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ARTICLES

Co-Operative Effect of c-Src and Ezrin in Deregulation of Cell–Cell Contacts and Scattering of Mammary Carcinoma Cells

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Abstract The non-receptor tyrosine kinase c-Src is activated in many human cancer types, and induces deregulation of cadherin-based cell–cell contacts and actin cytoskeleton. Because ezrin, a protein which cross-links the plasma membrane with the actin cytoskeleton, is often over-expressed in human cancers, and participates in cell adhesion, motility, and cell scattering, we therefore investigated whether c-Src co-operates with ezrin in regulating cell–cell contacts in a murine mammary carcinoma cell line, SP1. SP1 cells over-expressing wild type ezrin, or an activated c-Src mutant, formed loose aggregates which scattered spontaneously when plated on plastic. When wild type ezrin and activated c-Src were co-expressed, scattering was increased, cell–cell contacts disrupted, and cell aggregation prevented. Pre-treatment with the c-Src family kinase inhibitor PP2 partially restored aggregation of cells expressing activated c-Src and wild type ezrin, indicating that c-Src family kinases act co-operatively with ezrin in regulating cell–cell contacts. Furthermore, expression of a truncated NH_2-terminal domain of ezrin, which has dominant negative function, blocked the cell scattering effect of activated c-Src and promoted formation of cohesive cell–cell contacts. Together, these results suggest co-operativity between c-Src and ezrin in deregulation of cell–cell contacts and enhancing scattering of mammary carcinoma cells. J. Cell. Biochem. 92: 16–28, 2004.

Key words: ezrin; c-Src; cadherins; cell–cell contacts; cell scattering; carcinomas

Dissociation of cell–cell adhesion and loss of epithelial cell polarity are critical early steps in epithelial–mesenchymal transition (EMT) and tumorigenesis [Haefner et al., 1995]. A key regulator of EMT is the non-receptor tyrosine kinase c-Src, which shows sustained high level of activation in many cancer types [Ottenhoff-Kalff et al., 1992] with activating mutations reported in some metastases [Bjorge et al., 2000a,b]. c-Src is recruited at both focal adhesions [McLean et al., 2000] and cell–cell contacts [Owens et al., 2000; Frame, 2002], and is required for anchorage-independent growth and cell migration [Rahimi et al., 1998]. Sustained activation of c-Src has been shown to enhance tyrosine-phosphorylation of β-catenin [Owens et al., 2000; Frame, 2002], and to shift cadherin-based adhesion from a strong to a weak state [Takeda et al., 1995]. Recently, Avizienyte et al. [2002] showed that c-Src-induced deregulation of E-cadherin organization requires specific integrin signalling, demonstrating a reciprocal relationship between integrin-induced signals and cadherin-based cell adhesion.

Deregulation of cell–cell adhesion by c-Src also involves re-organization of the actin cytoskeleton. Several observations suggest that

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ezrin, a member of the ezrin-radixin-moesin (ERM) protein family [Bretscher et al., 2002] might be involved in the remodeling of the actin cytoskeleton induced by c-Src. Ezrin acts as a linker between the plasma membrane and the actin cytoskeleton [Bretscher et al., 2002]. ERM proteins are regulated by an intramolecular association of their NH_2-terminal and C-terminal domains that masks their protein–protein binding sites. Upon activation, the molecule unfolds allowing binding of membrane proteins and of polymerized F-actin to the C-terminal domain [Bretscher et al., 2000]. Phosphorylation of a conserved threonine residue (Thr-567) has been shown to cause opening of the molecule resulting in the formation of active monomers [Matsui et al., 1998; Gautreau et al., 2000]. Inactivation studies indicate that ezrin plays an important role in cell morphogenesis and cell polarity [Crepaldi et al., 1997; Lamb et al., 1997; Yonemura and Tsukita, 1999], and regulates cell adhesion and cell survival [Takeuchi et al., 1994; Martin et al., 1995; Crepaldi et al., 1997; Gautreau et al., 1999]. Following HGF stimulation of epithelial cells, ezrin is phosphorylated at specific tyrosine residues, and acts downstream of Met in several HGF-induced cell functions including cell motility and cell scattering [Crepaldi et al., 1997]. Recent gene expression studies have shown that ezrin is over-expressed in metastatic compared to corresponding nonmetastatic tumor cell lines from different tumor types [Khanna et al., 2001; Nestl et al., 2001], and is strongly expressed in a variety of invasive human cancers [Geiger et al., 2000; Tokunou et al., 2000]. In transformed cells, ezrin is associated with the cortical cytoskeleton and filopodia extensions, and facilitates increased cell survival, proliferation, spreading, and motility [Lamb et al., 1997; Reczek et al., 1997; Ng et al., 2001; Wick et al., 2001].

Since ezrin regulates cell functions similar to c-Src, we hypothesized that c-Src may interact co-operatively with ezrin in deregulating cell–cell adhesion and promoting increased cell scattering in malignancy. In the present study, we investigated co-operativity of c-Src and ezrin in the regulation of cell–cell contacts and cell scattering, using a mouse mammary carcinoma cell line, SP1, as a model of tumor progression. Using activated and inactive mutant forms of c-Src and ezrin, we demonstrated co-operativity between c-Src and ezrin in disassembly of cell–cell contacts and scattering of SP1 cells. We further showed that blocking ezrin function abrogated the scattering phenotype of an activated c-Src mutant in carcinoma cells. Thus, ezrin may be a key signaling molecule downstream of c-Src in the regulation of EMT and tumorigenesis.

MATERIALS AND METHODS

Antibodies and Reagents

Rabbit anti-sheep IgG conjugated with horseradish peroxidase was from Jackson Immuno-Research Laboratories (West Grove, PA). Mouse (monoclonal) anti-pan cadherin antibody was obtained from Sigma Immunochemicals (Oakville, Ont., Canada). Mouse monoclonal antibody against the vesicular stomatitis glycoprotein (VSVG) (clone P5D4) was previously described [Kreis, 1986]. Rabbit anti-ezrin IgG was prepared as described previously [Crepaldi et al., 1997]. Anti-phopho-tyrosine antibody (PY20) was obtained from Universal Biomedicals, Inc. (New York). Alexa-488-conjugated goat anti-mouse IgG was obtained from ICN Biomedicals (Toronto, ON). The PP2 c-Src family kinase inhibitor was obtained from Calbiochem (La Jolla, CA). Recombinant human HGF was a gift from Dr. R. Schwall (Genentech, Inc., San Francisco, CA).

Cell Lines and Tissue Culture

The SP1 tumor cell line was derived from a spontaneous poorly metastatic murine mammary intraductal adenocarcinoma, isolated from a female CBA/J retired breeder in the Queen's University Animal Facility [Elliott et al., 1992; Rahimi et al., 1996]. SP1 cells were cultured in RPMI 1640 medium (Invitrogen, Burlington, ON) supplemented with 2 mM L-glutamine and 7% FBS.

Cell Transfection

c-Src expression plasmids were constructed by subcloning activated (Y527F) and kinase dead (SRC-RF: K295F, Y527F) chicken c-Src cDNAs (gift from Dr. J. Brugge and Dr. D. Shalloway) into the EcoRI site of DNA polymerase I (Klenow fragment)-treated pBabe-Puro plasmid to generate pBabe SRC-Y527F and pBabe SRC-RF (puromycin resistant). The Y527F mutation prevents phosphorylation of the negative regulatory tyrosine Y527, thereby preventing intramolecular binding to the SH2

domain, and allowing c-Src to be constitutively active. The K295F mutation of SRC-RF inactivates the ATP-binding site in the kinase domain. The second mutation (Y527F) allows substrate binding without kinase activity. Clones of SP1 cells stably transfected with activated (SRC-Y527F) or kinase dead (SRC-RF) chicken c-Src mutants were previously established in our laboratory [Hung and Elliott, 2001]. We have shown that over-expression of SRC-Y527F in SP1 cells dramatically increases c-Src activity, whereas over-expression of SRC-RF inhibits endogenous c-Src activity, as determined by an in vitro kinase assay with enolase as substrate [Hung and Elliott, 2001].

The pCB6 vector containing cDNA encoding VSVG-tagged ezrin or the VSVG-tagged N-terminal truncated domain (aa 1-309, designated N-term) of ezrin were previously described [Algrain et al., 1993].

All transfections were carried out with Lipofectamine Plus reagent (Canadian Life Technology, Burlington, ON) according to manufacturer's instructions. Parental SP1 cells, or cells expressing activated c-Src or kinase dead c-Src, were plated at 70% confluence in 60 mm plates and transfected with 2 μg of plasmids expressing wild type or N-term ezrin. Puromycin (2 μg/ml, Sigma) and G418 (450 μg/ml, Sigma) was added to cells 24 h following transfection, and cultures were maintained until all cells in the mock transfection were killed. Drug resistant cells were then harvested and subcloned. Protein expression in the transfected cells was confirmed using indirect immunofluorescence and semi-quantitative Western blot analysis was performed to determine the fold increase relative to endogenous ezrin (see below). Two clones from each group were selected for further study.

Cell Aggregation Assay

SP1 cells were plated in monolayer culture at 30% confluence for 1 day before the assay. Cells were harvested using 5 mM EDTA/PBS and cell clusters were removed by sedimentation at unit gravity. Single cells (5×10^6) were added to a 25 ml siliconized Erlenmyer flask containing 5 ml of prewarmed 5% CO_2-adjusted RPMI with 7% FBS. The flasks containing the cell suspensions were sealed, and incubated at 37°C in a gyratory shaker at 80 rpm for the times indicated. Equal aliquots of cells were fixed in 1.5% paraformaldehyde, and representative fields were photographed using an inverted Leica DMIL inverted microscope equipped with an MCID-M4 image analysis system (Imaging Research, Brock University, St. Catherines, ON). The degree of aggregate formation was estimated according to the decrease in particle number [Dufour et al., 1999]: percent aggregation = 1−[no. of particles (at time = x)/initial number of particles) ×100%]. At least 200 cells were scored in three fields per group in each experiment. The results from two independent clones per group from two to three experiments were pooled, averaged, and expressed as the mean ± SE.

Cell Scattering Assay

Aggregates were made in RPMI medium supplemented with 7% FBS and incubated in a gyratory shaker for 12 h as described above. Aggregates of approximately 10–40 cells were enriched by sedimentation three times at unit gravity through RPMI with 0.5 mg/ml BSA to remove single cells. Equal numbers of aggregates were plated in 24-well Nunc tissue culture plates alone, or on fibronectin substratum (precoated on plates at 10 μg/ml), or with HGF (30 ng/ml), as indicated. Aggregates were allowed to adhere and scatter for the times indicated, followed by fixation in 1.5% paraformaldehyde. Representative fields (three per group) were photographed. Cells which had dispersed were identified by a spread-out morphology, and were counted visually. The proportion of dispersed cells was calculated as follows: [number of spread cells/total cells] ×100%. At least 10 aggregates per clone in each group were examined per experiment. The results from two independent clones per group from two to three experiments were pooled, averaged, and expressed as the mean ± SE.

Indirect Immunofluorescence

Cells were plated overnight on cover slips, rinsed three times in pre-warmed PBS with 0.1 μM $CaCl_2$ and 0.1 μM $MgCl_2$, and fixed for 20 min in 3% paraformaldehyde in PBS. Cells were permeablized by incubation for 5 min in 0.2% Triton X-100 in PBS, rinsed three times, and incubated for 10 min in 50 mM NH_4Cl in PBS. Cells were incubated with anti-VSVG, or anti-cadherin antibodies for 45 min, washed three times in PBS, and incubated with the appropriate secondary antibody. Preparations were observed using a Leica TCS SP2 confocal

microscope in the Queen's Cancer Research Institute and Protein Discovery and Function Facility. Image acquisitions were processed using Adobe Photoshop software.

Immunoprecipitation and Western Blotting

Cells were grown to 60% confluence, rinsed with PBS, and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM Na_3VO_4, 50 mM NaF, 1 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupetin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge, and supernates were subjected to protein determination using a bicinchoninic acid protein assay (Pierce, Rockford, IL). To assess relative amounts of exogenous and endogenous proteins, serial dilutions of total cell lysates (0.1–2 μg) were subjected to 8% SDS–PAGE under reducing conditions (with 2.5% β-2 mercaptoethanol), and transferred to nitrocellulose membranes. The membranes were probed with polyclonal rabbit anti-ezrin IgG (which detects the ezrin C-terminus), or monoclonal mouse anti-VSVG IgG (which detects VSVG-tagged ezrin). Immune complexes were detected using horse radish peroxidase-labelled donkey anti-rabbit IgG, followed by chemiluminescence with the Northern Lightning™ reagent (Perkin Elmer Life Sci., Inc., Boston). Relative densities of each band were compared visually with that of untransfected cells run at the same total protein concentrations to determine the approximate fold increase in exogenous versus endogenous ezrin expressed. The level of N-term ezrin expression was compared to that of the endogenous protein.

For immunoprecipitation experiments, equal amounts of protein from each cell lysate (400 μl) were precleared with 40 μl of a 50% (v/v) mixture of protein A sepharose (PAS) beads (Amersham Biosciences, Baie d'Urfé, QC) for 4 h at 4°C on a rotator. Cell lysates were clarified by centrifugation and incubated overnight with rabbit anti-ezrin IgG and additional PAS beads. Immunoprecipitates were washed five times in ice-cold lysis buffer and collected by centrifugation for 10 min at 4°C. Samples were eluted from PAS beads by boiling in 2× SDS sample buffer, subjected to reduced 8% SDS–PAGE as above, and transferred to nitrocellulose membranes. The membranes were probed with mouse anti-phospho-tyrosine IgG or rabbit anti-ezrin IgG, as described above.

RESULTS

Ezrin Acts Co-Operatively With c-Src in Disrupting Cell–Cell Aggregation in Carcinoma Cells

We have previously shown that overproduction of wild type ezrin enhances HGF-induced migration and morphogenesis of epithelial cells whereas over-production of N-term ezrin blocks the above response to HGF, suggesting that this domain exhibits a dominant negative effect [Crepaldi et al., 1997]. Spontaneous cell scattering was also observed with activated c-Src whereas expression of a kinase dead c-Src mutant in carcinoma cells promoted formation of cohesive cell aggregates and prevented HGF-induced cell scattering and motility [Elliott et al., 2002]. We therefore examined whether ezrin acts co-operatively with c-Src in regulating cell–cell aggregation and scattering of carcinoma cells.

We first generated stable transfectants expressing wild type or N-term ezrin alone, or in combination with activated c-Src in SP1 carcinoma cells. For exogenous ezrin expression, we used a pCB6 eukaryotic expression vector,

Fig. 1. (*Overleaf*) Effect of co-expressing an activated c-Src mutant and wild type or N-term ezrin on aggregation of carcinoma cells. **Panel A**: SP1 cells were transfected with the pCB6 expression vector only, or containing wild type ezrin or N-term (NT) ezrin tagged with VSVG, as described in ''Materials and Methods.'' Serial dilutions of total cell extracts (0.1–2 μg) were subjected to reduced 8% SDS–PAGE, and transferred to nitrocellulose membranes. The membranes were probed with polyclonal rabbit anti-ezrin IgG (**left three lanes**) or monoclonal anti-VSVG (P5D4) antibody (**right two lanes**), followed by peroxidase conjugated secondary antibody, and developed with chemiluminescence. Lanes from **left** to **right** contained equal protein amounts of cell lysates from: pooled SP1 cells transfected with the pCB6 empty vector; or with wild type ezrin (clones WT13 and WT16); or with N-term ezrin (clones NT16 and NT17). **Panel B**: A clone of SP1 cells expressing an activated c-Src mutant was transfected with vector only, or containing wild type or N-term ezrin, and expression of ezrin was determined by Western blotting as in panel A. Lanes from left to right contained equal protein amounts of lysates from: pooled SP1 cells expressing activated c-Src and transfected with the pCB6 empty vector; or with wild type ezrin (clones WT23 and WT35); or with N-term ezrin (clones NT19 and NT24). **Panels C–H**: Cells from each group in panels A and B were subjected to aggregate formation in gyratory suspension cultures with 7% FBS/RPMI medium for 12 h, as described in ''Materials and Methods.'' Cells were then fixed in 1.5% paraformaldehyde in PBS, and representative fields were photographed. Scale bar indicates 50 μm.

carrying cDNA encoding VSVG-tagged wild type ezrin or its N-terminal domain (aa 1-309, designated N-term), as previously described [Algrain et al., 1993]. The ezrin protein levels in the transfected cells were found to be 4–10 fold increased compared to endogenous ezrin, as determined by semi-quantitative Western blotting (Fig. 1A,B). Two clones from each group were selected for functional analysis.

Over-expression of wild type ezrin in SP1 cells caused formation of loose aggregates (Fig. 1E), similar to cells expressing activated c-Src

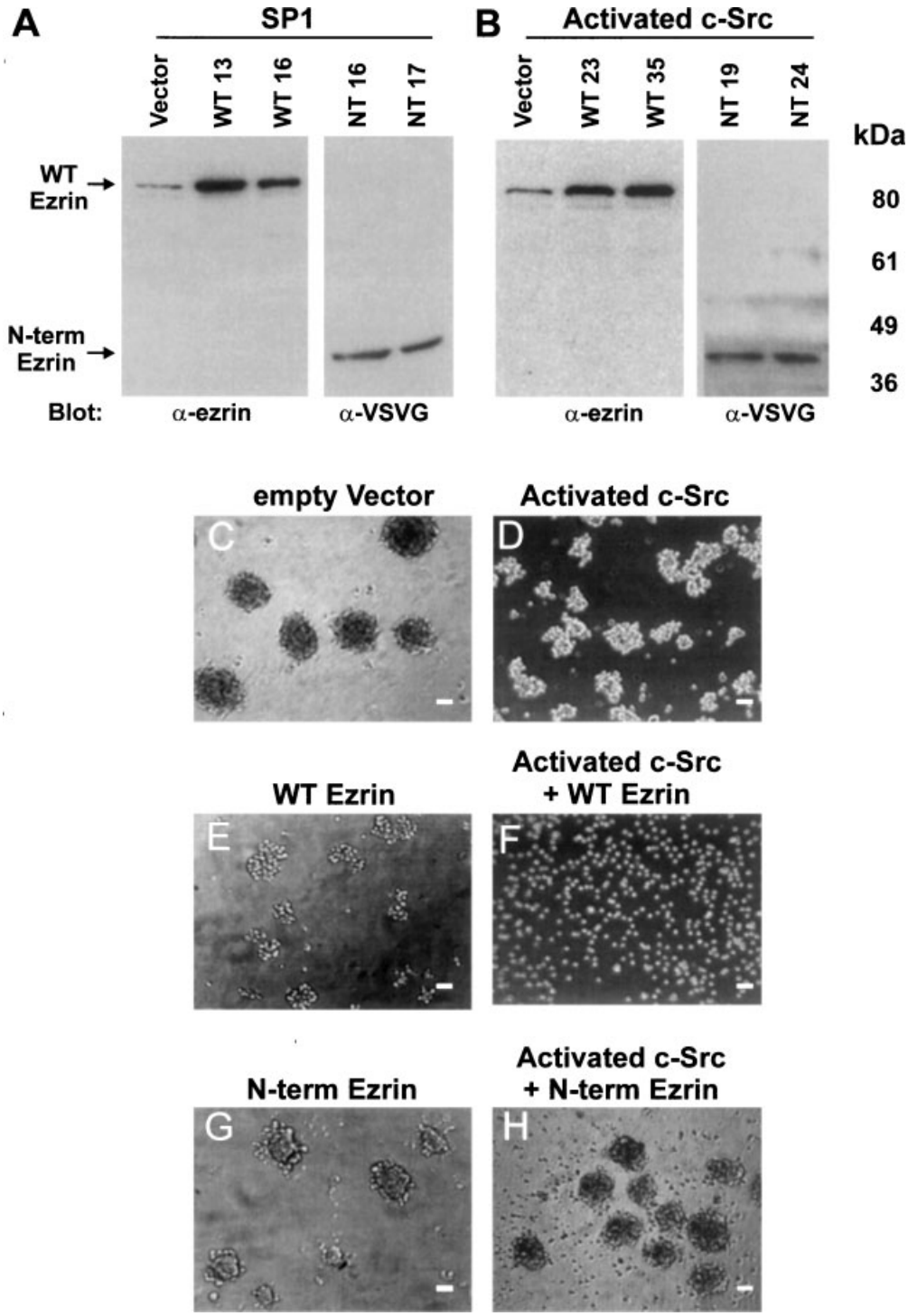

Fig. 1.

(Fig. 1D); however the total number of aggregates was similar in both groups (Fig. 2A). Interestingly, co-expression of wild type ezrin with activated c-Src completely disrupted cell–cell aggregation (Figs. 1F and 2A). In contrast, co-expression of N-term ezrin with activated c-Src restored formation of cohesive aggregates (Fig. 1H), similar to cells expressing N-term ezrin alone (Fig. 1G) or the pCB6 empty vector (Fig. 1C).

To further assess the co-operative role of c-Src and ezrin in disruption of cell aggregates, we examined the effect of an inhibitor (PP2) which blocks the ATP binding site of c-Src family kinases [Hanke et al., 1996]. We found that pretreatment (60 min) with the PP2 inhibitor significantly restored aggregation of cells co-expressing activated c-Src and wild type ezrin within 90 min, compared to control (DMSO-treated) cells (Fig. 2B). Only a marginal increase in aggregation of cells expressing activated c-Src or activated c-Src plus N-term ezrin following PP2 treatment was observed.

Together, these results show that c-Src kinase acts co-operatively with ezrin to disrupt aggregation of carcinoma cells.

Over-Expression of N-Term Ezrin Blocks c-Src-Induced Scattering of Carcinoma Cells

Our finding that N-term ezrin shifts cell–cell aggregation from a loose to a cohesive state in carcinoma cells expressing activated c-Src (Fig. 1H), suggests an important role of ezrin in c-Src-dependent scattering. To test this possibility, cells expressing various ezrin and c-Src mutants were tested for spontaneous cell spreading and scattering on plastic. Cells over-expressing wild type ezrin or activated c-Src alone, or both together, showed spontaneous scattering compared to empty vector cells, when plated on plastic (Fig. 3B,D,G). In these cells, ezrin localized at the cell membrane, and in actin-rich membrane extensions (data not shown). In contrast, cells over-expressing N-term ezrin showed a rounded cell morphology, with N-term ezrin localized primarily at the cell membrane and cell–cell contacts (data not shown). Furthermore, N-term ezrin prevented spontaneous scattering of SP1 cells alone, or expressing activated c-Src (Fig. 3F,G), or in response to HGF or fibronectin (data not shown). Thus over-expression of N-term ezrin blocks the cell scattering phenotype of activated c-Src in carcinoma cells.

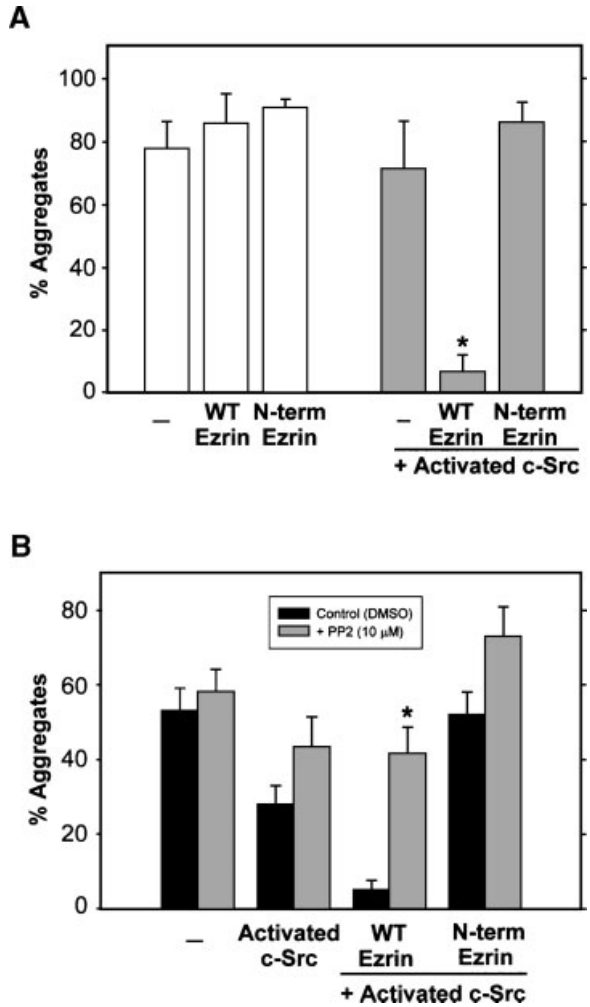

Fig. 2. Effect of wild type ezrin, activated c-Src and the c-Src kinase family inhibitor (PP2) on aggregation of carcinoma cells. **Panel A**: The percent aggregation of cells expressing ezrin and c-Src mutants from Figure 1C–H was determined as described in the ''Materials and Methods.'' The results from two independent clones per group from two (clear bars) and three (grey bars) experiments were pooled, averaged, and expressed as the mean ± SE. The asterisk indicates a significant reduction in percent aggregates ($P = 0.003$), as determined using a two-tailed Student's *t*-test. **Panel B**: Untransfected SP1 cells, and cells transfected with activated c-Src alone, or with wild type ezrin, or with N-term ezrin, were preincubated in monolayer cultures with PP2 (10 μM) or 0.1% DMSO (control) in 7% FBS/RPMI medium for 60 min. Cells were then harvested and subjected to aggregate formation in gyratory suspension cultures for 90 min with medium containing PP2 or DMSO as above. Cells were fixed with 1.5% paraformaldehyde, and the percent aggregate formation was determined as described in the ''Materials and Methods.'' The results from duplicate cultures per group from two independent experiments were pooled, averaged, and expressed as the mean ± SE. The asterisk indicates a significant increase in percent aggregates following PP2 treatment, compared to untreated cells ($P = 0.007$), as determined using a two-tailed Student's *t*-test.

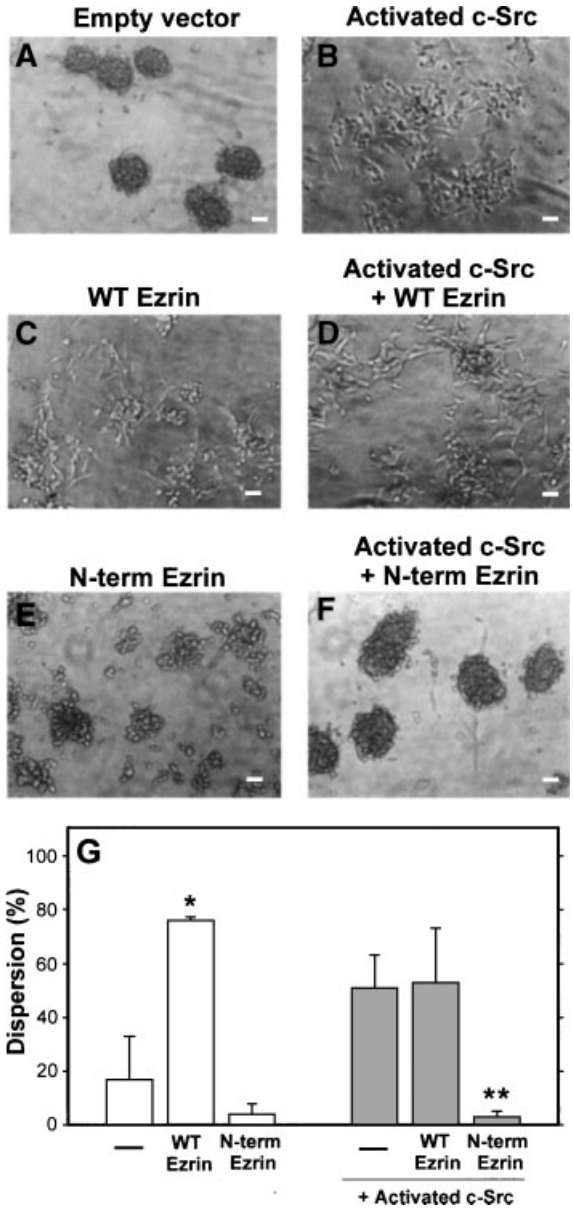

Fig. 3. Effect of ezrin mutants on scattering of carcinoma cells expressing activated c-Src. **Panels A–F**: Transfected SP1 carcinoma cells corresponding to the groups in Figure 1C–H were subjected to aggregate formation in gyratory suspension cultures with 7% FBS/RPMI medium for 12 h as described in ''Materials and Methods.'' Aggregates were isolated from single cells by sedimentation at unit gravity, and equal numbers of aggregates were plated in RPMI with 0.5 mg/ml BSA in 24-well tissue culture plates, and incubated for 6 h at 37°C. Cells were then fixed with 1.5% paraformaldehyde, and representative fields were photographed. Scale bar indicates 50 μm. **Panel G**: The percent of dispersed (spread) cells in each group was calculated as described in ''Materials and Methods.'' The results from two independent clones per group from at least two experiments were pooled, averaged, and expressed as the mean ± SE. Asterisks indicate a significant increase (*$P = 0.017$), or decrease (**$P = 0.003$) in percent of cell dispersion compared to corresponding control groups (−).

Over-Production of Wild Type-Ezrin Partially Reverses the Anti-Scattering Phenotype of Kinase Dead c-Src

The above results raise the question whether ezrin is downstream of c-Src in cell scattering function. We therefore determined the effect on cell–cell aggregation and scattering of over-production of wild type ezrin in carcinoma cells expressing kinase dead c-Src. The relative level of transfected ezrin versus endogenous ezrin protein was found to be 10–15 fold, as determined by semi-quantitative Western blotting (Fig. 4A). The results showed that over-production of ezrin in cells expressing kinase dead c-Src caused a twofold reduction in aggregate formation compared to the parent cell line (Fig. 4C,E). In contrast, cells co-expressing N-term ezrin with kinase dead c-Src formed very cohesive aggregates (Fig. 4D). Cells over-expressing both kinase dead c-Src and wild type ezrin showed only marginal spreading on plastic (Fig. 5D,J), however, a strong scattering response to HGF or fibronectin was restored in these cells (Fig. 5E,F,J). In contrast, cells expressing kinase dead c-Src alone, or kinase dead c-Src plus N-term ezrin, remained compact and showed no spreading or scattering under any of the above culture conditions (Fig. 5A–C,G–I,J). Thus, over-expression of exogenous ezrin partially reverses the anti-scatter phenotype of kinase dead c-Src in carcinoma cells.

Over-Production of Ezrin and Activated c-Src Causes Deregulation of Cadherin-Based Cell–Cell Contacts in Carcinoma Cells

Both c-Src [Owens et al., 2000] and ezrin [Hiscox and Jiang, 1999] have been shown to localize at cell–cell contacts in carcinoma cells. We therefore assessed the effect of over-expressing an activated c-Src and wild type ezrin on the formation of cell–cell contacts in SP1 cells grown in monolayer culture with 7% FBS/RPMI medium. We used immuno-staining with a pan-cadherin antibody to identify cell–cell contacts. Cells expressing activated c-Src (Fig. 6A) or wild type ezrin (not shown) alone displayed significant membrane localization of cadherins (Fig. 6A), consistent with the ability of these cells to form loose aggregates (Fig. 1D,E). However, over-expression of both wild type ezrin and activated c-Src markedly reduced membrane localization of cadherins and promoted a punctate cytoplasmic pattern of cadherin staining

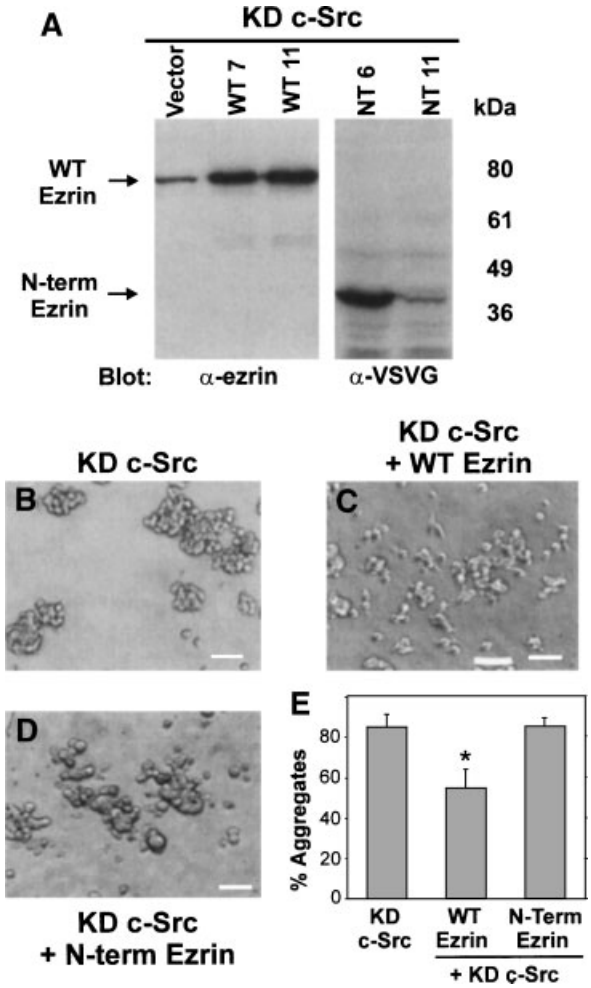

Fig. 4. Effect of ezrin mutants on aggregate formation by carcinoma cells expressing kinase dead c-Src. **Panel A**: A clone of SP1 cells expressing a kinase dead (KD) c-Src mutant (SRC-RF) was subjected to a second round of transfection with the pCB6 expression vector only, or containing wild type ezrin, or N-term ezrin, and the indicated clones were isolated. Serial dilutions of total cell extracts (0.1–2 μg) were subjected to reduced 8% SDS–PAGE and Western blotting, as described in Figure 1A. Lanes from **left** to **right** contained equal protein amounts of cell lysates from: pooled SP1 cells expressing kinase dead c-Src and transfected with the pCB6 empty vector; or with wild type ezrin (clones WT7 and WT11); or with N-term ezrin (clones NT6 and NT11). **Panels B–D**: Transfected cells from panel A were subjected to aggregate formation in gyratory suspension cultures for 12 h, as described in ''Materials and Methods.'' Representative images from each group are shown. Scale bar indicates 50 μm. **Panel E**: The histogram shows the percent aggregate formation expressed as the mean ± SE of triplicate fields, as described in ''Materials and Methods.'' The results from two independent clones per group from three experiments were pooled, averaged, and expressed as the mean ± SE. Asterisk indicates a significant decrease in percent aggregates, compared to cells expressing kinase dead c-Src ($P < 0.02$), as determined using a two-tailed Student's *t*-test.

with cell spreading (Fig. 6B). Thus, cadherin-based cell–cell contacts are abrogated following over-production of ezrin and activated c-Src in carcinoma cells, consistent with the inability of these cells to form aggregates (Fig. 1F). Conversely, co-expression of N-term ezrin with activated c-Src caused strong membrane localization of cadherins at cell–cell contacts (Fig. 6C). The latter cells formed compact aggregates, which were disrupted following incubation for 6 h in Ca^{2+}-free medium (data not shown).

Carcinoma cells expressing kinase dead c-Src alone (Fig. 6D) or with N-term ezrin (Fig. 6F) formed regular islets and showed strong membrane localization of cadherins at cell–cell contacts. In contrast, over-expression of wild type ezrin with kinase dead c-Src caused a shift of cadherin localization from the plasma membrane to the cytoplasm, and increased cell spreading (Fig. 6E). These findings are consistent with the decreased ability of these cells to form aggregates (Fig. 4C) and increased cell scattering on fibronectin substratum or with HGF (Fig. 5E,F).

Collectively, these results show that c-Src and ezrin act co-operatively in the regulation of cadherin-based cell–cell contacts.

Activated c-Src Induces Tyrosine Phosphorylation of Ezrin in Carcinoma Cells

Phosphorylation of tyrosine residues is important in regulating ezrin functions [Crepaldi et al., 1997]. In addition, cells expressing v-Src have been shown to exhibit increased tyrosine phosphorylation of several junctional proteins including β-catenin, Z0-1, and ERM proteins [Takeda et al., 1995]. We therefore examined whether SP1 cells expressing activated c-Src exhibit increased tyrosine phosphorylation of ezrin, using immunoprecipitation with anti-ezrin antibody and Western blot analysis. The results showed that expression of activated c-Src in SP1 carcinoma cells caused increased tyrosine phosphorylation of ezrin, compared to kinase dead c-Src or untransfected cells (Fig. 7). These findings support a role of c-Src in regulating ezrin function through tyrosine phosphorylation.

DISCUSSION

The previous demonstration by Crepaldi et al. [1997], that ezrin is an effector of

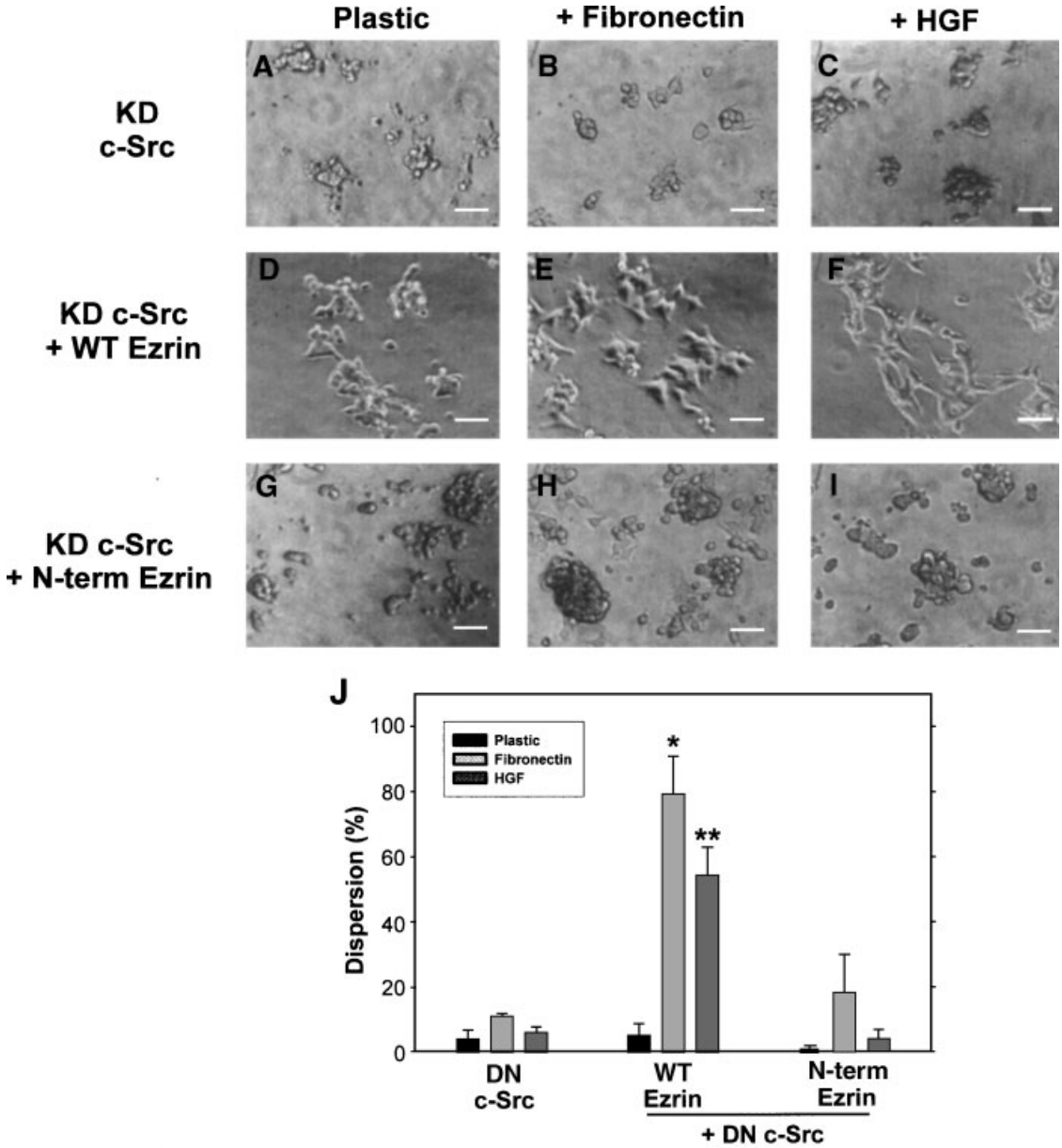

Fig. 5. Effect of ezrin mutants on fibronectin-, and HGF-induced scattering of carcinoma cells expressing kinase dead c-Src. **Panels A–I**: SP1 cells expressing kinase dead c-Src only, or kinase dead c-Src + wild type ezrin or kinase dead c-Src + N-term ezrin (see Fig. 4), were subjected to aggregate formation in gyratory suspension cultures for 12 h, as described in ''Materials and Methods.'' Aggregates were isolated, and plated in 24-well tissue culture dishes on plastic, fibronectin substratum (coated at 10 μg/ml), or with HGF (30 ng/ml). After 2 h (fibronectin) or 6 h (plastic or HGF) incubation at 37°C, cells were fixed with 1.5% paraformaldehyde in PBS, and representative fields were photographed. **Panel J**: The percent cell dispersion was calculated as described in ''Materials and Methods.'' Results from two independent clones per group from two experiments were pooled, averaged, and expressed as the mean ± SE. Asterisk indicates a significant increase in percent cell dispersion in response to fibronectin (*$P < 0.0005$) or HGF (**$P < 0.0005$), calculated using a two-tailed Student's *t*-test.

HGF-mediated functions and is part of a complex comprising c-Met and c-Yes, prompted us to examine a possible co-operative role of c-Src with ezrin in the regulation of aggregation and scattering of malignant cells. In this report, we show that combined increased activation of c-Src and over-production of ezrin caused disruption of cell–cell contacts and strongly reduced aggregation of carcinoma cells.

As a model, we used a mammary carcinoma cell line, SP1, which expresses both HGF and tyrosine phosphorylated Met under adherent

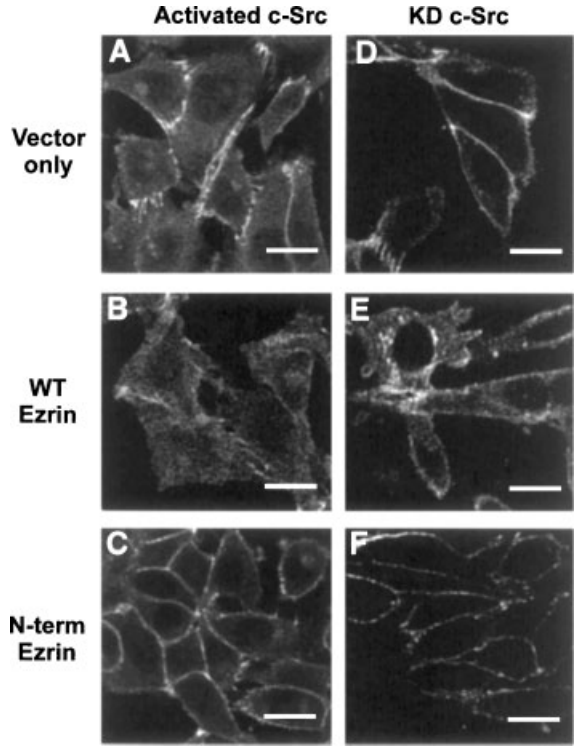

Fig. 6. Effect of c-Src and ezrin mutants on cadherin-based cell-cell contacts in carcinoma cells: SP1 cells expressing activated c-Src (**panels A–C**), or kinase dead c-Src (**panels D–F**) were co-transfected with wild type ezrin (panels B and E), or N-term ezrin (panels C and F). Each cell type was grown on glass cover slips in monolayer culture, and stained using indirect immunofluorescence with anti-pan cadherin antibody, as described in ''Materials and Methods.'' Representative images were digitized using a Leitz confocal microscope as described in ''Materials and Methods.'' Scale bar indicates 20 μm.

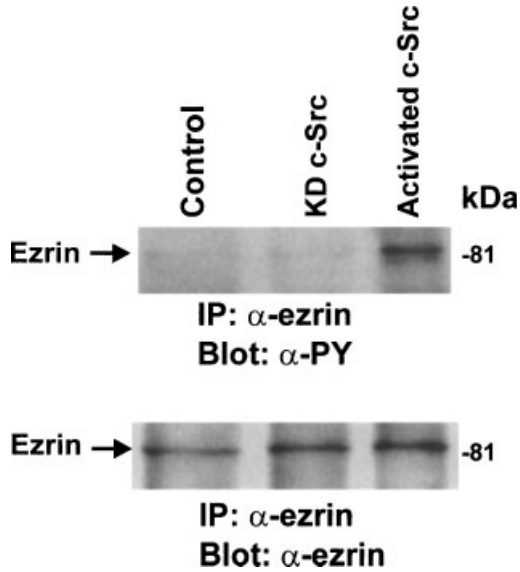

Fig. 7. Effect of c-Src mutants on tyrosine phosphorylation of endogenous ezrin in carcinoma cells: Untransfected SP1 cells (control), or SP1 cells expressing kinase dead c-Src or activated c-Src mutants were prestarved overnight in serum-free medium and lysed in 1% NP-40 lysis buffer as described in ''Materials and Methods.'' Equal protein amounts from each cell lysate were precleared with PAS beads for 4 h, and incubated with rabbit anti-ezrin antibody and additional PAS beads overnight at 4°C. Immunoprecipitates were washed four times, subjected to reduced 10% SDS–PAGE, and analyzed by Western blotting with anti-phospho-tyrosine (PY20) antibody (**upper blot**) or anti-ezrin antibody (**lower blot**).

conditions [Yang and Park, 1995]. Using activated and inactive c-Src mutants we previously demonstrated that c-Src activity is required for HGF-induced cell scattering and migration in SP1 cells [Rahimi et al., 1998; Elliott et al., 2002]. In this report, we show that over-production of wild type ezrin or activated c-Src alone in SP1 cells permitted formation of loose aggregates, which scattered spontaneously when plated on plastic. Over-production of ezrin thus shows an effect similar to c-Src in regulating cell scattering in carcinoma cells.

Remarkably, co-expression of activated c-Src and wild type ezrin in carcinoma cells caused complete disassembly of cell–cell contacts and disruption of cell aggregates, indicating a co-operative effect between c-Src and ezrin in cell scattering. This effect was reversed by pre-treating the cells with the c-Src family kinase inhibitor, PP2. Similarly, over-expression of N-term ezrin, which suppresses endogenous ezrin function [Crepaldi et al., 1997], increased cell aggregation and blocked spontaneous scattering of cells expressing activated c-Src. Thus, c-Src acts co-operatively with ezrin, and the activities of both molecules are required in deregulation of cell–cell contacts and scattering of malignant cells.

In contrast to wild type ezrin, expression of N-term ezrin reversed the scattering phenotype of activated c-Src in carcinoma cells cultured alone (Fig. 3), or with HGF or fibronectin (not shown). These findings indicate that ezrin is a key signalling molecule downstream of c-Src in the scattering response, and that blocking ezrin function may have a powerful tumor suppressor effect. How the inhibitory effect of N-term ezrin occurs is not known. Possible mechanisms include preventing the interaction of endogenous ezrin with membrane proteins or disruption

of F-actin binding function of endogenous ezrin [Crepaldi et al., 1997].

Interestingly, over-expression of wild type ezrin reduced cell aggregation and restored the HGF-, and fibronectin-, induced scattering phenotype in cells expressing kinase dead c-Src, although only marginal spreading in medium alone was observed. This finding suggests that ezrin over-expression allows partial recovery of cell scattering in cells expressing kinase dead c-Src and after growth factor stimulation or integrin engagement. Thus high levels of ezrin expression could lead to disruption of cell–cell contacts and scattering by a c-Src-independent mechanism. Pujuguet et al. [2003] have shown decreased trafficking of E-cadherin to the plasma membrane and extensive lamellipodia formation in cells producing a constitutively active form of ezrin (ezrin T567D), and that these effects were mediated through activation of Rac1. It is therefore possible that both c-Src-dependent and c-Src-independent ezrin pathways play a role in regulating cell–cell contacts and cell scattering.

Our observation, that activated c-Src causes increased tyrosine-phosphorylation of endogenous ezrin in carcinoma cells, suggests that ezrin function may be regulated in part through its tyrosine phosphorylation by c-Src. Phosphorylation of specific tyrosine residues on ezrin are known to regulate specific functions, such as PI3K/AKT-dependent survival which requires pY353 [Gautreau et al., 1999]. Recently, association of ezrin with the c-Src family member $p62^{yes}$ in epithelial cells stimulated with HGF [Crepaldi et al., 1997], and phosphorylation of the Y145 residue of ezrin by $p60^{lyk}$ in T cells [Autero et al., 2003] have been demonstrated. We also observed that activated c-Src and ezrin are co-localized at cell–cell contacts (data not shown) in SP1 carcinoma cells, indicating that these two molecules are in the same cellular compartment. However, co-immunoprecipitation of c-Src and ezrin from whole cell lysates was not readily demonstrated. Nevertheless, transient or low affinity interaction between c-Src and ezrin could occur directly or through a third party molecule such as FAK, which is known to associate through protein–protein interactions with both c-Src [Parsons et al., 2000] and ezrin [Poullet et al., 2001].

In this study, we have shown for the first time that c-Src acts co-operatively with ezrin in deregulation of cell–cell contacts and enhancing scattering of carcinoma cells. Using reciprocal combinations of active and inactive mutants, we further showed that ezrin is downstream of c-Src in these functions, and that tyrosine phosphorylation of ezrin by c-Src may be an important regulatory step in this process. We propose that signaling from integrins and the HGF receptor, Met, is channeled through c-Src and ezrin to the cytoskeleton, thereby regulating the cell scattering phenotype. Sustained high-level activation of the c-Src and ezrin pathways, as may occur in some malignancies, may be an important step in EMT and tumor progression.

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