Simultaneous Inhibition of Focal Adhesion Kinase and Src Enhances Detachment and Apoptosis in Colon Cancer Cell Lines

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Abstract
Focal adhesion kinase (FAK) and Src have been shown to be overexpressed in colon cancer. We have studied the role of these two kinases in resistance to apoptosis. Adenovirus-containing FAK-CD (Ad-FAK-CD), a dominant-negative, COOH-terminal portion of FAK, was used to inhibit FAK and cause apoptosis. Colon cancer cell lines were more resistant to Ad-FAK-CD-induced detachment and apoptosis than the breast cancer cell line, BT474. Colon cancer cell lines overexpressed highly active Src and FAK. Ad-FAK-CD-induced apoptosis was significantly increased by PP2, an inhibitor of Src family kinases. Activation of caspase-3, down-regulation of FAK, and Src and AKT activities were demonstrated in Ad-FAK-CD + PP2-treated colon cancer cells undergoing apoptosis. The results suggest that FAK and Src are both important survival factors, playing a role in protecting colon cancer cell lines from Ad-FAK-CD-induced apoptosis. Dual inhibition of these kinases may be important for therapies designed to enhance the apoptosis in colon cancers.

Introduction
Normal cells undergo apoptosis or “anoikis” when they lose adhesion to extracellular matrix (ECM) (1, 2). In contrast, cancer cells are more resistant to anoikis and can grow anchorage independently (1). One of the proteins implicated in the mechanism of anoikis is focal adhesion kinase (FAK), a nonreceptor tyrosine kinase that is involved in the control of cell-extracellular interactions such as spreading, migration, motility, and survival (3–5). FAK is a protein of Mr 125kDa that localizes to focal adhesions (5) and is activated and tyrosine phosphorylated in response to integrin clustering (6). Tyrosine 397 is an autophosphorylation site of FAK, and is a critical component in downstream signaling (7), providing a high-affinity binding site for the SH2 domain of Src family kinases (8, 9). This interaction between FAK and Src leads to a cascade of tyrosine phosphorylation of multiple sites in FAK (−576, −577, −925), as well as other signaling molecules such as p130CAS and paxillin, resulting in cytoskeletal changes and activation of other downstream signaling pathways (3).

FAK has been shown to become overexpressed in tumor cells (10–14), providing survival signals that suppress apoptosis in human breast cancer cells (15, 16). FAK inhibition by anti-sense oligonucleotides or adenoviral dominant-negative FAK, COOH-terminal part of FAK (human analogue of FRNK, FAK-related nonkinase; 17), has caused loss of cell adhesion, displacement of FAK from focal adhesions, degradation of FAK, and apoptosis (15, 18, 19). Furthermore, overexpression of a constitutively active form of FAK prevented apoptosis of epithelial cells (20). Similarly, microinjection of peptides impaired FAK-integrin binding and antibodies against FAK induced apoptosis (21). Finally, FAK has been shown to suppress etoposide and hydrogen peroxide-induced apoptosis in HL-60 cells (22) as well as chemically induced apoptosis in renal epithelial cells (23). Thus, FAK can suppress apoptosis in a number of different cellular systems, although the molecular mechanisms of apoptotic resistance in human cancer cells remain unclear (15).

In the present study, we studied apoptosis induced by FAK down-regulation in colon cancer cell lines and found them more resistant than other human breast cancer cell lines that we have studied (15, 19). Because many colon cancer cells overexpress both Src and FAK, in contrast to breast cancer cell lines, we hypothesized that Src may contribute additional survival signals to the colon cancer cells to make them more resistant to apoptosis. We have shown that simultaneous inhibition of both Src and FAK caused increased apoptosis in colon cancer cell lines. Thus, FAK and Src overexpression provide protection of colon cancer cell lines from apoptosis. Furthermore, we have shown that the mechanism of increased apoptosis involves blockade of the AKT survival pathway and activation of caspase-3. Thus, these data demonstrate that inhibition of both FAK and Src may be important for designing molecular colon cancer therapeutics.

Results
Colon Cancer Cell Lines Are More Resistant to Adenovirus-Containing FAK-CD (Ad-FAK-CD) Detachment Than Breast Cancer Cell Line BT474
We have previously shown that different breast cancer cell lines overexpress FAK detachment and undergo apoptosis after...
down-regulation of p125FAK expression by adenoviral delivery of a dominant-negative COOH-terminal FAK construct, Ad-FAK-CD (15, 19). Because colon carcinomas also express high levels of FAK (11, 13, 24, 25), we tested a colon cancer cell line model to study apoptosis induced by FAK down-regulation. To inhibit FAK, we used a dominant-negative construct for FAK, Ad-FAK-CD, and Ad-LacZ as a control that was used at the same multiplicity of infection. These conditions resulted in effective infectivity of different breast, BT474, BT474-EGFR and MCF-7 (15, 19) and colon, HT-29, LS180 and WIDR cancer cell lines as assessed by X-gal staining for Ad-LacZ (Fig. 1A) and by HA immunostaining for HA epitope-tagged Ad-FAK-CD (Fig. 1B). Ad-LacZ-infected HT-29 cells effectively expressed β-galactosidase, and the protein was shown by X-gal staining (Fig. 1A, right panel). Ad-FAK-CD-infected cells expressed high levels of HA-tagged FAK-CD protein, mainly localized at the focal adhesion sites (Fig. 1B, lower right panel).

The HT-29, LS180, and WIDR colon cancer cell lines had only 20 ± 2% detachment, while 100% of BT474 cells were detached (Fig. 1C). Furthermore, an average of only 8 ± 0.7% of the detached colon cancer cells underwent apoptosis, in contrast to the BT474 breast cancer cells that had a 78 ± 2% apoptotic rate. Previous reports in other breast cancer cell lines, MCF-7 and BT474-EGFR, have demonstrated effective detachment by Ad-FAK-CD with higher levels of apoptosis than seen in colon cancer cells [45 ± 2% apoptotic in case of MCF-7 cells (15) and 64 ± 17% apoptotic in case of BT474-EGFR cells (19)]. As determined by Western blot analysis, the colon cancer cell lines expressed high levels of p125FAK, comparable or higher than the levels in BT474 breast cancer cells. FAK had high autophosphorylation activity in colon cancer cell lines, as detected by Western blotting with anti-Y397-Y-FAK antibody (Fig. 2, upper panel). This endogenous activity was higher than in BT474 cancer cells. In addition, when we probed colon cancer cells for the level of Src activity and for c-Src expression by assessing the phosphorylation level of P-Y418-Src (autophosphorylation site and indicator of Src catalytic activity), all of these cell lines expressed higher levels of Src activity and expression than the BT474 cell line (Fig. 2), suggesting that Src may play a role in this resistance to detachment and apoptosis induced by FAK down-regulation.

### Inhibition of Src and FAK Increases Detachment and Apoptosis in HT-29 and WIDR Cell Lines

We used the Src-family kinase inhibitor, PP2, to inhibit Src in the two colon cancer cell lines that expressed the highest level of Src protein and activity, HT-29 and WIDR (Fig. 2). PP2 decreased the tyrosine phosphorylation of Src in both cell lines.
and also decreased the tyrosine phosphorylation of the Src substrate, FAK (Fig. 3A, upper panels). As expected, FAK and Src were associated in both cell lines, detected by co-immunoprecipitation analyses of each kinase (Fig. 3).

Next, we tested the effects of down-regulating FAK with and without simultaneous inhibition of Src in HT-29 and WIDR cells. We performed a time course study of both detachment and apoptosis at 24 and 48 h after Ad-FAK-CD infection. We found that treatment with control LacZ adenovirus alone or together with PP2 inhibitor caused negligible levels of detachment (Fig. 4). With Ad-FAK-CD treatment, we found that inhibition of Src by PP2 enhanced the rate of detachment in a time-dependent manner in each of the cell lines (Fig. 4). By 48 h, 77% of the HT-29 cells and 99% of the WIDR cells had detached, compared to 38% of the HT-29 cells and 75% of the WIDR cells with Ad-FAK-CD alone (Fig. 4).

When we examined apoptosis in these cells, we found that inhibition of Src with PP2 inhibitor, as well as FAK with Ad-FAK-CD together, gave the maximal rates of apoptosis (Fig. 5). Control Ad-LacZ alone or with PP2 caused low levels of apoptosis in both cell lines (Fig. 5). In HT-29 cells, the rate of Ad-FAK-CD-induced apoptosis increased dramatically with PP2 treatment, reaching a maximal level of 49% at 48 h (Fig. 5A). In the WIDR cells, apoptosis was increased from 6% to 14% at 24 h (Fig. 5B). By 48 h, the additional effect of Src inhibition was more pronounced in the WIDR cells, increasing the apoptotic rate from 14% with Ad-FAK-CD alone to 58% with Src and FAK inhibition (Fig. 5B).

To determine whether FAK inhibition or Src inhibition alone sensitizes cells more globally to apoptosis, we treated HT-29 cells that had high levels of active FAK and Src with another apoptotic agent, staurosporine (26), and performed inhibition of FAK or Src alone or FAK and Src together in these cells for 48 h (Fig. 5C). HT-29 cells showed low levels of staurosporine-
FAK activity by Ad-FAK-CD and Ad-FAK-CD + PP2 treatment as well as significant FAK down-regulation (Fig. 7A). Inhibition of p125FAK was significantly dephosphorylated by Ad-FAK-CD in HT-29 cells; #, P < 0.002 for Ad-FAK-CD + PP2 versus Ad-FAK-CD in WIDR cells; **, P < 0.03 for Ad-FAK-CD + PP2 versus Ad-FAK-CD in HT-29 cells; *, P < 0.02 for Ad-FAK-CD + PP2 versus Ad-LacZ + PP2 in both cell lines. No treatment; #, LacZ; ■, FAK-CD; □, FAK-CD + PP2.

Inhibition of FAK and Src in HT-29 and WIDR Cells Down-Regulates FAK and AKT and Activates Caspase-3

Next, we assessed the biochemical effects of FAK and Src inhibition in the HT-29 and WIDR cells. We immunoprecipitated FAK in HT-29 cells that had been treated for 48 h with Ad-FAK-CD in the presence and absence of PP2. Ad-LacZ was used as a control for non-specific adenoviral effects. In the HT-29 cells, p125FAK was significantly dephosphorylated by Ad-FAK-CD with and without Src inhibition, but not by Ad-LacZ (Fig. 7A, upper panel). However, in PP2-pretreated cells infected with Ad-FAK-CD, there was complete p125FAK dephosphorylation, as well as significant FAK down-regulation (Fig. 7A). Inhibition of FAK activity by Ad-FAK-CD and Ad-FAK-CD + PP2 treatment was also demonstrated by analyzing phosphorylation of the FAK substrate, paxillin (Fig. 7A, lower panel). Paxillin was significantly tyrosine dephosphorylated in Ad-FAK-CD-treated cells, and its phosphorylation was completely abrogated by both FAK and Src inhibition (Fig. 7A, lower panels).

We analyzed FAK activity in Ad-FAK-CD and Ad-FAK-CD + PP2-treated cells by Western blotting with Y397-FAK-specific antibody in HT-29 cells (Fig. 7B, upper panel) and in WIDR cells (Fig. 7C, upper panel). Ad-FAK-CD caused significant inhibition of FAK autophosphorylation activity versus LacZ-treated cells or Ad-LacZ + PP2-treated cells. Ad-FAK-CD plus PP2 treatment caused complete loss of FAK activity (Fig. 7, B and C). The same loss of FAK catalytic activity was detected by in vitro kinase assay in both cell lines (Fig. 7, B and C). There was additive down-regulation of total FAK in the HT-29 and WIDR cells treated with both Ad-FAK-CD and PP2 (Fig. 7, B and C).

We also performed analysis of Src activity with a Y-418-Src phospho-specific antibody. PP2 successfully inhibited Src activity in HT29 (Fig. 7B, middle panel) and in WIDR cells (Fig. 7C, middle panel). Ad-FAK-CD did not significantly change Src activity in HT-29 cells (Fig. 7B) and in WIDR cells (Fig. 7C). In both cell lines, Src activity was completely blocked in Ad-FAK-CD + PP2-treated cells, which was detected by decreased tyrosine phosphorylation on the Y418-e-Src site (Fig. 7B, middle panel, and Fig. 7C). The same inhibition of Src catalytic activity in Ad-FAK-CD + PP2-treated cells was demonstrated with the Src exogenous substrate enolase by in vitro kinase assay in HT-29 cells (Fig. 7B, lower panel) and in WIDR cells (Fig. 7C, lower panel). Thus, Ad-FAK-CD + PP2-treated cells completely lost Src and FAK catalytic activities in both cell lines (Fig. 7, B and C).

Furthermore, in response to FAK or Src alone inhibitions, colon cancer cell lines expressed highly active AKT that was serine 473 phosphorylated (Fig. 8, A and B). In contrast, in the HT-29 cells where both FAK and Src activities were down-regulated, there was reduced activity of AKT (P-Ser-473-AKT) and expression of AKT, a critical kinase in cell survival (27), as well as activation of caspase-3 (Fig. 8A). These results biochemically support the increased levels of apoptosis in the HT-29 cell line (Fig. 5A). Ad-FAK-CD + PP2-treated WIDR cells also showed reduced levels of AKT activity and expression and decrease of pro-caspase-3 expression, demonstrating increased activity of the apoptotic enzyme, caspase-3. (Fig. 8B).

**Discussion**

Colon cancer cells show increased resistance to chemotherapeutic agents compared to breast cancer cells (28, 29), and the molecular mechanisms of this resistance are not fully known. While colon cancer cells express high levels of Src family kinases (30), the survival signal pathways associated with its expression are not known (31). In the present study, we have demonstrated that colon cancer cell lines have survival signals operative through both FAK and Src activities, suggesting that the combination of these signals may contribute to their resistance to apoptosis. Furthermore, these results have shown for the first time that combined dual Src and FAK inhibition is effective for inducing apoptosis in colon cancer cell lines.
Several studies have demonstrated up-regulation of FAK expression in colorectal cancer (11, 13, 14, 24, 25, 32), and it appears that colon cells up-regulate expression of FAK at early stages of tumorigenesis, even before carcinoma has been detected (11). Our previous studies of the role of FAK in breast cancer cells have suggested that up-regulation of FAK in these cells has a dual function: (a) promoting adhesive properties of the tumor cells; and (b) promoting their survival (15). These results are consistent with a similar role for FAK in colon cancer, whereby inhibition of FAK alone did affect detachment and apoptosis. However, while the addition of Src inhibition enhanced the rate of detachment of the cells, it had a greater effect on enhancing the rate of apoptosis, operating through AKT-dependent pathways, with activation of caspase-3. Thus, these results demonstrate a cooperative role for FAK and Src in suppressing apoptosis in colon cancer cells and support a model whereby FAK and Src together provide survival signals that can be disrupted by dual inhibition of these kinases, similar to the cooperation between FAK and EGFR in breast cancer cell lines (19).

In this study, when FAK activity was inhibited by Ad-FAK-CD, Src activity was present in both colon cancer cell lines, and AKT remained active through a Src-dependent signaling pathway (33). Recently, it has been reported that Src can activate AKT directly through interaction of its SH3 domain and COOH-terminal regulatory proline-rich domain of AKT (34). This interaction leads to AKT phosphorylation at tyrosines 315 and 326 (35) and then to phosphorylation at Thr-308 and Ser-473 (34). When Src was inhibited by PP2

![Figure 5](image-url)
alone, FAK activity was enough to activate AKT, probably through PI-3 kinase (27, 36), suggesting that FAK and Src can activate AKT independently. Most significantly, when both FAK and Src kinase activities were inhibited, the survival AKT pathway was blocked, indicating its dependence on both FAK and Src. Following FAK and Src inhibition, caspase-3 was activated, leading to apoptosis of the colon cancer cells.

Inhibition of FAK and Src alone sensitized cells to staurosporine-induced apoptosis, which is consistent with other observation (26). This result shows that multiple cooperative kinase-dependent survival pathways are active in colon cancer cells (41–43), and the combination of Src inhibitor with FAK inhibitor enhances disruption of survival signaling pathways in colon cancer cells.

AKT (protein kinase B; 44) has important survival functions in cancer cells, and FAK can be upstream of phosphatidylinositol 3’-OH-kinase (PI-3 kinase) and AKT kinases (27). In BT474 cells, inhibition of FAK by Ad-FAK-CD caused apoptosis via pathways involving activation of caspase-8 and caspase-3, cleavage of poly(ADP-ribose)PARP, and caspase-3-dependent degradation of AKT (19). In a recent report, authors established a link between anoikis and AKT-mediated survival by demonstrating TNFR family FADD-dependent caspase-3 mediated AKT cleavage in detached epithelial cells (45). The decreased levels of total AKT seen with both FAK and Src inhibition in this report suggest that AKT can be cleaved by activated caspases-3.

It was shown that the FAK-Y397 auto-phosphorylation site is important for anti-apoptotic activity of FAK and activation of AKT pathways, as FAK-Y397F mutant cDNA caused apoptosis in human glioma T98G cells and inhibition of the AKT pathway (46). Src also has been shown to activate AKT (34). In the present report, colon cancer cell lines activated or expressed the active phospho-Ser-473 form of AKT in response to Ad-FAK-CD or PP2 alone (Fig. 8, A and B), causing cell resistance to stress-induced apoptosis, as seen with EGFR stably overexpressing the breast cancer BT474 cell line (19), and this resistance was abrogated only when FAK and Src were inhibited together. Activation of AKT was reported in cancer cells under stress-induced conditions, treated with chemotherapeutic drugs (doxorubicin, trastuzumab, or tamoxifen; 47) or in response to hypoxia (48). The constitutive activation of AKT was shown to be one of the mechanisms of tumor resistance to apoptotic agents. Thus, these data are also consistent with the recent reports that increased activity of Src was able to activate the AKT survival pathway in colon cancer cells (26) and FAK induces PI3K-AKT dependent anti-apoptotic pathways (37).

Src appears to have multiple roles in the pathogenesis and progression of colon cancer, and activation of Src in primary colorectal tumors was recently shown to be a marker of poor clinical prognosis (49). Decreased tumorigenicity of HT-29 cells has also been shown by antisense c-Src vector transfection (31), suggesting that Src activation can contribute to colon tumor progression. In addition, truncation c-Src mutations that had transforming activity and promoted metastasis were found in 12% of cases of advanced human colon cancer (30, 50). Elevated c-Src protein expression also has been shown to be an early event in colon neoplasia (51), consistent with a role for promoting tumor cell survival as a tumor becomes invasive and metastatic. It has been shown that Src regulated vascular endothelial growth factor (VEGF) expression in colon carcinoma cell lines indicating the role of c-Src in angiogenesis (52). Recently, Src has been shown to regulate the process of detachment-associated apoptosis, anoikis, in colon cancer cells, providing additional evidence that Src acts as a survival signal
FIGURE 7. Tyrosine phosphorylation and activities of FAK and Src are inhibited by Ad-FAK-CD + PP2 treatment. A. FAK dephosphorylation, down-regulation, and inhibition of activity is increased by Ad-FAK-CD + PP2 treatment. HT-29 cells were treated with Ad-FAK-CD and Ad-LacZ + PP2 for 48 h. Upper panel, immunoprecipitation of FAK with anti-FAK antibody was performed and the p125\textsuperscript{FAK} phosphorylation level was analyzed by Western blotting with phospho-tyrosine (RC20) antibody. Blot was reprobed with anti-FAK antibody. Lower panel, phosphorylation of FAK substrate, paxillin, is inhibited in Ad-FAK-CD + PP2-treated samples. Immunoprecipitation of paxillin (FAK substrate) with anti-paxillin antibody was performed and the paxillin phosphorylation level was analyzed by Western blotting with phospho-tyrosine-specific-HRP-conjugated (RC20) antibody. Blot was reprobed with anti-paxillin antibody. Phosphorylation of paxillin was inhibited by Ad-FAK-CD and more by Ad-FAK-CD + PP2 treatment. B. Activities of FAK and Src in HT-29 cells treated with Ad-FAK-CD + PP2. Upper left part, FAK activity was determined by Western blotting with anti-FAK-Y397 and FAK expression with anti-FAK-specific antibodies. Lower left part, in vitro kinase assay for analyzing Src activity in HT-29 cells. Whole cell lysates were immunoprecipitated with c-Src monoclonal antibody, and in vitro kinase assay with substrate enolase was done as described in “Materials and Methods.” Autoradiography of phosphorylated enolase is shown. The amount of c-Src in immunoprecipitates was determined with Western blot using anti-c-Src antibody and Src expression was analyzed with anti-c-Src antibody. The Western blot experiment was performed on bigger higher resolution gels than in A, and Src migrated as a doublet. Anti-\gamma-actin antibodies were used for loading control.
Our results are consistent with these observations, but provide evidence that inhibition of Src and FAK would provide more effective molecular cancer therapeutics than inhibition of either one alone.

Materials and Methods

Cells and Cell Culture
Breast carcinoma cells, BT474, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 5 \( \mu \)g/ml insulin, and 1 \( \mu \)g/ml penicillin/streptomycin. Colon cancer cell lines, HT-29, were maintained in McCoy’s 5A plus 10% FBS medium; WIDR cells were maintained in RPMI plus 10% FBS; LS180 cells were maintained in Earle’s minimum essential medium (EMEM) plus 10% FBS.

**Antibodies and Reagents**

Monoclonal anti-FAK (4.47) antibody to NH\(_2\)-terminal FAK and monoclonal anti-paxillin antibody were obtained from Upstate Biotechnology, Inc. (Charlottesville, VA). Polyclonal anti-phospho-Tyr-397-FAK and anti-phospho-Tyr-418-Src antibodies were from Biosource Inc. (Camarillo, CA). Monoclonal anti-caspase-3 antibodies were ordered from Transduction Labs (San Diego, CA). Monoclonal anti-\( \alpha \)-tubulin and \( \beta \)-actin antibodies were obtained from Sigma (St. Louis, MO). Monoclonal anti-HA antibody was from Roche Molecular Biochemical (Indianapolis, IN). PP2, inhibitor of Src phosphorylation, was obtained from Calbiochem (San Diego, CA) and used at concentration of 30 \( \mu \)M. Polyclonal anti c-Src antibody used for Western blotting was from Santa Cruz Inc. (Santa Cruz, CA). Monoclonal anti-Src antibody (clone 327) used for immunoprecipitation in kinase assay was from Oncogene Research Products Inc. (San Diego, CA). Staurosporine was purchased from Calbiochem and used at 100 nM dose.

**Adenoviral Infection, X-gal, and HA Staining**

Recombinant adenoviruses carrying the LacZ gene, Ad-LacZ, and HA-tagged FAK-CD gene, coding 693–1052 amino acids of FAK, and Ad-FAK-CD were obtained from Dr. J. Samulski and the Gene Therapy Center Virus Vector Core Facility of the University of North Carolina and described in Ref. 15. Cells were plated in six-well plates at 2 \( \times \) \( 10^5 \) and after 24 h of attachment were infected with adenoviruses at different viral concentrations and a viral titer that produced greater than 90% cell infectivity was used.

The optimal viral titer was 500 ffu/cell, obtained from the Gene Therapy Center Virus Vector Core facility that produced >90% cell infectivity and no toxic effects for Ad-LacZ transduction, checked by X-gal (5-bromo-4-chloro-3-indolyl-\( \beta \)-galactopyranoside) staining, as described in Ref. 15. The same viral titer was used for Ad-HA-tagged-FAK-CD infection, causing >90% of cell infectivity, determined by HA immunostaining, as described (15, 19).

**Treatment With the Src Inhibitor, PP2**

Cells were starved without serum for 1 h and PP2 was added at 30 \( \mu \)M for 15 min. This optimal PP2 dose was chosen in a dose-response experiment by blocking Src downstream phosphorylation in colon cancer cell lines used in the study. PP2 was added at 30 \( \mu \)M to the medium every 24 h in Ad-FAK-CD infection experiments.

**Immunoprecipitation and Western Blotting**

Cells were washed twice with cold 1 × PBS and lysed on ice for 30 min in a buffer containing: 50 mM Tris-HCl (pH 7.5),...
150 mM NaCl, 1% Triton X-100, 0.5% NaDOC, 0.1% SDS, 5 mM EDTA, 50 mM NaF, 1 mM NaVO₃, 10% glycerol, and protease inhibitors: 10 μg/ml leupeptin; 10 μg/ml phenylmethylsulfonyl fluoride; and 1 μg/ml aprotinin. The lysates were cleared by centrifugation at 10,000 rpm for 15 minutes at 4°C. Protein concentration was determined using Bio-Rad Kit. The cleared lysates with equivalent amount of protein were incubated with 5 μl of primary antibody for 1 hour at 4°C and 25 μl of protein A/G agarose beads (Oncogene Research Products). The precipitates were washed with the lysis buffer three times and resuspended in 30 μl of 2× Laemmli buffer. For Western blotting, boiled samples were loaded on Ready SDS-10% PAGE gels (Bio-Rad, Inc., Hercules, CA). Phosphorylation status of examined proteins was detected with horseradish peroxidase-linked anti-phosphotyrosine antibody, HRP-RC20 (Transduction Labs), in 1% BSA-TBST-buffer. The blots were visualized by autoradiography.

**Detachment Assay**

Detached and attached cells were counted in a hemocytometer. We calculated the percentage of detachment by dividing the number of detached cells by the total number of cells. The percentage of detached cells was calculated in three independent experiments.

**Apoptosis Assay**

Detached cells were collected and fixed in 3.7% formaldehyde in 1× PBS solution for the apoptosis assay. Detection of apoptosis was done with Hoechst 33342 staining or by TUNEL assay using ApopTag kit (Intergen, NY) according to the manufacturer’s protocol. Simultaneous staining and quantification of apoptotic cells with TUNEL assay and Hoechst methods produced very similar results. The percentage of apoptotic cells was calculated as a ratio of apoptotic detached cells divided by the total number of cells in three independent experiments in several fields with the fluorescent microscope. For each experiment, 300 cells per treatment were counted.

**In Vitro Src and FAK Activity Kinase Assay**

Immunoprecipitation with anti-Src monoclonal antibody was done as described above. In brief, 10 μCi of [γ³²P]ATP was added to antibody-bound c-Src protein in a kinase buffer [20 mM HEPES (pH 7.4), 5 mM MgCl₂, 5 mM MnCl₂, 0.1 mM NaVO₃] with 5 μg of substrate, acid-denatured rabbit muscle enolase (Sigma), for 15 minutes at room temperature. For the FAK activity kinase assay, immunoprecipitates with anti-FAK monoclonal antibody were incubated in the kinase buffer with 10 μCi of [γ³²P]ATP. The kinase reaction was performed for 15 minutes at room temperature and stopped by addition of 2× Laemmli buffer. Proteins were separated on Ready SDS-10% PAGE gels, and the phosphorylated enolase or phosphorylated FAK were visualized by autoradiography.

**Statistical Analysis**

The statistical significance of differences between means of samples in each assay was assessed by a Student’s t test. P values less than 0.05 were considered significant.

**References**


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