Activation of the \( \beta \)-Catenin/T-Cell–Specific Transcription Factor/Lymphoid Enhancer Factor-1 Pathway by Plasminogen Activators in ECV304 Carcinoma Cells

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Abstract

Besides its involvement in clot lysis, the plasminogen activator (PA) system elicits various cellular responses involved in cell migration, adhesion, and proliferation and plays a key role in the progression of cancers. \( \beta \)-Catenin interacts with E-cadherins and functions as transcriptional coactivator of the Wnt-signaling pathway, which is implicated in tumor formation when aberrantly activated. We report that tissue-type plasminogen activator (tPA) elicited tyrosine phosphorylation and cytosolic accumulation of an active (non–serine-threonin phosphorylated, nonubiquitinated) form of \( \beta \)-catenin in ECV304 carcinoma cells. tPA-dependent \( \beta \)-catenin activation is mediated through epidermal growth factor receptor (EGFR) transactivation (via Src), suggested by the inhibitory effects of AG1478 and PP2 (specific inhibitors of EGFR and Src, respectively) and by the lack of \( \beta \)-catenin activation in EGFR-negative B82 fibroblasts. EGFR phosphorylation and \( \beta \)-catenin activation were inhibited by plasminogen activator inhibitor 1 and pertussis toxin, two inhibitors of the urokinase-type plasminogen activator (uPA)/uPA receptor system. \( \beta \)-Catenin activation was correlated with the phosphorylation of glycogen synthase kinase-3\( \beta \) through a phosphatidylinositol 3-kinase/Akt-dependent mechanism. Gel shift experiments revealed the activation of \( \beta \)-catenin/T-cell–specific transcription factor (Tcf)/lymphoid enhancer factor-1 (Lef) transcriptional complex, evidenced by an increased binding of nuclear extracts to oligonucleotides containing the cyclin D1 Lef/Tcf site. \( \beta \)-Catenin silencing through small interfering RNA and antisense oligonucleotides inhibited both the tPA-mediated cyclin D1 expression and cell proliferation. A similar activation of the \( \beta \)-catenin pathway was triggered by amino-terminal fragment, the NH\(_2\)-terminal catalytically inactive fragment of tPA, thus suggesting that this effect was independent of the proteolytic activity of plasminogen activators. In conclusion, the \( \beta \)-catenin/Lef/Tcf pathway is activated by tPA and is involved in cell cycle progression and proliferation. (Cancer Res 2005; 65(2): 526-32)

Introduction

The plasminogen activator (PA) system is composed of two plasminogen activators [tissue-type (tPA) and the urokinase-type (uPA)], a specific uPA receptor (uPAR), and a major PA inhibitor, plasminogen activator inhibitor (PAI)-1 (1). tPA and uPA are well-characterized serine proteases that convert plasminogen to active plasmin and degrade fibrin and other constituents of the extracellular matrix (1, 2). Beyond their proteolytic role, PAs act as extracellular mediators that bind cell surface receptors and activate various cellular signaling pathways involved in cell migration, adhesion, and proliferation (3). PAs and uPAR play a major role in the pathophysiology of cardiovascular diseases, in cancer development, and in metastatic dissemination.

Upon binding to cell surface receptors, PAs trigger the activation of various signaling pathways, including Src family tyrosine kinases, \( \beta \)-integrins and cytoskeletal proteins, pertussis toxin–sensitive G\(_i\)-coupled proteins, epidermal growth factor receptor (EGFR) transactivation, Ca\(^{2+}\) influx, Janus-activated kinase/signal transducer and activator of transcription and Ras/extracellular signal-regulated kinases (ERK) (4–8). PAs can up-regulate the expression of the uPA receptor uPAR (9) whose transcription is activated by activator protein–1 (10).

Recent reports have underlined the existence of a concomitant increase in the expression of uPAR and of \( \beta \)-catenin (a key component of the canonical Wnt signaling pathway; ref. 11). in primary colon carcinoma, and in metastasis. Moreover, an overexpression of uPAR is observed in colorectal cell lines transfected with the \( \beta \)-catenin gene (12), suggesting that interactions could exist between these two signaling pathways.

\( \beta \)-Catenin is a signaling cytoplasmic transducer, that normally binds to the cytoplasmic domain of transmembrane cadherins and is involved in cell-cell adhesion (12–15). Usually, the \( \beta \)-catenin turnover is regulated in the cytosol by a large protein complex including axin, \( \beta \)-TrCP, the tumor suppressor gene product adenomatous polyposis coli, protein phosphatase 2A, casein kinase 1, and glycogen synthase kinase-3\( \beta \) (GSK3\( \beta \)). Phosphorylation of \( \beta \)-catenin by GSK3\( \beta \) prevents the nuclear translocation of \( \beta \)-catenin and promotes its ubiquitination and subsequent degradation by proteasome (for a review, see Giles et al., ref. 12). Upon stimulation through the canonical Wnt signaling pathway or following mutations on adenomatous polyposis coli (12), \( \beta \)-catenin accumulates into the cytosol and translocates to the nucleus where it may associate to the lymphoid enhancer factor-1/T-cell specific transcription factor (Lef-1/Tcf), thereby activating target gene transcription and biological responses (12, 16). The Wnt signaling pathway regulates cell growth and differentiation during embryonic development and plays a role in tumor formation when aberrantly activated (11, 16). Moreover, the nuclear translocation of \( \beta \)-catenin can also be stimulated by oncogenic factors including fibroblast growth factor 2, vascular endothelial growth factor, insulin-like growth factor I, amphiregulin and \( \beta \)-cellulin.
viral infection, and PKD1 overexpression (16–24). In addition, β-catenin could control EGF-mediated cell cycle progression by modulating cyclin D1 expression (25).

Because tPA/uPA and β-catenin pathways may play a role in cancer progression, we aimed to investigate whether their signaling pathways were interconnected. Moreover, as tPA/uPA is mitogenic to ECV-304 carcinoma cells (26), we examined whether the β-catenin pathway was implicated in this mitogenic effect. We report here that tPA induced the accumulation of the active form of β-catenin in ECV-304 and the activation of the β-catenin/LeF/Tcf transcription factor through an EGRF-dependent mechanism. Silencing β-catenin with small interfering RNA (siRNA) or antisense oligonucleotides strongly inhibited tPA-induced cyclin D1 overexpression, suggesting that the β-catenin pathway mediates the tPA-induced cell cycle progression and proliferation.

Materials And Methods

Chemicals. Alteplase (tPA) was obtained from Boehringer (Ingelheim, France), amino-terminal fragment (ATF) from Landing Biotech (Bighton, MA), anti-active and anti-total β-catenin, anti-phosphoGSK3β, antiphosphotyrosine protein (4G10), and anti-phosphoAkt antibodies from Upstate Biotechnology (Euromedex, Mundolsheim, France), anti-EGFR, anti-cyclin D1, and anti-total Akt from Santa Cruz (Tebu, France). Pertussis toxin, PAI-1, and PP2 were from Sigma (St. Louis, MO), AG1478 from Calbiochem (La Jolla, CA), and other reagents were from Sigma or Invitrogen-Life Technologies, Inc. (Carlsbad, CA).

Cell Culture. Human ECV-304 carcinoma cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 containing 10% FCS. Parental EGFR–negative B82 human fibroblasts (B82P) and EGFR-overexpressing (B82K+) fibroblasts were a generous gift from Dr. M. Weber (Department of Microbiology and Cancer Center, University of Virginia, Charlottesville, VA) and cultured as described (27). Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test or by the SYTO-13/IP test, as previously used (28).

siRNA Treatment. Cells were transiently transfected with 20 μmol/L siRNAs (Dharmacon, Lafayette, CO), corresponding to part of the β-catenin sequence 5'-CUAUCGCGUAUACGGGG-3' sense strand. In Optimem (Life Technologies, Inc.) containing oligofectamine (29). Alternatively, β-catenin expression was inhibited by antisense oligonucleotides as reported (ref. 14; coding sequence: 5'-TggTGCA C sCsTsT Cgc gT C TsC-3', sense strand, Proligo, Paris, France). Cell proliferation was determined on ECV304 cells previously serum starved in RPMI 1640 for 24 hours. Control, antisense-, and siRNA-treated cells were incubated with tPA (50 ng/mL) for 72 hours and labeled for the last 12 hours of the experiment with 0.5 μCi/mL 3H]thymidine (28).

Western Blot Analysis. After incubation with the different agents, the cells were lysed for 30 minutes at 4°C in solubilization buffer and 50 to 75 μg proteins from the cell lysate were subjected to SDS-PAGE as previously reported (28). When indicated, immunoprecipitation experiments were done using the indicated antibodies. Western blots were detected using enhanced chemiluminescence detection reagents (Amer sham, Les Ulis, France).

Electrophoretic-mobility Shift Assay. Cells were washed twice in cold PBS, scraped off, and resuspended for 15 minutes on ice in buffer A (10 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L MgCl2, 1 mmol/L dithiothreitol, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL aprotinin, 2 μg/mL pepstatin), incubated for 20 minutes at 4C, and centrifuged (13,000 × g, 5 minutes). Nuclear protein–containing supernatants were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% low-fat milk in PBS (10 minutes at RT), then incubated overnight with the different antibodies and visualized by ECL (Amersham Pharmacia Biotech, Uppsala, Sweden).

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Figure 1. tPA triggers the tyrosine phosphorylation and cytosolic accumulation of β-catenin. A, time course of tyrosine phosphorylation of β-catenin by tPA evaluated on β-catenin immunoprecipitates (IP) from ECV304 cells stimulated by tPA (50 ng/mL) using 4G10 antiphosphotyrosine (Anti-PY), and anti-total β-catenin antibodies. B, kinetics of cytosolic accumulation of β-catenin. After stimulation by tPA, cells were lysed in hypotonic buffer, as described in text, to separate cytosol from membrane fraction. After separation by SDS-PAGE, cytosol and membrane proteins were immunoblotted with anti-total β-catenin antibodies. Pretreatment of cells by LCI (1 mmol/L, 6 hours) was used as positive control. Alternatively, total cell extracts were immunoblotted using an anti-active (β-catenin (nonphosphorylated, nonubiquitinated β-catenin) antibody. C, confocal microscopy of β-catenin in ECV304 cells stimulated for 30 minutes by tPA (50 ng/mL). Representative of three separate experiments.

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then with a secondary FITC-conjugated anti-rabbit immunoglobulin G, and visualized using a Zeiss (Jena, German) LSM 510 confocal fluorescence microscope.

**Statistical analysis.** Data are given as mean ± SE. Estimates of statistical significance were done by ANOVA (one-way ANOVA, Tukey test, using SigmaStat software).

### Results

**tPA Triggers a Cytosolic and Nuclear Translocation of Active β-Catenin.** Because tyrosine phosphorylation of β-catenin is necessary for lowering its affinity for cadherins (30, 31), we first checked for the tyrosine phosphorylation of β-catenin mediated by tPA and its subsequent presence in the cytosol. As shown in Fig. 1A, treatment of ECV-304 cells with tPA led to a rapid tyrosine phosphorylation of β-catenin immunoprecipitated from ECV304 cells, starting after 5 minutes and maintained during 15 to 30 minutes. At the same time, we observed a progressive increase of β-catenin in the cytosol (maximal after 30 minutes), further identified as the active β-catenin (Fig. 1B). LiCl, which mimics Wnt signaling by inhibiting GSK3β activity, was used as positive control (32). As shown by confocal microscopy, the tPA treatment induced an increased nuclear localization of β-catenin in ECV-304 cells (Fig. 1C).

**Mechanism of β-Catenin Activation Elicited by tPA.** Because EGF-related growth factors may activate β-catenin (33) and because PA signaling has been shown to transactivate EGF/R (34), we investigated the role of EGF in the activation of β-catenin by tPA. In agreement with Guerrero et al. (34), tPA induced a rapid tyrosine phosphorylation of EGFR, mediated by a pertussis toxin-sensitive G protein and Src, as shown by the inhibitory

**Figure 2.** Role of EGFR and Src on β-catenin activation. A, EGFR and Src phosphorylation induced by tPA and effect of inhibitors. ECV304 cells were stimulated for 2 minutes by tPA (50 ng/mL) with or without AG1478 (AG, 2 μmol/L), pertussis toxin (PTX, 50 ng/mL), PAI-1 (50 ng/mL), and PP2 (10 μmol/L). Western blot experiments were done with anti-phosphotyrosine protein, anti-active phospho-Src, anti-EGFR, and anti-β-actin antibodies. B, β-catenin activation induced by tPA and effect of inhibitors. ECV304 cells were incubated for 30 minutes with tPA and with or without inhibitors (as in A). Western blot experiments were done using anti-active β-catenin. C, kinetics of β-catenin tyrosine phosphorylation induced by tPA (50 ng/mL), in B82K+ (EGFR-expressing cells) and in B82 (EGFR-nonexpressing cells). Immunoblots with anti-EGFR, anti-active β-catenin, and anti-β-actin as control. Representative of at least three separate experiments.

**Figure 3.** tPA and ATF trigger the phosphorylation of GSK3β through a PI3-kinase/Akt-dependent pathway in ECV304 cells. A, time course of Akt and GSK3β phosphorylation and β-catenin activation induced by tPA (50 ng/mL). Immunoblots with anti-phosphoAkt, anti-phosphorylated GSK3β antibodies, and anti-β-actin as control. B, effect of the PI3-kinase inhibitor LY290424 (LY, 1 μmol/L) on Akt activation, GSK3β phosphorylation, and β-catenin activation induced by tPA. C, effect of PTX, PAI-1, PP2, and AG1478 on Akt phosphorylation induced by tPA. D, activation of Akt and β-catenin by ATF (50 ng/mL, 30 min). Effect of inhibitors. Representative of three separate experiments.
were phosphorylated concomitantly to the catalytically inactive fragment of tPA (Fig. 3A, Lef/TCF site. Nuclear protein extracts from ECV304 cells stimulated by tPA (50 ng/mL) were subjected to gel shift analysis using a radiolabeled oligonucleotide containing the cyclin D1 Lef/TCF site. A, time course of Lef/TCF complex binding (arrow), quantified by Image Quant. B, EMSA supershift (arrow) of nuclear cell extracts (45-minute stimulation by tPA, 50 ng/mL) done in the presence of anti-active β-catenin and ERK1/2 as control. C, effect of inhibitors (PTX, PAI, and AG1478, same conditions as in Fig. 2) on the tPA-mediated Lef/TCF complex binding. A and C (bottom), data expressed as percent of control. Columns, mean of five separate experiments; bars, SE. *, P < 0.05.

Figure 4. EMSA of β-catenin/Lef/Tcf proteins to the Lef/TCF site of the cyclin D1 promoter. Nuclear protein extracts from ECV304 cells were phosphorylated concomitantly to EGFR and inhibited by pertussis toxin, PAI-1, PP2, and AG1478. The concomitant inhibition of tyrosine phosphorylation of EGFR and Src by both PP2 and AG1478 (Fig. 2A) strongly suggests that a cooperation exists between Src and EGFR, as previously shown and discussed (35, 36).

All these agents blocked the tyrosine phosphorylation of β-catenin and its accumulation as active form in the cytosol (Fig. 2B). To assess the role of EGFR in β-catenin activation, we used B82P fibroblasts, which do not express EGFR or genetically engineered B82K+ cells expressing EGFR (27). As shown in Fig. 2C, tPA treatment of B82K+ fibroblasts elicited tyrosine phosphorylation of β-catenin but not in parental B82P (EGFR deficient) fibroblasts (Fig. 2C). Altogether, these data indicate that β-catenin activation by tPA is subsequent to EGFR activation, which is mediated through pertussis toxin–sensitive G-protein and Src-dependent signaling pathways.

**tPA and ATF Activate Akt and GSK3β Phosphorylation.** Under basal conditions, β-catenin that is unbound by cadherins is complexed in the cytosol with adenomatous polyposis coli and axin, rapidly phosphorylated by GSK3β, and degraded by the proteasome (12). GSK3β activity is regulated by Akt-mediated phosphorylation, the phosphorylated form of GSK3β being inactive. On stimulation by tPA (Fig. 3A-C) or ATF, the NH2-terminal catalytically inactive fragment of tPA (Fig. 3D), Akt, and GSK3β were phosphorylated concomitantly to β-catenin activation (Fig. 3A and B). As expected, the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY290042 inhibited the phosphorylation of Akt and GSK3β and β-catenin activation (Fig. 3B). The tPA-induced Akt phosphorylation was strongly inhibited by EGFR and Src inhibitors AG1478 and PP2 (Fig. 3C). Similarly, ATF also triggered Akt phosphorylation and β-catenin activation that were inhibited by pertussis toxin and by AG1478 and LY290042 (Fig. 3D). This again suggests a role for EGFR and Src in β-catenin activation. Moreover, the activity of ATF indicates that the activation of β-catenin is independent of the proteolytic activity of tPA and is correlated with the phosphorylation of GSK3β by the PI3-kinase/Akt pathway.

**tPA Triggers the Activation of β-Catenin/Lef/Tcf Complex.** The nuclear translocation of the β-catenin/Lef/Tcf complex was investigated by analyzing the binding of nuclear protein extracts from cells stimulated by tPA on the consensus Lef/Tcf site of cyclin D1 promoter (18). Gel shift experiments (Fig. 4A) showed an increased binding of nuclear proteins to the cyclin D1 Lef/Tcf site, maximal after 45 minutes of stimulation by tPA. Supershift experiments done in the presence of the anti-active β-catenin antibody showed the presence of β-catenin in the complex, whereas an anti-ERK1/2 antibody (done as negative control) had no effect (Fig. 4B). The binding of β-catenin/Lef/Tcf complex was inhibited by pertussis toxin, PAI-1 and AG1478 (Fig. 4C).
cyclin D1 (25, 37–39). Incubation of ECV304 cells with tPA led to a rapid and sustained (up to 8 hours) overexpression of cyclin D1 protein (Fig. 5A). siRNA and antisense oligonucleotides markedly inhibited β-catenin expression (Fig. 5B) and considerably reduced the level of cyclin D1 expression (induced by tPA after 6 hours of pulse; Fig. 5C) and DNA synthesis (determined by [3H]thymidine incorporation; Fig. 5D), whereas nonspecific siRNA and scrambled oligonucleotides had no effect. This inhibitory effect did not result from any loss of viability of cells treated by siRNA and antisense oligonucleotides, as assessed by MTT test. The viability was 98 ± 5% and 93 ± 8% of controls with siRNA and antisense, respectively. It may be noted that the mitogenic effect of tPA and ATF was not restricted to ECV304 cells because it was also observed in smooth muscle cells (26) and also in Chinese hamster ovary cells in which treatment by 50 ng/mL of tPA or ATF induced an increase of DNA synthesis of 142 ± 5% (P = 0.03) and 198 ± 25% (P = 0.05) of controls, respectively.

Altogether, these data indicate that active β-catenin closely participates in the cell cycle progression and proliferation induced by tPA.

**Discussion**

PAs and uPAR are involved in tumor cell invasion and metastasis formation (2, 10). How PAs promotes cell invasiveness is associated with its involvement in ECM degradation, in association with metalloproteinases and cysteine proteases (40). Besides this protease activity, the PAs system interacts with various signaling pathways involved in cell migration, adhesion, and proliferation. Our results show that tPA and the catalytically inactive ATF stimulate the β-catenin pathway, thereby inducing a cytosolic and nuclear accumulation of β-catenin and the formation of the transcriptional complex β-catenin/Lef/Tcf, which in turn modulates cyclin D1 expression and cell cycle progression. To our knowledge, this is the first report providing evidence for an activation of β-catenin/Lef/Tcf transcriptional activity by PAs and giving a novel aspect of their pro-oncogenic properties.

Besides its classic activation by the (canonical) Wnt signaling pathway (12), β-catenin is stimulated by non-Wnt ligands, in particular by growth factors (19–23). We hypothesized that EGFR could be involved because EGF and EGFR are able to induce β-catenin signaling (33) and because EGFR is a known target of the uPA/uPAR system (34). In agreement with these reports, we found that EGFR is rapidly phosphorylated in the presence of tPA and is involved in β-catenin signaling, as suggested by (a) the inhibitory effect of AG1478 on EGFR phosphorylation and on β-catenin/Lef/Tcf transcriptional activity and (b) by the lack of β-catenin activation in EGFR-negative B82 parental fibroblasts. EGFR phosphorylation required Src activation through pertussis toxin- and PAI-sensitive mechanisms as previously reported (34–36). Note that a synergistic cooperation between Src and EGFR is likely because AG1478 inhibited Src phosphorylation by tPA, probably because phosphorylated EGFR may recruit Src and potentiate its enzymatic activity (35). Moreover, note that Src is essential for the tyrosine phosphorylation of β-catenin (30). The inhibitory effect of PAI-1 on Src and EGFR signaling (resulting in β-catenin inhibition) could be due to its inhibition of tPA-mediated proteolytic activity, but it is likely that PAI-1 inhibits the integrin pathway, which is also known to activate FAK and Src (41, 42). Moreover, the activation of β-catenin by tPA occurred independently of tPA-mediated proteolytic activity, as suggested by the similar effect of ATF, as previously reported for PAs signaling (2).

The accumulation of β-catenin as active form was correlated to the phosphorylation of GSK3β occurring through a PI3-kinase/Akt-dependent mechanism. GSK3β is constitutively active in resting cells and its activity is negatively regulated by a PI3-kinase/Akt-dependent phosphorylation, which leads to an accumulation of cytosolic and nuclear β-catenin as observed in the presence of Wnt ligands (12–16) and of PDK1 overexpression (which phosphorylates and activates Akt downstream PI3-kinase) or in presence of LiCl, which directly inhibits GSK3β (32, 43). We thus conclude that tPA stimulates an EGFR-dependent

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**Figure 5.** β-Catenin silencing by siRNA and antisense oligonucleotides reduce cyclin D1 expression and DNA synthesis. ECV304 were transfected with 20 μmol/L siRNAs β-catenin. After 24 hours, this medium was replaced by fresh RPMI medium containing 10% FCS. Alternatively, ECV304 cells were incubated for 72 hours in RPMI medium containing 10% FCS and 10 μm/L antisense (AS) or sense (S) oligonucleotides. Twelve hours before the stimulation by tPA, these media were replaced by FCS-free RPMI medium. A, time course of cyclin D1 overexpression induced by tPA (50 ng/mL) in ECV304. B, effect of siRNAs and S or AS oligonucleotides on β-catenin expression in ECV304 cells. C, effect of siRNAs and S or AS oligonucleotides on the level of cyclin D1 expression in ECV304 cells, after stimulation by tPA (50 ng/mL, 6 hours incubation). D, effect of siRNAs on DNA synthesis induced by tPA or ATF (50 ng/mL, 72 hours). Data are expressed as percent of the control. Columns, mean of four to seven separate experiments; bars, SE. *, P < 0.05.
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References


17. Garcia-Cazorla AL, Mezzina PE, Comoglio PM, Schlessinger J, Garcia-Cazorla AL, Mezzina PE, Comoglio PM, Schlessinger J. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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