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Tyrosine phosphorylation of vinexin in v-Src-transformed cells attenuates the affinity for vinculin

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ABSTRACT

Vinexin is an adaptor-type focal adhesion protein that interacts with vinculin. Here, we report the tyrosine phosphorylation of vinexin α in v-Src-transformed NIH3T3 cells. Point mutational analysis of vinexin α clarified that three tyrosine residues in vinexin α were phosphorylated. A non-phosphorylatable mutant of vinexin α had higher binding affinity for vinculin than its wild-type counterpart. In conclusion, vinexin α is tyrosine phosphorylated in v-Src-transformed cells, and this tyrosine phosphorylation of vinexin α attenuates the association of vinexin α with vinculin.

Introduction

Cell-to-ECM (extracellular matrix) adhesion is mediated by cell adhesion molecule integrins on the cell surface. Upon interaction with the ECM, integrins are activated and clustered, and then a group of cytoplasmic proteins, called focal adhesion proteins, accumulate at cell adhesion sites. As integrins have no enzymatic activity, these proteins seem to have important roles in transmitting adhesion signals to the cell [1–4].

There is growing body of evidence that tyrosine phosphorylation and dephosphorylation of focal adhesion proteins serve as a switch for modulating the cell adhesion structure during formation, maturation, and disruption of cell adhesions [5,6]. Focal adhesion proteins are tyrosine phosphorylated upon cell adhesion [7] or growth factor stimulation [8–10], mainly by tyrosine kinases Src and FAK (focal adhesion kinase), both of which also localize to focal adhesions. Genetic ablation of Src or PTP-PEST (protein tyrosine phosphatase-PEST), which acts as a tyrosine phosphatase for focal adhesion proteins, disrupts the turnover of focal adhesions, resulting in the formation of abnormally large focal adhesion and misregulation in cell motility [11,12].

In cells transformed by active Src or v-Src, focal adhesions are often disorganized, in part due to the elevated tyrosine phosphorylation of focal adhesion proteins. This leads to increased turnover of the adhesion structure and eventual elevated motility [13,14]. In addition, tyrosine phosphorylation of focal adhesion proteins has been reported to promote the formation of invasive structures known as invadopodia or podosomes [15–19]. From these observations, it is clear that tyrosine phosphorylation of focal adhesion proteins has important roles in the acquisition of increased motility and invasive potential in tumor cells; however, the functions of tyrosine phosphorylation of these proteins in the regulation of biochemical properties, such as an affinity to their binding partners, are not fully understood.

The focal adhesion protein vinexin is an adaptor-type protein that associates with cytoskeletal and signaling proteins, such as vinculin, lp-dlg, Sos, and ERK MAP kinase [20–24]. Overexpression of vinexin α enhances the actin cytoskeleton at focal adhesions [20,25], while vinexin β promotes anchorage-independent activation of ERK in response to growth factor stimulation or cell adhesion by associating with activated ERK [22,23]. EGFR activation is also sustained by vinexin β [26]. In several cancer tissues, including ovarian, lung and breast cancers, the expression of vinexin is down-regulated ([27] and Oncomine Research Platform (www.oncomine.org)). We recently clarified that vinexin expression is down-regulated in v-Src-transformed cells, and that this leads to increased cell motility [28]. Vinexin can be tyrosine phosphorylated by Abl tyrosine kinase [29]; however, whether vinexin is tyrosine phosphorylated in v-Src-transformed cells, and the

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significance of tyrosine phosphorylation of vinexin in cancer cells, are still unclear.

In this study, we demonstrated that vinexin α is tyrosine phosphorylated in v-Src-transformed cells. Point mutational analysis clarified that at least three tyrosine residues of vinexin α are tyrosine phosphorylated and that blockage of tyrosine phosphorylation by mutations increased the binding affinity of vinexin α to vinculin.

Materials and methods

Cell culture. Wild-type and v-Src-transformed NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (HyClone), and Cos-7 cells were cultured in DMEM with 10% fetal calf serum (Invitrogen). The cells were maintained under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Antibodies and reagents. Monoclonal (M2) and polyclonal antibodies against FLAG epitope tag and monoclonal antibody against vinculin were purchased from Sigma. Monoclonal antibodies against Abl and phosphotyrosine (pY20) were obtained from BD Pharmingen. Polyclonal antibodies against ERK2 and phospho-Src (Y418) were purchased from Santa Cruz Biotechnologies and Biosource, respectively. The polyclonal antibody against vinexin was described previously [20]. Pervanadate was prepared as follows; 50 mM sodium orthovanadate and 1.7 mM H_2O_2 were incubated for 20 min in HEPES buffer (20 mM, pH 7.3), followed by additional incubation with 200 μg/ml catalase for 5 min. The resulting solution was added to culture medium at 1:100 dilution and incubated for 3 h prior to cell lysis.

Plasmids, site-directed mutagenesis, and transfection. Expression plasmids for FLAG-tagged vinexin α and β were described previously [20]. Mutants of vinexin α, in which tyrosine residues (Y127, 170, 196, 269, and 394) were replaced with phenylalanine, have been described [29] or were generated using the Quick Change Site-directed Mutagenesis Kit (Stratagene). Transfection into NIH3T3 cells and Cos-7 cells was performed with Lipofectamine Reagent and Plus Reagent (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation. Cells were lysed with either RIPA buffer or 1% Triton X-100 containing 10 μg/ml aprotinin, 100 μg/ml *p*-amidinophenyl methanesulfonyl fluoride hydrochloride, 10 μg/ml leupeptin and phosphatase inhibitor cocktail I and II (Sigma). Equal amounts of cell lysates were subjected to incubation with anti-FLAG antibody, anti-vinexin antibody, or control rabbit IgG for 1 h at 4 °C, followed by further incubation with protein G Sepharose beads (Sigma). Precipitated proteins were separated by SDS–PAGE and subjected to immunoblotting with the respective antibodies.

In vitro kinase assay. FLAG-vinexin α was immunoprecipitated using anti-FLAG M2 monoclonal antibody from lysates of Cos-7 cells transiently expressing the protein. Immunoprecipitated beads were resuspended in reaction buffer (10 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.01% Triton X-100, 1 μM DTT, 300 μM ATP) containing purified active Src (Millipore), and incubated at 30 °C for 30 min. The beads were washed with ice-cold 1% Triton X-100 in PBS. Phosphorylation of FLAG-vinexin α was detected by immunoblotting with anti-phosphotyrosine antibody.

Results

Tyrosine phosphorylation of vinexin α in v-Src-transformed NIH3T3 cells

To examine whether vinexin is phosphorylated in wild-type and v-Src-transformed cells, FLAG-tagged vinexin α and its N-terminal truncated isoform vinexin β were transfected into wild-type and v-Src-transformed NIH3T3 cells, and then immunoprecipitated with anti-FLAG antibody. Tyrosine phosphorylation of each protein was examined by immunoblotting with anti-phosphotyrosine antibody (Fig. 1A). In v-Src-transformed cells, tyrosine phosphorylation of vinexin α was detected, while tyrosine phosphorylation of vinexin β was not. In wild-type NIH3T3 cells, vinexin α was scarcely phosphorylated (Fig. 1A). To explore the tyrosine phosphorylation of endogenously expressing vinexin, cell lysate extracted from v-Src-transformed NIH3T3 cells was immunoprecipitated with anti-vinexin antibody and immunoblotted with anti-vinexin and anti-phosphotyrosine antibodies. Comparable amounts of both vinexin α and vinexin β were included in immunoprecipitates, and tyrosine phosphorylation of vinexin α, but not vinexin β, was detected (Fig. 1B). These results indicated that vinexin α is tyrosine phosphorylated in v-Src-transformed cells.

To determine whether Src can directly phosphorylate vinexin α, FLAG-vinexin α was partially purified from vinexin α-transfected Cos-7 cells and incubated with or without purified active Src *in vitro*. Tyrosine phosphorylation of FLAG-vinexin α was not detected in the absence of active Src, but was detected after incubation with active Src (Fig. 1C). This result indicates that v-Src can directly phosphorylate vinexin α in v-Src-transformed NIH3T3 cells.

Tyrosine phosphorylation of vinexin α in wild-type NIH3T3 cells

Many focal adhesion proteins are tyrosine phosphorylated not only in transformed cells, but also in normal cells to regulate adhesion dynamics [7,11,30]. To examine whether vinexin α is also tyrosine phosphorylated in non-transformed cells, tyrosine phosphorylation of FLAG-vinexin α was evaluated in wild-type NIH3T3 cells. Under normal conditions, vinexin α was scarcely tyrosine phosphorylated as demonstrated in Fig. 1A. However, when cells were treated with tyrosine phosphatase inhibitor pervanadate (PV), we detected moderate tyrosine phosphorylation of vinexin α, although it was weaker than that in v-Src-transformed NIH3T3 cells (Fig. 1D). Taken together, these results imply that vinexin α is also tyrosine phosphorylated in normal cells.

Cell detachment attenuates tyrosine phosphorylation of vinexin α

Tyrosine phosphorylation of several focal adhesion proteins depends on cell adhesion and subsequent integrin activation [7]. Interestingly, some Src substrates are phosphorylated in an adhesion-dependent manner even in v-Src-transformed cells [31]. To examine whether tyrosine phosphorylation of vinexin α depends on cell adhesion, v-Src-transformed NIH3T3 cells expressing FLAG-vinexin α were cultured in an adherent state, kept in suspension, or replated on fibronectin-coated dishes, and then tyrosine phosphorylation of vinexin α was evaluated (Fig. 2). Tyrosine phosphorylation of vinexin α fell after cells were kept in suspension. Phosphorylation then significantly increased 4 h after incubation on fibronectin. Interestingly, v-Src activity was not affected by cell adhesion during the experiment, judging from Western blotting using anti-active Src (phosphorylated Src at tyrosine 418) (Fig. 2). This result indicates that the tyrosine phosphorylation of vinexin α is regulated by cell adhesion even in v-Src-transformed cells, although the v-Src activity is unchanged.

Identification of phosphorylated tyrosine residue(s) in vinexin α

The observation that vinexin α, but not vinexin β, was phosphorylated in v-Src-transformed NIH3T3 cells suggests that the N-terminal region of vinexin α that does not overlap vinexin β contains phosphorylated tyrosine residue(s). Vinexin α contains five tyrosine residues in the N-terminal region (Fig. 3A). To examine

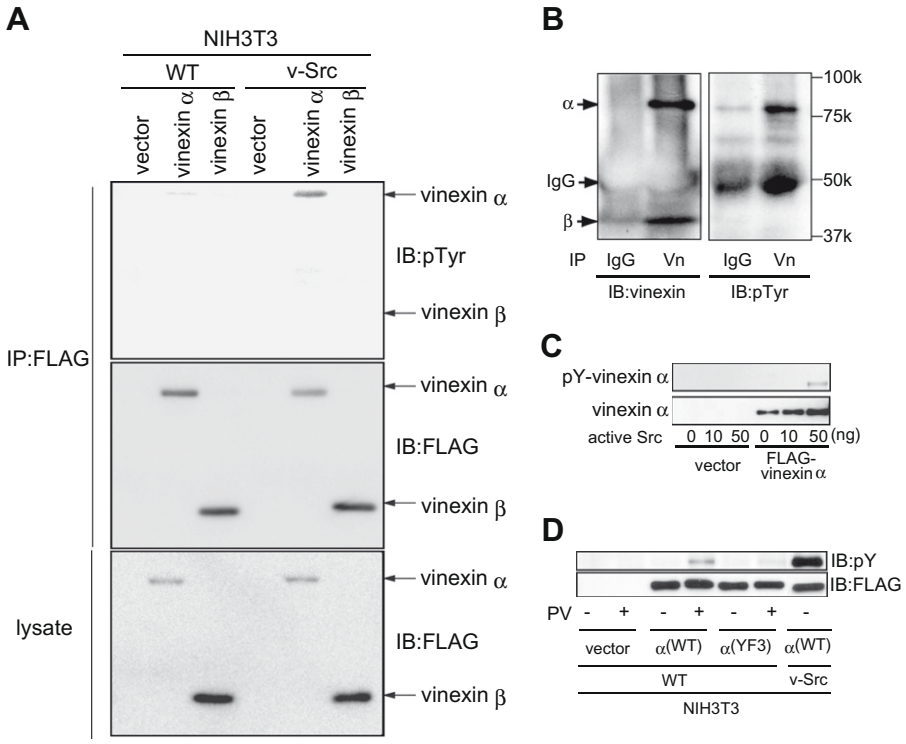

Fig. 1. Vinexin α, but not vinexin β, was tyrosine phosphorylated in v-Src-transformed NIH3T3 cells. (A) FLAG-tagged vinexin α or vinexin β was overexpressed in either wild-type (WT) or v-Src-transformed NIH3T3 cells. Overexpressed protein was immunoprecipitated with anti-FLAG antibody, and tyrosine phosphorylation was detected by Western blotting using anti-phosphotyrosine (pTyr) antibody. (B) Lysates from v-Src-transformed NIH3T3 were immunoprecipitated with either preimmune rabbit IgG or anti-vinexin antibody (Vn). Immunoprecipitated endogenously expressed vinexin was examined using anti-vinexin or anti-phosphotyrosine (pTyr) antibody. (C) FLAG-tagged vinexin α was overexpressed in Cos-7 cells, and partially purified by immunoprecipitation with anti-FLAG antibody. The proteins were incubated with the indicated amount of active Src. Phosphorylation of immunoprecipitated protein was detected by Western blotting using anti-phosphotyrosine antibody. (D) Empty vector, FLAG-vinexin α (WT) or its mutant (YF3) was transfected into wild-type or v-Src-transformed NIH3T3 cells. After transfection, cells were treated (+) or not treated (−) with 50 mM pervanadate (PV) for 3 h. Tyrosine phosphorylation of vinexin α was determined as in (A).

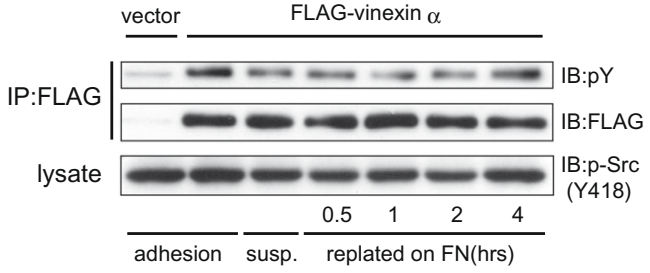

Fig. 2. Tyrosine phosphorylation of vinexin α depended on cell adhesion. v-Src-transformed NIH3T3 cells transiently expressing FLAG-vinexin α were either kept adhered to culture dishes (adhesion), detached and kept in suspension (susp.), or replated on fibronectin-coated dishes (FN) for the indicated hours. FLAG-vinexin α was immunoprecipitated from cell lysate and tyrosine phosphorylation was evaluated by anti-phosphotyrosine antibody (upper panel). Activity of Src was evaluated by antibody against phosphotyrosine 418 of Src (lower panel).

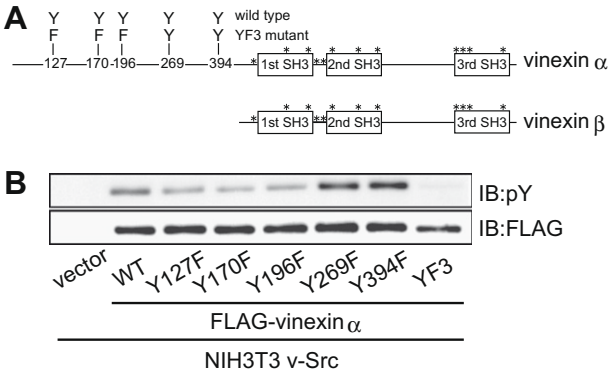

Fig. 3. Three tyrosine residues in N-terminal half of vinexin α were phosphorylated in v-Src-transformed NIH3T3 cells. (A) Schematic of vinexin α and vinexin β. Numbers indicate the positions of tyrosine residues located in N-terminal half of vinexin α. Each tyrosine (Y) residue was mutated to phenylalanine (F) to create mutants. As for YF3 mutant, tyrosines 127, 170, and 196 were replaced with phenylalanine. Asterisks indicate tyrosine residues in the region overlapping vinexin α and β. (B) Empty vector or plasmids encoding FLAG-tagged vinexin α or mutants were transfected into v-Src-transformed NIH3T3 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody. Tyrosine phosphorylation of immunoprecipitated protein was evaluated by Western blotting with anti-phosphotyrosine (pY) antibody.

which tyrosine residue is phosphorylated, five vinexin α mutants were generated, in which one of these tyrosine residues was mutated to phenylalanine. When overexpressed in v-Src-transformed NIH3T3 cells, tyrosine phosphorylation of Y127F, Y170F, and Y196F mutant was lower than that of wild-type vinexin α (Fig. 3B). Substitution of these three tyrosine residues to phenylalanine (YF3) almost totally suppressed the phosphorylation of vinexin α (Fig. 3B). YF3 mutation also impaired the phosphorylation of vinexin α in pervanadate-treated wild-type NIH3T3 cells (Fig. 1D). The results indicated that these three tyrosine residues are major phosphorylation sites both in v-Src-transformed and wild-type cells.

Tyrosine phosphorylation of vinexin α affects binding affinity for vinculin

Vinexin functions as an adaptor protein and binds to vinculin, ERK, Abl, and several other cytoskeletal and signaling molecules

[20,23,29]. To explore the role of tyrosine phosphorylation of vinexin α in v-Src-transformed cells, we examined the affinity for these binding partners using wild-type and YF3 mutant of vinexin α. FLAG-tagged wild-type and YF3 vinexin α were transfected into v-Src-transformed NIH3T3 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and the amount of co-precipitated proteins was assessed (Fig. 4). Vinculin, ERK2 and Abl were co-precipitated with wild-type FLAG-vinexin α, as we reported previously for other cell types. ERK2 and Abl were co-precipitated comparably with YF3 mutant; however, vinculin was co-precipitated with YF3 mutant more efficiently than with the wild-type (Fig. 4). Interestingly, no significant difference in binding affinity to vinculin was detected in wild-type NIH3T3 cells, in which vinexin α was scarcely tyrosine phosphorylated (data not shown). Although vinexin associates with WAVE2 as well as vinculin through its first and second SH3 domains [20,32], co-precipitation of WAVE2 with wild-type or YF3 mutant vinexin α was not detected in v-Src-transformed cells (data not shown). These observations imply that tyrosine phosphorylation of vinexin α specifically attenuates the binding affinity for vinculin, not for ERK2 and Abl.

Discussion

In this study, we demonstrated that vinexin α, but not vinexin β, is tyrosine phosphorylated in v-Src-transformed NIH3T3 cells. We also observed moderate tyrosine phosphorylation of vinexin α in wild-type cells treated with a phosphatase inhibitor (Fig. 1). Active Src can phosphorylate purified vinexin α *in vitro,* indicating that vinexin α was phosphorylated by v-Src in v-Src-transformed cells (Fig. 1C). The non-phosphorylatable (YF3) mutant of vinexin α showed higher binding affinity for vinculin than wild-type vinexin α in v-Src-transformed cells. These observations suggest that vinexin α is phosphorylated at tyrosine residues in v-Src-transformed cells and that these phosphorylations attenuate the binding affinity for vinculin.

We have shown here that tyrosines 127, 170, and 196 in vinexin α were phosphorylated in v-Src-transformed cells (Fig. 3B). Point mutations at these tyrosine residues resulted in increased binding affinity to vinculin in v-Src-transformed cells, but not in wild-type cells, in which vinexin α was only marginally tyrosine phosphorylated without phosphatase inhibitor (Fig. 4 and data not shown). These results indicate that tyrosine phosphorylation plays a role in regulating vinexin affinity to vinculin. Although the first and second SH3 domains of vinexin α, which are located in the C-terminal half of vinexin α and apart from phosphorylated tyrosine residues, are essential for this interaction [20,25], the N-terminal region of vinexin α is suggested to promote the interaction with vinculin [20]. Thus, tyrosine phosphorylation of vinexin α is likely to attenuate this promotion.

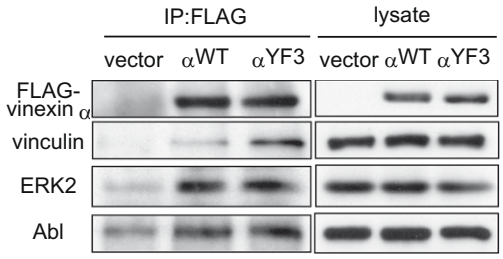

Fig. 4. Binding affinity of vinexin α YF3 mutant for vinculin was greater than that of wild-type vinexin α in v-Src-transformed NIH3T3 cells. FLAG-tagged vinexin α (wild-type or YF3 mutant) was transfected into v-Src-transformed NIH3T3 cells. Exogenously expressed protein was immunoprecipitated with anti-FLAG antibody, and co-precipitated endogenously expressing binding partners of vinexin α (vinculin, ERK2, and Abl) were detected by Western blotting.

Our previous observations have shown that v-Src-mediated transformation of NIH3T3 cells suppresses the expression of vinexin α, resulting in enhanced cell migration [28]. Here, we showed that increased tyrosine phosphorylation of vinexin α attenuated the binding affinity to vinculin in v-Src-transformed cells. These observations indicate that the amount of vinculin interacting with vinexin α in v-Src-transformed cells is much lower than in wild-type NIH3T3 cells. Loss of vinculin expression promotes focal adhesion turnover and cell motility, as well as increasing the ability of anchorage-independent growth and resistance to apoptotic stimuli [33–35]. Thus, it is possible that the decreased expression of and the increased tyrosine phosphorylation of vinexin α contribute to v-Src-mediated transformation through the interaction with vinculin.

We previously demonstrated that Abl, another non-receptor-type tyrosine kinase, also tyrosine phosphorylates vinexin α in Cos-7 cells [29]. This phosphorylation depends on the interaction with Abl through the third SH3 domain of vinexin α. It is reported that Abl can be activated downstream of v-Src [36,37]. Thus, it is possible that Abl activated by v-Src mediates the tyrosine phosphorylation of vinexin α in v-Src-transformed cells. Indeed, Abl kinase inhibitor STI571 inhibited the phosphorylation partially, suggesting that Abl may be involved in tyrosine phosphorylation in part (data not shown). However, Abl mainly phosphorylates tyrosine 127 but not tyrosine residues 170 and 196 [29]. Mutation in the third SH3 domain of vinexin α, which disrupts Abl-mediated phosphorylation [29], did not decrease the phosphorylation of vinexin α in v-Src-transformed cells (data not shown). Furthermore, Src directly phosphorylated vinexin α *in vitro* (Fig. 1C). Taken together, these results suggest that direct phosphorylation by v-Src mainly contributes to the phosphorylation of vinexin α in v-Src-transformed cells.

We have shown that tyrosine phosphorylation of vinexin α in v-Src-transformed cells depended on cell adhesion (Fig. 2). Loss of cell adhesion decreased and replating on fibronectin increased tyrosine phosphorylation. Interestingly, the activity of v-Src was not affected by cell adhesion (Fig. 2). This is consistent with a previous report that tyrosine phosphorylation of certain types of Src substrates, including tensin, talin, and paxillin, decreased in suspension even in v-Src-transformed cells, although other substrates, such as FAK, p130CAS, and p190RhoGAP, are strongly tyrosine phosphorylated irrespective of the adhesion condition [31]. Protein tyrosine phosphatase was suggested to regulate this adhesion-dependent phosphorylation in v-Src-transformed cells; therefore, adhesion-regulated protein tyrosine phosphatases can be predicted to regulate the phosphorylation of vinexin α in v-Src-transformed cells.

In conclusion, we demonstrated that vinexin α is tyrosine phosphorylated at Y127, 170, and 196 in v-Src-transformed cells in a cell adhesion-dependent manner, and that elevation in tyrosine phosphorylation decreases the binding affinity for vinculin.

Acknowledgments

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