cell adhesion between T cells and antigen antigen-presenting cells, leading to stronger T cell co-stimulation (Shibagaki *et al.*, 1998). Therefore, it seems likely that CD82 co-stimulates T cells not only by sending intra-T cell signals but also by inducing enhanced T cell-antigen presenting cell interaction. Cell adhesion molecules such as LFA-1, VLA integrins and ICAM have also shown to deliver co-stimulatory signals for full T cell activation (Wingren *et al.*, 1995; Udagwa *et al.*, 1996). Besides immune cells, we here demonstrated the signal transducing role of *KAI1*/CD82 in cells of epithelial origin. When *KAI1*-transfected DU 145 human prostate cancer cells was treated with anti-CD82 mAb, a significant increase in homotypic cell adhesion was observed (Figure 1B). The enhancement of homotypic cell adhesion by ligation of *KAI1*/CD82 was blocked by treatment of PP1, an inhibitor of Src family kinases, and overexpression of mutant Src lacking kinase activity, implicating the functional involvement of Src kinase in *KAI1*/CD82 signaling pathway(s) for the induction of homotypic adhesion of prostate cells (Figures 3 and 5).

Although the ligand(s) for *KAI1*/CD82 is not yet known, cross-linking of *KAI1*/CD on cell surface with anti-CD82 mAb clearly increases endogenous Src kinase activity of the *KAI1*-transfected prostate cells (Figure 4). The involvement of Src family kinases (SFKs) in CD82 signaling pathway was also observed in T cell activation process (Lagaudrière-Gesbert *et al.*, 1998). CD82 ligation with anti-CD82 mAb when used in association with suboptimal doses of anti-CD3 mAb leads to high levels of IL-2 production in Jurkat cells. Jurkat cells upon co-activation via CD82 associated with suboptimal doses of anti-CD3 mAb produces much higher amount of IL-2 than the cells upon strong stimulation with high doses of anti-CD3 mAb alone. However, JCam-1 cells, p56^{lck}-deficient mutant of Jurkat cells, produces 20-fold less IL-2 than the parental Jurkat cells, upon co-engagement of CD82 and low numbers of CD3. The decrease in the co-stimulatory activity of CD82 on IL-2 production in JCam-1 cells strongly suggests that CD82 delivers T cell co-stimulating signal(s) for IL-2 production through

a member of Src family kinases, p56^{lck}. On the other hand, tyrosine phosphorylation by endogenous SFKs and v-Src located at adherent junctions has been suggested to be associated with disruption of epithelial cell-cell adhesions (for reviews, Frame, 2002; Frame *et al.*, 2002). For example, Madin-Darby canine kidney (MDCK) cells lose cell-cell contact and epithelial differentiation by vanadate treatment and v-Src activity (Volberg *et al.*, 1992; Behrens *et al.*, 1993). In addition, v-Src activity in rat 3Y1 cells perturbs cadherin-mediated cell-cell adhesion (Hama-guchi *et al.*, 1993). Overexpression of a naturally occurring, activated Src-531 mutant decreases homotypic adhesion in human colon cells and src-transformed rat fibroblasts, possibly by disrupting the cadherin/catenin complex (Irby and Yeatman, 2002). c-Src activity is also required for the disruption of cadherin-dependent homotypic cell adhesion. In rat bladder carcinoma cells, EGF-induced cell scattering requires c-Src activity (Boyer *et al.*, 1997). Cadherin-dependent keratinocyte cell-cell contacts are also disrupted by catalytic activity of c-Src (Owens *et al.*, 2000). It therefore seems that Src kinase located at cell-cell contact sites plays a negative role in cadherin-dependent homotypic cell adhesion. However, there are conflicting reports regarding the role of endogenous SFKs in the regulation of cell-cell adhesion. Keratinocytes of Fyn-deficient mouse and the epidermis of mice with a disruption in both the *fyn* and *c-src* genes display impaired cell-cell adhesion, suggesting that tyrosine phosphorylation by SFKs has a positive role in the assembly of cell-cell adhesions between normal keratinocytes (Calautti *et al.*, 1998). p120 catenin (p120^{CTN}), a substrate of Src kinase, has also been controversially suggested to mediate both cell-cell assembly and strengthening, and also adhesion disassembly (Yap *et al.*, 1998; Aono *et al.*, 1999). These discrepant observations as to how SFKs contribute to cell-cell adhesion dynamics may reflect the dual role of SFKs in the modulation of cell-cell adhesion, possibly due to the multidomain nature of the Src kinases. Intracellular location of endogenous SFKs might be one of the important factors determining the role of SFKs in the modulation of homotypic cell adhesion; that is, Src kinases located at cell-cell contact sites disrupts cadherin-dependent adherent junction assembly, possibly by phosphorylating β -catenin and p120^{CTN} (Roura *et al.*, 1999; Thoreson *et al.*, 2000). On the other hand, Src kinases associated with signal transducing molecules such as CD3 and CD82 deliver intracellular activation signals, leading to increased expression of certain genes, as exemplified in cytokine production of T lymphocytes. It is therefore possible that Src-mediated signaling pathway(s) triggered by KAI1/CD82 may up-regulate or activate cell adhesion molecule(s) directly participating

in homotypic adhesion of epithelial cells. Although it is uncertain how KAI1/CD82 signaling pathway(s) induces homotypic adhesion of prostate cells, the data in this study suggest that Src kinase is one of the molecules which can promote homotypic adhesion of prostate cells by mediating signal transduction pathway(s) triggered by KAI1/CD82.

Acknowledgement

This study was supported by a research fund from Korea Research Foundation (KRF-99-042-F00039).

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