Src Induces *Urokinase Receptor* Gene Expression and Invasion/Intravasation via Activator Protein-1/p-c-Jun in Colorectal Cancer

Jörg H. Leupold,[1] Irfan Asangani,[1] Gabriele D. Maurer,[1] Ernst Lengyel,[3] Stefan Post,[2] and Heike Allgayer[1]

[1]Department Experimental Surgery Mannheim Faculty, University of Heidelberg, and Molecular Oncology of Solid Tumors Unit, Deutsches Krebsforschungszentrum; [2]Department Surgery, Mannheim Faculty, University Heidelberg, Heidelberg, Germany; and [3]Section of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of Chicago, Chicago, Illinois

Abstract

The urokinase receptor [urokinase plasminogen activator receptor (u-PAR)] promotes invasion and metastasis and is associated with poor patient survival. Recently, it was shown that Src induces *u-PAR* gene expression via Sp1 bound to the *u-PAR* promoter region −152/−135. However, *u-PAR* is regulated by diverse promoter motifs, among them being an essential activator protein-1 (AP-1) motif at −190/−171. Moreover, an *in vivo* relevance of Src-induced transcriptional regulators of u-PAR–mediated invasion, in particular intravasation, and a relevance in resected patient tumors have not sufficiently been shown. The present study was conducted (*a*) to investigate if, in particular, AP-1–related transcriptional mediators are required for Src-induced *u-PAR*–gene expression, (*b*) to show *in vivo* relevance of AP-1–mediated Src-induced *u-PAR* gene expression for invasion/intravasation and for resected tissues from colorectal cancer patients. Src stimulation of the *u-PAR* promoter deleted for AP-1 region −190/−171 was reduced as compared with the wild-type promoter in cultured colon cancer cells. In gelshifts/chromatin immunoprecipitation, Src-transfected SW480 cells showed an increase of phospho–c-Jun, in addition to JunD and Fra-1, bound to region −190/−171. Src-transfected cells showed a significant increase in c-Jun phosphorylated at Ser^{73} and also Ser^{63}, which was paralleled by increased phospho–c-jun-NH_2-kinase. Significant decreases of invasion/*in vivo* intravasation (chorionallantoic membrane model) were observed in Src-overexpressing cells treated with Src inhibitors, u-PAR–small interfering RNA, and dominant negative c-Jun (TAM67). In resected tissues of 20 colorectal cancer patients, a significant correlation between Src activity, AP-1 complexes bound to *u-PAR* region −190/−171, and advanced pN stage were observed. These data suggest that Src-induced *u-PAR* gene expression and invasion/intravasation *in vivo* is also mediated via AP-1 region −190/−171, especially bound with c-Jun phosphorylated at $Ser^{73/63}$, and that this pathway is biologically relevant for colorectal cancer patients, suggesting therapeutic potential. (Mol Cancer Res 2007;5(5):485–96)

Introduction

The complex process of tumor invasion and metastasis requires a network of different proteases. Among them, the urokinase plasminogen activator (u-PA) system plays a critical role in enabling cells to overcome anatomic barriers, intravasate, and form metastases (1). The u-PA system comprises the serine protease u-PA, its substrate plasmin, the serpin inhibitors PAI-1 and PAI-2, and the u-PA receptor (u-PAR; ref. 2). u-PA leads to an activation of plasminogen and further proteases such as matrix metalloproteinases-2 (MMP-2) and MMP-9, and to the degradation of main components of the extracellular matrix such as fibrin, fibronectin, and laminin. This effect of u-PA is potentiated by binding to its specific cell receptor u-PAR, which has been shown to be overexpressed in diverse human tumors, such as gastric, colorectal, breast, and other cancers (3, 4). Several studies support the notion that an overexpression of u-PAR, a highly glycosylated glycosylphosphatidylinositol-anchored receptor, is mainly regulated at the transcriptional level, although further means of additional control such as posttranscriptional modifications, mRNA stability, and receptor internalization have been described (5). It has been shown that *u-PAR* gene expression can be induced by diverse transcription factors [e.g., Sp1, activator protein-1 (AP-1), AP-2, or nuclear factor-κB (NF-κB)] bound to different promoter motifs, following stimulation with different growth factors or cytokines (6). We and others have characterized two main cis-elements of the *u-PAR*

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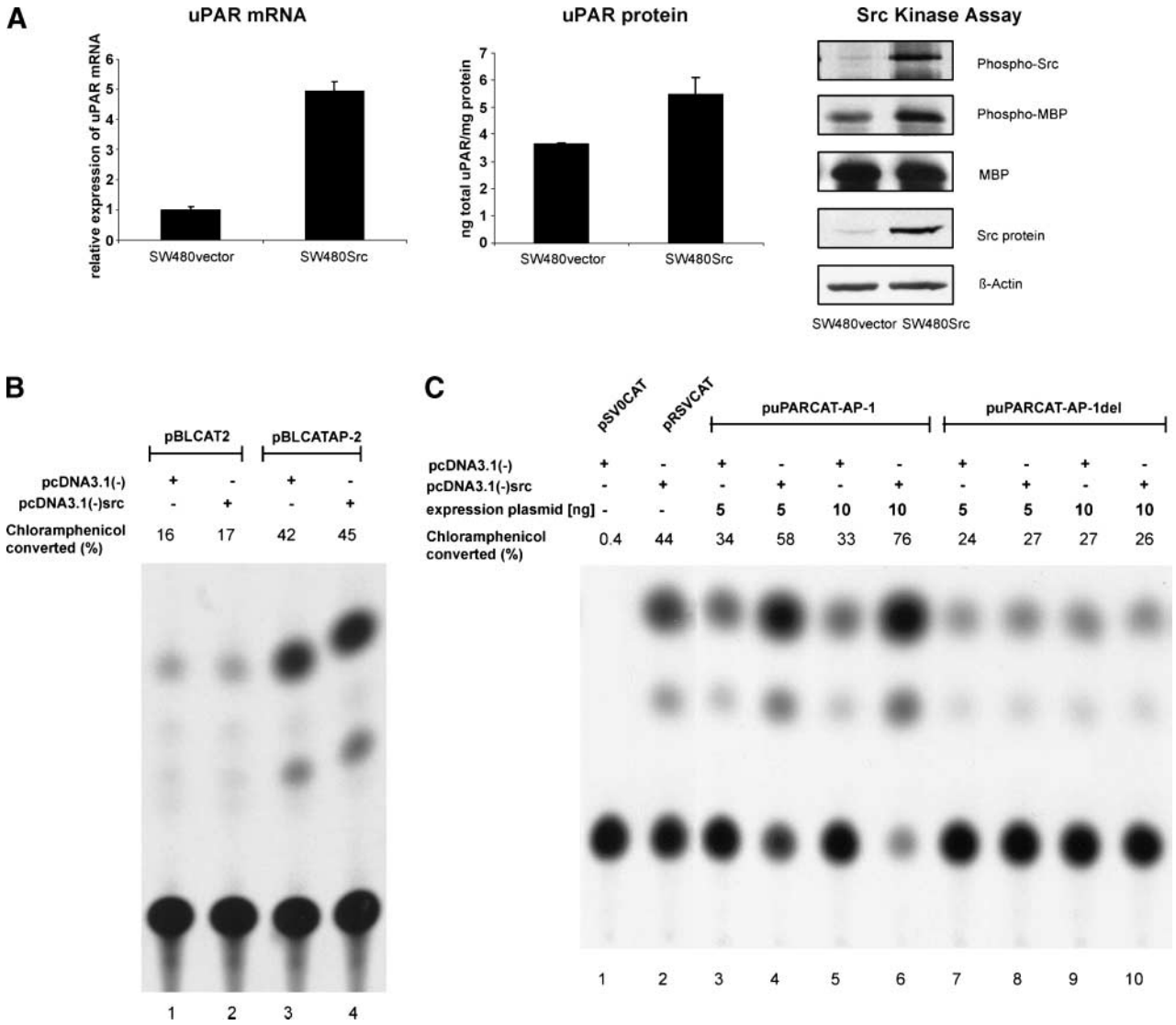


FIGURE 1. The prevention of AP-1 binding to region −190/−171 inhibits Src-mediated *u-PAR* promoter induction, whereas AP-2 is not mediating Src-induced promoter activity. **A.** Total RNA was extracted from vector and transiently Src-transfected SW480 cells, reverse transcribed, and quantified by real-time PCR using specific TaqMan primers and probes for human *u-PAR*. Samples were analyzed in quadruplicates, and relative *u-PAR* expression levels normalized against β-actin. u-PAR amounts are shown as a relative quantification, with corresponding expression levels of vector-transfected SW480 cells as a reference. Additionally, ELISA and Src kinase assays show increased u-PAR protein and Src activity in the transfected cells. After normalization, the fold increase of the Src-transfected cells compared with the vector control was 9.3-fold for phospho-Src and 4.2-fold for phospho-MBP. **B.** SW480 cells were transiently cotransfected with a CAT reporter driven by three tandem repeats of an AP-2 consensus (pBLCAT2AP2) or the corresponding control (pBLCAT2) and either the empty vector [pcDNA3.1(−)] or the vector expressing constitutively active Src [pcDNA3.1(−)src]. **C.** SW480 cells were transiently cotransfected with a CAT reporter driven by the wild-type *u-PAR* promoter containing 435 bp of the 5′-flanking sequence (uPARCAT-AP-1) or a reporter plasmid deleted for AP-1 region −190/−171 (uPARCAT-AP-1del) and either the empty vector [pcDNA3.1(−)] or the vector expressing constitutively active Src [pcDNA3.1(−)src]. After normalization for transfection efficiency, the lysates were assayed for CAT activity. pRSVCAT, positive control, pSV0CAT, negative control. Data are representative of three different experiments, the range of which did not exceed 10%.

promoter, which are highly relevant for diverse means of *u-PAR* gene expression in colorectal cancer. One of them (−190/−171) spans an AP-1 consensus motif that mediates constitutive, phorbol 12-myristate 13-acetate (PMA)−, and K-Ras−inducible expression of the gene (7, 8). A second motif (−152/−135) bound with an AP-2α−like transcription factor, Sp1 and Sp3, has been shown to mediate constitutive, PMA-, and Src-inducible *u-PAR* gene expression (9).

Src and other Src family protein-tyrosine kinases play key roles in cell differentiation, motility, proliferation, and survival (10). The activity of Src depends on the phosphorylation status of the protein, brought about by autophosphorylation, or the phosphorylation of an inhibiting phosphotyrosine by COOH-terminal Src kinase (Csk), and Csk homologous kinase (11, 12). Previous studies have shown that an elevated activity of c-Src increases *in vitro* invasion of cultured colon cancer cells, and that Src activity increases from benign colonic polyps to primary colon cancers and to colon cancer metastases (13, 14). Moreover, own studies have indicated an elevated Src activity as a poor prognostic factor in colorectal cancer patients (15).

Furthermore, our studies showed that active Src transcriptionally induces expression of *u-PAR*, this being mediated, at least in part, via increased Sp1 binding to the combined AP-2/Sp1/Sp3 u-PAR promoter region −152/−135 (16). However, because this motif is bound by additional transcription factors, for the present study, we were interested as to whether the AP-2–like protein bound to this combined AP-2/Sp1/Sp3 region is an additional parameter at this motif mediating Src-induced u-PAR gene expression. Moreover, because *u-PAR* is also regulated by other promoter motifs, especially the AP-1 site at −190/−171, we were interested to provide evidence for, in particular, AP-1–related transcriptional mediators required for Src-induced *u-PAR* gene expression. Finally, an *in vivo* relevance of Src-induced transcription factors regulating u-PAR–mediated invasion, in particular intravasation, has not yet been shown, nor has a relevance been investigated for resected tumors from patients. Therefore, a further aim of this study was to show an *in vivo* relevance of AP-1–mediated Src-induced *u-PAR* gene expression for invasion/intravasation and for resected tissues from colorectal cancer patients. Our present study is the first to suggest that Src-induced *u-PAR* gene expression and invasion/intravasation *in vivo* is also mediated via the AP-1 promoter region −190/−171 and, in particular, by c-Jun phosphorylated at Ser^{73} and Ser^{63}, that this pathway can be targeted to suppress invasion, and that this pathway is biologically relevant for colorectal cancer patients as judged from *ex vivo* studies in resected tissues.

Results

u-PAR *Promoter Induction by a Constitutively Active Src Is Mediated via AP-1, but not AP-2*

To show that c-Src overexpression leads to an increase in u-PAR-mRNA and protein, SW480 were transfected with constitutively active c-Src. A 9.3-fold induction of phospho-Src and 4.2-fold induction of phospho–myelin basic protein (phospho-MBP) were observed in the transfected cells. Furthermore, u-PAR mRNA and protein were significantly increased in c-Src–transfected cells (Fig. 1A). As published, Sp1 bound to a combined AP-2/Sp1 motif (−152/−135) is mediating, in part, Src-induced *u-PAR* gene expression (16). To investigate whether the AP-2α–like protein, bound to the same *u-PAR* promoter motif, plays an additional role in Src-induced *u-PAR* promoter activity, a chloramphenicol acetyltransferase (CAT)–reporter driven by three tandem repeats of an AP-2 consensus motif was cotransfected with either a pcDNA3 control, or constitutively active Src. As shown in Fig. 1B, Src was unable to induce activity of this AP-2 reporter as compared with the empty vector control. Furthermore, dominant negative AP-2 (AP-2αB) was unable to counter Src-induced *u-PAR* promoter activity in a second reporter assay (data not shown). These experiments suggested that AP-2 transcription factors have no additional role in mediating Src-induced *u-PAR* promoter activity.

To investigate as to whether the AP-1 binding site (−190/−171) is mediating Src-induced *u-PAR* promoter activity, a second set of CAT-reporter assays was done comparing the ability of Src to induce the wild-type *u-PAR* promoter, with a

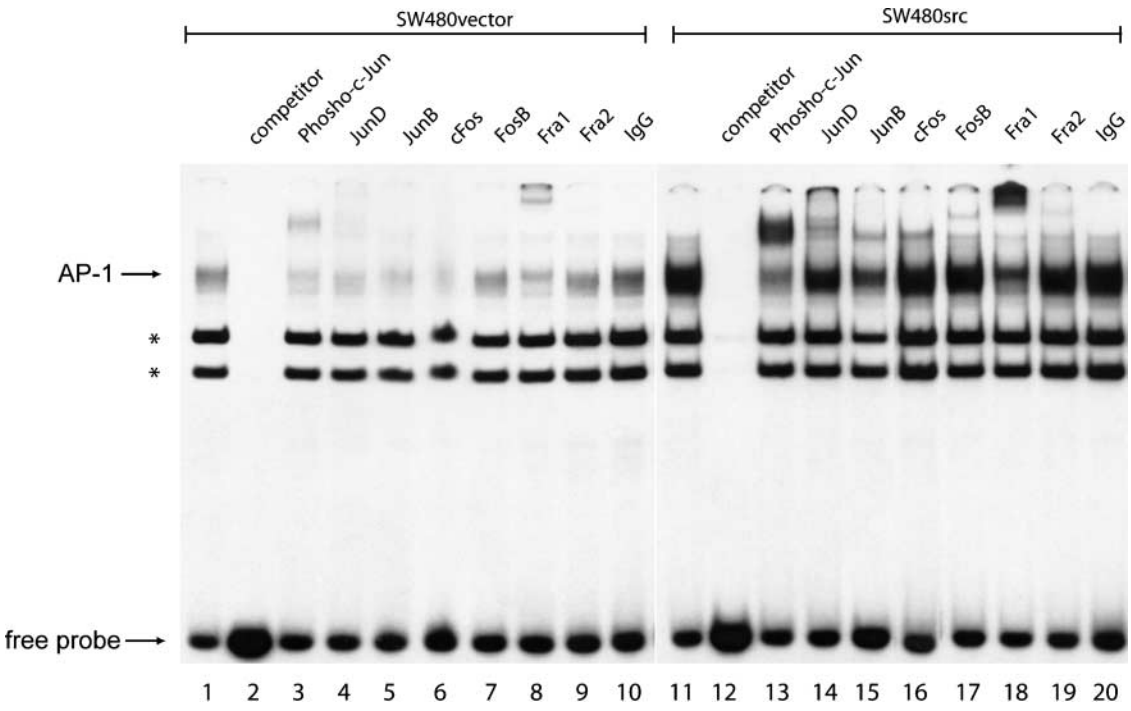


FIGURE 2. Nuclear extracts from c-*src*–expressing SW480 cells show increased binding of AP-1 transcription factors (c-Jun, JunD, and Fra-1) to an oligonucleotide spanning region −190/−171. Equal amounts of nuclear extract from vector control SW480 cells or Src-transfected SW480 cells were incubated with an end-labeled oligonucleotide (spanning −190/−171 bp region of the *u-PAR* promoter) in the presence or absence of a 100-fold excess of the unlabeled competitor sequence and the indicated antibodies. EMSA was carried out using 10 μg of nuclear extract, 1 μg of poly(deoxyinosinic-deoxycytidylic acid), 1×10^6 cpm of ^{33}P oligonucleotide, and 1 μg of antibody. Complexes were subsequently analyzed by gel electrophoresis. Top arrow, AP-1 complexes. *, nonspecific binding. Bottom arrow, free probes.

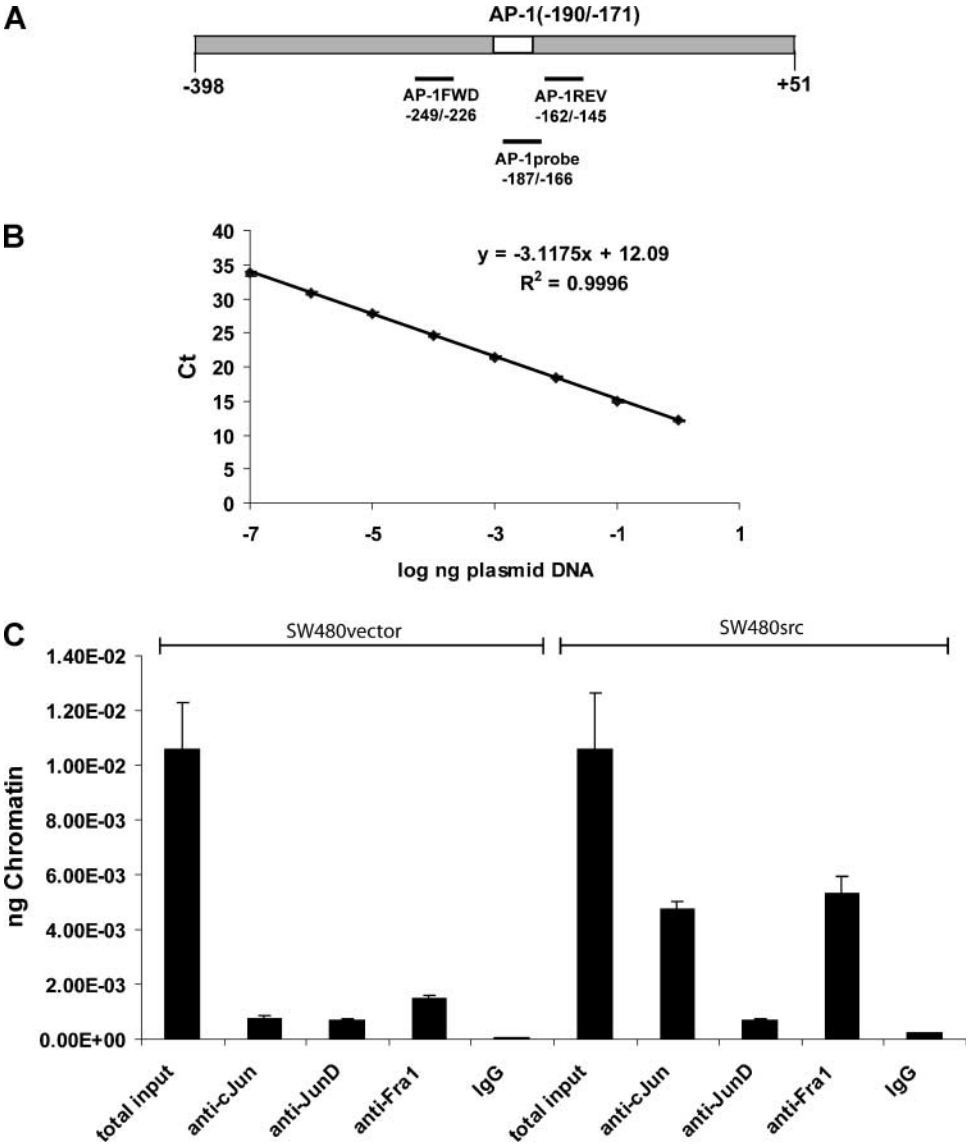


FIGURE 3. Recruitment of p-c-Jun, JunD, and Fra-1, to the AP-1 responsive cis-element within the endogenous region −190/−171 of the *u-PAR* promoter as indicated by quantitative ChIP. **A.** Schematic presentation of the region of the *u-PAR* promoter and the primers/probe used for amplification. **B.** To calculate the amount of immunoprecipitated chromatin, serial dilutions (1 to 10^{-7} ng) of pGL3-398 plasmid containing the specific AP-1 binding region were used as a standard curve. The fluorescent signal is shown as the mean of quadruplicate experiments. **C.** Quantitative measurement of immunoprecipitated chromatin. Equal amounts of chromatin from vector- or *c-src*–transiently transfected SW480 cells were incubated with the indicated antibodies and quantified by real-time PCR.

construct in which the AP-1 binding site (−190/−171) had been deleted (Fig. 1C; ref. 7). In contrast to the wild-type promoter, which was induced several-fold by Src (Fig. 1C, *lane 4* and *6*), the AP-1–deleted promoter was almost insensitive to Src induction (Fig. 1C, *lanes 8* and *10*). These results, together with observations that TAM67 can inhibit *u-PAR* promoter activity (17), imply AP-1 transcription factors, and specifically the AP-1 region −190/−171, as further mediators of Src-induced *u-PAR* promoter activity.

Expression of Constitutively Active Src Leads to Enhanced Binding of AP-1 Family Members to u-PAR *Promoter Region −190/−171*

Gelshift and supershift analysis was conducted to investigate whether AP-1 binding to region −190/−171 is increased by Src transfection and to differentiate AP-1 family members bound to this region in response to Src induction. Nuclear extracts of the colon carcinoma cell line SW480, characterized by an almost undetectable endogenous Src activity (16), were compared with transiently Src-transfected SW480 cells, resulting in a high Src activity. As shown in Fig. 2, AP-1 binding to region −190/−171 was considerably higher in Src transfectants. In supershift analysis, especially, phospho–c-Jun, in addition to JunD and Fra-1, was identified as the major AP-1 family protein bound to this region. Furthermore, there was a significantly increased binding of these components in Src-transfected SW480 cells as compared with the vector-transfected controls (Fig. 2, *lanes 11, 13, 14*, and *18*, respectively). These results support the notion that AP-1 transcription factors bound to region −190/−171 are transcriptional mediators of Src-induced *u-PAR* promoter activity, the main parameter being p-c-Jun and, less intense, JunD and Fra-1.

In vivo *Evidence for the Physical Interaction of Phospho–c-Jun and JunD and Fra-1 with the AP-1 Motif −190/−171 at the Natural* u-PAR *Promoter*

Gelshift analysis had implicated specific AP-1 family members bound to an oligonucleotide corresponding to the

sequence of region −190/−171 of the *u-PAR* promoter. To further determine which AP-1 transcription factors bind to this region of the natural *u-PAR* promoter following Src transfection, we did chromatin immunoprecipitation (ChIP) analysis, applying anti-phospho–c-Jun, JunD, and Fra-1 antibodies in Src-transfected and vector-transfected cells. Quantitative PCR amplification of the immunoprecipitated DNA using the primers specific for region −190/−171 showed a clear increase of especially phosphorylated c-Jun and also Fra-1 binding (Fig. 3C, *lanes 2* and *4* compared with *lanes 7* and *9*) to this natural promoter region following Src transfection. The quantity of these transcription factors bound to the natural promoter region reflects the band intensities found in previous gelshift analysis (Fig. 2). Taken together, these data indicate that phosphorylated c-Jun, and also Fra-1, are the main mediators of Src-induced *u-PAR* promoter activity at this motif.

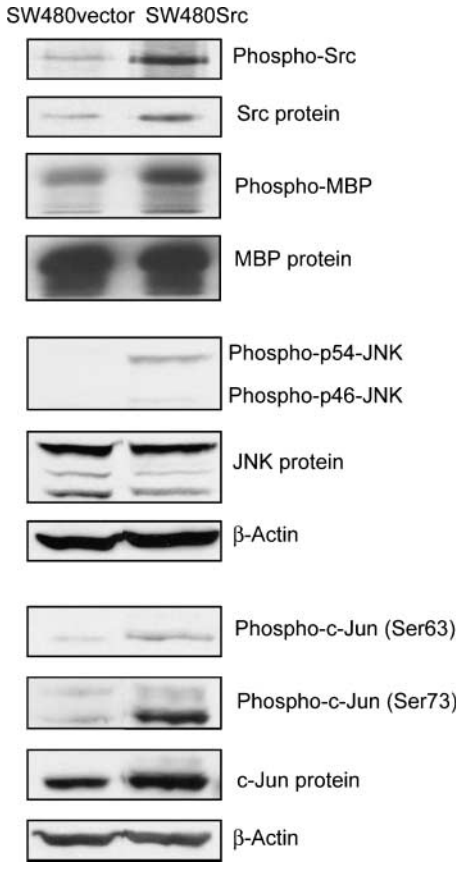


FIGURE 4. Src induces JNK activity and phosphorylation of c-Jun at Ser^{63} and, especially, Ser^{73}. Cellular extracts (equal protein) were assayed for Src activity by immune complex kinase assay using MBP as a substrate. The corresponding blots were reprobed after decay with Src and MBP antibodies. For all other proteins, Western blotting was done on cell lysates with phospho-JNK, phospho–c-Jun (Ser^{63}), and phospho–c-Jun (Ser^{73}) antibodies. The corresponding blots were stripped and reprobed with JNK, c-Jun, and actin antibodies. After normalization, the fold increase of the Src-transfected cells compared with the vector control was 2.5-fold for phospho-Src, 1.6-fold for phospho-MBP, 2.4-fold for phospho-JNK(p54), 1.2-fold for phospho–c-Jun(Ser^{63}) and 2.8-fold for phospho-c-Jun(Ser^{73}).

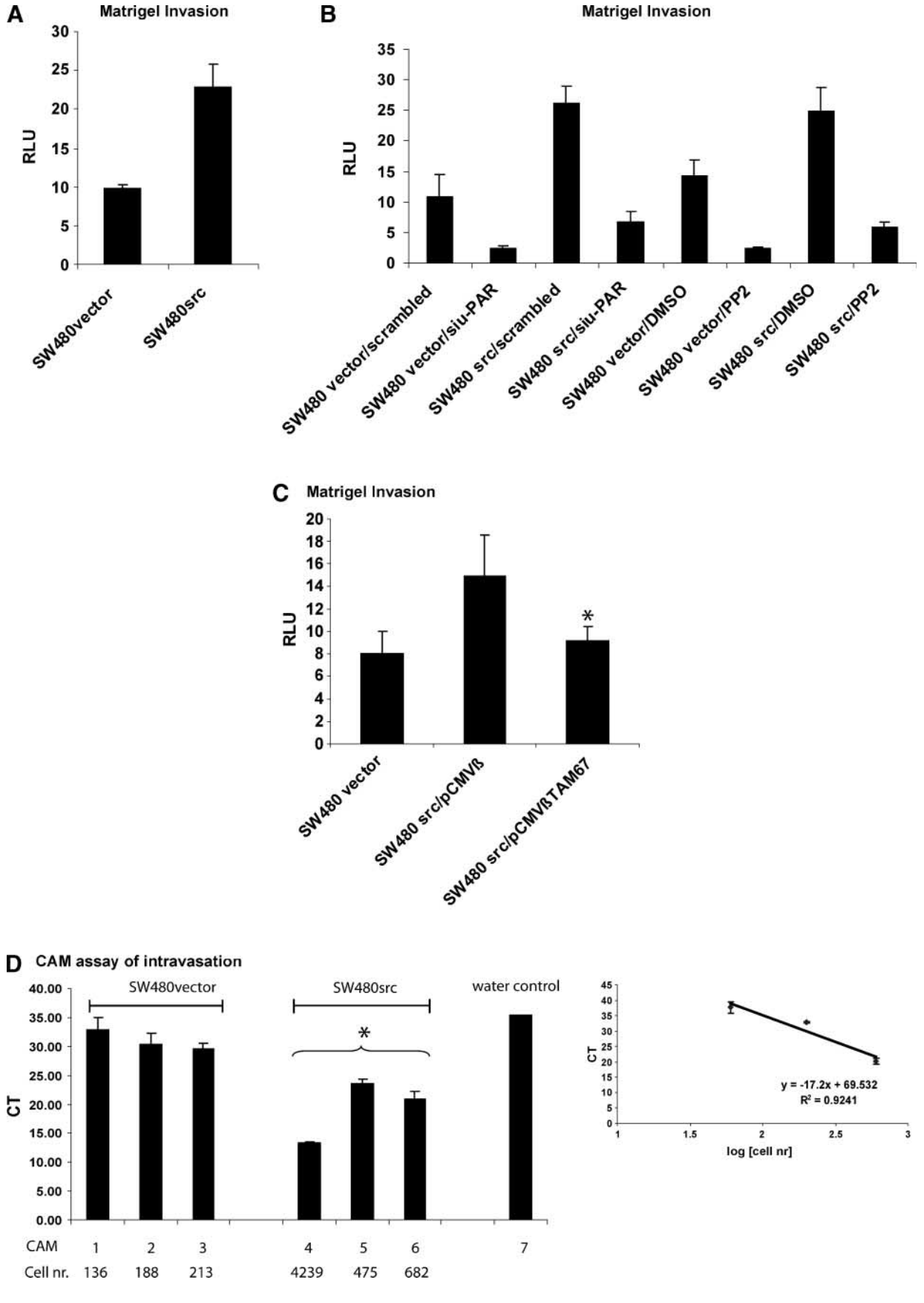
Src Induces the c-Jun-NH_2-Kinase Signal Transduction Pathway, Leading to a Specific Phosphorylation of c-Jun

Because gelshift and ChIP experiments implicated c-Jun as the main transcription factor mediating Src-induced *u-PAR* gene expression at this promoter motif, we speculated that Src might induce *u-PAR* promoter activity via c-jun-NH_2-kinase (JNK), leading to specific phosphorylation of c-Jun. To support this notion, cell lysates from SW480 cells overexpressing constitutively active c-Src and vector-transfected cells were compared for phospho-activated JNK and c-Jun phosphorylations at specific sites (Fig. 4). The amounts of endogenous phospho–p54-JNK were noticeably higher (2.4-fold) in Src-transfected than in vector-transfected SW480 cells, whereas the amount of total endogenous JNK protein remains the same with and without c-Src expression (Fig. 4). This increase of specifically active JNK was paralleled by a significant increase of Src activity in the Src-transfected cells as shown by the phosphorylation of Src (2.5-fold) and MBP (1.6-fold; Fig. 4), indicating that JNK phosphorylation was specifically brought about by an enhanced specific Src activity in these cells. Because c-Jun is known to be a direct substrate of active JNK, which leads to specific amino-terminal phosphorylation at Ser^{63} and Ser^{73} (18), we compared Src-transfected with vector-transfected cells for the phosphorylation status of these specific amino acids. As shown in Fig. 4, Src overexpression and increased activity leads to elevated amounts of phospho-c-Jun, both at Ser^{63} and Ser^{73}. The amount of phosphorylation at position 73 was higher (2.8-fold) than at position 63 (1.2-fold), indicating that Src overexpression and activation induce c-Jun mainly via pathways resulting in the phosphorylation of Ser^{73}.

Overexpression of Constitutively Active c-Src Increases Invasion In vitro *and* In vivo *and Can Be Specifically Inhibited by Src Inhibitors, u-PAR* Small Interfering RNA, *and Overexpression of a Dominant Negative c-Jun (TAM67)*

Our next objective was to investigate whether *in vitro* invasion and *in vivo* intravasation were increased by Src and whether Src inhibition and AP-1 inhibition could specifically diminish these effects. We did a Matrigel invasion assay using Src-transfected and SW480 parental cells transfected with empty control vector. When compared with vector-transfected cells, Src-overexpressing cells showed a substantially increased invasive capacity into Matrigel (2.4-fold, Fig. 5A).

Next, we asked whether specific Src inhibition, and also specific inhibition of AP-1, prevents invasion. Treatment of Src-overexpressing SW480 cells by Src inhibitor PP2 led to a significant decrease in u-PAR protein (Supplementary Fig. S1A) and to a significant reduction of Matrigel invasion (Fig. 5B, *lanes 5-8*) as compared with vector-transfected parental cells. Because there are other AP-1–dependent, Src-inducible genes relevant for invasion, we investigated whether knockdown of u-PAR mRNA via specific small interfering RNA (siRNA) is also able to inhibit Src-induced invasion. Transfection of Src-overexpressing SW480 cells with siRNA targeting the u-PAR mRNA led to a significant decrease of u-PAR mRNA and protein amounts (Supplementary Fig. S1B and C) and a significant reduction of invasion into Matrigel (Fig. 5B, *lanes 1-4*).



Furthermore, treatment of Src-overexpressing cells with dominant negative c-Jun (TAM67) substantially countered Src-induced invasive capacity (Fig. 5D). Taken together, these data indicate that Src-induced invasion can be blocked specifically by a synthetic Src inhibitor and by dominant negative c-Jun, which is mediated, at least in part, via u-PAR in this cell system.

To further analyze the impact of Src on the specific step of intravasation *in vivo*, we did the chicken embryo chorionallantoic membrane (CAM) assay quantitatively, as we described (19). Src-transfected SW480 cells were compared with empty vector–transfected parental cells in their ability to invade the upper CAM, followed by intravasation into embryonic blood vessels as measured by human Alu sequences of tumor cells arriving in the lower CAM. As observed in Matrigel, Src-transfected cells, characterized by a high *u-PAR* gene expression and high Src activity (see Figs. 1A and 4), showed an increased proportion of intravasated cells as represented by a significantly earlier detection of human Alu sequences within the chicken embryo background ($P < 0.01$; Fig. 5D). Vector-transfected parental SW480 cells showed no significant intravasation compared with the water control (Fig. 5D). Taken together, these data from two different *in vitro/in vivo* models indicate that Src regulates at least two specific steps of the metastatic cascade, which are local invasion, and intravasation.

Src Specifically Correlates with AP-1 Binding to u-PAR *Promoter Motif −190/−171 in Resected Colorectal Cancers of Patients*

Previous data had indicated that Src induces invasion and intravasation by AP-1 bound to region −190/−171 of the *u-PAR* promoter. To support this notion by further *ex vivo* data from resected tissues of patients, the tumor and corresponding normal tissues of 20 patients undergoing surgery for colorectal cancer were analyzed for endogenous Src activity and for the binding of AP-1 complexes to the *u-PAR* promoter region −190/−171, with gelshift and Src assay methods (15, 20, 21). A significant association was found between specific Src activities and AP-1 binding in resected tumor ($P = 0.01$, linear regression, Fig. 6) and also normal tissues ($P = 0.01$). In addition, significant correlations were found for u-PAR, Src, and AP-1 binding with advanced clinical tumor stages, especially with lymph node involvement (pN; Spearman, Table 1; Supplementary Fig. S2). These data give further *in vivo* support for the biological significance of Src-induced *u-PAR* gene expression specifically mediated by this AP-1 promoter motif.

Discussion

In the present paper, we provide evidence that Src induces *u-PAR* promoter activity via an AP-1 consensus motif (−190/−171) bound especially with phospho–c-Jun, in cultured colon cancer cells, this being paralleled by specific JNK activation and phosphorylation of c-Jun at Ser^{73}. Furthermore, applying two different models, the present work gives evidence for an induction of invasion and intravasation *in vivo* by Src, this being specifically blocked by synthetic Src inhibitors, siRNA against u-PAR, and by dominant negative c-Jun (TAM67). Finally, further *ex vivo* data from resected tumor and normal tissues of colorectal cancer patients clearly support the biological relevance of Src-induced *u-PAR* gene expression as mediated by this AP-1 motif of the promoter. This adds new knowledge on molecular mechanisms by which Src regulates this important invasion-related gene because in previous studies, we implicated Sp1 bound to the *u-PAR* promoter region −152/−135 as a transcription factor mediating Src-induced *u-PAR* regulation.

AP-1 transcription factors have been identified as mediators of Src in the regulation of other genes, such as CD11c, prostaglandin synthase 2, matrilysin, and different oncogenes (22-25). In the case of CD11c, AP-1 cooperated with Sp1 to mediate Src-induced gene regulation in myeloid cells (24). This is reminiscent of the present situation in which Src-induced *u-PAR* gene expression is mediated by both AP-1 transcription factors and Sp1. Certainly, our present study does not give proof for a functional synergism of AP-1 bound to region −190/−171 and Sp1 bound to region −152/−135 in the Src-induced situation, as we have shown for JunD bound to the former and the AP-2–like protein bound to the latter, to induce constitutive *u-PAR* promoter activity synergistically (9). However, the observation that a *u-PAR* promoter either deleted for the AP-1 region (Fig. 1C) or mutated for Sp1 binding to region −152/−135 (16) is almost unresponsive to Src stimulation suggests that both AP-1 binding to region −190/−171 and Sp1 binding to region −152/−135 are required for Src inducibility of the *u-PAR* promoter. Thus, Src is comparable to other stimuli that also can activate *u-PAR* gene expression,

FIGURE 5. Cells expressing constitutively active c-Src show an increased invasion/intravasation, which can be reduced by treatment with Src-kinase inhibitors, u-PAR knockdown, and TAM67. **A.** A Matrigel assay was used to assess the invasive capacity of Src-transfected SW480 colon cancer cells versus vector-transfected control cells. The transwell filters were coated with 25 μg of Matrigel. Cells (0.5×10^6) were added to the upper chambers. Conditioned NIH-3T3 medium was added to the bottom chambers. After 24 h, the cells that had migrated to the lower chamber of the filter were trypsinized and quantified. **B.** For si-RNA–mediated u-PAR knockdown, SW480 cells were transiently transfected with an siRNA specific for u-PAR (ID289) or with an unspecific (scrambled) siRNA control at a final concentration of 40 nmol/L. After 24 h, cells were additionally transfected with Src-expressing plasmid or vector control and subjected to Matrigel after an additional 48 h. For PP2-induced inhibition, SW480 cells were transiently Src-transfected, preincubated 12 h with 30 μmol/L PP2, and after 72 h, subjected to matrigel. During the whole assay, the same concentration of PP2 was added to the upper chamber. **C.** Down-regulation of the invasive capacity (Matrigel) of Src-transfected SW480 cells by dominant negative c-Jun (TAM67). Cells were transiently transfected using the Src expression plasmid either in combination with empty vector (*pCMVβ*) or the TAM-67 expression plasmid (*pCMVβTAM67*) and compared with vector-transfected control (pcDNA3.1(−)). *, $P < 0.01$, differences between vector-transfected and Src-expressing SW480 cells were statistically significant. **D.** A CAM assay was done to assess the intravasation capacity of Src-transfected SW480 cells versus vector-transfected control cells. 0.33×10^5 cells were inoculated onto the upper CAM of a 9-day-old chicken embryo. After 48 h, the lower CAM was removed, and the genomic DNA was isolated. Human Alu sequences were amplified and quantified by quantitative PCR. To back-calculate the number of intravasated cells, human genomic DNA (equivalent to 600, 200, and 60 cells of SW480) was diluted in 0.5 μg CAM genomic DNA and quantified in parallel. The fluorescent signal is shown as the mean of triplicate experiments. The R^2 value is at least 92% for the standard curve. Conversion of cycle threshold (*CT*) to cell numbers (*Cell nr.*) is shown. Differences between vector-transfected and Src-transfected cells were significant ($P < 0.01$).

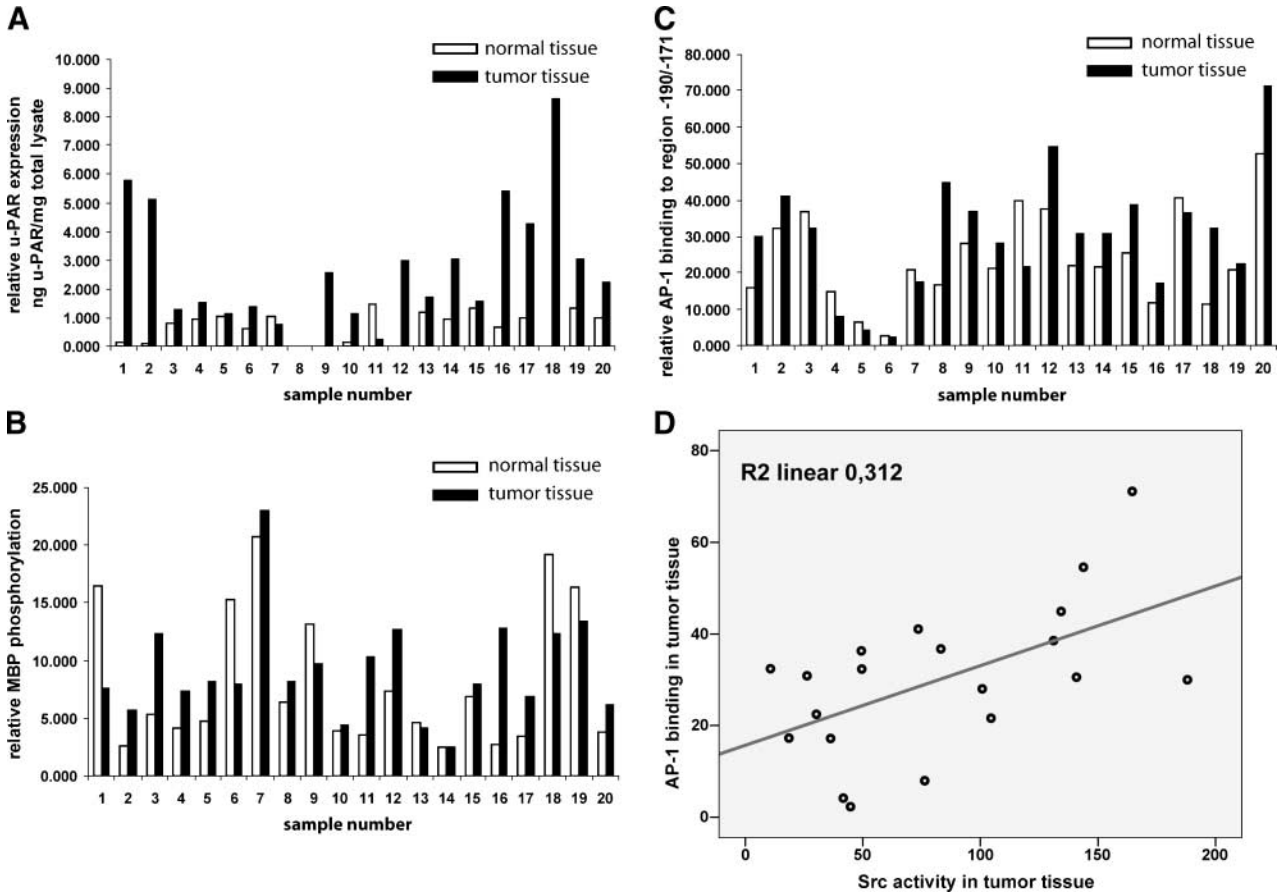


FIGURE 6. Src activity correlates significantly with binding of AP-1 transcription factors in resected tumor tissues of 20 patients with colorectal cancer. Gelshift analysis was done using resected tumor and corresponding normal tissues for the binding of transcription factors to *u-PAR* promoter region −190/−171, and specific Src activity was measured in the same resected tissues. Activities were correlated with densitometric measures of AP-1 complexes in the EMSAs. **A.** u-PAR protein amounts in normal and tumor tissues of 20 colorectal cancer patients as measured by ELISA. **B.** Src activity as measured by the ratio of Src autophosphorylation to Src protein for the same resected 20 normal and tumor tissues. Both parameters were quantified by densitometry. **C.** AP-1 binding as quantified densitometrically from gelshifts done with the same resected 20 normal and tumor tissues. **D.** Linear regression analysis shows a significant positive correlation between AP-1 binding to this specific *u-PAR* promoter motif and Src activity ($P = 0.01$; $R^2 = 0.312$).

such as the epidermal growth factor, fibroblast growth factor, and transforming growth factor-β1 (6), which can induce gene expression via AP-1 and/or Sp1 transcription factors (26-30). The fact that Src can regulate *u-PAR* via different transcription factors also explains our observation that although there was an overall correlation of Src activity and AP-1 binding, in a few of the patients investigated, there was no association. Furthermore, there are other pathways that up-regulate u-PAR in individual cases. For example, five patients (patients 9, 13, 14, 18, and 19; Fig. 6A-C) showed more binding of AP-1 to the promoter and high u-PAR protein amounts, but low Src activity. This can be explained by the fact that, for example, the small GTPase K-Ras can also activate AP-1 in colorectal cancer leading to *u-PAR* up-regulation (8). Also, further pathways independent of AP-1 have been shown to regulate *u-PAR*, this being one potential explanation for a lack of correlation between AP-1 and u-PAR protein in the small patient series investigated here. However, because in another recent publication we see a significant correlation of Src activity, AP-1 binding, and u-PAR protein in a larger patient series ($n = 92$; ref. 31), the most likely explanation for the lack of significance in the present paper is the small case number. Still, the significant correlation between Src and AP-1 and between u-PAR, Src, AP-1 and pN stage supports the *in vivo* relevance of our present work for colorectal cancer patients.

Signaling cascades employed by Src to induce AP-1 activation have been implicated in a recent paper (32), showing AP-1 activation by a synergism of TRAF6 and Src, which was mediated, in part, by phosphoinositide-3-kinase, Akt, and JNK. In our present work, phosphorylated c-Jun was the essential transcription factor bound to *u-PAR* promoter motif −190/−171 following Src induction. Specifically, an increase of c-Jun phosphorylation especially at Ser^{73} and also, to a lesser extent, at Ser^{63} was observed, which was paralleled by an increase of phosphorylation-activated JNK, implicating that, for AP-1–mediated *u-PAR* gene induction, Src employs a JNK-mediated pathway as shown for PMA-induced *u-PAR* gene expression in ovarian cancer (33). The present report specifically shows phospho–c-Jun as the main mediator of Src-induced *u-PAR* gene expression at this particular motif −190/−171 in colon cancer. Phosphorylated c-Jun bound to this motif had been implicated to mediate RalA-induced *u-PAR* gene expression in HEK293

embryonic kidney cells, and a mediating role of a Src intermediate had been speculated but not precisely followed (34). In contrast to previous situations of *u-PAR* gene expression mediated by this AP-1 motif −190/−171 in colon cancer, in which JunD was seen to be the main AP-1 transcription factor mediating constitutive and PMA-induced expression (7, 8), phospho−Ser$^{63/73}$−c-Jun has now been implicated to be a main mediator of Src-induced *u-PAR* gene expression in colon cancer. This is another example of the notion that different transcription factors bound to the same promoter motif are of different functional importance in terms of mediating diverse pathways toward the same gene. Certainly, the finding that Src induced the JNK/c-Jun pathway is not new, but it is novel in the specific context of Src-induced *u-PAR* gene expression.

In addition to modulating *u-PAR* gene expression, previous studies suggested that the viral form of Src up-regulates the production of several other proteases, such as MT1-MMP at the promoter level (35), the 92-kDa type IV collagenase (32, 33), and cathepsin L (36). Equally important, v-*src* also increases the expression of urokinase, which is the ligand for the u-PAR, and this induction requires the catalytic activity of the protein tyrosine kinase and its plasma membrane localization (37). Transforming growth factor-β1− and Smad2/3-dependent synthesis of the u-PA−specific inhibitor PAI-1 has been shown to be negatively regulated by Src (38), suggesting Src to promote proteolytic activity. In addition to (tumor-associated) proteases, Src has been reported to regulate other molecules mediating further phenomena important for the metastatic cascade, such as adhesion-regulating molecules. For example, a recent paper showed that Src mediates phosphorylation of the 140-kDa glycoprotein Trask (transmembrane and associated with Src kinases) in a mitosis-dependent manner, which in turn interacts with cadherins, syndecans, and other adhesion-regulating molecules, thus causing a deregulation of adhesion signaling following abundant Src activation (39). Furthermore, Src has been shown to deregulate cell-cell contacts in cooperation with Ezrin in breast cancer cells (40). The observation that Src can up-regulate the u-PAR, the u-PA system, and several additional proteases and deregulate adhesion and cell-cell contacts is of particular interest because it suggests a coordinate means of regulating a set of genes required for invasion and metastasis. Thus, it is not surprising that Src has been found to enhance *in vitro* invasion and motility in tamoxifen-resistant breast cancer cells (41), to mediate hepatocyte growth factor−induced *in vitro* invasion of cultured human cholangiocarcinoma cells (42), or to mediate c-Met−induced *in vitro* invasion of PC3 cells, which can be countered by the metastasis suppressor CD82(KAI1) (43). In addition, different Src inhibitors have been shown to reduce *in vitro* invasiveness, for example, in colon cancer (13), prostate cancer (44), or breast cancer (41), to inhibit angiogenesis of cultured pancreatic cancer (45) and metastasis formation in lung cancer cell lines (46).

Our present study is the first, however, to show that *in vivo* intravasation as represented in the CAM model is increased after Src transfection, this being, at least in part, brought about by *u-PAR* gene expression. Furthermore, TAM67 has been implicated in the prevention of tumor growth (47), transformation (48), in cell cycle arrest (47, 49) and migration (50), but rarely in invasion thus far. The notion that u-PAR plays a crucial role in Src-mediated invasion is further supported by the fact that specific siRNAs targeting the u-PAR mRNA significantly reduce the invasive potential of cells. The biological and potentially clinical importance of Src/c-Jun−induced *u-PAR* gene expression and invasion/intravasation is further supported substantially by our present *ex vivo* correlational data in resected tissues of colorectal cancer patients, suggesting that this specific pathway is activated in patient tumors and might be a good target.

Together with our previously published report that Src activity and also cooperation of transcription factors and Src regulating u-PAR are indicators of poor prognosis in colorectal cancer patients (15, 31), these results corroborate the notion that Src inhibition and, specifically, an inhibition of Src-induced *u-PAR* gene expression mediated by phospho−c-Jun bound to AP-1 motif −190/−171 might be promising strategies to prevent tumor progression and metastasis in colorectal cancer, specifically by inhibiting the decisive initial metastatic steps of invasion and intravasation into the systemic circulation.

Table 1. *P* Values for the Correlation of Clinical Tumor Stages and Histopathologic Grading

	pT	pN	M	Grading
AP-1 binding T/N	n.s.	0.024	n.s.	(0.058)
Src activity T/N	n.s.	0.045	n.s.	n.s.
Src protein T/N	n.s.	0.039	n.s.	n.s.
u-PAR in tumor tissue	0.029	0.037	n.s.	0.009

NOTE: Molecular parameters were analyzed [transcription factor binding to the −190/−171 *u-PAR* promoter motif, Src activity, and Src protein (ratio tumor tissue/normal mucosa), u-PAR protein in tumor tissue (ng u-PAR/mg protein)]. High expression/activity of all molecular parameters in the tumors correlate significantly with positive lymph node stages, u-PAR additionally correlating with advanced pT stages (local tumor invasion) and dedifferentiation (grading), and AP-1 binding to the *u-PAR* promoter also correlating with pN and, in trend, grading.
Abbreviations: pT, local tumor invasion; pN, lymph node status; M, distant metastases; pN, positive lymph node stages; n.s., not significant.

Materials and Methods

Materials

Media were purchased from Invitrogen/Life Technologies, fetal bovine serum, and PP2 from Sigma. Antibodies raised against the following proteins used for supershift experiment were from Santa Cruz Biotechnology: p-c-Jun, JunD, JunB, c-Fos, Fra-1, Fra2, FosB, Western blot antibodies against p-c-Jun(Ser^{73} and Ser^{63}), c-Jun, p-JNK, and JNK were from Cell Signaling Technology, and oligonucleotides were provided by Metabion. The probe for the quantitative CAM assay was from Eurogentec, TaqMan Universal PCR Master Mix was from Applied Biosystems. Transwell chambers (1 cm^2, 12-μm pore size) were from Costar, TLC plates (SIL G-25 UV254) were from Machery-Nagel, and Matrigel Basement Membrane Matrix were from BD Biosciences. The cell line SW480 was obtained from the American Type Culture Collection, fertilized special pathogen-free (SPF) eggs were from Charles River.

Patients and Tumors

Twenty patients prospectively followed underwent surgery for primary colorectal cancer between March 1999 and October 2003 (tumor characteristics, Supplementary Table S1). The study was approved by the Institutional Ethical Review Board, and informed consent was obtained from patients. Tissue specimens (tumor, normal mucosa) were collected after verification by a pathologist and frozen immediately in liquid nitrogen. Tissues were processed and analyzed for AP-1 binding to *u-PAR* promoter region −190/−171 and Src activity as described (15, 20).

Plasmids

The u-PARCAT-398 reporter, pCAT-AP-1 and the corresponding AP-1 deletion construct (pCATAP-1del), the Src construct containing *c-src* coding sequence harboring a tyrosine/phenylalanine substitution at codon 527, and the TAM67 plasmid (pCMVβ) were described previously and were, in part, gifts from G.E. Gallick (Department of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX) and M. Birrer (Biomedicum, Helsinki, Finland; refs. 9, 16, 51). pGL3-398 was obtained by subcloning the basal *u-PAR* promoter from u-PARCAT-398 into pGL3Basic (Promega). The AP-2 pBLCAT2 constructs containing three consensus AP-2 motifs are described in refs. (9, 52), and pRSVCAT and pSV0CAT are described in refs. (53, 54).

Reporter Assay, RNA Interference, and Transfection

Cells were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. CAT assays were done as described previously (7), and luciferase assays were done according to the manufacturer's protocol (Promega). The amount of acetylated [^{14}C]chloramphenicol was determined with a phosphoimager BAS1500 (Fuji) using AIDA software. Cells were transfected with siRNAs from Ambion specifically targeting u-PAR (ID289377 and ID289) or a nonsilencing control (4611G).

Electrophoretic Mobility Shift Assays

Nuclear extracts, electrophoretic mobility shift assays (EMSA), and Supershift Analysis were carried out as described previously (20). The sequence of the upstream AP-1 consensus–containing region −190/−171 of the *u-PAR* promoter is 5′-GTGATCACAACTCCATGAGTCAGGGCCGAG-3′. Gelshift analysis for AP-1 binding to region −190/−171 in resected tissues were done as described previously (20).

Total RNA Extraction, cDNA Synthesis, and Real-time PCR

Total RNA for each sample was extracted using RNeasy following the manufacturer's instructions. Total RNA was resuspended in RNase-free water, and the concentration of each sample was determined from absorbances at 260 nm. RNA quality was evaluated by 1% agarose gel electrophoresis. For cDNA synthesis, reverse transcription using 500 ng total RNA was done in a volume of 25 μL using an oligo-dT primer. For real-time PCR, cDNA was diluted 1:50, and 5 μL were used for quantification using ABI PRISM 7000 (Applied Biosystems) according to the manufacturer's instructions [TaqMan Gene Expression Assay for *uPAR* (Hs00182181_m1)]. As internal positive control, the human *β-actin* (human *ACTB*) TaqMan predeveloped assay was used.

Western Blotting and ELISA

Cell or tissue lysis, quantification of lysate protein, and Western immunoblotting were done as described (55). u-PAR in resected tissues was assayed using the Imubind-u-PAR-ELISA kit (American Diagnostica) according to the manufacturer's instructions.

Src Kinase Assay

Cells or tissues were lysed in 1 mL lysis buffer [50 mmol/L HEPES (pH, 7.2), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 100 mmol/L sodium fluoride, 10 mmol/L sodium PPi, 5 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate], the lysate was centrifuged for 15 min at 12,500 × *g*, and Src protein immunoprecipitated from supernatant using 1 mg total lysate for 4 h at 4°C on a rotating wheel in the presence of 30 μL of a 1:1 ratio of Protein G-Sepharose (Amersham Pharmacia Biotech), with the respective antibody and 500 μL 1× HNTG [50 mmol/L HEPES (pH, 7.5), 75 mmol/L NaCl, 10% glycerol, 0.1% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 mmol/L sodium PPi, 5 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate]. Precipitates were washed twice with 1× HNTG followed by kinase buffer [100 mmol/L HEPES (pH 7.2), 5 mmol/L $MnCl_2$, 5 mmol/L $MgCl_2$, 5 mmol/L DTT, 0.5 mmol/L sodium orthovanadate] and subjected to the kinase assay in the presence of 20 μL reaction buffer (kinase buffer supplemented with 5 μg MBP) and 2 μCi γ-^{33}P-ATP. Finally, Laemmli buffer was added, and the mix was boiled for 4.5 min, size separated by 10% SDS-polyacrylamide, transferred to nitrocellulose filters, and exposed to X-ray film. Src activities were calculated as described previously per amount Src protein as measured in Western blotting (15). Src kinase assays for resected patient tissues were done as described previously (15).

ChIP Assay

ChIP was done according to the manufacturer's protocol (Upstate). In brief, SW480 cells were plated in 10-cm plates and transfected with empty vector or a c-Src expression plasmid. Cells were washed after 48 h and cultured for 1 h with fresh medium supplemented with 10% FCS. Cells were fixed with 1% formaldehyde for 10 min and lysed, and the genomic DNA was sheared to between 300 bp and 1.5 kb. ChIP with 2 μg specific (p-c-Jun, JunD, Fra-1-ab) and nonspecific immunoglobulin G was carried out overnight using aliquots of precleared lysates. The immunocomplexes were heated at 65°C for 4 h in the presence of NaCl to reverse the cross-linking between DNA and proteins. Samples were digested with appropriate amounts of proteinase K at 45°C for 1 h and purified by repeated phenol/chloroform extraction/ethanol precipitation. The DNA was dissolved in 100 μL Tris buffer [10 mmol/L Tris (pH, 8.5)]. DNA isolated using the same procedure with omission of the immunoprecipitation step was designated as input DNA. Fluorogenic TaqMan QPCR probe and primers were designed by using PRIMER EXPRESS software (Applied Biosystems) to amplify 101-bp amplicons, based on the sequence from GenBank S78532. DNA copy numbers of bound

and input DNA were quantified using the ABI PRISM 7000. Product amounts were determined relative to a standard curve of serially diluted pGL3-398 plasmid. Sequences of primers and probe: forward, 5′-GAAGAGAGAGAACTGGGATTTGCA-3′; reverse, 5′-GCCGGCTGGTGGTGAAG-3′; probe, 5′-TCCATGAGTCAGGGCCGAGCCA-3′. PCR conditions are 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min.

Invasion Assay

Matrigel invasion assays were done as described previously with some modifications (56). Briefly, 3×10^5 cells were plated on transwell chambers precoated with 10 μg Matrigel. In the case of inhibition with Src kinase inhibitor, cells were pretreated (30 min) with 5 μmol/L PP2. Conditioned NIH-3T3 medium was used as the chemoattractant, and 5 μmol/L PP2 was added to the medium in the upper chamber. Following 16 h incubation, noninvading cells were removed with cotton swabs, and invading cells were trypsinized and counted using the ATP Luminescence-Based Motility Invasion Assay as described previously (57).

Chicken Embryo Metastasis (Chorionallantoic Membrane) Assay

The CAM assay was done as previously described (19). Genomic DNA from chicken CAMs was prepared using the Puregene DNA purification system according to the manufacturer's instructions (GentraSystems). Purified DNA was quantified spectrophotometrically and controlled for high molecular weight on agarose gels. Quantification of human cells in the lower CAM was done as in ref. (19). Fluorogenic TaqMan QPCR probes were applied as above, and DNA copy numbers were quantified using Applied Biosystems 7000.

Statistical Analysis

Statistical analysis was done using SPSS version 14.0 (SPSS). To determine correlations between variables, Spearman's correlation coefficient was calculated; a P value <0.05 was considered significant. Linear regression analysis was done to determine correlations between AP-1 binding and Src activity in resected tumor/normal tissues.

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Jörg H. Leupold, Irfan Asangani, Gabriele D. Maurer, et al.

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