Integrin $\alpha_2\beta_1$-dependent EGF receptor activation at cell-cell contact sites

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SUMMARY

Certain integrins including $\alpha_2\beta_1$ and $\alpha_3\beta_1$ localize to intercellular binding sites, and thus may participate in cell-cell interactions. We demonstrated here the physical and functional associations of integrin $\alpha_2\beta_1$ with epidermal growth factor receptor (EGFR) at intercellular adhesion sites. Immunoprecipitation with anti-integrin $\alpha_2$ antibodies or anti-integrin $\beta_1$ antibody resulted in preferential coprecipitation of EGFR from A431 cell lysates, while anti-EGFR antibody coprecipitated integrin $\alpha_2\beta_1$. Chemical crosslinking confirmed the association of integrin $\alpha_2\beta_1$ and EGFR. Colocalization of integrin $\alpha_2\beta_1$ and EGFR at cell-cell contact sites was observed by double immunofluorescence staining of A431 cells. EGF-induced EGFR stimulation did not affect the association of integrin $\alpha_2\beta_1$ and EGFR. However, immunostaining with the antibody specific to activated-EGFR revealed that EGFR localized at cell-cell contact sites are phosphorylated even in serum-depleted conditions, while EGFR localized to other sites is totally dephosphorylated in the same conditions. The EGFR phosphorylation in cell-cell contact sites observed in a serum-depleted culture was abrogated with a function-blocking antibody of integrin $\alpha_2$, but not with a non-function-blocking $\alpha_2$ antibody or function-blocking $\alpha_3$ antibody. Moreover, the EGFR phosphorylation in serum-depleted conditions was not observed in suspended cells, or largely abrogated in sparse cells, indicating that cell-cell adhesion is required for EGFR phosphorylation. These results indicate that integrin $\alpha_2\beta_1$ not only physically associates with EGFR but also functions in serum-independent EGFR activation at cell-cell contact sites. The present results shed a new light on the role of intercellular integrins in cell-cell interactions.

Key words: Integrin, EGF, A431 cell, Phosphorylation

INTRODUCTION

Integrins are cell surface adhesive receptors formed by $\alpha$ and $\beta$ subunits. Integrin-mediated cell adhesion triggers a variety of signal events including cytoplasmic alkalization, calcium influx, potassium channel activation, activation of MAP kinases cascade and tyrosine phosphorylation (Clark and Brugge, 1995; Schwartz et al., 1995), and consequently regulates cell migration, proliferation, differentiation and cell survival (Hynes, 1992; Schwartz et al., 1995). The major ligands for integrins are extracellular matrix (ECM) proteins, thus integrin-mediated signaling has been mainly studied by integrins interacting with ECM proteins on substratum.

Integrins, however, are not only expressed at the cell basement membrane but also expressed at the cell-cell contact sites. For example, immune system integrins such as Mac-1 and LFA-1 mediate cell-cell interactions by binding to counter molecules on adjacent cells (Diamond et al., 1990; Elices et al., 1990). In epithelial cells, some of the $\beta_1$ integrins localize at the cell-cell boundaries (Larjava et al., 1990) as well as at the basement membrane. Immunofluorescence studies and immunoelectron microscope observation showed the localization of integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ at cell-cell contact sites (Kaufmann et al., 1989; Carter et al., 1990; Symington et al., 1993; Jones et al., 1995), suggesting a role for these integrins in cell-cell interaction. Monoclonal antibodies blocking integrin $\alpha_3$ or $\beta_1$ inhibited keratinocyte aggregation (Carter et al., 1990), while other anti-integrin $\alpha_3$ antibodies may induce cell-cell adhesion (Weitzman et al., 1993). Integrin $\alpha_2\beta_1$ has been known to be a receptor for laminin and collagen, while integrin $\alpha_3\beta_1$ can bind laminin 5 and other ECM proteins. However, these ECM proteins have not been observed at cell-cell contact sites. By utilizing immobilized purified integrins, affinity chromatography, and blocking mAb, it was suggested that integrin $\alpha_2$, $\alpha_3$ heterophilic (Symington et al., 1993) or integrin $\alpha_3$ homophilic interaction may exist (Sriramarao et al., 1993). Although these studies implied that intercellular integrins may participate in cell-cell adhesion, transfection of integrin $\alpha_2$ or $\alpha_3$ into cells did not result in enhancement of these integrin-mediated intercellular adhesions (Weitzman et al., 1995). Hence, sufficient evidence to demonstrate the role of integrins in cell-cell interaction is still lacking.

Recent studies have revealed that integrins can form cis associations with other cell surface proteins on the same cell (Porter and Hogg, 1998; Hemler, 1998). The list of cell surface...
proteins associated with integrins includes integrin-associated protein (IAP; CD47), tetrameric-4 super family (TM4SF), GPI-linked receptors and EMMPRIN. We have previously shown that CD9, a member of the TM4SF, associates with integrin α3β1 and that these complexes localize at cell-cell contact sites (Nakamura et al., 1995). HB-EGF is an EGF family growth factor, which binds to and stimulates EGF receptor (EGFR; HER-1; Higashiyama et al., 1991). In addition to CD9, the membrane-anchored form of HB-EGF (proHB-EGF) also associates with the integrin α3β1/CD9 complex, suggesting a cooperative role among these proteins in cell-cell interaction (Nakamura et al., 1995; Higashiyama et al., 1995). Moreover, association of integrins with growth factor receptors is also suggested by co-clustering (Miyamoto et al., 1996; Sundberg and Rubin, 1996; Jones et al., 1997) and direct co-precipitation studies (Schneller et al., 1997; Falcioni et al., 1997). Not only physical association but also functional cooperativity between integrins and growth factor receptors are demonstrated (Mainiero et al., 1996; Cybulsky et al., 1994; Schneller et al., 1997; Moro et al., 1998; Wang et al., 1998).

Consistent with the localization of proHB-EGF complexes at cell-cell contact sites, EGFR is also localized at cell-cell boundaries (Fukuyama and Shimizu, 1991; Nakamura et al., 1995). Although the association of intercellular integrins with growth factor receptors at cell-cell boundaries has not been demonstrated, it is tempting to assume that EGFR also associates with integrins at cell-cell contact sites to form a functional complex for interaction with neighboring cells. In the present study we demonstrate the association of EGFR with integrin α2β1 in A431 cells and that this complex localizes at cell-cell contact sites. Results also indicate the functional cooperation between EGFR and integrin α2β1.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Human anti-EGFR mAb was purchased from Boehringer Mannheim Co., rabbit anti-EGFR Ab from Santa Cruz Biotechnology, Inc., mouse anti-EGFR mAb (clone LAI) from Upstate Biotechnology Inc., mouse anti-activated EGFR mAb (clone 74) from Transduction Lab and mouse anti-integrin β1 mAb (4B4) from Coulter Immunology. Mouse anti-integrin β3 mAb (2B11), β4 mAb (3E11), α1 mAb (FB12), α2 mAb (P1E6), α3 mAb (P1B5), rabbit anti-β1 and α2 Ab were purchased from Chemicon International Inc. Mouse anti-α2 mAb (12F1) and anti-α5 mAb (KH33) were from PharMingen International and Seikagaku Co., respectively. FITC-conjugated goat anti-rabbit IgG, Cy3-conjugated donkey anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were from Chemicon International Inc. HRP-conjugated goat anti-mouse IgG was from Zymed Laboratories, Inc., FITC-conjugated goat anti-mouse IgG2a from Southern Biotechnology associates, Inc. and goat IgG fraction to anti-mouse IgG Fc from ICM Pharmaceuticals, Inc. Cy3-conjugated Streptavidin and HRP-conjugated Streptavidin were from Pierce Chemical, Co. Anti-diphtheria toxin mAb (Hayakawa et al., 1983) was used as a control antibody.

**Cell culture**

A431 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS. For serum-depleted conditions, cells were cultured in DMEM containing 0.5% FCS. For cell-suspended conditions, cells were trypanosized with EDTA, and the detached cells were cultured for 4 hours with DMEM containing 10% FCS on bacterial culture dishes.

**Cell surface labeling and chemical cross-linking**

For cell surface biotinylation, cells were grown in 100 mm dishes and washed three times with PBS, then incubated with 0.25 mg/ml Sulfo-NHS-Biotin (Pierce Chemical Co.) in biotinylation buffer (0.15 M NaCl, 0.2 mM CaCl2, 0.2 mM MgCl2, 10 mM HEPES, pH 8.0) at 4°C for 30 minutes. Reaction was stopped by DMEM medium and washed three times with TBS buffer (0.1 M NaCl, 20 mM Tris-HCl, pH 7.5). For cross-linking assay, cells were washed three times with PBS, incubated with 1 mM diethyloxydisulfosuccinimidylpropionate (DTSSP; Pierce Chemical Co) in PBS at 4°C for 30 minutes, followed by washing three times with TBS and then used for further studies.

**Immunoprecipitation and immunoblot**

Cells were lysed by 15 mM CHAPS in lysis buffer (0.15 M NaCl, 2 mM EDTA, 1 mM PMSF, 1 mM NaVO4, 50 mM Tris-HCl, pH 7.5) or 1% Triton X-100 in lysis buffer, then lysates were centrifuged for 20 minutes at 15,000 g. The supernatants were preclarified with Sepharose 4B for 4 hours and then incubated with primary antibody for 4 hours followed by incubation with Sepharose 4B-conjugated secondary antibody. The Sepharose beads were washed three times with 15 mM CHAPS or 1% Triton solution and once with deionized water, then boiled for 5 minutes in SDS-PAGE sample buffer with or without 50 mM DTT. Samples were subjected to SDS-PAGE and western blotting as described previously (Nakamura et al., 1995). For biotinylated samples, the membrane was blocked in 3% BSA in TBS and detected by HRP-streptavidin. To analyze EGFR phosphorylation, cells were cultured in DMEM containing 0.5% FCS for 24 hours, then incubated with 100 nM EGF for 3 minutes.

**Immunofluorescence microscopy**

Cells grown on coverslips were fixed with 3% paraformaldehyde in PBS for 45 minutes at 4°C and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 minutes, followed by blocking with 0.2 M glycine, 50 mM Tris-HCl, pH 7.5, for 45 minutes at 4°C, and with 5% skim milk in PBS at room temperature for 45 minutes. A solution containing 5% BSA, instead of 5% skim milk, was used as the blocking solution when cells were stained with Streptavidin-Cy3. Samples were incubated with the primary antibody at room temperature for 1 hour. After washing three times with PBS, cells were incubated with FITC-conjugated secondary antibody or Streptavidin-Cy3. The samples were observed under a fluorescence microscope, or a confocal microscope. Serum starvation of cells grown on coverslips was achieved by incubation with DMEM containing 0.5% FCS for 24 hours.

**RESULTS**

**Integrin α2β1 associates with EGFR**

Association of integrins with EGFR was studied using epithelial-like A431 cells derived from human adenocarcinoma. Biotinylation and immunoprecipitation with anti-integrin subunit antibodies showed that A431 cells express predominantly integrins β1 and β4 for β subunits, and α1, α2 and α3 for α subunits (Fig. 1A). Integrin β3 subunit was not detected, while α5 was observed as a faint band. To examine the association of EGFR with these integrin subunits, the cell lysate solubilized with 15 mM CHAPS solution was immunoprecipitated with anti-integrin subunit antibodies, and co-precipitation of EGFR was tested by immunoblot analysis. As shown in Fig. 1B, anti-β1 antibody and anti-α2 antibody, but not other integrin subunits antibodies, co-precipitated...
EGFR, suggesting the association of integrin α2β1 with EGFR. Conversely, anti-EGFR antibody co-precipitated integrin α2β1 with EGFR (Fig. 2), further supporting the association of integrin α2β1 with EGFR. Integrin α6 subunit is also expressed in A431 cells but the coprecipitation assay with EGFR was not performed because α6 mainly associates with β4 in this cell line (Berditchevski et al., 1996).

In order to confirm the association of integrin α2β1 and EGFR, chemical cross-linking was performed. Intact A431 cells were first incubated with DTSSP, a cleavable homobifunctional reagent, and lysed with a solution containing 1% Triton X-100. Then the cell lysate was precipitated with the anti-integrins β1, β4, α1, α2, α3 or α5 mAb, followed by immunoblotting with anti-EGFR mAb. Because EGFR dissociates from integrin α2β1 in a Triton X-100 solution, coprecipitation of EGFR with integrin α2β1 was not observed without the crosslinking reagent (Fig. 3A). However, when cells were treated with the cross-linking agent, EGFR was coprecipitated with integrins α2 and β1, but not with α1, α3, α5 and β4 (Fig. 3B and C). From these cross-linking studies, together with co-precipitation using cell lysate solubilized with CHAPS, we concluded that EGFR physically associates with integrin α2β1 in A431 cells. Since the crosslinker DTSSP cannot penetrate plasma membrane, and thus only links cell surface proteins, it also proves that EGFR and integrin α2β1 form a complex on the cell surface.

**Integrin α2β1 colocalizes with EGFR at cell-cell contact sites**

A431 cells were immunofluorescently stained with an anti-EGFR antibody or one of the integrin subunit antibodies. Fig. 4a shows the phase contrast and fluorescence micrographs of immunostained A431 cells. EGFR was observed at cell-cell contact sites, free surfaces and microspikes. Integrin α2 was observed predominantly at cell-cell contact sites. Integrin α3 also localized at the cell-cell boundary, but it localized at free surface and microspikes. Integrins α1 and α5 were observed at the basement membrane and their localization and staining patterns were quite different from those of EGFR.

Co-localization of EGFR and integrin α2 at the cell-cell contact sites was demonstrated by observation with a confocal microscope (Fig. 4b). Sectioned images of double stained A431 cells revealed that EGFR localized from the basement layers to the apical tips including the lateral surface, while integrin α2 predominantly localized at cell-cell contact sites of the lateral surface. The fluorescence of integrin α2 overlapped well with that of EGFR at cell-cell contact sites.

**EGF stimulates EGFR phosphorylation but does not induce association of EGFR with integrin α2β1**

In this paper we showed that integrin α2β1 and EGFR have physical interaction at cell-cell contact sites. To explore
whether phosphorylation of EGFR influences the association of EGFR with integrin α2β1, co-precipitation experiments were carried out using A431 cells cultured in different growth conditions. In a normal growth medium containing 10% FCS, EGFR was moderately phosphorylated (Fig. 5A, lane 2). When A431 cells were cultured for 24 hours in the absence of serum, phosphorylation of EGFR was greatly diminished (Fig. 5A, lane 3). Addition of EGF (100 nM) into a serum-free medium resulted in strong phosphorylation of EGFR (Fig. 5A, lanes 1 and 3). Irrespective of EGFR phosphorylation, however, the amounts of EGFR coprecipitated with anti-integrin α2 antibody were similar between serum-depleted conditions and EGF-stimulated conditions (Fig. 5B), suggesting that EGFR phosphorylation by EGF does not induce association between EGFR and integrin α2.

Integrin α2β1-dependent EGFR phosphorylation at cell-cell contact sites

As shown in Fig. 5B, EGFR was co-precipitated with anti-integrin α2 antibody from samples prepared in serum-depleted conditions and the amount of EGFR was not influenced by addition of EGF. Interestingly, immunoblotting with anti-phosphotyrosine antibody showed this co-precipitated EGFR was significantly tyrosine-phosphorylated without EGF stimulation (Fig. 5C). The level of EGFR phosphorylation in serum-depleted conditions was similar to that in normal culture conditions, but much less than that in EGF-stimulated conditions. These results implied that integrin α2β1-associated EGFR would be phosphorylated even in the absence of EGF stimulation.

To examine the phosphorylation state of α2β1-associated EGFR more clearly, we utilized an antibody to the activated form of EGFR (actEGFR) which specifically reacts with the tyrosine phosphorylated-form of EGFR (Couet et al., 1997; Luttrell et al., 1997; Emlet et al., 1997; You and Zhao, 1997; Tsai et al., 1997). When cells were cultured in DMEM medium containing 10% FCS, phosphorylated EGFR was observed all around the cell surface as well as at cell-cell contact sites (Fig. 6A). The staining pattern coincides with that of the standard anti-EGFR antibody. Double staining with anti-actEGFR antibody and anti-α2 antibody showed that the activated EGFR colocalizes with integrin α2β1 at cell-cell contact sites (Fig. 6B). When cells were cultured with serum-depleted medium, the anti-actEGFR antibody predominantly stained EGFR localized at cell-cell contact sites, but not EGFR localized at other sites (Fig. 6C). Double staining with standard anti-EGFR antibody proved the presence of EGFR at other cell surface areas as well as at cell-cell contact sites, eliminating the possibility that redistribution of EGFR to cell-cell contact sites was induced in serum-depleted conditions. Thus, anti-actEGFR antibody stained almost all of the cell-surface EGFR when EGF was added to serum-depleted cultures (Fig. 6F). From these results it was concluded that even under serum-depleted conditions EGFR which localized at cell-cell contact sites, and where it associated with integrin α2β1, was phosphorylated, while EGFR not localized at cell-cell contact sites was dephosphorylated.

In order to examine the involvement of integrin α2β1 in this EGFR phosphorylation, we next tested whether integrin α2 antibody inhibits the phosphorylation of EGFR localized at cell-cell contact sites. Addition of a function-blocking anti-α2 mAb (P1E6; Carter et al., 1990) in the serum-depleted conditions almost completely inhibited the phosphorylation of EGFR localized at cell-cell contact sites, but did not change the distribution of EGFR as was shown by staining with standard anti-EGFR antibody (Fig. 6D), whereas, non-function-blocking anti-α2 mAb (12F1; Takada et al., 1988) had no inhibitory effect (Fig. 6E). These results indicate that EGFR phosphorylation at cell-cell contact sites in serum-depleted conditions is integrin α2β1 dependent.
Effects of various culture conditions on integrin-associated EGFR phosphorylation

In order to know the biological significance of EGFR phosphorylation at cell-cell contact sites in serum-depleted cells, effects of various culture conditions on integrin-associated EGFR phosphorylation were examined by immunoblot assay. A431 cells were cultured in serum-depleted medium. Cell lysates from the confluent culture ($1 \times 10^6$ cells/10 cm dish) were precipitated with anti-integrin α2 antibody and the immunoprecipitated materials were analyzed by western blotting using anti-EGFR or anti-actEGFR antibody. Similar to data shown in Fig. 5, integrin-associated EGFR was phosphorylated even in serum-depleted conditions (Fig. 7A, lane 1). Consistent with immunofluorescence studies shown in Fig. 6, function-blocking anti-α2 mAb (P1E6) did not affect the association of integrin α2 with EGFR, but abrogated EGFR phosphorylation (Fig. 7A, lane 5), while function-blocking anti-α3 mAb (P1B5) neither inhibited association of integrin α2 with EGFR nor EGFR phosphorylation (Fig. 7, lane 6). To examine whether cell-cell contact is a requisit for EGFR activation in serum-depleted conditions, phosphorylation levels of EGFR in sparse conditions ($3 \times 10^5$ cells/10 cm dish) were compared with those in standard confluent conditions ($1 \times 10^6$ cells/10 cm dish). In sparse conditions, most A431 cells were singles or doublets on the dish. Although similar amounts of EGFR were coprecipitated with integrin α2 from cells cultured in sparse conditions and in confluent conditions, the phosphorylation level of integrin-associated EGFR was greatly diminished in sparse conditions compared to that in confluent conditions (Fig. 7A, lanes 1 and 3). Cell density-dependent phosphorylation of integrin-associated EGFR was confirmed by another series of experiments (Fig. 7B). These results suggested that cell-cell contact is not necessarily required for association of EGFR with integrin α2 but is required for EGFR phosphorylation in serum-depleted conditions.

Next, we examined whether cell-substratum adhesion affects the association of integrin α2 with EGFR and EGFR phosphorylation. When cells were cultured on collagen type-I dishes, phosphorylation levels of integrin-associated EGFR
were slightly increased as compared with cells cultured on non-coated tissue culture dishes (compare Fig. 7A, lanes 1 and 2). A more striking effect was seen when cells were cultured in suspension with serum-depleted medium. Under these culture conditions, neither EGFR phosphorylation, nor association of integrin \(\alpha_2\) with EGFR were seen, even though integrin \(\alpha_2\) and EGFR were expressed in A431 cells similarly to the case in normal plating conditions (Fig. 7, lane 4). These results indicate that cell-substratum adhesion is required for association of integrin \(\alpha_2\) with EGFR, and for phosphorylation of integrin-associated EGFR.

**DISCUSSION**

Integrins and growth factor receptors share some down stream signal transduction pathways (Fantle et al., 1993; Clark and Brugge, 1995; Schwartz et al., 1995; Schlaepfer and Hunter, 1998; Howe et al., 1998). Both activate the same signal molecules such as Shc, Src family kinases, mitogen-activated protein (MAP) kinase, jun kinase (JNK), protein kinase c (PKC), phosphatidylinositol (PI) 3-kinase, the small GTPase and cell skeleton protein. Both can also induce early gene expression, such as c-fos, c-myc, c-jun (Fantle et al., 1993; Dike and Ingber, 1996), mediate cell transit from G1 to S phase in cell cycle progression (Giancotti, 1997; Howe et al., 1998), and block cell apoptosis (Frish and Ruoslahti, 1997; Meredith and Schwartz, 1997). Several lines of evidence indicate that integrin and receptor tyrosine kinase (RTK) also cooperate with each other. For example: (a) enhanced activation of growth factor receptors has been observed when integrins were aggregated and occupied (Miyamoto et al., 1996); (b) growth factor stimulates cell movement through integrin-mediated cell-substrate interaction (Matsumoto et al., 1995); (c) integrins and growth factor receptors also synergistically regulate cell differentiation (Sastry et al., 1996); and (d) growth factor-induced cell proliferation and antiapoptosis require cell adhesion (Meredith et al., 1993; Renshaw et al., 1997). This evidence suggests that direct interaction may exist between individual integrins and growth factor receptors. Indeed, associations of integrin \(\alpha \beta_3\) with PDGF\(\beta\) receptor or insulin receptor (Schneller et al., 1997), integrin \(\alpha \delta \beta_1\) or \(\alpha \delta \beta_4\) with ErbB2 (Falcioni et al., 1997), \(\beta_1\) integrin with EGFR (Mor et al., 1998), and integrin \(\alpha \beta_3\) with VEGF receptor (Soldi et al., 1999) have been observed.

We have demonstrated here that EGFR associates with integrin \(\alpha \beta_1\) on the cell surface of A431 cells by showing the co-precipitation, crosslinking and co-localization of both molecules. More importantly, immunofluorescence studies revealed that the EGFR-integrin complex predominantly localizes at cell-cell contact sites. These results provide the first evidence of the physical interaction of RTK with integrins at...
cell-cell contact sites. Integrin $\alpha_3\beta_1$ is expressed on the cell surface of A431 cells and also localized at cell-cell contact sites, but integrin $\alpha_3\beta_1$ did not associate with EGFR, suggesting a clear discrimination in association partners. Present studies also indicate the requirement of cell-substratum adhesion in the association of integrin $\alpha_2\beta_1$ with EGFR. Thus, although the mechanism remains to be clarified, co-precipitation of EGFR with integrin $\alpha_2$ was not observed in suspension, while it was strengthened by culture of the cells on collagen-coated dishes.

The present studies also demonstrated that integrin $\alpha_2\beta_1$-associated EGFR, which localized at cell-cell contact sites, is phosphorylated even in the absence of serum stimulation, while EGFR localized at other sites of the cell surface was totally dephosphorylated. Function-blocking anti-integrin $\alpha_2$ antibody P1E6, which binds to the I-domain of the $\alpha_2$ subunit (Tuckwell et al., 1995), abrogated the EGFR phosphorylation in serum-depleted conditions, while non function-blocking anti-$\alpha_2$ antibody 12F1, or function-blocking anti-$\alpha_3$ antibody P1B5 did not abrogate EGFR phosphorylation. These results constitute evidence that integrin $\alpha_2\beta_1$ is involved in EGFR phosphorylation at cell-cell contact sites in serum-depleted conditions by either a direct or an indirect mechanism.

The molecular mechanisms of integrin-dependent EGFR activation remain to be elucidated. The most simple and plausible explanation of integrin $\alpha_2\beta_1$-dependent EGFR activation is that laterally associated-integrin $\alpha_2\beta_1$ directly activates EGFR on the cell surface by an as yet unknown mechanism. The result, that phosphorylation of integrin-associated EGFR was largely diminished in a sparse conditions, indicates that cell-cell contact is required for EGFR activation. Therefore, although there is no evidence which suggests the presence of ECM ligands

![Fig. 6. Integrin $\alpha_2\beta_1$-dependent phosphorylation of EGFR in serum-depleted conditions. A431 cells were cultured in normal culture conditions containing 10% FCS (A,B) or in serum-depleted conditions (C-F) for 24 hours. During this culture, anti-$\alpha_2$ function-blocking mAb P1E6 (1 $\mu$g/ml), anti-$\alpha_2$ non function-blocking mAb 12F1 (2 $\mu$g/ml), and EGF (100 nM) were added (D,E,F, respectively). Then cells were fixed, double stained with botinylated anti-actEGFR antibody and either rabbit anti-EGFR Ab (A,C,F) or anti-$\alpha_2$ mAb 12F1 (B), followed by Cy3-conjugated streptavidin for the staining of actEGFR and FITC-conjugated appropriate second antibodies for the staining of either EGFR or integrin $\alpha_2$. Upper left, phase contrast; lower left, actEGFR; upper right, EGFR or integrin $\alpha_2$; lower right, overlaped image of two colours.]
for α2β1 at cell-cell contact sites, interaction of integrin α2β1 with its known or unidentified ligand at cell-cell contact sites may activate the integrin-associated EGFR without EGFR ligands. Recently, integrin-induced phosphorylation of EGFR has been reported for a cell-substratum integrin αvβ3 or αvβ3 (Moro et al., 1998), indicating that a novel signaling mechanism of integrin-dependent EGFR activation may exist between integrins and EGFR. Additionally, the model in which ligand-stimulated integrin activates laterally associated RTK is supported by recent reports on association and activation of other RTKs with integrins at cell-substratum adhesion (Schneller et al., 1997; Moro et al., 1998; Soldi et al., 1999).

Since A431 cells express proHB-EGF on the cell surface, proHB-EGF•CD9•integrin αβ1 complex probably localized at cell-cell contact sites (Nakamura et al., 1995). In the context of the juxtacrine activity of proHB-EGF (Higashiyama et al., 1995), another explanation for the mechanism of integrin αβ1-dependent EGFR activation could be possible. ProHB-EGF, and/or other membrane-anchored EGFR ligands such as amphiregulin, may phosphorylate EGFR of adjacent cells by a juxtacrine mechanism without soluble EGFR ligands in the medium. Integrin α2β1 of one cell may serve to bind to adjacent cells and to stabilize the interaction of the membrane-anchored ligands with EGFR on the opposite cell. Thus integrin α2β1 may indirectly support EGFR activation at cell-cell contact sites. Whatever the mechanism, identification of the ligand or molecule binding to integrin α2β1 at the cell-cell contact site is important.

The biological role of integrin-dependent EGFR activation at cell-cell contact sites is not clear. ECM-induced EGFR activation observed in the αβ1•EGFR complex protects cells from apoptosis (Moro et al., 1998). Thus, EGFR activation at cell-cell contact sites may serve as an anti-apoptotic signal in A431 cells. In A431 cells, however, excess stimulation of EGFR has been known to result in growth inhibition (Gill and Lazar, 1981). Thus, it is also plausible that activation of EGFR at cell-cell contact sites may generate growth inhibition signals in A431 cells.

Although fairly large amounts of integrins α2β1 and α3β1 are localized at cell-cell contact sites on the cell surface of epithelial cells and keratinocytes (Symington et al., 1993; Nakamura et al., 1995), the functions of these integrins remains to be clarified. The present study gives new clues to understanding the role of intercellular integrins in cell-to-cell interactions.

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