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Involvement of Src Family Kinases in N-Cadherin Phosphorylation and β-Catenin Dissociation during Transendothelial Migration of Melanoma Cells[D]

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N-cadherin is recruited to the heterotypic contact during transendothelial migration of melanoma cells in a coculture system with tumor cells seeded on top of a monolayer of endothelial cells. However, β-catenin dissociates from N-cadherin and redistributes to the nucleus of transmigrating melanoma cells to activate gene transcription. In this report, we demonstrate that Src becomes activated at the heterotypic contact between the transmigrating melanoma cell and neighboring endothelial cells. Src activation shows close temporal correlation with tyrosine phosphorylation of N-cadherin. Expression of a dominant-negative Src in melanoma cells blocks N-cadherin phosphorylation, β-catenin dissociation, and nuclear translocation in transmigrating cells, consistent with the involvement of Src family kinases. In in vitro binding assays, Src-mediated phosphorylation of the N-cadherin cytoplasmic domain results in a significant reduction in β-catenin binding. Although five phospho-tyrosine residues can be identified on the N-cadherin cytoplasmic domain by mass spectrometry, site-specific mutagenesis indicates that Tyr-860 is the critical amino acid involved in β-catenin binding. Overexpression of N-cadherin carrying the Y860F mutation inhibits the transmigration of transfected cells across the endothelium. Together, the data suggest a novel role for tyrosine phosphorylation of N-cadherin by Src family kinases in the regulation of β-catenin association during transendothelial migration of melanoma cells.

INTRODUCTION

Cancer metastasis is a complex multistep process, involving the detachment of cancer cells from the primary tumor, intravasation, survival in the bloodstream, extravasation, and establishment of new foci in remote organs (Orr *et al*., 2000; Fidler, 2003). Extravasation is one of the least known steps in cancer metastasis, and it involves dynamic interactions between cancer cells and the endothelium. We have investigated the mechanism of tumor cell transendothelial migration (TEM) using a coculture assay (Sandig *et al*., 1997; Voura *et al*., 1998). Our results have highlighted the role of chemokine and cell adhesion molecules (CAMs) in the transmigration process (Voura *et al*., 2001b; Ramjeesingh *et al*., 2003).

The attachment of melanoma cells on a monolayer of endothelial cells induces localized dissolution of two major CAMs, vascular/endothelial-cadherin (VE-cadherin) and platelet/endothelial cell adhesion molecule-1, in the endothelial junction beneath the cancer cell (Sandig *et al*., 1997; Voura *et al*., 2001a). As VE-cadherin redistributes from the endothelial junction, N-cadherin moves in and becomes concentrated in the heterotypic contacts between the melanoma cell and its adjacent endothelial cells, promoting TEM of melanoma cells (Sandig *et al*., 1997; Qi *et al*., 2005).

Interestingly, β-catenin dissociates from the N-cadherin adhesion complex in the heterotypic contact during TEM (Qi *et al*., 2005). The regulation of assembly and disassembly of the cadherin–β-catenin complex is thought to underlie the dynamics of the adhesive interactions between cells during tissue development and cancer metastasis (Gumbiner, 2000; Takeichi and Abe, 2005). Because the strength of cadherin-mediated cell–cell interaction depends on the association of its cytoplasmic domain to the actin cytoskeleton through β-catenin (Takeichi, 1995), the loss of β-catenin may result in a reduction in N-cadherin-mediated adhesiveness and facilitate the migration of tumor cells across the endothelial junction.

In addition to cell–cell adhesion, β-catenin is known to be a key signal transducer in the Wnt signaling pathway. Cytoplasmic β-catenin is normally recruited to the multiprotein complex containing GSK3β. Phosphorylation of β-catenin by GSK3β promotes its ubiquitination and subsequent proteasomal degradation (Peifer and Polakis, 2000). However, in the presence of the Wnt signal, GSK3β activity is inhibited, leading to the accumulation of β-catenin in the nucleus where it interacts with the LEF/TCF family of transcription factors to regulate gene transcription (Peifer and Polakis, 2000; Nelson and Nusse, 2004). During TEM, the dissociated β-catenin is also capable of escaping degradation to accu-

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Abbreviations used: CAM, cell adhesion molecule; HMVEC, human lung microvascular endothelial cells; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; SFK, Src family kinase; TEM, transendothelial migration.

mulate in the nucleus of melanoma cells and activating β-catenin-mediated gene transcription (Qi *et al.*, 2005). Among the known β-catenin induced genes are N-cadherin and those that code for proteins involved in the invasive process of cancer cells (Brabletz *et al.*, 1999; Mann *et al.*, 1999; Wielenga *et al.*, 1999; Hiendlmeyer *et al.*, 2004; Qi *et al.*, 2005).

A key question to be addressed is the mechanism involved in the dissociation of β-catenin from the heterotypic contact between melanoma cells and the endothelium. Our previous study has implicated a role for tyrosine phosphorylation of N-cadherin (Qi *et al.*, 2005), although the enzyme and mechanism remain to be determined. Several reports have implicated a regulatory role for Src in the disassembly of the cadherin–β-catenin complex (Matsuyoshi *et al.*, 1992; Behrens *et al.*, 1993; Hamaguchi *et al.*, 1993). In these studies, Src-induced phosphorylation of β-catenin has been correlated with the disruption of cadherin-mediated cell–cell adhesion. However, other reports indicate that β-catenin can be dispensable in the disassembly process (Takeda *et al.*, 1995; Irby and Yeatman, 2002; Wrobel *et al.*, 2004), suggesting that other Src substrates may be involved.

In this report, we demonstrate that activation of Src associated with the heterotypic contact leads to the phosphorylation of N-cadherin and subsequent dissociation of β-catenin during TEM of melanoma cells. Further studies lead to the identification of Tyr-860 in the cytoplasmic domain of N-cadherin as the critical amino acid involved in the regulation of its interaction with β-catenin.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies (mAbs) against human N-cadherin, β-catenin, p120ctn, and anti-phospho-tyrosine mAb (PY-20) were purchased from BD Transduction Laboratories (Lexington, KY). For immunostaining of nuclear β-catenin, mouse mAb against the unphosphorylated form of human β-catenin was used (A.G. Scientific, San Diego, CA). Rabbit anti-human N-cadherin antibody (H-36) was purchased from R&D Systems (Minneapolis, MN) for immunoprecipitation studies. Mouse mAbs against Src and phospho-Src (Tyr-416) were purchased from Upstate Biotechnology (Lake Placid, NY). Mouse anti-myc antibody was purchased from Invitrogen (Carlsbad, CA). Rabbit anti-human β-catenin antibody was purchased from Sigma-Aldrich (St. Louis, MO). The Alexa 488- or Alexa 568-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Molecular Probes (Eugene, OR).

Cell Lines

Human lung microvascular endothelial cells (HMVECs) were purchased from Clonetics (San Diego, CA) and cultured in the endothelial medium EGM-2 MV (Clonetics) supplemented with penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively). Routinely, HMVECs between passages 6 and 8 were used in the experiment. The human melanoma cell line WM239 (obtained from Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

cDNA Constructs

Both wild-type and dominant-negative (DN)-Src cDNAs in the pUSEamp vector were purchased from Upstate Biotechnology. The DN-Src contains two point mutations (K296R/Y528F), resulting in loss of kinase activity and adoption of an open conformation. They were subcloned into *Xho*I/*Bam*HI sites of pEGFP-N1 vector. The cDNA encoding the cytoplasmic domain of N-cadherin was obtained by reverse transcription-PCR using total RNA isolated from WM239 melanoma cells and then cloned into the *Sph*I/*Bam*HI sites of pQE-70 vector containing a C-terminal 6xHis tag or into the *Bam*HI/*Eco*RI sites of pGEX-2T vector containing an N-terminal glutathione S-transferase (GST) tag. Point mutations that convert tyrosine into phenylalanine in the N-cadherin cytoplasmic domain were performed using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). β-Catenin cDNA was cloned into the *Bam*HI/*Sal*I sites of the pTrcHis2 vector containing a C-terminal 6xHis tag. The complete coding region of N-cadherin cDNA was purchased from Invitrogen and then subcloned into the *Bam*HI/*Xho*I sites of the pcDNA3.1 myc-His vector containing a C-terminal myc-tag. The N-cadherin Y860F mutation was generated using Stratagene's QuikChange II kit. All mutations and cDNA constructs were confirmed by DNA sequencing.

Cell Transfection

Plasmid DNA was transfected into WM239 melanoma cells using Lipofectamine 2000 (Invitrogen). For transient expression of N-cadherin-myc, cells were analyzed 48 h after transfection. For stable transfection of Src-GFP, the selection media were applied 48 h after transfection (1 mg/ml G418). Stable colonies were screened by fluorescence microscopy.

Transendothelial Migration Assay

The in vitro TEM assay was carried out as described previously (Sandig *et al.*, 1997; Voura *et al.*, 1998). HMVEC cells (1.5×10^5 cells, passages 5–8) in 200 μl of endothelial medium were placed on top of a Matrigel-coated glass coverslip (12 mm in diameter) and allowed to settle for 5–6 h. Coverslips were then transferred to a new 24-well plate and incubated in 0.5 ml of endothelial medium containing 10 ng/ml tumor necrosis factor (TNF)-α (Invitrogen/BRL, Burlington, Ontario, Canada) for 12 h to promote CAM expression. The monolayer was washed three times and then incubated in 0.5 ml of fresh endothelial medium without TNF-α. Melanoma cells were labeled with 10 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Molecular Probes) for 5 min at 37°C. After washing, cells were resuspended at 2.5×10^6 cells/ml in the endothelial medium. Melanoma cells (5×10^4 cells) were added to each HMVEC monolayer. Cocultures were fixed at different time points with paraformaldehyde and stained for F-actin using BODIPY-phallacidin (Molecular Probes). Quantitative analysis of transmigration was carried out using a Wild Leitz Orthoplan epifluorescence microscope. For each coverslip, three sets of random fields for a total of 45 fields were scored for transmigrated cells. Each set of 15 fields contained 100–200 melanoma cells, and >1000 melanoma cells were routinely scored for each data point.

In inhibition studies, cocultures were carried out for 5 h in the presence of AG1478 (epidermal growth factor receptor inhibitor; 10 μM), K252a (c-Met inhibitor; 50 nM), SU5402 (fibroblast growth factor receptor inhibitor; 50 μM), tyrphostin AG1295 (platelet-derived growth factor receptor inhibitor; 10 μM), SU5416 (vascular endothelial growth factor receptor-2 inhibitor; 10 μM), I-Ome-tyrphostin AG538 (insulin-like growth factor-1 receptor inhibitor; 10 μM), or PP2 (Src family kinase [SFK] inhibitor; 10 μM). In all inhibition studies, the total number of melanoma cells associated with the endothelial monolayer was estimated to ensure that any reduction in the number of transmigrated cells was not due to a reduction in cell attachment.

Immunofluorescence Staining for Confocal Microscopy

Melanoma cells were labeled with 2 μM Cell Tracker Orange (Molecular Probes) and then deposited on a monolayer of endothelial cells cultured on Matrigel-coated coverslips. Cells were fixed with 3.5% paraformaldehyde at 22°C for 5 min or 100% methanol (prechilled to −20°C) on ice for 3 min. After three washes in phosphate-buffered saline (PBS), the paraformaldehyde-fixed cells were permeabilized for 5 min in 0.1% Triton X-100. After blocking in 1% bovine serum albumin (BSA), cells were incubated with the primary and secondary antibodies at room temperature for 45 min each. After washing, coverslips were mounted on slides and examined under a Zeiss LSM410 confocal microscope.

Luciferase Reporter Assay

The TCF reporter plasmid kit, which contained the TOPflash, FOPflash, and TK vectors, was purchased from Upstate Biotechnology. Melanoma cells were cotransfected transiently with TOPflash (or FOPflash) and TK vectors at a ratio of 30:1 using Lipofectamine 2000. Two days later, melanoma cells (3×10^5) were seeded on top of a HMVEC monolayer (3×10^5 cells) in a Matrigel-coated 24-well plate. Cells were collected at different times and analyzed using the dual luciferase reporter assay system (Promega, Madison, WI). Luminescence was measured using a Lumat LB luminometer (Berthold Technologies, Bad Wildbad, Germany). The ratio between firefly luciferase activity (TOPflash or FOPflash) and *Renilla* luciferase activity (TK vector) was used to estimate changes in β-catenin-mediated transcription. The fold increase in luciferase activity was calculated by normalizing the data to that at 0 h of coculture.

Immunoprecipitation and Immunoblot Analysis

For biochemical analysis, 4×10^6 endothelial cells were cultured on a Matrigel-coated 60-mm-plate in endothelial medium containing 10 ng/ml TNF-α for 12 h. The cells were washed three times and then incubated in fresh endothelial medium. Melanoma cells (4×10^6) were deposited on the endothelial monolayer and cocultured for 5 h before lysis. To prepare 0-h coculture samples, melanoma cells were deposited on the Matrigel for 5 h before lysis, and the lysate was mixed with the lysate of endothelial monolayer that has been cultured on Matrigel for 17 h (12 h + 5 h).

For immunoprecipitation, cells were lysed in immunoprecipitation (IP) lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, a protease inhibitor cocktail, and 1% NP-40). The supernatant was incubated overnight with 3 μg of primary antibody at 4°C. Protein A-Sepharose (10 mg) was added before further incubation for 1 h at 4°C. The Sepharose beads were pelleted and washed

three times in IP lysis buffer. The proteins on the beads were solubilized in sample buffer at 100°C for 5 min before SDS-PAGE.

For immunoblot analysis of total cell lysate, cells were lysed in gel lysis buffer (50 mM Tris-HCl, pH 7.5, 2% SDS, and 2 mM phenylmethylsulfonyl fluoride). After SDS-PAGE, proteins were transferred to nitrocellulose membrane and then blocked with 5% skim milk in PBS before incubation with primary antibodies for 1 h. To probe for phospho-tyrosine and phospho-Src, the membranes were blocked in 1% BSA in Tris-buffered saline and incubated with corresponding mAbs overnight at 4°C. The membranes were washed and incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h and then developed with the ECL reagents (Amersham Biosciences, Baie d'Urfe, Quebec, Canada). For quantification, the blots were analyzed in a Fluor-S imager (Bio-Rad, Hercules, CA), and the pixel value of each band was obtained by subtracting a blank region of the blot with the same size.

In Vitro Phosphorylation and Analysis of Recombinant Cadherin Proteins

In vitro phosphorylation was performed in a final volume of 40 μl containing 25 mM Tris-HCl, pH 7.2, 30 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mM EGTA, 0.25 mM VaO_4Na_3, 0.5 mM dithiothreitol (DTT), 0.1 mM ATP, 5 μg of His-tagged or GST-tagged N-cadherin cytoplasmic domain, and 50 U of recombinant Src protein (Upstate Biotechnology). Reactions were performed at 30°C for 10 min to 5 h. Control reactions were performed under the same conditions either in the presence of 10 μM PP2 or in the absence of ATP.

After in vitro phosphorylation, the His-tagged N-cadherin cytoplasmic domain was analyzed by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry to determine the total number of phospho-tyrosine residues. Then tryptic peptides of the phosphorylated protein were prepared and analyzed by mass spectrometry to map the specific phospho-tyrosine residues.

Protein Binding Assay

For binding studies, 0.6 pmol of His-tagged β-catenin and 1.2 pmol of phosphorylated or nonphosphorylated GST-tagged N-cadherin cytoplasmic domain (wild-type or mutant recombinant protein) were incubated in 200 μl of binding buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.1% NP-40) for 1 h at 4°C. The protein complexes were allowed to bind to 40 μl of 50% slurry of glutathione-Sepharose beads for 1 h at 4°C. Then, the beads were washed three times with the binding buffer. Proteins bound on the beads were solubilized in the sample buffer and boiled for 5 min before SDS-PAGE and immunoblot analysis.

RESULTS

Src Family Kinase Inhibitor Prevents β-Catenin Dissociation during TEM of Melanoma Cells

Our previous work suggests that tyrosine phosphorylation plays a role in regulating the dissociation of β-catenin from N-cadherin (Qi *et al*., 2005). To identify the tyrosine kinase involved in this process, we tested the effects of several specific tyrosine kinase inhibitors, including AG1478, K252a, SU5402, tyrphostin AG1295, SU5416, I-Ome-tyrphostin AG538, and PP2 on melanoma cells during TEM. Melanoma cells were labeled with Cell Tracker Orange and subjected to the TEM assay. Melanoma cells exhibited a round morphology at the initial stage of attachment on the endothelial monolayer (Figure S1A). Then, they began to insert pseudopodia into the endothelial junction (Figure S1B). Transmigrating cells generally adopted an elongated spindle shape (Figure S1C), whereas cells underneath the endothelium displayed a fibroblastic morphology (Figure S1D). To compare effects of different kinase inhibitors, transmigrating cells with a spindle-shaped morphology were examined. Among these kinase inhibitors, only PP2 was effective in abolishing the phospho-tyrosine staining at the heterotypic contact between transmigrating melanoma cells and their surrounding endothelial cells. PP2 also prevented β-catenin dissociation and its accumulation in the nucleus (Figure 1A). These results suggest the involvement of SFKs in the tyrosine phosphorylation event at the heterotypic contact.

To examine the effects of PP2 on the phosphorylation state of the N-cadherin adhesion complex, melanoma cells were cultured on an endothelial monolayer for 5 h in the absence or presence of PP2. The N-cadherin complex was immunoprecipitated and subjected to immunoblot analysis. In the absence of PP2, N-cadherin became tyrosine phosphorylated, and the amount of β-catenin coprecipitating with N-cadherin was reduced by >60% at 5 h of coculture (Figure 1B). Because p120ctn can be tyrosine-phosphorylated (Mariner *et al*., 2001) and it migrates right before N-cadherin in SDS gels, it was necessary to determine whether the phosphorylated band was indeed N-cadherin and not p120ctn. When immunoprecipitates of N-cadherin were subjected to immunoblot analysis, N-cadherin and p120ctn were found to be separated by only a small distance. However, the tyrosine-phosphorylated band clearly comigrated with N-cadherin (Figure S2A). In a separate experiment, the N-cadherin complex in immunoprecipitates was disrupted with 1% SDS and then reprecipitated using either anti-N-cadherin or anti-p120ctn antibodies. Immunoblot analysis showed that N-cadherin and not p120ctn was tyrosine-phosphorylated at 5 h of coculture (Figure S2B).

However, tyrosine phosphorylation of N-cadherin was abolished in the presence of PP2 and the level of β-catenin association remained constant during the coculture period. For comparison, the epidermal growth factor receptor inhibitor AG1478, representative of the other noninhibitory reagents, did not affect N-cadherin phosphorylation or the dissociation of β-catenin from N-cadherin (Figure 1B). In all cases, phosphorylation of β-catenin was not detected.

When the TEM assay was carried out in the presence of PP2, transmigration of melanoma cells was reduced by 60%, although the number of cells attached on the monolayer of endothelial cells was comparable with that of the control. However, AG1478 did not have any adverse effect on TEM (Figure 1C). These results implicate a crucial role for SFKs in the phosphorylation of N-cadherin and the transmigration process.

Src Is Activated at the Heterotypic Contact between Transmigrating Melanoma Cells and Endothelial Cells

The Src family of kinases includes nine members, among which Src has been shown mostly to be involved in the regulation of the cadherin–catenin complex as well as cancer metastasis (Nelson and Nusse, 2004; Yeatman, 2004). Therefore, we investigated the potential role of Src in TEM of cancer cells. Melanoma cells were stably transfected with green fluorescent protein (GFP)-tagged wild-type (WT) Src or DN-Src (Figure 2A). The WT-Src-GFP was localized in the cell–cell contact region as well as the cytoplasm but clearly absent from the nucleus (Figure 2B). In TEM assays, cells from the initial attachment stage (characterized by their round shape) and from the transmigration stage (characterized by their spindle shape or fibroblastic morphology) were examined (Figure S1, A and C). A high level of WT-Src-GFP was observed in heterotypic contacts between melanoma cells and their surrounding endothelial cells during the stages of initial attachment and transmigration (Figure 2C).

Because our previous work indicates that tyrosine phosphorylation of the N-cadherin complex occurs only during transmigration and not at the cell attachment stage (Qi *et al*., 2005), the above-mentioned results suggest that Src might not be active at the cell attachment stage but become activated in heterotypic contacts during transmigration. To test this hypothesis, cocultures were stained with a mAb directed against the active p-Src (Src phosphorylated on Tyr-416). Weak punctate staining of p-Src in the heterotypic contact region was detected when melanoma cells were crossing the endothelial junction (Figure 2D, c). To maximally visualize the p-Src staining, cocultures were treated with the tyrosine phosphatase inhibitor pervanadate for 5

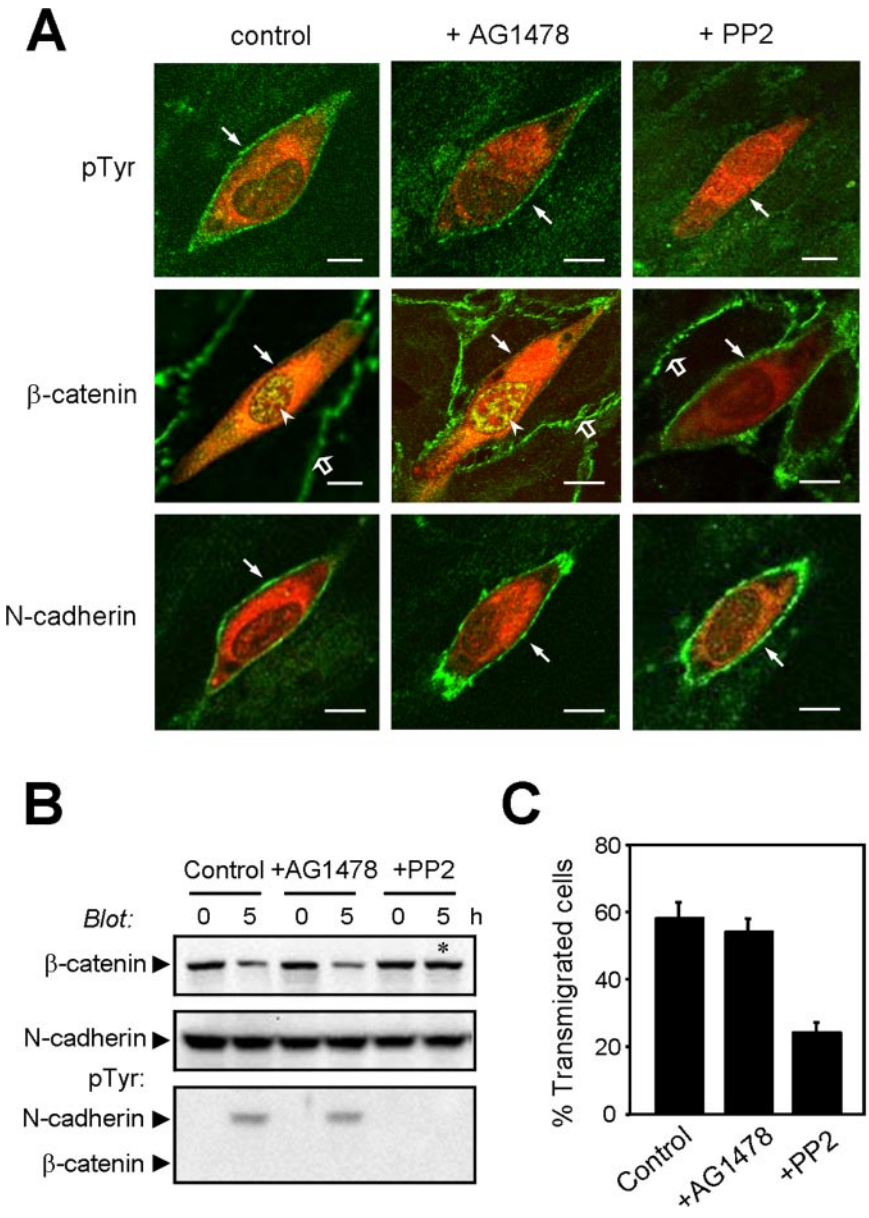

Figure 1. Effects of the Src-specific inhibitor PP2 on tyrosine phosphorylation of N-cadherin and TEM of melanoma cells. Labeled melanoma cells (red) were cultured on an endothelial monolayer in the presence of different kinase inhibitors. Only PP2 inhibited tyrosine phosphorylation of N-cadherin and β-catenin dissociation. The data of AG1478 are shown as representative of the other noninhibitory compounds. (A) TEM assay coverslips were fixed at 5 h of coculture and immunostained for phospho-tyrosine, β-catenin, or N-cadherin (green). Arrows indicate the heterotypic contact between melanoma cell and endothelial cells, and open arrows indicate β-catenin labeling of endothelial junctions. Arrowheads indicate the accumulation of nuclear β-catenin in transmigrating melanoma cells. Bars, 10 μm. (B) Cocultures were performed in the presence of AG1478 or PP2. Samples were collected at 0 and 5 h for immunoprecipitation with an N-cadherin antibody. The immunoprecipitates were analyzed by immunoblotting against N-cadherin, β-catenin, or phosphotyrosine. *, inhibition of β-catenin dissociation by PP2. (C) TEM assay was performed in the presence of AG1478 or PP2. The percentage of transmigrated cells was scored at 5 h. The data represent the mean ± SD (n = 3).

min before fixation. The p-Src staining became clearly visible in the heterotypic contact regions of transmigrating cells as well as the homotypic contact regions between endothelial cells (Figure 2D, d). In contrast, p-Src staining was absent in the heterotypic contact at the cell attachment stage (Figure 2D, a and b). Consistent with these results, immunoblot analysis showed a fivefold increase in the p-Src level at 5 h of coculture. However, this increase was abolished when cocultures were carried out in the presence of PP2 (Figure 2E).

Further studies of Src activation were carried out using stably transfected melanoma cells overexpressing either WT-Src-GFP or DN-Src-GFP. Immunoblot analysis showed that overexpression of either WT-Src or DN-Src abolished the basal level of endogenous Src activation (Figure 2F). WT-Src-GFP displayed a clear p-Src signal, whereas p-Src was not detectable in the DN-Src band (Figure 2F). To examine whether WT-Src became activated like the endogenous Src, the transfected cells were cultured on an endothelial monolayer and immunoblots of 0- or 5-h samples were probed for Src and p-Src. WT-Src showed >3-fold increase in p-Src level at 5 h of coculture (Figure 2G). Both WT-Src and DN-Src inhibited partially the activation of endogenous Src at 5 h (Figure 2G). Because WT- or DN-Src blocked only the activation of endogenous Src in transfectants, the low level of endogenous Src activation in 5-h cocultures might reflect Src activation in endothelial cells.

Expression of DN-Src Inhibits β-Catenin Signaling and TEM

To examine the effects of DN-Src expression on β-catenin distribution during TEM, both WT- and DN-Src transfectants were examined in the coculture assay (Figure 3A). The expression of DN-Src led to a significant reduction in p-Src staining in heterotypic contacts and a high level of β-catenin remained associated with the heterotypic contact during TEM. In contrast, WT-Src transfectants showed an enrichment of p-Src, but not β-catenin, in heterotypic contacts.

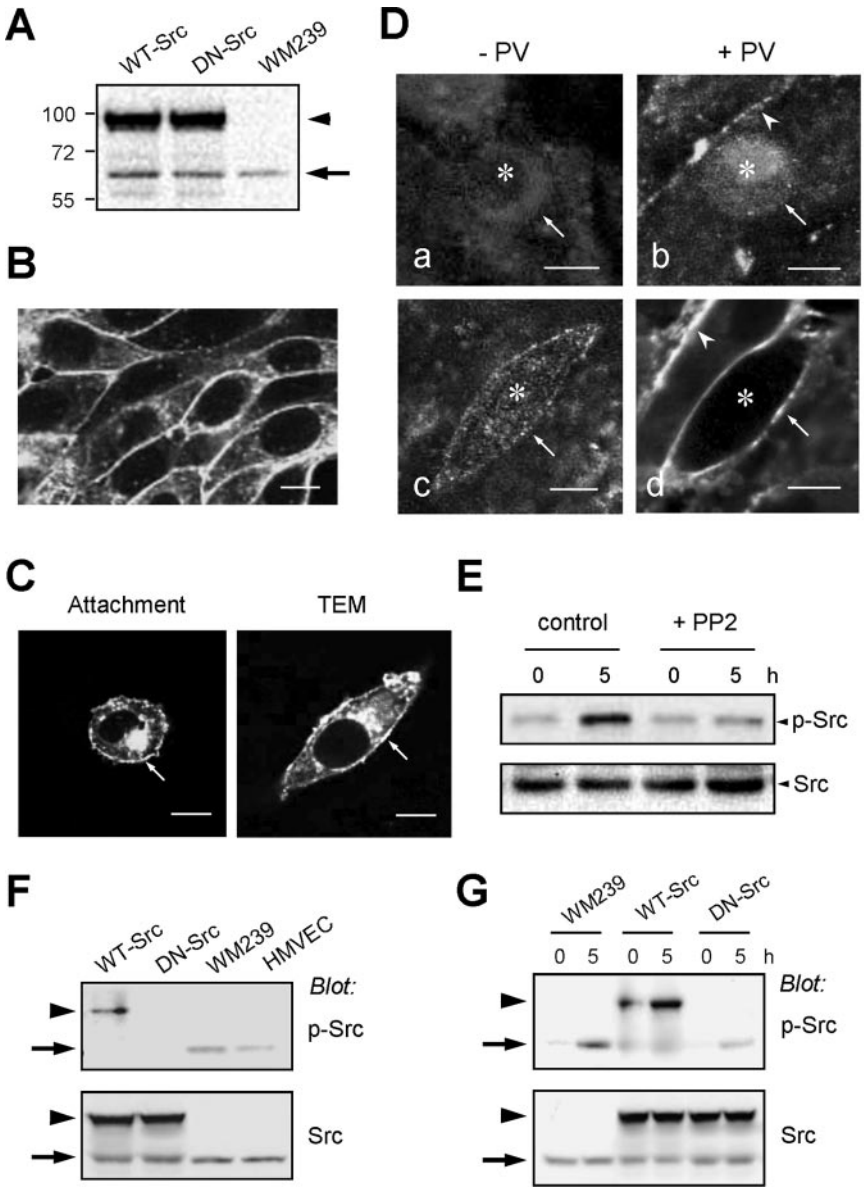

Figure 2. Src activation at the heterotypic contact during TEM of melanoma cells. (A) Immunoblots of stable transfectants expressing GFP-tagged WT and DN Src in WM239 melanoma cells. The endogenous Src is indicated by an arrow and GFP-tagged Src by an arrowhead. (B) Confocal image showing the localization of WT-Src-GFP in melanoma cell transfectants. (C) Confocal images showing the concentration of WT-Src-GFP at the heterotypic contact during both cell attachment and transmigration. Arrows indicate the heterotypic contact between the transfected melanoma cell and endothelial cells. (D) Immunolocalization of activated Src (p-Src) at the heterotypic contact during TEM of nontransfected melanoma cell. (a and b) Cocultures fixed at 1 h showing melanoma cells at the attachment stage. (c and d) Cocultures fixed at 5 h showing the transmigration stage. Control cocultures (a and c) and cocultures pretreated for 5 min with 1 mM pervanadate before fixation (b and d) were immunostained for p-Src. Melanoma cells are marked by an asterisk. Arrows indicate the heterotypic contact between melanoma cells and endothelial cells and arrowheads indicate the homotypic endothelial cell junction. Bars, 10 μm (B, C, and D). (E) Immunoblots showing that PP2 abolished Src activation during TEM. Melanoma cells were cultured on an endothelial monolayer in the presence or absence of PP2. Cells were collected at 0 or 5 h, and immunoblots were probed against p-Src and Src. (F) Immunoblots showing the p-Src level in WT-Src and DN-Src transfectants. (G) Src transfectants were cultured on an endothelial monolayer, and cells were collected at 0 or 5 h for immunoblot analysis of p-Src. In F and G, arrowheads indicate WT- or DN-Src-GFP, whereas arrows indicate the endogenous Src.

Thus, the dissociation of β-catenin from heterotypic contacts seems to be closely correlated with Src activation.

Consistent with our previous observation (Qi *et al.*, 2005), the above-mentioned results also show an abundance of β-catenin present in the perinuclear region and inside the nucleus of WT-Src transfectants, whereas β-catenin was not detectable in the nucleus of DN-Src transfectants (Figure 3A). The accumulation of β-catenin in the nucleus is known to regulate gene expression. To assess the role of Src activation on β-catenin-dependent transcription, melanoma cells were transiently transfected with the TOPflash vector, which contained TCF/LEF binding sites before a luciferase reporter gene. These cells were then cultured on an endothelial monolayer for 5 h. An increase in luciferase activity would reflect an increase in β-catenin-dependent gene transcription. In the absence of PP2, a sixfold increase in luciferase activity was observed at 5-h coculture in WM239 melanoma cells, GFP control transfectants, and WT-Src transfectants (Figure 3B). Addition of PP2 to the cocultures abolished β-catenin-mediated transcription in all the transfectants and control cells. In contrast, the luciferase activity in DN-Src transfectants remained at the background level in the absence or presence of PP2 (Figure 3B).

In the TEM assays, DN-Src transfectants transmigrated poorly, with only 20% of cells transmigrated by 5 h in the presence or absence of PP2 (Figure 3C). In contrast, control transfectants and WT-Src transfectants showed 55 and 70% transmigration, respectively. In all cases, PP2 treatment inhibited TEM, resulting in 20–25% of transmigration (Figure 3C).

In Vitro Phosphorylation of N-Cadherin by Src Disrupts β-Catenin Binding

To provide direct evidence that Src is capable of phosphorylating N-cadherin, His-tagged N-cadherin cytoplasmic domain was prepared and subjected to an in vitro phosphorylation reaction using recombinant Src. Immunoblot analysis showed that the N-cadherin cytoplasmic domain was phosphorylated efficiently by Src and that the phosphorylation reaction became almost saturated within 10

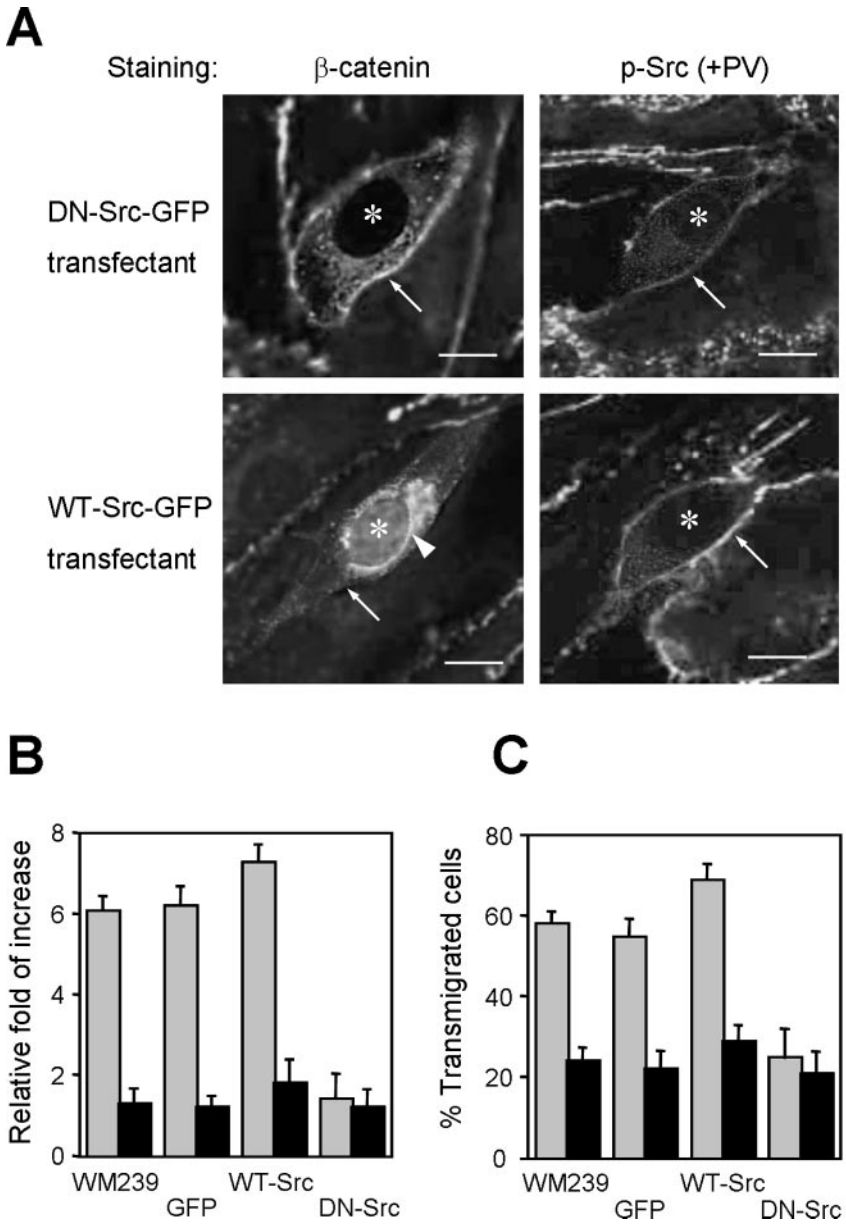

Figure 3. Effects of DN-Src expression on Src activation, β-catenin signaling, and TEM of melanoma cells. (A) Confocal images showing the immunolocalization of β-catenin and p-Src during TEM of Src-GFP transfectants (asterisks). Cells were treated with pervanadate for 5 min before immunostaining of p-Src. Arrows indicate the heterotypic contact between the Src-GFP transfectant and endothelial cells, and the arrowhead indicates the nuclear and perinuclear labeling of β-catenin. Bars, 10 μm. (B) Inhibition of β-catenin-mediated gene transcription in transmigrating melanoma cells by PP2 or DN-Src transfection. Cells were transiently transfected with a TOPflash vector and then cultured on top of an endothelial monolayer in the presence (■) or absence (□) of PP2. Cells were collected at 5 h of coculture and assayed for luciferase activity. The luciferase activity at 5-h of coculture was normalized to that at 0-h coculture and the relative fold-increase in luciferase activity was calculated. Data represent the mean ± SD (n = 3). (C) Inhibition of TEM of melanoma cells by PP2 or DN-Src transfection. Cells were subjected to the TEM assay in the presence (■) or absence (□) of PP2. The percentage of transmigrated cells was scored at 5 h. The data represent the mean ± SD (n = 3).

min. The phosphorylation reaction was dependent on ATP and sensitive to PP2 inhibition (Figure 4A).

Next, the effect of Src-mediated tyrosine phosphorylation of N-cadherin on the binding of β-catenin to N-cadherin was examined. An in vitro binding assay was set up to investigate the interaction between GST-tagged N-cadherin cytoplasmic domain and His-tagged β-catenin. The protein complexes were isolated using glutathione beads and then subjected to immunoblot analysis. β-Catenin bound to the cytoplasmic domain of N-cadherin but not to GST. However, the binding of β-catenin to the Src-phosphorylated N-cadherin cytoplasmic domain was significantly reduced (Figure 4B).

Identification of the Tyrosine Residues on N-Cadherin That Are Phosphorylated by Src

MALDI-TOF mass spectrometry was used to identify the Src-phosphorylated tyrosine residues. The His-tagged N-cadherin cytoplasmic domain, which contains eight tyrosine residues, was phosphorylated in vitro by Src. Before phosphorylation by Src, N-cadherin cytoplasmic domain showed a single peak of ~19 kDa in the mass spectrum. After phosphorylation, five new distinct peaks occurred, and the separation between the adjacent peaks was ~80 Da (Figure 5A). Because the mass of a phosphate group is 80 Da, the results indicate that five tyrosine residues on the N-cadherin cytoplasmic domain were phosphorylated in vitro by Src.

To locate the specific phospho-tyrosine residues, the Src-phosphorylated N-cadherin cytoplasmic domain was digested with trypsin before analysis by MALDI-TOF mass spectrometry. Peptides that contained phospho-tyrosine residues were identified based on the appearance of pairs of peaks that differed by 80 Da. Four of the five phospho-tyrosine residues resided within a long peptide, AADNDPTAPPYDSLLVFDYEGSGSTAGSLSSLNSSSSGGEQDYDYLNDWGPR (5448 Da) (Figure 5B), which gave rise

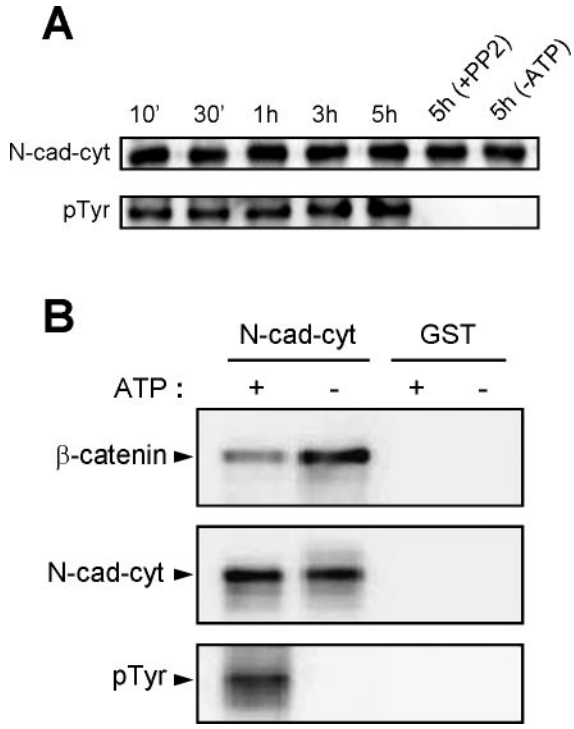

Figure 4. In vitro phosphorylation of the N-cadherin cytoplasmic domain disrupts its interaction with β-catenin. (A) Phosphorylation of recombinant N-cadherin cytoplasmic domain (N-cad-cyt) by an active Src. The phosphorylation reaction was carried out for different times, and aliquots of the reaction were subjected to SDS-PAGE. The immunoblots were probed with antibodies against N-cadherin and phospho-tyrosine. (B) Reduction of β-catenin binding to phosphorylated N-cadherin cytoplasmic domain (N-cad-cyt). GST-tagged N-cad-cyt was phosphorylated in vitro by Src in the presence or absence of ATP and then incubated with His-tagged β-catenin. The protein complex was isolated by glutathione beads, and immunoblots were probed for β-catenin, N-cadherin, and phospho-tyrosine.

to phosphopeptides of 5528, 5608, 5688, and 5767 Da. Because there were only four tyrosine residues (Y852, Y860, Y884, and Y886) in this peptide, all of them were phosphorylated. The fifth phospho-tyrosine residue occurred in two peptides, MDERPIHAEPQYPVR (1838 Da) or RMDERPIHAEPQYPVR (1995 Da), which were produced by differential digestion of the same peptide and gave rise to phosphopeptides of 1918 and 2075 Da, respectively (Figure 5C). Because there was only one tyrosine residue in these peptides, Y820 was phosphorylated.

Mapping of the Phospho-Tyrosine on N-Cadherin Responsible for β-Catenin Dissociation

To identify the specific phospho-tyrosine residue(s) that disrupted the β-catenin association with N-cadherin, the five tyrosine residues identified by mass spectrometry were mutated individually to phenylalanine. GST-fusion proteins carrying these mutations were subjected to in vitro phosphorylation by Src. Both phosphorylated and nonphosphorylated proteins were assayed for their ability to pull down His-tagged β-catenin. Mutation of any of the five tyrosine residues did not significantly alter the phosphorylation level of the recombinant proteins by Src (Figure 6A), suggesting that the five tyrosine residues are phosphorylated equally in vitro. Among all the phosphorylated mutant proteins, only the Y860F mutant protein did not show a compromised level of β-catenin binding (Figure 6, A and B), indicating that phosphorylation of Y860 in the N-cadherin is responsible for the disruption of its interaction with β-catenin. Tyr-860 resides within a highly conserved sequence corresponding to the core region involved in cadherin-β-catenin binding (Huber and Weis, 2001; Xu *et al*., 2002) (Figure 6C).

Inhibitory Effects of the Y860F Mutation in N-Cadherin on TEM

To provide further evidence that the phosphorylation of Y860 was responsible for β-catenin dissociation during TEM, melanoma cells were transiently transfected with either a myc-tagged wild-type N-cadherin construct or a myc-tagged mutant (Y860F) N-cadherin construct. Immunoblot analysis was performed to ascertain the expression of myc-tagged N-cadherin (Figure 7A). Immunostaining results showed that generally only 10–15% of the transfected cells express the myc-tagged protein.

Both types of transfectants were subjected to cocultures with endothelial cells and stained for myc and β-catenin (Figure 7B). Myc-staining allowed the positive identification of the transfected melanoma cells. Similar to the parental WM239 cells (Figure 1A), the wild-type N-cadherin transfectants showed little β-catenin staining at the heterotypic contact between the melanoma cell and its surrounding endothelial cells, whereas nuclear β-catenin staining was evident. In contrast, transfectants expressing the Y860F mutant N-cadherin displayed prominent β-catenin staining in the heterotypic contact regions but little β-catenin staining in the nucleus.

To further analyze the N-cadherin–myc complex, transiently transfected melanoma cells were cultured on an endothelial monolayer at a cell ratio of 3:1 so that sufficient myc-tagged N-cadherin could be immunoprecipitated for immunoblot studies. At 5 h of coculture, the wild-type N-cadherin-myc became tyrosine phosphorylated and a reduced level of β-catenin was associated with the complex (Figure 7C), with a pattern similar to that of WM239 cells (Figure 1B). In contrast, the Y860F N-cadherin-myc showed a reduction in tyrosine phosphorylation at 5 h of coculture, but there was no effect on β-catenin dissociation.

To assess the effects of the mutant N-cadherin on TEM, melanoma cells transiently expressing either wild-type N-cadherin-myc or mutant-N-cadherin-myc were cultured on an endothelial monolayer. The fixed specimens were doubly immunostained for myc to identify the transfected melanoma cells and β-catenin to visualize endothelium. Only the myc-positive melanoma cells were scored. Melanoma cells expressing the mutant N-cadherin showed ~50% reduction in the number of transmigrated cells in comparison with cells transfected with either the vector or the wild-type N-cadherin-myc construct (Figure 7D). Together with the in vitro data, these results indicate that phosphorylation of Y860 on N-cadherin leads to β-catenin dissociation during TEM of melanoma cells.

DISCUSSION

In this article, we present evidence that one or more of the Src family kinases are involved in the regulation of N-cadherin-mediated cell adhesion and signaling during melanoma cell TEM. Specifically, we find that Src is localized at the heterotypic contacts and becomes activated when melanoma cells are transmigrating across the endothelium. The

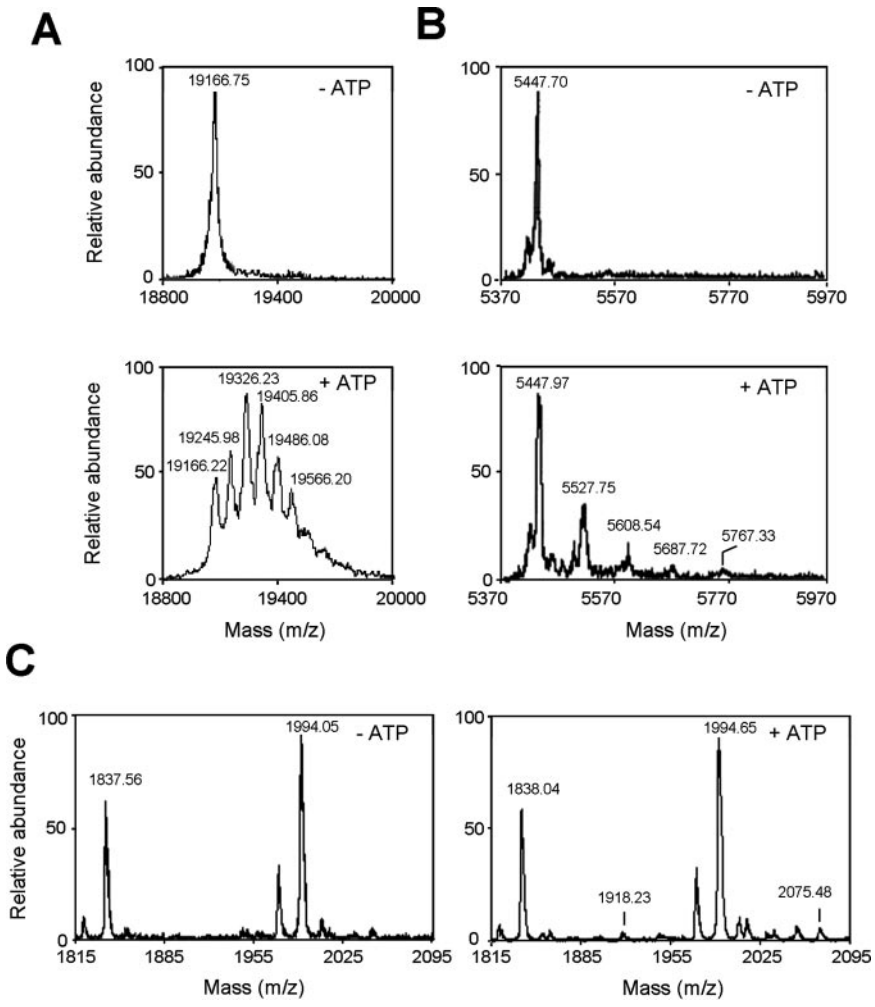

Figure 5. Identification of phospho-tyrosine residues on the N-cadherin cytoplasmic domain after in vitro phosphorylation by Src. The spectra are shown in pairs showing peptides that had been subjected to the phosphorylation reaction either in the presence or the absence of ATP. Phosphorylated peptides occurred as new peaks with an extra 80 Da in mass. (A) The His-tagged N-cadherin cytoplasmic domain was phosphorylated in vitro by Src and then subjected to MALDI-TOF mass spectrometry analysis. (B and C) His-tagged N-cadherin cytoplasmic domain was phosphorylated in vitro by Src and then digested by trypsin. The tryptic peptides were subjected to MALDI mass spectrometry analysis. (B) Identification of the phosphorylated Y852, Y860, Y884, and Y886 residues in the peptide AADNDPTAPPYDSLLVFDYEGSGSTAGSLSSLNSSSSGGEQDYDYLNDWGPR (5448 Da). (C) Identification of phospho-Y820 in peptides MDERPIHAEPQYPVR (1838 Da) and RMDERPIHAEPQYPVR (1994 Da).

cytoplasmic domain of N-cadherin is an in vitro substrate of Src and phosphorylation of Tyr-860 in N-cadherin disrupts β-catenin binding, whereas overexpression of N-cadherin with the Y860F mutation in melanoma cells has a dominant-negative effect.

The proto-oncogene c-Src belongs to the Src family of nonreceptor tyrosine kinases, among which Src has been most frequently associated with tumor progression (Frame, 2002; Summy and Gallick, 2003; Yeatman, 2004). Src is known to promote cell proliferation, survival, adhesion, motility, invasiveness, and angiogenesis. Because Src plays a critical role in many signal transduction pathways, elevated expression or activity of Src has been linked to cancer metastasis to distant sites (Boyer *et al*., 2002; Myoui *et al*., 2003).

Consistent with the notion of Src involvement in TEM, either the SFK inhibitor PP2 or the overexpression of DN-Src inhibits β-catenin dissociation and signaling. The transmigration capability of melanoma cells is also compromised. However, both PP2 and DN-Src inhibit other kinases of the Src family. Several reports have shown that SFKs, such as Fyn and Yes, may also regulate cadherin-mediated adhesion (Owens *et al*., 2000; Piedra *et al*., 2003). Therefore, we cannot exclude the potential involvement of SFKs other than Src during TEM of melanoma cells.

Significantly, our results show that the endogenous Src is activated in a stringently regulated manner during TEM. Src is associated with the heterotypic contact between melanoma cells and their surrounding endothelial cells right from the initial stages of cell attachment. But activated Src is only detectable at the heterotypic contact when the melanoma cells penetrate the endothelial junction. The timing of Src activation corresponds closely to the phosphorylation of N-cadherin and the dissociation of β-catenin from heterotypic contacts (Qi *et al*., 2005).

Structural studies have revealed that Src consists of an N-terminal myristylation site that links it to the inner plasma membrane, two Src homology domains (SH3 and SH2), a tyrosine kinase domain, and a C-terminal tail (Xu *et al*., 1999). Src activity is regulated by phosphorylation and intramolecular interactions involving the SH2 and SH3 domains (Frame, 2002). Phosphorylation of Tyr-527 leads to the binding of C-terminal region of Src to its SH2 domain, giving rise to a "closed" and inactive conformation. Src is known to be activated by a variety of mechanisms in the cell, such as intramolecular displacement, dephosphorylation of Tyr-527, and phosphorylation of Tyr-416 (Frame, 2002; Yeatman, 2004). Immunoblot analyses suggest that at least a portion of the Src associated with the heterotypic contacts is phosphorylated at Tyr-416. Several protein kinases and phosphatases have been found associated with cadherin complexes (Brady-Kalnay *et al*., 1995; Lilien and Balsamo, 2005). However, it is not known which ones are involved in the activation of Src during TEM or how the heterotypic

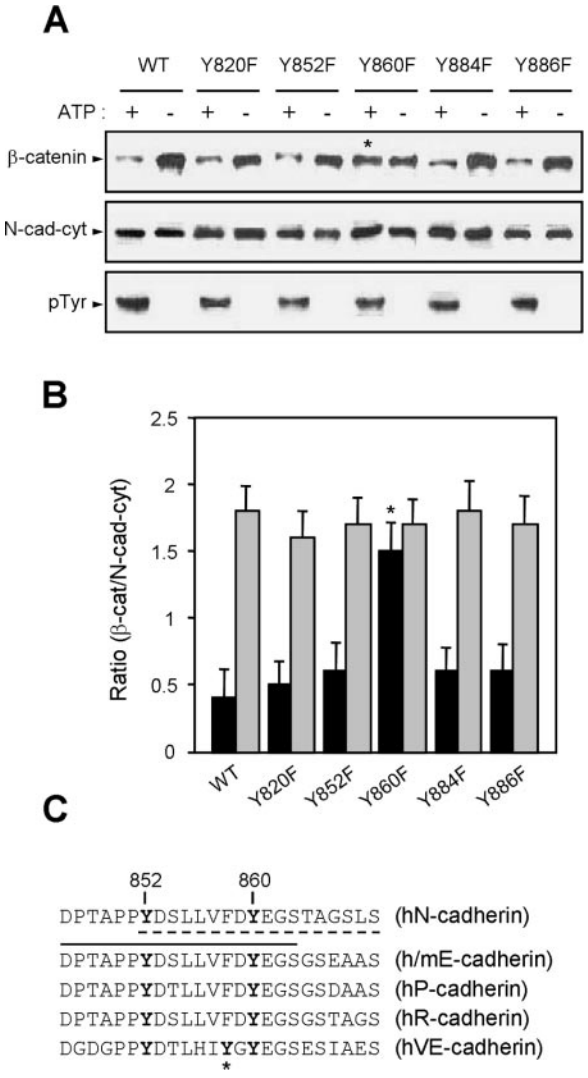

Figure 6. Effects of N-cadherin Y860 phosphorylation on β-catenin binding in vitro. (A) The five tyrosine residues identified on the N-cadherin cytoplasmic domain were mutated to phenylalanine individually. GST-tagged mutant proteins were purified from bacteria and then phosphorylated in vitro by Src. The phosphorylated proteins were subjected to the binding assay with His-tagged β-catenin. The protein complex was isolated by glutathione-Sepharose beads and the immunoblots were probed with antibodies against β-catenin, N-cadherin, and phospho-tyrosine. The phosphorylation of the protein carrying the Y860F mutation did not affect its binding with β-catenin (asterisk). (B) Quantification of the binding assays between mutant N-cadherin cytoplasmic domain and β-catenin. Before the binding assay, the mutant N-cadherin cytoplasmic domain was subjected to an in vitro Src phosphorylation reaction in the presence (black bars) or absence (gray bars) of ATP. The *y*-axis shows the ratio of bound β-catenin to N-cadherin cytoplasmic domain in the pull-down complex. *, Student's *t* test with $p > 0.1$, indicating that the level of β-catenin binding was not significantly different between the phosphorylated and the nonphosphorylated proteins. Data represent the mean ± SD (n = 3). (C) Conservation of the core β-catenin binding region in different classic cadherins. Tyr-852 and Tyr-860 of human N-cadherin and corresponding residues in the other cadherin sequences are shown in bold. The solid line indicates the core interacting region in the murine E-cadherin–β-catenin complex as identified by x-ray crystallography (Huber and Weis, 2001). The dash line indicates the core region identified in N-cadherin by peptide competition (Xu *et al.*, 2002). *, the tyrosine residue essential for VE-cadherin–β-catenin interaction (Potter *et al.*, 2005).

interactions between melanoma cells and endothelial cells would lead to Src activation.

Src has been implicated to regulate the dynamics of cell–cell interactions (Frame, 2002). An increase in Src kinase activity usually leads to a down-regulation of cadherin adhesive function (Gumbiner, 2000; Owens *et al.*, 2000; Wrobel *et al.*, 2004). However, the identity of the Src substrate within the cadherin adhesion complex remains controversial. Our in vitro and cell culture studies point to N-cadherin as a direct substrate of activated Src. The phosphorylation of N-cadherin seems to be a highly specific event, because no phosphorylated tyrosine has been detected in other proteins that coimmunoprecipitate with N-cadherin.

N-cadherin and E-cadherin are not phosphorylated or rather weakly phosphorylated on tyrosine residues in most cells (Matsuyoshi *et al.*, 1992; Behrens *et al.*, 1993; Hamaguchi *et al.*, 1993; Shibamoto *et al.*, 1994), although transfection of an active c-Src (Src531) in colon cancer cells causes tyrosine phosphorylation of N-cadherin and E-cadherin but not β-catenin (Irby and Yeatman, 2002). In contrast to the classic I cadherins, VE-cadherin, a classic II cadherin, can be heavily tyrosine phosphorylated (Lampugnani *et al.*, 1997; Esser *et al.*, 1998). Activation of Src by vascular endothelial growth factor induces the tyrosine phosphorylation of both VE-cadherin and β-catenin, leading to the disruption of VE-cadherin-to-β-catenin interaction (Weis *et al.*, 2004a,b). Interestingly, dephosphorylation of VE-cadherin but not the dephosphorylation of β-catenin has been correlated with the enhancement of cell contact integrity (Nawroth *et al.*, 2002). The latter observation is consistent with our finding that the phosphorylation state of N-cadherin regulates its interaction with β-catenin.

Results from our studies have highlighted the critical role of Tyr-860 of N-cadherin in β-catenin binding. Crystallographic studies show that the interaction interface between the murine E-cadherin cytoplasmic domain and β-catenin is extensive, involving the entire length of the β-catenin armadillo repeats and the C-terminal 100 residues of the E-cadherin cytoplasmic domain (Huber and Weis, 2001). However, a core region conserved among classic I cadherins seems to be essential to the interaction between E-cadherin and β-catenin. Both Tyr-852 and Tyr-860 of human N-cadherin are located in this region (Figure 6C). Immediately next to these two tyrosine residues are an aspartate and a glutamate, respectively, which are involved in salt bridge formation with two lysine residues in β-catenin. Mutating either one of the two β-catenin lysine residues to glutamate disrupts the binding to cadherin, indicating the essential role of these two ionic bonds in cadherin-β–catenin interaction (Graham *et al.*, 2000). On the other hand, Tyr-860 is located in the middle of the core β-catenin binding site of N-cadherin, which has been determined by peptide competition studies (Lilien *et al.*, 2002). It is conceivable that phosphorylation of Tyr-860 in N-cadherin might disrupt the binding with β-catenin by introducing negative charges that interfere with the adjacent salt bridge. Although this tyrosine residue is conserved in VE-cadherin, a recent report shows that it is not involved in the binding of β-catenin to VE-cadherin (Potter *et al.*, 2005). Instead, the tyrosine residue one amino acid ahead on its N-terminal side is found to cause β-catenin dissociation after phosphorylation (Figure 6C). However, this tyrosine is not present in classic I cadherins. Other differences in amino acid composition are also observed in the β-catenin binding region between VE-cadherin and classic I cadherins. These differences might account for variations in the molecular detail of β-catenin interactions with classic I and classic II cadherins.

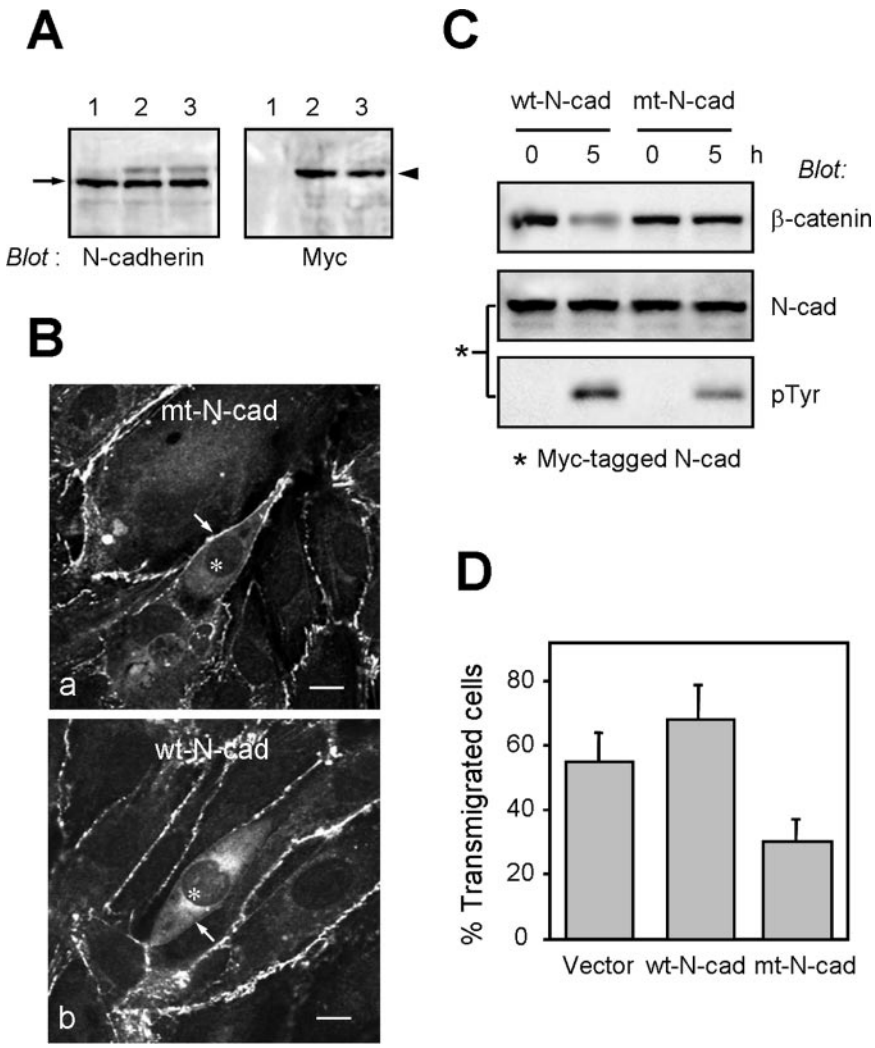

Figure 7. Effects of the expression of mutant N-cadherin on β-catenin dissociation and TEM of melanoma cells. (A) WM239 cells were transiently transfected with a myc-tagged wild-type or mutant (Y860F) N-cadherin. The expression of myc-tagged protein was confirmed by immunoblot analysis. The arrow indicates the endogenous N-cadherin, and the arrowhead indicates the expression of myc-tagged wild-type N-cadherin (lane 2) or myc-tagged mutant N-cadherin (lane 3). Control cells are shown in lane 1. (B) The transfectants were cocultured with an endothelial monolayer for 5 h before fixation and double staining using a myc mAb and a rabbit antibody against β-catenin. Myc staining was used to identify transfected cells (asterisks), and the confocal images show only β-catenin staining: melanoma cell expressing myc-tagged mutant N-cadherin (a) and melanoma cell expressing myc-tagged wild-type N-cadherin (b). Arrows indicate the heterotypic contact between transfectants and endothelial cells. Bars, 10 μm. (C) Melanoma cell were transiently transfected with wild-type N-cadherin-myc or mutant N-cadherin-myc and cocultured with an endothelial monolayer in a 3:1 ratio. Samples were collected at 0 or 5 h for immunoprecipitation with an antibody against myc. Protein blots of the immunoprecipitates were probed with antibodies against β-catenin, N-cadherin, and phospho-tyrosine. The pTyr-positive bands correspond to myc-tagged N-cadherin. (D) Transfected cells transiently expressing either wild-type or mutant myc-tagged N-cadherin were subjected to the TEM assay. Transmigration of myc-positive cells was scored at 5 h of coculture. Data represent the mean ± SD (n = 3).

When β-catenin is not bound to cadherin, it will be sequestered by the GSK3β–APC–Axin complex in the cytoplasm and targeted for degradation (Jamora and Fuchs, 2002). In contrast, we find that the total level of β-catenin shows no change between 0- and 5-h coculture (Qi *et al.*, 2005), suggesting the presence of a stabilizing mechanism for the dissociated β-catenin. GSK3β is constitutively active, but it can be rapidly inhibited by Wnt or growth factors. However, soluble factors such as Wnt and growth factors are probably not involved in stabilization of β-catenin during TEM because conditioned media from cocultures have little effect on the β-catenin level in either melanoma cells or endothelial cells (Qi and Siu, unpublished data). We speculate that the activation of Src might provide a potential mechanism to stabilize the β-catenin that has been released from N-cadherin. Src is known to activate both mitogen-activated protein kinase and phosphoinositide 3-kinase pathways (Penuel and Martin, 1999). Several recent reports show that both of these pathways can lead to the phosphorylation of GSK3β at Ser-9 and inactivate its enzyme activity, thereby inhibiting the degradation of β-catenin (Hashimoto *et al.*, 2002; Ding *et al.*, 2005; Maupas-Schwalm *et al.*, 2005). It would be of interest to determine whether one or both of these pathways are involved in the stabilization of β-catenin during TEM of melanoma cells.

As β-catenin dissociates from the N-cadherin adhesion complex, it begins to accumulate in the nucleus and activate gene transcription in transmigrating melanoma cells. Nuclear β-catenin is also frequently observed in colon cancer cells at the invasion front (Brabletz *et al.*, 2001; Hiendlmeyer *et al.*, 2004), suggesting the possible involvement of β-catenin signaling in invasion. Among the known β-catenin target genes are those that code for proteins involved in the invasive process of cancer cells, such as CD44 (Wielenga *et al.*, 1999), urokinase plasminogen activator (Hiendlmeyer *et al.*, 2004), urokinase-type plasminogen activator receptor (Mann *et al.*, 1999), matrix metalloproteinase (MMP)-7 (Brabletz *et al.*, 1999), and membrane-type matrix 1-MMP (Takahashi *et al.*, 2002). Upregulation of invasion-related genes might augment the transmigration process by promoting invasion of the basement membrane. As the molecular details of a major signaling pathway involved in the diapedesis of tumor cells are being elucidated, new possibilities are now open for the future development of therapeutic modalities to prevent metastasis.

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