The Nonreceptor Tyrosine Kinase Fer Mediates Cross-talk between N-Cadherin and β1-Integrins

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Abstract. Cadherins and integrins must function in a coordinated manner to effectively mediate the cellular interactions essential for development. We hypothesized that exchange of proteins associated with their cytoplasmic domains may play a role in coordinating function. To test this idea, we used Trojan peptides to introduce into cells and tissues peptide sequences designed to compete for the interaction of specific effectors with the cytoplasmic domain of N-cadherin, and assayed their effect on cadherin- and integrin-mediated adhesion and neurite outgrowth. We show that a peptide mimicking the juxtamembrane (JMP) region of the cytoplasmic domain of N-cadherin results in inhibition of N-cadherin and β1-integrin function. The effect of JMP on β1-integrin function depends on the expression of N-cadherin and is independent of transcription or translation. Treatment of cells with JMP results in the release of the nonreceptor tyrosine kinase Fer from the cadherin complex and its accumulation in the integrin complex. A peptide that mimics the first coiled-coil domain of Fer prevents Fer accumulation in the integrin complex and reverses the inhibitory effect of JMP. These findings suggest a new mechanism through which N-cadherin and β1-integrins are coordinately regulated: loss of an effector from the cytoplasmic domain of N-cadherin and gain of that effector by the β1-integrin complex.

Key words: cadherin • integrin • Trojan peptides • adhesion • neurite outgrowth

Introduction

The temporal and spatial regulation of cell–cell and cell-substrate adhesion molecules is thought to direct tissue formation, axon guidance, cell migration, and tumor cell metastasis. Type I cadherins and the β1-integrins play a decisive role in all of these processes (Varner and Cheresh, 1996; Beauvais-Jouneau, 1997; Semb and Christofori, 1998; Vleminckx and Kemler, 1999). Type I cadherins function as cis-homodimers (Brieher et al., 1996; Yap et al., 1997a, 1998; Takeda et al., 1999) to mediate homophilic cell–cell adhesion, forming adherens junctions (Marrs and Nelson, 1996; Yap et al., 1997b). Strong adhesion formation by cadherins depends on their association with the actin-containing cytoskeleton, a connection mediated by α- and β- or γ-catenin (Aberle et al., 1996; Wheelock et al., 1996). Other potential regulatory molecules are associated either directly or indirectly with the cytoplasmic domain: p120ctn (Reynolds et al., 1994; Ohkubo and Ozawa, 1999; Thoreson et al., 2000); the nonreceptor protein tyrosine kinase Fer (Kim and Wong, 1995; Rosato et al., 1998); Shc (Xu et al., 1997; Xu and Carpenter, 1999); and the nonreceptor tyrosine phosphatase PTP1B (Balsamo et al., 1996, 1998). Integrins function as heterodimers, mediating adhesion between cells and the extracellular matrix (Hynes, 1992; Cheresh, 1993). Integrin function also depends on association with the actin-containing cytoskeleton, forming well defined focal adhesion complexes at points where actin fibers are anchored (Critchley et al., 1999). The activity of integrins and their association with actin is mediated and regulated by an extensive set of structural and regulatory proteins associated directly or indirectly with the cytoplasmic domain of the β1 subunit (Aplin et al., 1998; Hemler, 1998; Schoenwaelder and Burridge, 1999).

It is apparent that the activity of these two receptor systems, and possibly many others, must be coordinated in time and space for the proper development of tissue architecture, and many of the regulatory proteins associated with the cytoplasmic domain of these two adhesion receptors are candidate cross-regulatory molecules. Although there is a growing body of literature documenting coordinate regulation between cadherin- and integrin-mediated adhesion systems, little is known about the levels or
mechanisms through which such coordination takes place (Hodivala and Watt, 1994; Finnemann et al., 1995; M onier-Gavelle and D uband, 1995, 1997; Z u et al., 1996; H uttenlocher et al., 1998; L u et al., 1998; W u et al., 1998).

We speculated that coordinate regulation of cadherin and integrin function during development might be affected by the exchange of regulatory molecules associated with the cytoplasmic domain of these receptors. Thus, we sought to create reagents that have the potential to interfere specifically with the binding of such effectors to the cytoplasmic domain of cadherin. Two regions of the cytoplasmic domain of cadherin have been shown to be functionally important: a juxtamembrane region that acts as a dominant negative, blocking cadherin function when introduced into X enopus blastomeres (K intner, 1992) or eye primordia (R iehl et al., 1996); and the β-catenin-binding region, which has been defined by cadherin deletions that result in the loss of β-catenin binding (N agafuchi and T akeichi, 1989; S tappert and K emler, 1994; J ou et al., 1995). Here, we show that peptides mimicking these two regions of N-cadherin attached to a cell permeation sequence enter cells and disrupt adhesion and neurite outgrowth. The peptide mimicking the juxtamembrane region (JMP) causes inhibition of both cadherin and integrin function, whereas the peptide mimicking the β-catenin-binding region causes inhibition of only cadherin function. We further show that inhibition of integrin function by JMP requires the presence of cadherin and the complex of proteins associated with its cytoplasmic domain. Finally, inhibition of integrin function is accompanied by a loss of the nonreceptor protein tyrosine kinase Fer from the cadherin complex of proteins and its concomitant appearance in the integrin complex of proteins, and blocking the association of Fer with the integrin complex reverses the effect of JMP on integrin function.

Materials and Methods

Antibodies

The anti–N-cadherin antibody N C D-2 (H atta and T akeichi, 1986) was purified from hybridoma cultures as described previously (B alsamo et al., 1991). The anti–β1-integrin antibodies are J G 22, a mouse monoclonal IgG (G reve and G ottlieb, 1982; T omaSELLI et al., 1986), and a commercial anti–β1-integrin antibody (T ransduction L aboratories). The anti–F A K antibody was a monoclonal mouse IgG from C hemicon International, I nc. A nti-Fer antisemur, a rabbit polyclonal antibody, was a gift from D r. N ora H eisterkamp (U niVersity of S outhern C alifornia, L os A ngeles, C A.). A nti-p120ctn, an anti–β-catenin, anti–P T P 38, anti–p320r ( h), and anti-phosphotyrosine ( P Y 20) were products of T ransduction L aboratories. A t nactin was from B oehringer-M annheim. H RP-conjugated secondary antibodies were from C appell L aboratories (I CN B iomedicals). The secondary antibodies conjugated to magnetic beads, used in immunoprecipitations, were from P erSeptive D iagnostics.

Other Materials

N-Caderhin, which was used as a substrate for neurite outgrowth, was purified from embryonic chick brains as described previously (B ixby and Z hang, 1990; B alsamo et al., 1991). The laminin and fibronectin, which were used as substrates for neurite outgrowth or adhesion, were from G IBCO-B RL. For preparation of recombinant N-cadherin cytoplasmic domain and recombinant β-catenin, the cDNA sequence for the cytoplasmic domain of N-cadherin or the full-length cDNA for β-catenin were cloned into pGEX, expressed as glutathione-S-transferase fusion proteins, and purified on glutathione-4S-Sepharose columns (P harmacia B iotech).

Preparation of Synthetic Peptides

The following peptides from the N-cadherin cytoplasmic domain and the nonreceptor tyrosine kinase Fer were synthesized as fusions with the antenapedia homeodomain cell permeation sequence (D erosso et al., 1994; P rochiantz, 1996) and purified to >90% by H PLC ( G enemed B iotechnologies, I nc.; see F ig. 1): control antennapedia peptide (C OP ), R QIK W F Q- N R M K W K K K ; c adherin binding peptide ( C B P ), R QIK W F Q N R M K W K K S L L V F D Y E G S G S T A G S S L L ; j uxtamembrane peptide ( J M P ), R QIK W F Q N R M K W K K R Q A K O L L D P E D V R N L K ; Shc bind- ing peptide region ( S B P ), R QIK W F Q N R M K W K K R L R D P I H A E P O Y P V R S A ; F er coiled-coil domain peptide ( F CC ), R QIK W F Q N R M K W K K E K Y E K A L A K G K E T E K A ; and F er NH2-terminal peptide ( F NT ), R QIK W F Q N R M K W K K S H E A V L K L Q D W L R. A ll peptides were dissolved in sterile deionized water to a concentration of 10–30 mg/ml and stored in small aliquots at –80°C. Peptide activity was assessed in neurite outgrowth and adhesion assays din a range of 0.2–50 μM. Biotinylation of peptides was carried out using Sulfo-NHS-biotin according to the manufacturer’s instructions (Pierce C hemical C o.).

Preparation of Retina Explants and Cell Culture

Neural retina explants were prepared from 7-d chick embryos (E 7). Intact retinas were whole mounted onto black filters (M illipore) with the vitreous surface facing up, and cut with a tissue sectioner to squares of ~0.09 mm2. Retina sections were resuspended in culture medium (F 12 supplemented with 5 mM Hepes, pH 7.2, 0.6% glucose, 5 μg/ml insulin, 100 μg/ml transferrin, 5 ng/ml sodium selenite, and 5 μg/ml gentamycin) and plated on coverslips coated with the appropriate substrate. 2 h later, non-adherent sections were removed by washing. Single cells were prepared from chick retina as described previously (B alsamo et al., 1995). P C12 cells were cultured in D M E supplemented with 5% F BS, 0.3% horse serum and antibiotics, and 100 μg/ml penicillin and streptomycin. L cells expressing N-cadherin ( L N cells) were prepared by transfecting the full-length N-cadherin cDNA into L cells as previously described (B alsamo et al., 1998).

Neurite Outgrowth and Cell Adhesion Assays

Neural retina explants were cultured overnight in the presence of 2 μM peptides. Neurite outgrowth was visualized using phase optics. Neurite length was quantitatively assessed in sparse single cell cultures of E 7 neural retina to prevent neurite fasciculation. Peptides were added 2 h after cell plating. A fiber overnight culture, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. The longest neurite per cell was measured using a Z eiss microscope equipped with a C CD camera at- tached to the M etamorph image analysis system (U niversal I maging C orp.). F or each assay condition, the length of neurites from >100 cells bearing neurites longer than two cell diameters were categorized into three groups: 0-49, 50-99, and >100 μM.

Cell adhesion assays were carried out as described (B alsamo et al., 1996, 1998; A rregui el al., 1998). A fter a starvation period of 2 h for P C12 cells or overnight for L and L N cells, cells were resuspended by a brief treatment with 0.03% trypsin in H BS containing 1 mM CaCl23+. T rypsin treatment was terminated by adding the trypsin inhibitor E SBF at 1 mM (C albiochem). C ess in suspension were incubated for 2 h (retina cells, P C12 cells) or 6 h (L and L N cells) with peptides in D M E+H epes before plat- ing onto the indicated substrates. F or assays evaluating the effect of sequential addition of peptides, cells were incubated for 1 h with the first peptide (2 μM ) before addition of the second (2 μM ). C ell adhesion was evaluated after 45 min.

Substrates for neurite outgrowth and adhesion were prepared by coating glass coverslips (neurite outgrowth) or 96-well plates (adhesion) with poly-L-lysine for 2 h at room temperature, followed by the appropriate substrate (cadherin, 5 μg/ml; N C D-2, 50 μg/ml; laminin, 20 μg/ml; fi- bronectin, 20 μg/ml).

Peptide Penetration and Assays for Cytotoxicity

To assess peptide penetration into cells and tissue explants, biotinylated peptides were incubated with explants for 2, 4, and 16 h at a concentration...
of 2 μM. The explants were fixed, permeabilized with 0.5% Triton X-100 (10 min), and reacted with FITC-streptavidin. Fluorescent cells in different layers of the explant were examined using a confocal microscope.

To determine cytotoxicity, retina explants were incubated overnight in the presence of peptides (0.2, 2, 4, 10, and 50 μM). The medium was replaced by Hepes-buffered saline with added glucose and potassium (HBSG K: 20 mM HEPES, pH 7.2, 150 mM NaCl, 2 mM glucose, and 3 mM KCl) containing 1:50 dilution of the green fluorescent SYTO 10 and the red fluorescent DEAD Red (Molecular Probes). A filter 15 min, explants were washed twice, fixed with paraformaldehyde, and observed using a fluorescence microscope. The fluorescence of SYTO 10 and DEAD Red was analyzed using FITC and rhodamine filters, respectively.

**Binding of Peptides to Recombinant Cadherin and β-Catenin**

The antennapedia fusion peptides were biotinylated using sulfo-NHS-biotin (Pierce Chemical Co.). 4 μg of each peptide, or inactivated biotinyl reaction mix alone, were incubated overnight with 10 μg of recombinant cadherin cytoplasmic domain or β-catenin in 200 μl PBS, at 4°C. The reaction mixture was loaded into streptavidin-coated wells blocked with BSA. Protein bound to biotinylated peptide was detected using an antibody to the cytoplasmic domain of cadherin or a monoclonal anti–β-catenin antibody and the appropriate HRP-conjugated secondary antibody. Triplicate wells were measured for each peptide.

**In Vitro Competition by CBP for Cadherin–β-Catenin Interaction**

Neural retina homogenates were prepared in mild lysis buffer (MLB: 20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM NaVO₄, 50 mM NaF, 1 mM A EBSF, 10 μg/ml DNase, and 5 μg/ml each leupeptin, pepstatin, and aprotinin). The lysates were cleared by centrifugation at 14,000 g, and aliquots of the supernatant were incubated with increasing concentrations of JMP, CBP, or SBP for 2 h at 37°C with mixing. The lysates were immunoprecipitated with the anticadherin antibody NCD-2, the immunoprecipitates collected using goat anti–rat IgG conjugated to magnetic beads, and the supernatants were further immunoprecipitated with polyclonal anti–β-catenin antibody (Balsamo et al., 1995, 1998). A control, to analyze protein associated with β-catenin to cadherin was determined.

**Immunoprecipitation and Immunoblot Analysis**

For analysis of the proteins associated with N-cadherin, cells were homogenized in MLB; the lysates were centrifuged at 14,000 g and the supernatants immunoprecipitated for 4 h at 4°C with the anti–N-cadherin antibody NCD-2. The NCD-2 immunoprecipitates were collected using goat anti-rat IgG conjugated to magnetic beads, and the supernatants were further immunoprecipitated with polyclonal anti–β-catenin antibody (Balsamo et al., 1995, 1998). A control, to analyze protein associated with β1-integrin, cell lysates were immunoprecipitated with anti–FAK antibody. The immunoprecipitates were fractionated by SDS-PAGE and the Western transfers were probed with the indicated antibodies.

For analysis of tyrosine phosphorylation of p130cas, cells were lysed in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 50 mM NaF, 1 mM PM SF, 5 μg/ml leupeptin, and 1 mM NaVO₄) immunoprecipitated with anti-p130cas antibody, fractionated by SDS-PAGE, and Western transfers were probed with PY 20.

**Results**

**Peptides Containing a Cell Permeation Sequence Fused to Sequences Mimicking Specific Regions of the N-Cadherin Cytoplasmic Domain Enter Cells and Are Nontoxic**

To introduce peptide competitors into living cells, amino acid sequences mimicking several domains of the cytoplasmic region of N-cadherin were fused to the 16-amino acid, cell permeable peptide derived from the third helix of the antennapedia homeodomain (D’erosi et al., 1994; Prochiantz, 1996; Hall et al., 1996; see Fig. 1 for diagram and M tetra and M ethods for peptide sequences). These sequences correspond to 20 amino acids in the juxtapositional region of N-cadherin (JMP) shown to act as a dominant negative when introduced into X enusop blastomeres (Kintner, 1992); 20 amino acids in the catenin binding region (CBP) (Nagafuchi and Takeichi, 1989; Stappert and Kemler, 1994; Jou et al., 1995) and 20 amino acids in the central region close to the binding region for the adaptor protein Shc (SBP) (Xu et al., 1997; Xu and Carpenter, 1999).

To establish that the antennapedia fusion peptides are indeed able to penetrate cells, we incubated E7 chick neural retina explants of ~1 mm² with each of the fusion peptides covalently linked to biotin. The explants were incubated with fluorescein-conjugated streptavidin and peptide penetration was assessed in confocal, planar images through the explant as well as cross-sectional reconstructions. All peptides penetrate equally well (Fig. 2 shows penetration of CBP as an example). In addition, the antennapedia fusion peptides are not toxic to cells, as determined by assessing cell death using a SYTO/10 DEAD Red (Fig. 2, live cells are labeled green and dead cells are red). Very few cells are dead after 24 h of incubation of the explant either in the absence or presence of antennapedia peptides, and those that do appear dead are at the edge of the explant and may have been damaged during dissection and initial placement of the explant.

**JMP and CBP Have Unique and Specific Effects on N-Cadherin and Integrin-mediated Neurite Outgrowth and Adhesion**

To determine the effect of the cell permeable peptides on N-cadherin– or integrin-mediated neurite outgrowth, explants from 7-d embryonic chick retinas were plated on N-cadherin (or anti-N-cadherin antibody NCD-2) or laminin in the presence of JMP, CBP, SBP, antennapedia peptide alone (COP), or no peptides (NOP). Treatment with either JMP or CBP results in a dramatic reduction of neurite outgrowth on cadherin substrates (Fig. 3). However, when explants are plated on laminin, where neurite extension depends on β1-integrin function and is inhibited by the function blocking anti–β1-integrin antibody JG 22 (Greve and Gottlieb, 1982; Tomase et al., 1986; not shown), numerous long neurites develop in the presence of CBP.
but only a few short neurites develop in the presence of JMP (Fig. 3). In contrast, neither SBP (not shown) nor COP (Fig. 3) have any effect on either N-cadherin or laminin substrates. Thus, while JMP was designed to mimic the cadherin juxtamembrane sequence, it nevertheless influences integrin-mediated neurite outgrowth.

To quantify the results, we cultured E7 retina cells on N-cadherin or laminin-coated slides and determined the length of neurites after 20 h in the presence of JMP, CBP, and COP, or in the absence of peptide (NOP). When cells are plated on N-cadherin, in the absence of peptide or in the presence of control peptides, \( \approx \)80% of neurites are between 50 and 99 \( \mu \)m in length (Fig. 4). In contrast, in the presence of JMP or CBP, only \( \approx \)20% of the neurites fall in this category (Fig. 4). When cells are plated on laminin, CBP has no discernible effect; however, treatment with JMP reduces the number of neurites in the \( >100 \mu \)m class to \( \approx \)10% (Fig. 4).

Consistent with the effect of JMP and CBP on neurite outgrowth from explants or single cells, N-cadherin-mediated adhesion of E7 retina cells is inhibited by JMP and CBP, whereas only JMP inhibits integrin-mediated adhesion (Fig. 5). That adhesion is indeed mediated by N-cadherin or \( \beta_1 \)-integrin is demonstrated by the ability of the function blocking antibodies NCD-2 (Hatta and Takeichi, 1986) and JG 22 (Greve and Gottlieb, 1982; Tomaselli et al., 1986) to specifically inhibit adhesion to N-cadherin or laminin, respectively (Fig. 5). JMP also inhibits integrin-mediated adhesion among PC12 cells, which differ from retina cells in expressing distinct \( \alpha \) chains, in combination with \( \beta_1 \)-integrin, as receptors for laminin (see Fig. 8 D), indicating that the effect of JMP is specific for \( \beta_1 \)-integrin, independent of the attached \( \alpha \) chain (Tomaselli et al., 1990; de Curtis and Reichardt, 1993). A ditionally, treatment of cells with cycloheximide or actinomycin D, to specifically inhibit protein or RNA synthesis, respectively, has no effect on the inhibition of cadherin or \( \beta_1 \)-integrin function by JMP (not shown). A nd finally, adhesion to poly-L-lysine, which does not involve cadherins or integrins, is unaffected by CBP or JMP (Fig. 5).

To determine the effect of COP, CBP, and JMP on cell-surface expression, N-cadherin and \( \beta_1 \)-integrin were immunoprecipitated from cells on which cell-surface proteins were biotinylated. A ditionally, cells were incubated with anti-\( \beta_1 \)-integrin antibody, followed by fluorescein-conjugated second antibody, and the amount of cell-bound fluo-
and SBP have little effect, even at concentrations as high as 12.5 μM (Fig. 6 B). On the other hand, JMP binds in vitro to the cytoplasmic domain of N-cadherin (Fig. 6 A), and biotin-labeled JMP localizes to areas of cell–cell contact, where cadherin is enriched (Fig. 6 C). This suggests that the juxtamembrane region of cadherin may participate in homodimer formation through a direct interaction, which is consistent with reports suggesting that the juxtamembrane domain of cadherin is indeed involved in cis-homodimerization (Ozawa and Kemler, 1998; Yap et al., 1998).

JMP and CBP Are Targeted to Specific Components of the Cadherin Adhesion Complex

CBP and JMP, but not control peptides, have specific effects on cadherin and integrin function, presumably based on their ability to compete for specific protein–protein interactions at the cytoplasmic domain of cadherin. To further demonstrate the specificity of these effects, we examined the interaction of the peptides with their putative targets. The CBP sequence corresponds to the binding site for β-catenin and, indeed, CBP binds directly to recombinant β-catenin (Fig. 6 A). In addition, CBP competes for up to 50% of the N-cadherin–bound β-catenin in cell lysates, as determined by immunoprecipitation with NCD-2 and immunodetection of β-catenin (Fig. 6 B). In contrast, JMP and SBP have little effect, even at concentrations as high as 12.5 μM (Fig. 6 B). On the other hand, JMP binds in vitro to the cytoplasmic domain of N-cadherin (Fig. 6 A), and biotin-labeled JMP localizes to areas of cell–cell contact, where cadherin is enriched (Fig. 6 C). This suggests that the juxtamembrane region of cadherin may participate in homodimer formation through a direct interaction, which is consistent with reports suggesting that the juxtamembrane domain of cadherin is indeed involved in cis-homodimerization (Ozawa and Kemler, 1998; Yap et al., 1998).

Treatment of Cells with JMP or CBP Alters the Array of Proteins Associated with the Cytoplasmic Domain of N-Cadherin

The targeting of CBP to β-catenin and its ability to compete for binding of β-catenin to cadherin in vitro, and the targeting of JMP to cadherin itself suggest that treatment of cells and tissues with these peptides disrupts interactions crucial to cadherin function. To examine the in situ effects of these fusion peptides, cells were pretreated with JMP, CBP, or a control peptide, and the array of polypeptides coimmunoprecipitated with anti-N-cadherin antibody was examined. Preincubation of cells with either CBP or JMP, but not control peptide, reduces the amount of actin immunoprecipitated with anti-N-cadherin antibody, suggesting that both peptides uncouple N-cadherin from its association with the actin-containing cytoskeleton (Fig. 7 A). The association of actin with the cytoplasmic domain of cadherin is mediated by β-catenin interacting with α-catenin, which in turn interacts with actin, either directly or through α-actinin (Wheelock et al., 1996). Binding of JMP does not alter the amount of β-catenin associated with N-cadherin (Fig. 7 A). On the other hand, CBP reduces the amount of β-catenin associated with cadherin (Fig. 7 A), as expected from results shown in Fig. 6. Thus, CBP uncouples cadherin from the cytoskeleton with a concomitant loss of function, probably through competition with the endogenous β-catenin binding site.

We also examined the distribution of p120ctn (Reynolds et al., 1994) and the nonreceptor tyrosine phosphatase PTP1B (Balsamo et al., 1996, 1998), which are bound directly to the cytoplasmic domain of cadherin, and the protein tyrosine kinase Fer, which is associated with p120ctn (Kim and Wong, 1995) and possibly β-catenin (Rosato et al., 1998). Treatment with CBP reduces the amount of all three components precipitated with cadherin (Fig. 7 A). Additionally, after treatment with CBP, p120ctn and Fer are coimmunoprecipitated with β-catenin from the N-cadherin–free β-catenin pool (Fig. 7 B), indicating that β-catenin, Fer, and p120ctn are released as a complex. In contrast, binding of JMP directly to cadherin results in a reduction in the amount of Fer, leaving p120ctn and PTP1B largely unaffected (Fig. 7 A). Thus, JMP and CBP act through different mechanisms to differentially alter the association of effector proteins with the cytoplasmic domain of cadherin. The differences are noteworthy: competition for β-catenin results in the release of a complex of associated proteins including β-catenin, p120ctn, and Fer, suggesting that this set of bound components depends on β-catenin for stable association, in spite of the fact that at least one, p120ctn, binds directly to cadherin (Roura et al., 1999; Thoreson et al., 2000). In contrast, direct binding of JMP to cadherin

Figure 4. Quantitative determination of neurite outgrowth by single E7 cells in the presence and absence of peptides. Data are expressed as the percent of neurites in each of three length categories. More than 100 cells bearing neurites were counted for each treatment. NOP, no peptide.

Figure 5. The effect of peptides on cadherin- and integrin-mediated cell adhesion by E7 retina cells. Cells preincubated in the absence or presence of peptides were plated on the indicated substrates, the wells were washed, and adherent cells were counted. Values for each condition represent the percentage of attached cells as compared with controls lacking peptide. Each bar is the average of three measurements, and the error bars represent the SD. The substrate is indicated in the top right of each panel: NCD-2, anti–N-cadherin antibody; LM, laminin; and PL, poly-L-lysine.
specifically causes the release of Fer, other components remaining associated with cadherin. As Fer is not known to associate directly with cadherin, the binding of JMP appears to alter the association of Fer through a change in its binding partner(s), p120<sub>ctn</sub> and/or β-catenin. Thus, interactions among the components associated with cadherin are complex and mutually interdependent.

The Effect of JMP on Integran Function Requires N-Cadherin and the Complex of Proteins Associated with the Cadherin Cytoplasmic Domain

A database search reveals no close similarity between the amino acid sequence of JMP and that of β1-integrin or any other integrin-associated peptide, suggesting that the effect of JMP on integrin function may occur through cadherin. To determine if this is indeed the case, we compared the effects of JMP on L cells, which express β1-integrins but not N-cadherin, and L-cells stably transfected with N-cadherin (Balsamo et al., 1998), which express both β1-integrins and N-cadherin. In this case, we used fibronectin as a substrate, since L cells adhere poorly to laminin. Both L cells and LN cells adhere equally well to fibronectin (see A585 values in Fig. 8, A and B) and this adhesion is integrin-dependent, as >90% of attachment is inhibited by an arginine, glycine, aspartic acid–containing peptide (Arregui et al., 1998). However, similar concentrations of JMP inhibit LN cell adhesion to fibronectin but have little or no effect on L cell adhesion (Fig. 8, A and B). CBP and control peptides have little or no effect on integrin-mediated adhesion of L-cells or LN-cells (Fig. 8, A and B) but, as expected, CBP does inhibit cadherin-mediated adhesion of LN cells (Fig. 8 B). Thus, inhibition of integrin function by JMP requires the expression of cadherin. Furthermore, perturbation of the array of proteins associated with the cytoplasmic domain of cadherin by prior treatment with CBP, which causes the release of a complex of proteins containing β-catenin, p120<sub>ctn</sub>, and Fer, eliminates the effect of JMP on integrin-mediated adhesion. This is true for all three cell types tested: LN (not shown), E7 retina (Fig. 8 C), and PC12 (Fig. 8 D). Thus, JMP acts through N-cadherin to affect integrin function, and specific alterations in the cadherin complex induced by CBP abolish the effect of JMP on integrin function.

Inhibition of β1-Integran Function by JMP Correlates with an Increase in Tyrosine Kinase Fer Associated with the Integrin Complex

Inhibition of integrin function depends on a specific configuration of effector molecules associated with the cytoplasmic domain of N-cadherin and is independent of protein and RNA synthesis. This suggests that one or more effectors, originally associated with the cytoplasmic do-

Figure 6. Interaction of peptides with presumptive targets. (A) In vitro binding of peptides to the N-cadherin cytoplasmic domain and to β-catenin. 10 μg of recombinant GST-N-cadherin cytoplasmic domain or GST-β-catenin were incubated with 4 μg of biotinylated CBP or JMP. The amount of protein bound to immobilized peptide was determined as described in Materials and Methods. Each point is the average of three measurements and bars represent the SD. (B) The ability of CBP to disrupt the association of cadherin with β-catenin. The ratio of β-catenin to cadherin was measured in neutral detergent extracts in the presence of increasing concentrations of CBP and JMP, and reported as a percentage of the value in the absence of peptide. The results shown are one experiment representative of many. (C) Localization of JMP to regions of cell–cell contact. The left frame labeled DIC (differential interference contrast microscopy) and the middle frame labeled JMP-bio are the same field. The frame on the right shows the distribution of biotin-labeled control peptide (COP-bio). Individual cells are ∼8 μm in diameter.
Coiled-coil Domain in Fer Reverses the Effect of JMP

A Peptide that Mimics a Sequence in the First Coiled-coil Domain in Fer Reverses the Effect of JMP on Integrin Function

The protein kinase Fer contains three presumptive coiled-coil domains in its NH₂-terminal region, two of which, CC1 and CC2 are involved in oligomerization, that have the potential to mediate targeting (Craig et al., 1999). To definitively implicate Fer in the inhibition of integrin function by JMP, we attempted to interfere with targeting of Fer to the integrin complex using a cell permeable peptide that mimics a coiled-coiled domain. We hypothesized that if Fer were prevented from targeting to the integrin complex, JMP should lose its inhibitory effect on integrin function. Two Fer peptides were prepared: one mimicking the 16-amino acid sequence (amino acids 152–168) in coiled-coil domain 1 (FCC) and the second mimicking a 16-amino acid sequence at the NH₂-terminus (FNT; Fig. 1).

Both of these peptides show little sequence similarity to other members of the c-Fes family (Smithgall et al., 1998). Neural retina cells were plated on laminin-coated slides and allowed to adhere for 1 h. FCC, FNT, or control peptide (COP) were added and the cells were incubated for an additional hour before addition of JMP. The cells were incubated overnight and neurite growth was assessed. Cells incubated in the presence of FCC, before the addition of JMP, are able to extend neurites as well as control cells or cells treated with control peptides, whereas few neurites are seen on cells treated with JMP alone or JMP after COP or FNT (Fig. 10).

To determine if targeting of Fer to the integrin complex is disrupted in the presence of FCC as we predicted, cells were incubated for 1 h with FCC, FNT, or COP, followed by another hour in the presence of JMP. The cells were lysed in mild detergent and immunoprecipitated with anti-Fer antibody. As expected, Fer coprecipitates with FA K after treatment with JMP alone and FNT or COP followed by JMP (Fig. 11 A). However, when cells are treated with FCC before JMP, Fer is not found in the anti-FAK precipitate (Fig. 11 A). Indeed, FCC prevents targeting of Fer to the integrin complex and, thus, prevents the inhibitory effect of JMP on integrin function. In contrast, FCC has no effect on the association of Fer with the cadherin complex (Fig. 11 B) or on cadherin function (not shown).

Discussion

We have used cell permeable peptides containing the cell permeation antennapedia sequence (Derossi et al., 1994;
Prochiantz, 1996), fused to peptides mimicking regions of the cytoplasmic domain of N-cadherin, to specifically perturb protein–protein interactions essential to cadherin and integrin function. These peptides rapidly enter cells and are nontoxic. One peptide, CBP, mimics the cadherin binding site for β-catenin, which is conserved among all Type I cadherins. CBP is targeted to β-catenin, as shown by direct binding and dissolution of β-catenin-cadherin complex both in situ and in vitro. In situ, this peptide causes the release of a complex of proteins containing β-catenin, p120 ctn, and FAK from the cytoplasmic domain of cadherin and loss of cadherin function. A second peptide, JMP, is based on the juxtamembrane region of cadherin, which is conserved in all N- and R-cadherins, and was shown to act as a dominant negative when introduced into Xenopus blastomeres (Kintner, 1992). It binds directly to the cadherin cytoplasmic domain and localizes to sites of cell–cell association. This peptide causes release of the nonreceptor tyrosine kinase Fer from the N-cadherin complex and loss of N-cadherin and β1-integrin function. The loss of integrin function is accompanied by accumulation of Fer in the integrin complex, reversing the effect of Fer on integrin-mediated adhesion. Thus, Fer appears to be a critical component of an epigenetic pathway through which cadherin regulates integrin function.

Figure 11. The effect of peptides mimicking specific regions of Fer on its association with the integrin and cadherin complex. (A) Peptide FCC reverses the effect of JMP on targeting of Fer to the integrin complex. The indicated peptide pairs were added to retina cells sequentially with a 1-h incubation after the addition of the first peptide, and a 1-h incubation after the second. The cells were homogenized in neutral detergent and immunoprecipitated with anti-FAK antibody. The immunoprecipitates were fractionated by SDS-PAGE, and Western transfers were blotted with anti-Fer and anti-FAK antibodies. (B) FCC does not affect the association of Fer with the cadherin complex. The indicated peptides were added to retina cells for 1 h, and all cells bearing neurites longer than two cell diameters were considered positive. Bar, 20 μm.

What Is the Role of Fer in the Cadherin Complex?

We have previously reported that the presence of the nonreceptor protein tyrosine phosphatase PTP1B in the cadherin complex is essential for dephosphorylation of β-catenin and, therefore, N-cadherin function, and that binding of PTP1B to cadherin requires phosphorylation on tyrosine residues (Balsamo et al., 1996, 1998). We have also reported that hyperphosphorylation of β-catenin is not only correlated with the loss of PTP1B from the cadherin complex, but also with reduced tyrosine kinase activity associated with the cadherin complex (Balsamo et al., 1995).
This loss of kinase activity from the cadherin complex may reflect the loss of Fer. Therefore, Fer may be required to phosphorylate PTP1B, targeting it to the cadherin complex. We are investigating this possibility. Additionally, overexpression of Fer has been shown to result in increased phosphorylation of p120<sup>ctn</sup> and β-catenin with concomitant dissolution of the cadherin–catenin complex, a state consistently correlated with loss of cadherin function (D'aniel and Reynolds, 1997; Lilien et al., 1997).

Fer may also play a role in cis-dimerization of cadherin through phosphorylation of p120<sup>ctn</sup>. cis-dimerization is required for strong adhesion (Brieher et al., 1996; Yap et al., 1997, 1998; Takeda et al., 1999) and the juxtamembrane region of cadherin has been implicated in regulation of cis-dimerization (O'zawa and Klemper, 1998) and in binding of p120<sup>ctn</sup> (Thoreson et al., 2000). Okubo and O'zawa (1999) have reported that the presence of p120<sup>ctn</sup> interferes with homodimerization, whereas Thoreson et al. (2000) report that p120<sup>ctn</sup> is essential for the formation of strong adhesions. In fact, this apparent paradox may be a result of changes in the affinity of p120<sup>ctn</sup> for cadherin because of different states of p120<sup>ctn</sup> phosphorylation (Thoreson et al., 2000), possibly mediated by Fer. Indeed, increased phosphorylation on tyrosine residues has been reported to increase the affinity of p120<sup>ctn</sup> for cadherin (Roura et al., 1999). The direct binding of JMP to the cytoplasmic domain of N-cadherin suggests that this region mediates dimerization, whereas the immediate COOH-terminal sequences, through interaction with p120<sup>ctn</sup>, perform a regulatory function that may be modulated by Fer. The effect of JUMP on N-cadherin-mediated neurite outgrowth and adhesion, thus, may be because of direct inhibition of dimerization, or indirectly a result of release of Fer and disruption of the phosphorylation of p120<sup>ctn</sup>. This suggests that Fer's role is in the formation and/or maintenance of cadherin dimers. In this same vein, Fer may be specifically recruited to cadherin dimers and any circumstances that inhibit dimerization might prevent binding of Fer, leaving it free to associate with integrin. However, this suggests a pool of free Fer, but this seems unlikely as integrin function would be continuously compromised. There is a pool of Fer associated with cortactin (Kim and Wong, 1998) and it seems likely that Fer is sequestered by cortactin, p120<sup>ctn</sup> or other targets preventing its interaction with the β1-integrin complex.

If Fer is in fact bound to p120<sup>ctn</sup> as Kim and Wong (1995, 1998) suggest, the ability of JUMP to specifically effect its release suggests that interactions among the components associated with the cytoplasmic domain of cadherin are complex and interdependent. This complexity is also revealed by the effect of CBP. CBP competes for β-catenin binding, resulting in the release of a complex of proteins that includes p120<sup>ctn</sup>, β-catenin, and Fer. Indeed, a recent report (Huber et al., 1999) indicates that the cytoplasmic tail of E-cadherin folds upon binding of β-catenin, and that folding of the cytoplasmic tail may facilitate the binding of p120<sup>ctn</sup>. The converse may also hold; i.e., competition for β-catenin may cause the unfolding of the cytoplasmic tail affecting the binding of all components, resulting in their release as a complex. While these suggestions are speculative, they do raise interesting questions concerning interactions among the cadherin effectors and cadherin.

How Does Fer Affect Integrin Function?

The loss of integrin function on interaction of Fer is consistent with the loss of cell–substrate adhesion on overexpression of Fer (Rosato et al., 1998). Our observations and those of Rosato et al. (1998), that Fer-mediated loss of integrin function is correlated with a reduction in phosphorylation of p130<sup>cas</sup> are consistent with reports that phosphorylation of p130<sup>cas</sup> is correlated with assembly into focal adhesions (Nogima et al., 1995; Petch et al., 1995; Voiu and Ruoslahti, 1995). p130<sup>cas</sup> is at the nexus of a subarray of effector molecules that regulate integrin function. It is an adaptor or docking protein with multiple interaction domains; an SH 3 domain, multiple proline-rich regions, and critical tyrosine residues (Sakai et al., 1994). Both the tyrosine phosphatases PTP-PEST (Garton et al., 1997) and PTP1B (Li et al., 1996), as well as the tyrosine kinase FA K (Polte and Hanks, 1995) interact with p130<sup>cas</sup> through its SH 3 domain, and it has been suggested that these components compete with each other to regulate the tyrosine phosphorylation of p130<sup>cas</sup> and, therefore, assembly of focal adhesions (Garton et al., 1997). Tyrosine phosphorylated p130<sup>cas</sup> also interacts with Crk through an SH 2 domain (Matsuda and Kurata, 1996). This interaction has two ramifications: it has the potential to protect p130<sup>cas</sup> from dephosphorylation (Birge et al., 1999) and appears to act through R ac to stimulate cell migration (Klemke et al., 1998). While the loss of focal adhesions correlates with the loss of tyrosine phosphorylation of p130<sup>cas</sup>, it is not clear how the presence of Fer in the integrin complex causes a reduction in p130<sup>cas</sup> phosphorylation. It is obvious that the effect of Fer is indirect; however, it is not clear through what partners Fer affects the dephosphorylation of p130<sup>cas</sup>. The simplest explanation is that phosphorylation of one of the p130<sup>cas</sup> binding partners, PTP-PEST, PTP1B, FA K, or even Crk, alters their activity or their interaction with p130<sup>cas</sup>, changing the balance of phosphorylated tyrosine residues.

The Interaction of Fer with the Cadherin and Integrin Complexes

Fer has the potential to interact with multiple target sites. It has three coiled-coil domains (Craig et al., 1999), which have the potential to mediate multiple protein–protein interactions (Lupas, 1996), and it has the potential to interact with SH 2 or PTP (phosphotyrosine-binding) domains through phosphorylated tyrosine residues (Craig et al., 1999). Fer is presumed to interact directly with p120<sup>ctn</sup> (Kim and Wong, 1995), and p120<sup>ctn</sup> has a predicted coiled-coil domain at its NH<sub>2</sub> terminus (Reynolds et al., 1994), which has been suggested to interact with a coiled-coil domain of Fer (Kim and Wong, 1995; Craig et al., 1999). We find that FCC has no effect on the association of Fer with the cadherin complex or on cadherin function; thus, if coiled-coil interactions do mediate the interaction, it is not through CC 1. CC1 does appear to be involved in targeting to the integrin complex. However, it is not clear whether FCC blocks targeting through an effect on oligomerization of Fer itself (Craig et al., 1999) or through blocking formation of heteromultimers. These results suggest that targeting of Fer to the two complexes is mediated by distinct domains.
Our results demonstrate for the first time that specific regulatory alterations in a cadherin complex of proteins affect integrin function through an epigenetic mechanism: translocation of regulatory proteins from one adhesion receptor to another, or reequilibration and redistribution of a pool of effectors. The protein tyrosine kinase Fer, and possibly other effectors, may thus coordinately regulate cadherin and integrin function. This provides a mechanism for rapid, specific, and coordinate regulation of cell–cell and cell–substrate adhesion during development.

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