Increased Src Activity Disrupts Cadherin/Catenin-mediated Homotypic Adhesion in Human Colon Cancer and Transformed Rodent Cells

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ABSTRACT

Src has been implicated in the development and progression of human colon cancer. Because the capacity for tumor cells to dissociate from the primary tumor is a critical step in the development of metastases, the effect of a naturally occurring, activated Src-531 on intercellular adhesion was examined. Homotypic adhesion was assessed using dissociation assays on Src-transformed rat fibroblasts and human colon cancer cell lines. The data indicate that both rodent and human cells expressing the mutant Src protein display up to 7-fold less homotypic adhesion than do wild-type cells (P < 0.01). Experiments demonstrated that cadherin was phosphorylated in cells transfected with activated Src and that cadherin/catenin complexes were disrupted as a result. Experiments using dominant negative (DN) Src or an Src-specific inhibitor (PD 180970), demonstrated that adhesion was restored when Src activity was inhibited in Src-531 transfectants, confirming that Src is a causal factor in the decreased homotypic adhesion observed. In addition, DN Ras, DN focal adhesion kinase (FAK), but not Stat3Δβ, restored intercellular adhesion, which suggested that Ras and FAK may be downstream effectors of Src-mediated homotypic adhesion. Collectively, these data support a role for Src, Ras, and FAK in the regulation of intercellular adhesion, which may in turn regulate metastatic potential of human colon cancer cells.

INTRODUCTION

Colorectal cancer is a common disease that results in ~65,000 deaths each year (1), often as a result of metastatic liver tumors. Although numerous events leading to metastasis, such as adhesion, migration, invasion, angiogenesis, and growth, have been identified, the mechanism underlying these processes is still poorly understood. Src has been implicated in the development and progression of human colon cancer and has been the subject of numerous studies (2–7). Nearly all colon carcinomas display an increased level of Src kinase activity (6, 8). Src protein levels are modestly increased in adenomatous polyps and increase further during the progression of colon cancer and the development of metastatic disease (8–11). More importantly, dramatic increases in the Src protein kinase activity during the progression of colon cancer have been observed (7). Studies have shown that the increase in Src activity is greater in liver metastases secondary to colorectal cancer than in those from primary tumors of other origins (12). Whether this increased Src activity is an event that caused the metastasis or whether it resulted from the influence of hepatic environment on the tumor is still unclear. Recently we reported an uncommon but novel mutation, termed Src-531, that occurred in 12% of human colorectal liver metastases (13). The mutation produces a truncated protein in which a stop codon occurs immediately COOH-terminal to the regulatory Tyr 530, eliminating the last six amino acids. The mutation is both activating and transforming, with modest Src activity, would provide a better model for studying this problem. Moreover, we propose that Src activation will induce a reduction of homotypic adhesion in human colon cancer cells, a process critical to the development of metastatic potential.

To assess the potential role for cadherins and catenins in Src-mediated metastasis, transfected cells were assayed to determine their adhesive characteristics via a calcium-dependent mechanism. Transfected cells were examined in an intercellular adhesion assay in the presence of Src inhibition to determine their capacity to maintain homotypic adhesions both in the presence and absence of calcium. Cells were transiently transfected with a DN Src construct to inhibit exhibit metastatic behavior. Presumably, in addition to increased protein levels, the increased Src activity in transfected cells is a result of the inability of the truncated Src-531 protein COOH terminus to interact with the Src homology 2 domain to inactivate the protein, maintaining a constitutively active Src protein (13). For these reasons, Src-531 may be used to model human colon cancer progression.

Critical to the metastatic process is the release of cells from the primary tumor to foster cellular entry into the lymphovascular systems. Because increased Src activity is associated with metastasis, we hypothesized that Src activation might contribute to the process of tumor dissociation. As a measure of the potential of a primary tumor to release individual tumor cells, the degree of homotypic adhesion in cells expressing activated Src was examined using calcium-dependent dissociation assays. Src-531-transformed rodent cells or human colon cancer cell lines expressing low levels of endogenous Src but stably transfected with a mutant Src-531 construct, were used for these studies.

Proteins known to be involved in homotypic adhesion of cells include cadherins (14), catenins, FAK (15), and members of the cell cytoskeleton. Cadherins are transmembrane proteins that establish calcium-dependent homophilic protein–protein attachments between cells and are involved in embryonic morphogenesis and cell-cell adhesion in solid tissues (14, 16–20). Cadherins contain an extracellular domain, which is responsible for adhesion to similar cadherins on neighboring cells, and a cytoplasmic domain, which binds β-catenin and creates a link to the actin cytoskeleton. It has been suggested that by using a highly activated form of v-Src, inactivation of this complex may be induced through phosphorylation of cadherins. This results in the reduction of homotypic cell-cell adhesion (21–22). Conversely, increased expression of active cadherins decreases invasiveness of tumor cells (23). Hamaguchi et al. (24) also demonstrated that v-Src caused phosphorylation of N-cadherin and its associated catenins, suppressing homotypic adhesion between cells. Likewise, Behrens et al. (25) showed that phosphorylation of E-cadherins and the associated β-catenin by v-Src caused a decrease in homotypic adhesion and an increase in invasive properties of the cells. Despite these compelling results using the highly activated v-Src, this relationship has not been explored using other forms of less-activated Src associated with human tumorigenesis. For this reason, we hypothesized that Src-531, a naturally occurring mutant form of human c-Src with modest Src activity, would provide a better model for studying this problem. Moreover, we propose that Src activation will induce a reduction of homotypic adhesion in human colon cancer cells, a process critical to the development of metastatic potential.

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3 The abbreviations used are: FAK, focal adhesion kinase; v-SRC, avian Src; DN, dominant negative; RIPA, radioimmunoprecipitation assay (buffer); TRITC, tetramethylrhodamine isothiocyanate; STAT, signal transducer and activator of transcription.
Src activity in an attempt to return cells to the wild-type adhesion phenotype and to demonstrate that altered homotypic adhesion was a specific effect of Src activity. The nature of the influence on the cadherin/catenin complex was also examined. Because v-Src is known to induce phosphorylation of the cadherin/catenin complex, causing dissociation of this complex with resulting decrease in homotypic adhesion, we explored the possibility that Src-531 would elicit a similar response. Finally, the possible roles of Ras, FAK, and STAT 3, on homotypic adhesion were examined in dissociation assays. Each of these proteins is known to be regulated, at least in part, by Src (26–29), and, thus, may be downstream components in the pathway through which Src regulates adhesion. The activity of each of these proteins was examined in an attempt to elucidate pathways through which Src mediates homotypic adhesion, which may enhance metastatic potential of cancer cells.

MATERIALS AND METHODS

Cell Culture and Transfections. SW 480 and HCT 116 human colon tumor cells were grown in RPMI 1620 (Life Technologies, Inc.) with 10% fetal bovine serum at 37°C, 5% CO₂. 3Y1 cells were cultured in DMEM (Life Technologies, Inc.) with 10% FBS. Transfections were done according to the manufacturer’s directions with Fugene (Roche) using 2 μg of pcDNA 3.1-src-531. Transfectants were selected with 50 μg/ml G418 (Life Technologies, Inc.) for expansion according to their levels of Src expression and activity. Analysis of phosphotyrosine activity in stably transfected cell clones expressing Src-531 showed increased kinase activity of Src with respect to control cells (Fig. 1). Kinase assays support these data; mock transfectants display very little basal, endogenous Src kinase activity in comparison with Src 531-transfected cell lines, which show activity in stably transfected cell clones expressing Src-531 demonstrates an increased kinase activity over empty vector (mock)-transfected cells (Fig. 1). Kinase assays support these data; mock transfectants display very little basal, endogenous Src kinase activity in comparison with Src-531-transfected cell lines, which show a high level of kinase activity with the exogenous substrate enolase (Fig. 2a) relative to Src protein levels (Fig. 2b). Several clones of Src-531-transfected cells were examined to eliminate the possibility of clonal artifact. In every case, cells expressing Src 531 displayed an increase in phosphotyrosine activity.

RESULTS

Src Expression Decreases Homotypic Adhesion. Both rat fibroblast and human colon cancer cells transfected with pcDNA-Src-531 (activated form of mutant c-Src) or pcDNA3.1 (empty vector) were selected in G418 (Life Technologies, Inc.) for expansion according to their levels of Src expression and activity. Analysis of phosphotyrosine activity in stably transfected cell clones expressing Src-531 demonstrates an increased kinase activity over empty vector (mock)-transfected cells (Fig. 1). Kinase assays support these data; mock transfectants display very little basal, endogenous Src kinase activity in comparison with Src-531-transfected cell lines, which show a high level of kinase activity with the exogenous substrate enolase (Fig. 2a) relative to Src protein levels (Fig. 2b). Several clones of Src-531-transfected cells were examined to eliminate the possibility of clonal artifact. In every case, cells expressing Src 531 displayed an increase in phosphotyrosine activity.
Src Activity Disrupts Homotypic Adhesion

Increased level of kinase activity over cells transfected with empty vector, yet less activity than cells expressing v-Src. (data not shown).

To determine whether increased Src activity reduces the homotypic adhesion characteristics of transfected cells, dissociation assays were performed on both mock- and Src-transfected cells according to Naga-fuchi et al. (30). Cells lose active cadherins on removal of calcium during trypsinization of cells. When calcium is added to the trypsin solution, adhesion molecules requiring calcium for function are preserved. Calcium-dependent homotypic adhesion assays demonstrated that Src-531 transfected cells displayed in up to a 7-fold reduction in homotypic adhesion compared with wild-type cells (Fig. 3). The addition of calcium restores homotypic adhesion in a dose-dependent manner but is less effective in Src-531-expressing cells (Fig. 3b). To determine whether Src-specific inhibition would result in a restoration of normal levels of homotypic adhesion, cells that express Src-531 and show reduced adhesion, were transiently transfected with a plasmid containing a DN Src clone. Fig. 4a shows that the DN Src increases the adhesion properties of cells in a dose-dependent manner using 0.5 μg/ml and 1.0 μg/ml. DN Src at 1.0 μg/ml reduces Src protein kinase activity by 2- to 3-fold in Src 531-transfected cells (Fig. 4b). Similarly, incubation of cells with the Src-specific inhibitor, PD180970 (125 nM), yields similar results: increased homotypic adhesion concordant with the inhibition of Src activity (Fig. 5a).

Src-dependent Homotypic Adhesion May Use Ras and FAK. Ras, a signaling molecule regulated by Src, has been implicated as a potential downstream target of Src (26, 31). In this regard, we hypothesized that Ras may be involved in the adhesion functions affected by Src. A role for this protein in Src-mediated adhesion was explored using a DN construct for Ras (N-17; 1.0 μg/ml) in a transient transfections of cells stably expressing Src-531. Adhesion assays were then performed on the N-17-transfected cells and compared with DN Src- and PD180970-treated treated cells in the same experiment to determine relative effects (Fig. 5). Ras inhibition by the DN construct resulted in increased homotypic adhesion at levels similar to the DN Src and PD180970 inhibitor, which suggested that Src may exert regulatory effects on adhesion through Ras.

The possible involvement of both FAK and STAT 3β in homotypic adhesion was also explored because both molecules are known substrates of v-Src. Moreover, both molecules are thought to play significant roles in oncogenesis and tumor progression. Dissociation assays were performed on cells stably transfected with either Src-531 or an empty vector, and transiently transfected with DN FAK (1.0 μg/ml) or STAT 3β (1.0 μg/ml). Transfection with DN FAK results in a reduced adhesion at levels similar to those elicited by DN Src and DN Ras, which indicates that FAK may also be involved in Src-mediated homotypic adhesion. On the other hand, STAT 3β seems to have very little effect on adhesion and serves as a reasonable control for these experiments.

Src Activity Causes Dissociation of the Cadherin/Catenin Complex. We explored the possibility that the reduction in cell-cell adhesion is a result of alteration in the cadherin/catenin complex known to be operational in homotypic adhesion (18, 24). Previous reports have suggested a role for v-Src in dissociating this complex. Fluorescence microscopy revealed a partial colocalization of N-cadherin and increased calcium levels.

Fig. 3. In a, a calcium-dependent dissociation assay demonstrates that highly active Src-531 reduces intercellular adhesion by up to 6-fold over mock-transfected cells. Assays were performed on rat 3Y1, and SW480 and HCT 116 human colon cancer cells, both mock- and Src-531-transfected. Results are expressed as NcNsc, comparing the number of particles in TC-treated cells, virtually a single-cell suspension, to the number present in TC-treated samples. Each assay was performed twice in triplicate. Results represent the mean ± SD (P < 0.01). In b, dissociation assays were performed on HCT116 cells with both 0.5 and 1.0 molar calcium added to the trypsin. Increased association occurs with the increased calcium levels.

Fig. 4. In a, dissociation assay on cells treated with DN Src shows that inhibition of Src activity with DN Src restores homotypic adhesion in a dose-dependent manner. Both mock-transfected cells and Src-531-transfected cells were transiently transfected with either 0.5 or 1.0 μg/ml DN Src, a kinase inactive Src, and allowed to grow for 72 h. Each assay was performed twice in triplicate. Assays were performed as in Fig. 3. Results expressed as NcNsc, P < 0.01. In b, kinase assays performed on transfected cells show the effective inhibition of Src activity by DN Src constructs. Results represent the mean ± SD.

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Flow cytometry was performed on both wild-type and Src-531-transfected SW480 cells labeled with N-cadherin. Results show that N-cadherin is present on the surface of wild-type cells, however, Src-531 expressing cells do not show surface N-cadherin. Immunofluorescence data provides a possible explanation for this occurrence. Whereas the N-cadherin staining occurs on the surface only of every wild-type cell in the sample, the Src-531 expressing cells show significant amounts of N-cadherin in the cytoplasm, and, furthermore, not every cell expresses surface N-cadherin. These data suggest that the increased Src activity from an activated mutant Src-531 is able to disrupt the cadherin/catenin complex and that the disruption of this complex may be responsible for the decrease in calcium-dependent homotypic adhesion. In addition, Src activity may be responsible for the translocation of N-cadherin to the cytoplasm, further reducing its ability to perform the role of an intercellular adhesion molecule.

Src Activity Results in Phosphorylation of N-Cadherin Complex Proteins. Using v-Src-transfected fibroblasts, Hamaguchi et al. (24) reported that increased Src activity results in phosphorylation of the N-cadherin/catenin complex. These studies showed that phosphorylation of the N-cadherin/catenin complex greatly reduced cell-cell adhesion properties resulting in the disaggregation of cells. Investigation of the phosphorylation state of N-cadherin in cells overexpressing Src indicates that activated Src-531 results in tyrosine phosphorylation of N-cadherin in SW480 cells (Fig. 7d). The same experiments were performed on HCT116 cells to examine the phosphorylation state of E-cadherin expressed in these cells (Fig. 7e). Western blotting was performed on supernatants from immunoprecipitated samples to assure that immunoprecipitated proteins were fully precipitated and not discarded with the supernatant in both kinase assays and cadherin/catenin complex assays (data not shown).

DISCUSSION

Although high levels of Src protein kinase activity have been associated with the development and progression of human colon cancer, the precise role for Src in this process is still poorly understood. For many years, v-Src has been used as the prototypical model of Src activation in human cancer; however, v-Src activation is greatly exaggerated in these models, which leads to concerns that they may not accurately reflect the biology of the human cancer cell. Because we recently identified Src 531 as a naturally occurring mutant form of Src in human colon cancer, we hypothesized that it might prove to be a more relevant model for evaluating the role of Src in this biologically complex disease. We propose using both the classic rodent model of Src transformation in combination with two human colon cancer cell lines transfected with the mutant Src 531 to assess the role of Src in tumor progression.

In this study, the effects of activated mutant Src on the adhesive properties of human cancer cells were examined in an effort to identify a mechanism by which increased Src activity enhances the metastatic phenotype of human colon cancer cells. We hypothesized that Src expression and/or Src activation may lead to decreased intercellular adhesion, which would permit a primary tumor to release metastatic tumor cells. The SW 480 cell line was chosen because it has a low level of endogenous Src expression and activity and a normal β-catenin, and it is known to be poorly metastatic. Src-531 was used as a natural model of Src activation. SW480 cells express N-cadherin, whereas HCT116 cells express E-cadherin. These two cell lines were used with the activated Src protein to determine whether the data represent a common phenomenon that will be widely applicable to human colon cancer. These data provide evidence that Src occurring naturally in colon tumors enhances the metastatic phenotype through reduced adhesion. Other forms of activated Src, including v-Src and the chicken Src-527, are not known to occur naturally in mammalian systems, and, therefore, may be less relevant to...
typic adhesion activity is required for the disruption of cadherin-dependent homo-
typic adhesion of tumor cells. Moreover, v-Src produces very high levels of Src activity that are often toxic to cells and make stable transfection difficult. These data provide evidence that Src protein occurring naturally in human tumors enhances metastatic potential by reducing homotypic adhesion of tumor cells.

Recently, Owens et al. (32) reported that c-Src family kinase activity is required for the disruption of cadherin-dependent homotypic adhesion in vitro, a finding that supports our hypothesis. On the other hand, Takeda et al. (33) provided evidence that, although v-Src activity causes β-catenin phosphorylation to increase as cadherin-mediated adhesion is disrupted, β-catenin phosphorylation is not necessary and, furthermore, is not involved in this disruption. Taken together, these data implicate the presence of yet another factor in the homotypic adhesion process, perhaps p-120 Cas, vinculin, integrins, or other junction proteins. These issues may need to be addressed to obtain a clear understanding of the role of Src in cell-cell adhesion.

β-catenin plays a dual role in the structure and function of cells. It functions both in cell adhesion as a link between cell surface cadherin molecules and the actin cytoskeleton and as a signaling molecule for gene transcription (34). On stimulation by Wnt [vertebrate homologue of the molecule wingless (Wg) an extracellular glycoprotein important in regulating cell growth and differentiation], β-catenin is translocated to the nucleus, in which it binds to lymphoid-enhancing factor/T-cell factor transcription factors that control the transcription of genes involved in cell proliferation (35, 36). Inappropriate activation of this pathway can lead to the development of various tumors, as is the case with colon cancer mediated by an APC mutation (37). Localization of catenin to the nucleus is reduced by the binding of catenins to molecules in the cytoplasm such as cadherins (36) and platelet-endothelial cell adhesion molecules (38). Therefore, participation of β-catenin in a cadherin/catenin complex counteracts the ability of β-catenin to function as a transcription regulator as it enables homotypic adhesion.

Results from immunofluorescent assays and immunoprecipitation experiments indicated that the cadherin/catenin complex is disrupted in cells expressing Src-531. N-cadherin also appears to be partially translocated into the cytoplasmic region of the cell. Brabletz et al. (39) reported that E-cadherin expressed by SW480 cells appears in the cytoplasm and localizes to the membrane after 3–4 days growing in culture. N-cadherin in mock-transfected cells appears to be localized in the membrane after 3–4 days in culture. However, in Src-531-transfected cells, a pool of N-cadherin appears in the cytoplasm under identical growth conditions, it enables homotypic adhesion.

Fig. 6. In a, fluorescence microscopy of cadherin and β-catenin in SW480 cells shows the subcellular localization of N-cadherin and β-catenin. N-cadherin is localized at the cell membrane, whereas β-catenin is located both in the nucleus and at the cell membrane in mock-transfected cells but only in the nucleus in Src-531-transfected cells. In addition, N-cadherin is located in the cytoplasmic region of the SW 480 cells expressing Src-531. SW 480 cells transfected with an empty vector or with Src-531 were probed with mouse anti-cadherin (Zymed Laboratories) and rabbit anti-β-catenin followed by antimouse FITC-conjugated and antirabbit TRITC-conjugated secondary antibodies. Cadherin (green); β-catenin (red). In b, flow cytometry results show expression of N-cadherin on the surface of mock SW480 cells (indicated by the shift of the gray line), but not on the surface of Src-531 expressing cells.

Fig. 7. Western blots after immunoprecipitation of cadherins show the presence of cadherin/catenin complex in mock-transfected cells and lack or complex in Src-531-transfected cells. Western blots show equal levels of cadherin and catenin in samples. a, immunoprecipitation of SW480 cell extracts with anti-N-cadherin, Western blotting of membrane with anti-β-catenin. Mock lane shows communoprecipitation of cadherin and catenin. b, Western blot of immunoprecipitated β-catenin. c, Western blot of immunoprecipitated N-cadherin. d, mock- and Src-531-transfected SW480 cell extracts, immunoprecipitated with anti-N-cadherin monoclonal antibody and Western blot-probed with anti-β-tyrosine antibody, indicate phosphorylation of N-cadherin in Src-531-transfected cells only. e, mock- and Src-531 HCT116-transfected cell extracts, immunoprecipitated with anti-E-cadherin and Western blot-probed with p-tyrosine, show phosphorylation of Src-transfected cells only. f, Western blot confirms the presence of E-cadherin in the cells. kDa, molecular weight (M_r) in thousands.
cadherin expression. Phosphotyrosine Western blots of immunoprecipitated proteins indicated that the dissociation of the complex may have been a result of the phosphorylation of N-cadherin in SW480 cells and of E-cadherin in HCT116 cells. Cell extracts were immunoprecipitated with β-catenin antibodies, and Western blots were performed. The membranes were probed with phosphotyrosine and yielded no bands, indicating that β-catenin is not phosphorylated in these cells. This may be a result of the fact that, once phosphorylated in a Src-regulated pathway, β-catenin is targeted to the ubiquitin-mediated degradation pathway (40). Multiple cell lines were examined to ensure that phenotypic changes in cells were the result of altered Src expression and not merely limited to a specific cell line. In every case, the mutant Src-531 stimulated a reduction in adhesion. Adhesion studies were performed under the influence of DN Src, a kinase-inactive Src protein that appears to compete with active Src for substrate binding. The DN Src restored adhesion characteristics in a dose-dependent manner, which indicated that the Src activity is responsible for decreased adhesion. Ras is known to be a downstream element in v-Src transformation and may, therefore, play a role in Src-mediated adhesion properties of cells (26, 41). This hypothesis was tested using the DN H-Ras (N-17). Data showing that the inhibition of Ras function in Src-transfected cells increases adhesion indicated that Ras is a downstream target for Src involvement in the pathway regulating homotypic adhesion. src is also involved in the regulation of FAK, a molecule associated with integrins in the adhesion process. The FAK pathway may play an additional role in determining Src-mediated cell adhesion, independent of the cadherin/catenin pathway.

Collectively, these data suggest that activated Src expression may be a critical factor in the release of viable cells from the primary tumor as an initial step in metastasis. This process is linked to the disruption of the catenin/cadherin complex as a result of cadherin phosphorylation and is mediated through both Ras and FAK pathways.

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