

# Membrane-anchored Heparin-binding EGF-like Growth Factor (HB-EGF) and Diphtheria Toxin Receptor-associated Protein (DRAP27)/CD9 Form a Complex with Integrin $\alpha 3\beta 1$ at Cell-Cell Contact Sites

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**Abstract.** Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family of growth factors, which interact with EGF receptor to exert mitogenic activity. The membrane-anchored form of HB-EGF, proHB-EGF, is biologically active, providing mitogenic stimulation to neighboring cells in a juxtacrine mode. ProHB-EGF forms a complex with diphtheria toxin receptor-associated protein (DRAP27)/CD9, a tetra membrane-spanning protein that upregulates the juxtacrine mitogenic activity of proHB-EGF. We explored whether other proteins associate with DRAP27/CD9 and proHB-EGF. Immunoprecipitation with anti-DRAP27/CD9 resulted in preferential coprecipitation of integrin  $\alpha 3\beta 1$  from Vero cell, A431 cell and MG63 cell lysates. Anti-integrin  $\alpha 3$  or anti-integrin  $\beta 1$  coprecipitated DRAP27/CD9 from

the same cell lysates. Chemical cross-linking confirmed the physical association of DRAP27/CD9 and integrin  $\alpha 3\beta 1$ . Using Vero-H cells, which overexpress HB-EGF, we also demonstrated the association of proHB-EGF with DRAP27/CD9 and integrin  $\alpha 3\beta 1$ . Moreover, colocalization of proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$  at cell-cell contact sites was observed by double-immunofluorescence staining. At cell-cell contact sites, DRAP27/CD9 was highly coincident with  $\alpha$ -catenin and vinculin, suggesting that DRAP27/CD9, proHB-EGF, and integrin  $\alpha 3\beta 1$  are colocalized with adherence junction-locating proteins. These results indicate that direct interaction of growth factors and cell adhesion molecules may control cell proliferation during the cell-cell adhesion process.

CELL-CELL interactions regulating cell growth and differentiation play a vital role in embryonic development, morphogenesis of tissue, and maintenance of the adult organism (Whitman and Melton, 1989; Edelman and Crossin, 1991; Takeichi, 1991). These interactions are mainly mediated by two categories of proteins, growth factors and cell adhesion molecules. Growth factors are generally secreted as diffusible proteins and transduce proliferation and differentiation signals by binding to specific receptors on the target cell membrane (Carpenter and Cohen, 1979), while cell adhesion molecules assemble animal cells into tissues by their adhesive properties and also regulate cell growth and differentiation by transducing a signal directly or via intracellular molecules associated with their cytoplasmic domains (Geiger and Ayalon, 1992; Juliano and Haskill, 1993). Thus, it has been postulated that proper expression and interaction of growth fac-

tors and cell adhesion molecules is essential for normal development and maintenance of multicellular organisms. However, direct interaction between growth factors and cell adhesion molecules has not been demonstrated.

Heparin-binding EGF-like growth factor (HB-EGF)<sup>1</sup> is a member of the EGF family (Higashiyama et al., 1991), which encompasses a number of structurally homologous mitogens including EGF, TGF- $\alpha$ , vaccinia virus growth factor (Carpenter and Wahl, 1990), amphiregulin (Shoyab et al., 1989) and  $\beta$ -cellulin (Shing et al., 1993). Like EGF, TGF- $\alpha$ , and amphiregulin, HB-EGF binds to and stimulates phosphorylation of the EGF receptor. Secreted mature HB-EGF is a potent mitogen for NIH3T3 cells, bovine aortic smooth muscle cells, rat hepatocytes, and human keratinocytes (Higashiyama et al., 1991).

An important feature of HB-EGF is that it is a mem-

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1. *Abbreviations used in this paper:* DRAP27, diphtheria toxin receptor-associated protein; DSP, Dithiobis(succinimidylpropionate); DT, diphtheria toxin; HB-EGF, heparin-binding EGF-like growth factor; proHB-EGF, membrane-anchored form of HB-EGF.

brane-anchored growth factor. Analysis of the nucleotide sequence of human HB-EGF cDNA predicts a precursor protein of 208 amino acids composed of signal peptide, heparin-binding, EGF-like, transmembrane, and cytoplasmic domains (Higashiyama et al., 1991). Although the membrane-anchored form of HB-EGF (proHB-EGF) can be cleaved to yield a mature biologically active protein containing 75–86 amino acids, a considerable amount of proHB-EGF remains uncleaved on the cell surface. We have shown that proHB-EGF transduces biological signals in a nondiffusible manner to neighboring cells (Higashiyama et al., 1995). Such “juxtacrine stimulation” has been demonstrated for other membrane-anchored growth factors and lymphokines, including TGF- $\alpha$  (Brachmann et al., 1989; Wong et al., 1989), tumor necrosis factor  $\alpha$  (Perez et al., 1990), colony-stimulating factor 1 (Stein et al., 1990), and c-kit ligand (Flanagan et al., 1991).

ProHB-EGF is not only a membrane-anchored growth factor or a precursor for soluble growth factor, but also acts as the specific receptor for diphtheria toxin (DT) and mediates endocytosis of the receptor-bound toxin (Naglich et al., 1992; Iwamoto et al., 1994). Studies of the DT receptor revealed that proHB-EGF forms a complex with another membrane protein known as DRAP27, a 27-kD DT receptor-associated protein in Vero cells, a monkey kidney cell line (Iwamoto et al., 1991, 1994). DRAP27 is identical to human CD9 (Mitamura et al., 1992), which was first identified as a cell surface antigen on lymphohemopoietic cells (Kersey et al., 1981). Anti-DRAP27 antibodies inhibit the binding of DT to proHB-EGF (Iwamoto et al., 1991) and coprecipitate proHB-EGF with DRAP27/CD9 (Iwamoto et al., 1994), indicating that these two proteins are closely associated in the cell membrane. Furthermore, coexpression of DRAP27/CD9 with proHB-EGF markedly upregulates juxtacrine growth stimulation activity of proHB-EGF (Higashiyama et al., 1995) as well as DT binding and DT sensitivity of cells (Mitamura et al., 1992; Iwamoto et al., 1994).

The number of DRAP27/CD9 molecules on the surface of Vero cells is at least 100-fold higher than the number of proHB-EGF molecules (Iwamoto et al., 1991). This suggests that a large fraction of DRAP27/CD9 may not be associated with proHB-EGF and that DRAP27/CD9 could have other functions. Recent studies by other groups imply that DRAP27/CD9 may function in the cell adhesion process (Letarte et al., 1993; Masellis-Smith and Shaw, 1994) and cell motility (Ikeyama et al., 1993). Moreover, DRAP27/CD9 belongs to the newly identified tetramembrane-spanning protein family, of which several members may be involved in the cell adhesion system (Bell et al., 1992; Imai and Yoshie, 1993). These results prompted us to examine whether DRAP27/CD9, and proHB-EGF associate with cell adhesion molecules.

In this article, we provide evidence that DRAP27/CD9 and proHB-EGF associate with integrin  $\alpha 3\beta 1$ . Integrin  $\alpha 3\beta 1$  differs from typical focal contact integrins and is found in cell–cell adhesion sites (Carter et al., 1990) as well as in cell–substrate adhesion sites. We observed by double immunofluorescence staining that proHB-EGF, DRAP27/CD9 and integrin  $\alpha 3\beta 1$  are colocalized at cell–cell contact sites. Together with the results of coprecipitation and chemical cross-linking experiments, these data

show a direct link of the membrane-anchored growth factor and cell adhesion molecules at cell–cell contact sites.

## Materials and Methods

### Cells and Cell Cultures

Vero cells from monkey kidney were maintained in MEM with nonessential amino acids (MEM-NEAA) supplemented with heat-inactivated 10% calf serum. Vero-H cells were isolated by transfection with human HB-EGF cDNA into Vero cells (Goishi, K., S. Higashiyama, M. Klagsbrun, N. Nakano, T. Umata, M. Ishikawa, E. Mekada, and N. Taniguchi, manuscript in preparation). Human adenocarcinoma A431 cells and human osteosarcoma MG63 cells were cultured in DMEM supplemented with 10% FCS and MEM-NEAA with 10% FCS, respectively.

### Proteins and Antibodies

DT and CRM107 were produced as described previously (Uchida et al., 1973), and anti-DT antiserum was raised by immunizing rabbits with formalin-fixed diphtheria toxoid. The antiserum was purified by ammonium sulfate precipitation and affinity chromatography with immobilized DT. Anti-DRAP27/CD9 mAb 007 (IgG2a) was prepared and purified as previously described (Iwamoto et al., 1991). Other anti-CD9 mAbs, TP82 (IgG1), ALB6 (IgG1), and BU16 (IgG2a), were obtained from Nichirei Co., Ltd. (Tokyo, Japan), MBL Co., Ltd. (Nagoya, Japan), and The Binding Site Inc. (Birmingham, England), respectively.

Rabbit anti-HB-EGF antisera H6 and M6 were obtained as described previously (Iwamoto, et al., 1994; Higashiyama et al., 1995). Mouse anti-integrin  $\beta 1$  mAbs 4B4 (IgG1, Morimoto et al., 1985) and K20 (IgG2a, Amiot et al., 1986) were purchased from Coulter Immunology (Hialeah, FL) and MBL Co., Ltd., respectively. Rabbit anti- $\alpha 3$  and anti- $\beta 1$  antisera were from Chemicon International, Inc. (Temecula, CA). Mouse mAbs to integrin  $\alpha 2$  (P1E6),  $\alpha 3$  (P1B5),  $\alpha 5$  (P1D6) (Wayner et al., 1988) and  $\alpha v$  (VNR147; Freed et al., 1989) were purchased from Telios Pharmaceuticals, Inc. (San Diego, CA).

Mouse anti-vinculin IgG was purchased from Chemicon International, Inc. and mouse anti-EGF receptor from Genzyme Corp. (Cambridge, MA). Rat anti- $\alpha$  catenin was a generous gift from Dr. Shoichiro Tsukita (Kyoto University, Kyoto, Japan).

### Cell Surface Labeling and Chemical Cross-Linking

Cell surface iodination was carried out by the lactoperoxidase method. Cells were grown in 100-mm dishes and washed with Hepes-buffered HBSS three times. Then cells were radiolabeled with 0.5 mCi  $Na^{125}I$  (Amersham International, Amersham, UK), 0.2 U lactoperoxidase (Sigma Chemical Co., St. Louis, MO), and 20  $\mu l$  of 1:20,000-diluted 30% hydrogen peroxide in 625  $\mu l$  of HBSS for 5 min. The reaction was stopped by addition of excess tyrosine and 500 nM DTT, and cells were washed with HBSS.

Cell surface biotinylation was carried out as follows. Cells were washed with HBSS and incubated in a solution containing 150 mM NaCl, 10 mM Hepes, 0.2  $\mu g/ml$  NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL), 0.2 mM  $CaCl_2$  and  $MgCl_2$ , pH 8.0, for 30 min at 4°C. The reaction was stopped by the addition of 40 mM glycine, and cells were washed with HBSS.

Chemical cross-linking was carried out as described previously (Iwamoto et al., 1994) with minor modifications. Briefly, the cells were washed with PBS (137 mM NaCl, 0.67 mM KCl, 8 mM  $Na_2HPO_4$ , 1.4 mM  $KH_2PO_4$ ) and incubated for 30 min at 4°C with 0.8 mM Dithiobis(succinimidylpropionate) (DSP; Pierce Chemical Co.), or 1% DMSO for control, in 20 mM sodium bicarbonate buffer, 150 mM NaCl, 0.2 mM  $CaCl_2$ , 0.2 mM  $MgCl_2$ , pH 8.5. DSP is cleavable under reducing conditions. Cells were washed twice with TBS (20 mM Tris-HCl, 100 mM NaCl, pH 7.5), once with PBS, and used for further studies.

### Immunoprecipitation and SDS-PAGE

Cells were lysed with HBS (10 mM Hepes, 150 mM NaCl, pH 7.0) containing 10 mM CHAPS, 10  $\mu g/ml$  chymostatin, and 20  $\mu g/ml$  antipain, unless otherwise mentioned, and kept for 30 min at 4°C. The lysates were cleared of insoluble material by centrifugation at 40,000 g for 30 min, and the supernatant was precipitated with primary antibodies at a concentra-

tion of 5  $\mu\text{g/ml}$  followed by the addition of Sepharose 4B-conjugated second antibodies (Cappel Laboratories, Durham, NC). Protein A-Sepharose 4B (Pharmacia LKB Biotechnology, Tokyo, Japan) was also used for some experiments. The gel was washed with washing buffer (HBS containing 10 mM CHAPS) and then boiled with SDS-PAGE sample buffer. Material recovered from the gel was analyzed by SDS-PAGE with or without 50 mM DTT. Unless otherwise indicated, gradient gels from 4 to 20% acrylamide (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) were used for SDS-PAGE. For the iodinated samples, gels were dried, exposed to imaging film, and analyzed using an image analyzer (model BAS2000; Fujix, Tokyo, Japan).

In the reprecipitation experiment,  $^{125}\text{I}$ -labeled lysates were precipitated with Sepharose 4B-conjugated anti-DRAP27/CD9 mAb 007. After the gel was washed with washing buffer, it was treated with elution buffer (10 mM Hepes, 500 mM NaCl, 10 mM CHAPS, 1% Triton X-100, 0.02% SDS) for 1 h at 4°C. The eluted material was immunoprecipitated again with the indicated anti-integrin mAb and Sepharose 4B-conjugated goat anti-mouse IgG in the presence of the detergents mentioned above. Precipitated material was subjected to SDS-PAGE.

### Western Blotting

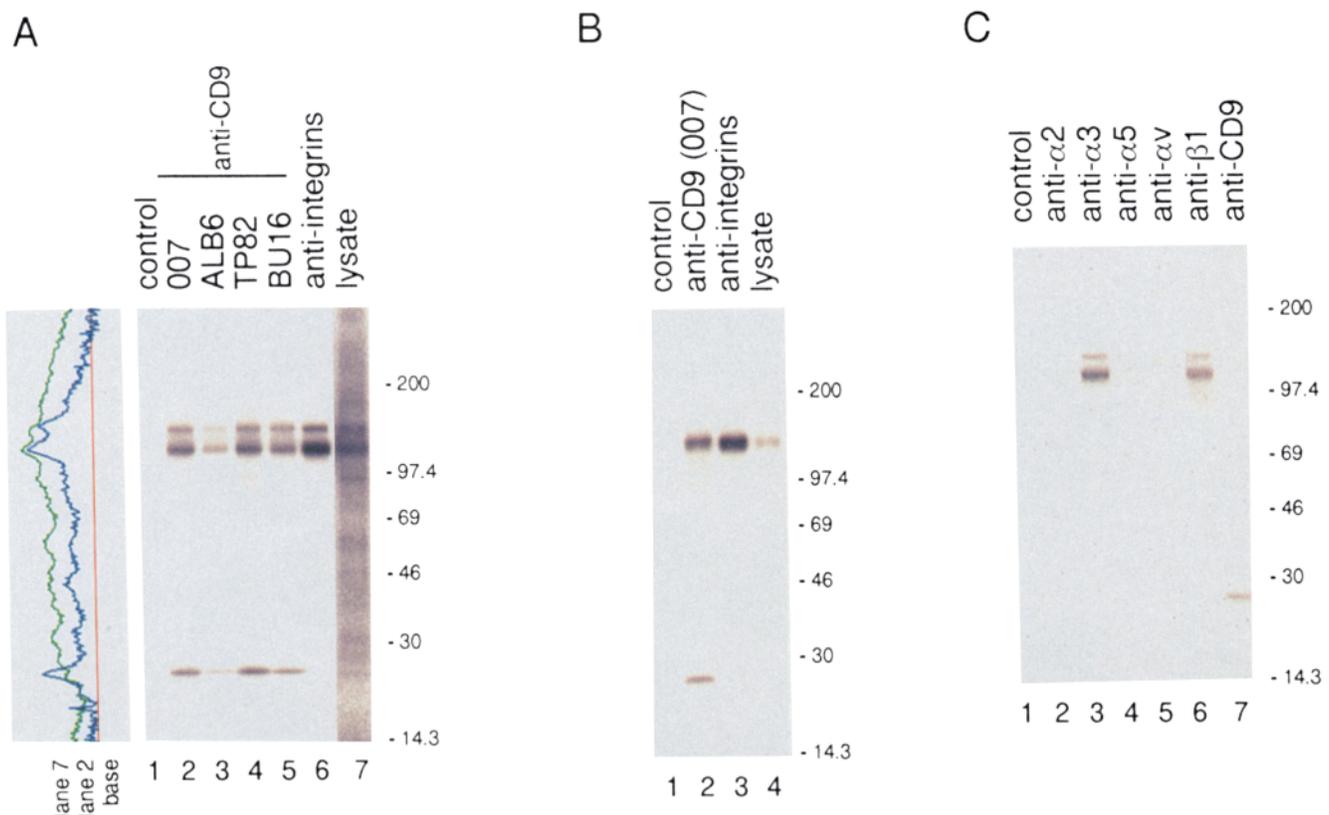
Samples subjected to SDS-PAGE were electrotransferred to an Immobilon membrane. The membrane was blocked with TBS containing 3% skim milk at room temperature for 1 h, then incubated at 4°C overnight with anti-integrin  $\alpha 3$  mAb (100 ng/ml) or anti-HB-EGF antiserum H6 (1:20,000 dilution) in TBS containing 1% skim milk and 200 mM NaCl. After

thorough washing with TTBS (TBS containing 0.05% Tween 20), the membranes were incubated with HRP-conjugated donkey anti-rabbit IgG (Chemicon International, Inc.) at a concentration of 400 ng/ml, washed with TTBS, and analyzed with an ECL-Western blotting kit (Amersham International). For biotinylated samples, the membrane was blocked with 3% BSA in TBS, and proteins were detected by incubation with 100 ng/ml HRP-streptavidin (Pierce Chemical Co.).

### Indirect Immunofluorescence Microscopy

Cells grown on coverglasses were fixed with 3.7% paraformaldehyde in HBSS for 30 min at 4°C and permeabilized with 0.1% Triton X-100 at 4°C for 2 min if needed. Then cells were blocked with blocking solution (HBSS containing 10% skim milk) at room temperature for 30 min and incubated with primary antibodies (5  $\mu\text{g/ml}$ , or a dilution of 1:100) in blocking solution. After washing, cells were treated with a fluorescent second antibody or biotinylated second antibody followed by streptavidin-Cy3 (Pierce Chemical Co.). The samples were mounted in HBSS containing 1  $\mu\text{g/ml}$  *p*-phenylenediamine and observed under a fluorescence microscope (Nikon MICROPHOT-FXA, Nikon Co. Ltd., Tokyo, Japan) or laser scanning confocal microscope (Carl Zeiss LSM 10, Carl Zeiss, Oberkochen, Germany). Confocal images were obtained and analyzed using an LSM 10 microscope equipped with analysis software. For double staining using mouse mAbs, subclass specific anti-mouse IgG antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) were used as second antibodies at 1:100 dilution.

For indirect staining of proHB-EGF with DT, Vero and Vero-H cells



**Figure 1.** Anti-DRAP27/CD9 antibodies coprecipitate integrin  $\alpha 3\beta 1$ . (A) Immunoprecipitated material analyzed by SDS-PAGE under nonreducing conditions.  $^{125}\text{I}$ -labeled Vero cells were lysed with 10 mM CHAPS solution, and lysates were immunoprecipitated with various antibodies: irrelevant antibody (lane 1), anti-CD9 mAb 007 (lane 2), ALB6 (lane 3), TP82 (lane 4), BU16 (lane 5), or a mixture of anti-integrin  $\alpha 3$  and anti-integrin  $\alpha 5$  mAbs (lane 6). Lane 7 contained total cell lysate. Gels were analyzed with a BAS2000 image analyzer. Bars on the right margin show molecular mass markers in kilodaltons. Scans of the signal intensity of lanes 2 and 7 are shown to the left. (B) Immunoprecipitated material analyzed by SDS-PAGE under reducing conditions. Antibodies used: irrelevant antibody (lane 1), anti-CD9 mAb 007 (lane 2), mixture of anti-integrin  $\alpha 3$  and  $\alpha 5$  mAbs (lane 3). Lane 4 contained total cell lysate. (C) Reprecipitation of coprecipitated material.  $^{125}\text{I}$ -labeled Vero cell lysates were immunoprecipitated with Sepharose 4B-conjugated anti-DRAP27/CD9 mAb. Precipitated materials were eluted from the gels as described in Materials and Methods, and each aliquot was immunoprecipitated again with irrelevant mAb (lane 1), anti- $\alpha 2$  (lane 2), anti- $\alpha 3$  (lane 3), anti- $\alpha 5$  (lane 4), anti- $\alpha v$  (lane 5), anti- $\beta 1$  (lane 6), or anti-CD9 mAb (lane 7). Precipitated material was analyzed by SDS-PAGE under nonreducing condition.

were incubated for 4 h on ice with 100 ng/ml DT in Hepes-buffered MEM-NEAA supplemented with 10% calf serum. Cells were washed and then incubated with rabbit anti-DT (1:100 dilution) for 1 h at 4°C and fixed with 3.7% paraformaldehyde for 1 h on ice. Then cells were incubated with biotinylated goat anti-rabbit IgG (Zymed Laboratories Inc., South San Francisco, CA), followed by streptavidin-Cy3. In the double-staining experiment, DT-treated Vero-H cells were incubated with rabbit anti-DT antibody and mouse anti-integrin  $\alpha 3$  for 1 h on ice and fixed. Then cells were incubated with FITC-labeled goat anti-mouse IgG (Chemicon International, Inc.) and biotinylated goat anti-rabbit IgG followed by streptavidin-Cy3 and observed under a fluorescence microscope.

## Results

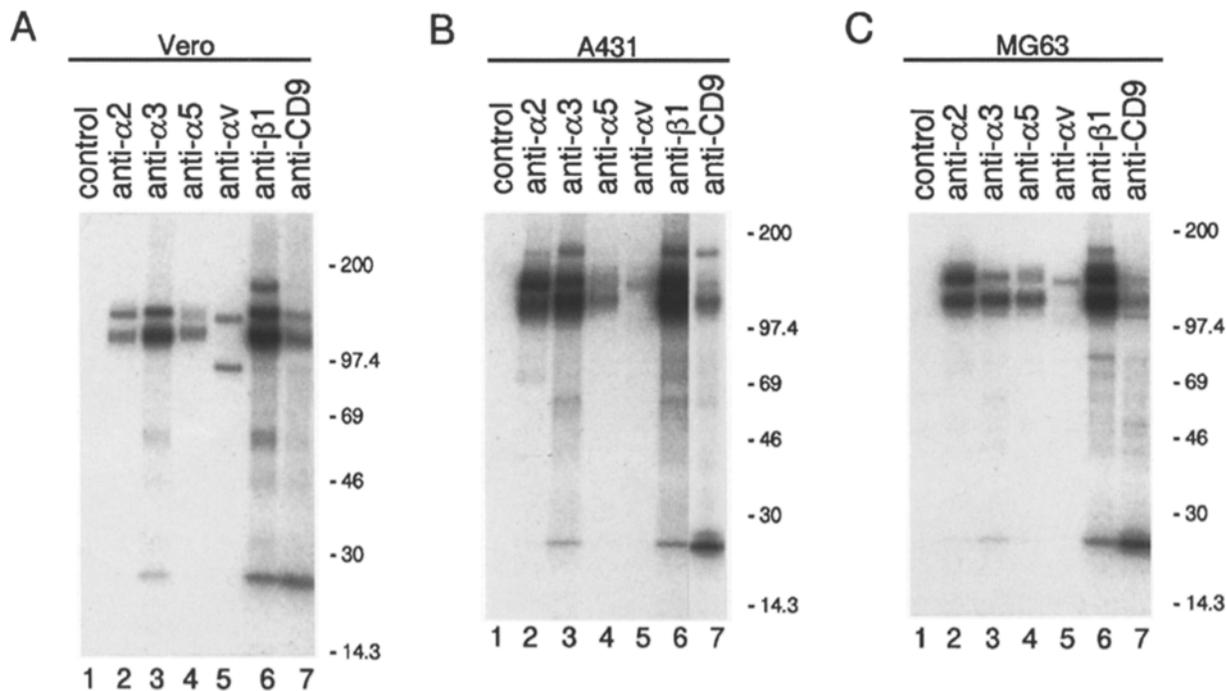
### Association of DRAP27/CD9 with Integrin $\alpha 3\beta 1$

To identify proteins that associate with DRAP27/CD9, Vero cell lysate was immunoprecipitated with anti-DRAP27/CD9 antibody, and polypeptides coprecipitated with DRAP27/CD9 were analyzed. Surface-iodinated Vero cells were lysed with 10 mM CHAPS solution, and the lysate was immunoprecipitated with anti-DRAP27/CD9 mAb, followed by incubation with immobilized goat anti-mouse IgG. The precipitated material was analyzed by SDS-PAGE under reducing or nonreducing conditions. Anti-DRAP27/CD9 mAb (clone 007) precipitated 150- and 115-kD proteins in addition to DRAP27 itself (27 kD) under nonreducing conditions (Fig. 1 A, lane 2). Under reducing conditions, the 150- and 115-kD bands were both shifted to  $\sim 135$  kD (Fig. 1 B, lane 2). Control antibody did not precipitate any polypeptides (Fig. 1, A and B, lanes 1). The 150- and 115-kD polypeptides were major polypeptides of surface-iodinated Vero cells (Fig. 1 A, lane 7, and Fig. 1 B, lane 4), so it was possible that these polypeptides might be nonspecific contaminants. However,

this was not the case, because radiograms of the electrophoresed gels indicated that 150- and 115-kD bands were specifically concentrated by precipitation with anti-CD9 antibody (Fig. 1 A, radiogram). Precipitation of 150- and 115-kD polypeptides was also observed with other independently isolated anti-human CD9 mAbs (ALB6, TP82, BU16) (Fig. 1 A, lanes 3–5), which rules out precipitation of the 150- and 115-kD polypeptides due to an irrelevant cross-reaction of mAb 007.

The mobilities of the coprecipitated proteins in SDS gel and the shifts of these proteins in reducing conditions are quite similar to those of some  $\beta 1$ -containing integrins (Fig. 1 A, lane 6, and Fig. 1 B, lane 3). To examine whether the polypeptides were integrins, material recovered from anti-CD9/DRAP27 precipitates was precipitated again with mAbs specific for each integrin subunit, and the precipitated material was subjected to SDS-PAGE (Fig. 1 C). Only mAb directed to integrin  $\alpha 3$  or  $\beta 1$  precipitated the 150- and 115-kD polypeptides (Fig. 1 C, lanes 3 and 6). As shown in Fig. 2 A, Vero cells also express  $\alpha 2$ ,  $\alpha 5$ , and  $\alpha \nu$  subunits, but these integrin  $\alpha$  subunits were not included in the material precipitated with anti-DRAP27/CD9 antibody. Western blotting analysis confirmed that the 150-kD band is integrin  $\alpha 3$  (see Fig. 4 C, lane 4) and the 115-kD band is integrin  $\beta 1$  (data not shown).

Next, in a reciprocal experiment, Vero cell lysate was immunoprecipitated with each of the anti-integrin subunit-specific antibodies. As shown in Fig. 2 A, anti- $\alpha 3$  antibodies precipitated 150- and 115-kD bands of integrin and also coprecipitated a 27-kD polypeptide. Anti- $\beta 1$  antibodies also precipitated the 27-kD polypeptide in addition to 180- and 150-kD integrin  $\alpha$  subunits and the 115-kD  $\beta 1$



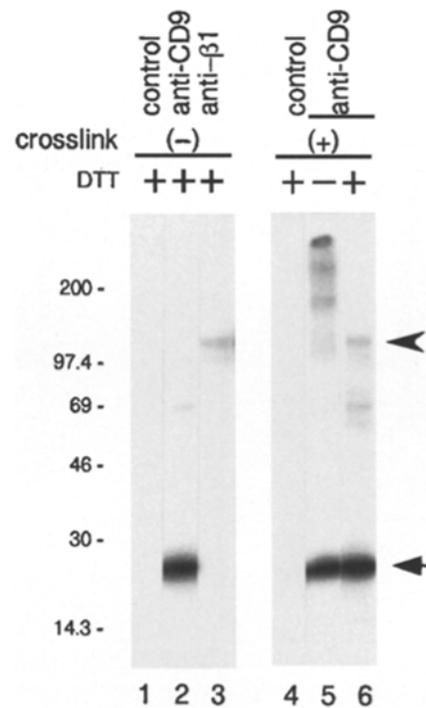
**Figure 2.** Coprecipitation of DRAP27/CD9 and  $\alpha 3\beta 1$  in Vero, A431, and MG63 cells.  $^{125}\text{I}$ -labeled cell lysates of Vero cells (A), A431 cells (B), and MG63 cells (C) were immunoprecipitated with irrelevant mAb (lanes 1), anti- $\alpha 2$  (lanes 2), anti- $\alpha 3$  (lanes 3), anti- $\alpha 5$  (lanes 4), anti- $\alpha \nu$  (lanes 5), anti- $\beta 1$  (lanes 6), or anti-CD9 (lanes 7) and Sepharose 4B-conjugated anti-mouse IgG. Precipitated material was analyzed by SDS-PAGE under nonreducing condition.

subunit. Immunoblotting with anti-CD9 antibody confirmed that the 27-kD polypeptide is DRAP27/CD9 (data not shown). Immunoprecipitation with anti-integrin  $\alpha 2$ ,  $\alpha 5$ , or  $\alpha \nu$  antibodies indicated that Vero cells contain these integrins, but coprecipitation of DRAP27/CD9 was not observed (Fig. 2 A). Coprecipitation of DRAP27/CD9 with anti- $\alpha 3$  antibody or  $\beta 1$  antibody was also observed when different antibodies directed against  $\alpha 3$  (11G5, M-Kid) or  $\beta 1$  (DF5, K20, P4C10) were used (data not shown).

There is no reason to expect immunological cross reactivity between integrin  $\alpha 3$  or  $\beta 1$  and DRAP27/CD9, as integrin  $\alpha 3$  and  $\beta 1$  have no sequence homology with DRAP27/CD9. In fact, almost no coprecipitation was observed when cells were lysed with Triton X-100 (data not shown). Thus, coprecipitation of DRAP27/CD9 and integrin  $\alpha 3\beta 1$  indicates physical association of these molecules.

To exclude the possibility of artifactual association after cell lysis, we carried out cross-linking studies of DRAP27/CD9 and integrin molecules. When Vero cells were lysed with a buffer containing 500 mM NaCl, 1% Triton X-100, and 0.02% SDS, anti-DRAP27/CD9 mAb 007 precipitated DRAP27/CD9 (Fig. 3, arrow) but not integrin  $\alpha 3\beta 1$ , due to the dissociation of the proteins under these conditions (Fig. 3, lane 2). However, integrin  $\alpha 3$  and  $\beta 1$  subunits are noncovalently linked in this condition (see Fig. 1 C). When cells were treated with the cleavable bifunctional cross-linker DSP before cell lysis, high molecular mass bands were observed in addition to the 27-kD DRAP27/CD9 band after SDS-PAGE under nonreducing conditions (Fig. 3, lane 5). Under reducing conditions, the high molecular mass bands shifted to 135 kD (Fig. 3, lane 6, arrow-head), the mobility of integrin  $\alpha 3$  subunit and  $\beta 1$  subunits (Fig. 3, lane 3). These results indicate that coprecipitation of DRAP27/CD9 and integrin does not result from incomplete solubilization or from aggregation of the molecules after cell lysis. We conclude that DRAP27/CD9 and integrin  $\alpha 3\beta 1$  are physically associated in Vero cell membrane.

To examine whether association of DRAP27/CD9 and integrin  $\alpha 3\beta 1$  is generally seen, we carried out coprecipitation experiments using other cell lines. A431 cells, a cell line derived from human adenocarcinoma, express predominantly integrins  $\alpha 2$ ,  $\alpha 3$ , and  $\beta 1$ , and also small amounts of  $\alpha 5$  and  $\alpha \nu$  subunits (Fig. 2 B). Anti-DRAP27/CD9 mAb coprecipitated 150- and 115-kD protein bands as well as an unidentified 180-kD band (Fig. 2 B, lane 7). Reprecipitation experiments showed that these 150- and 115-kD bands were  $\alpha 3$  and  $\beta 1$  subunits. Reciprocally, 27-kD polypeptide was coprecipitated when anti-integrin  $\alpha 3$  or  $\beta 1$  was used (Fig. 2 B, lanes 3 and 6), but not with anti- $\alpha 2$ ,  $\alpha 5$  or  $\alpha \nu$  (Fig. 2 B, lanes 2, 4, and 5). These results indicate that DRAP27/CD9 associates with integrin  $\alpha 3\beta 1$  in A431 cells. Coprecipitation of  $\alpha 3\beta 1$  but not  $\alpha 2\beta 1$  with anti-DRAP27/CD9, despite expression of similar amounts of these integrins by A431 cells, provides further evidence that coprecipitation of  $\alpha 3\beta 1$  with DRAP27/CD9 is due to specific association of these proteins. MG63 cells, a human osteosarcoma cell line, express integrin  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha \nu$ , and  $\beta 1$  (Fig. 2 C). In these cells, expression of integrin  $\alpha 2$  and  $\alpha 5$  is higher than expression of  $\alpha 3$ . The 150- and 115-kD bands again coprecipitated with

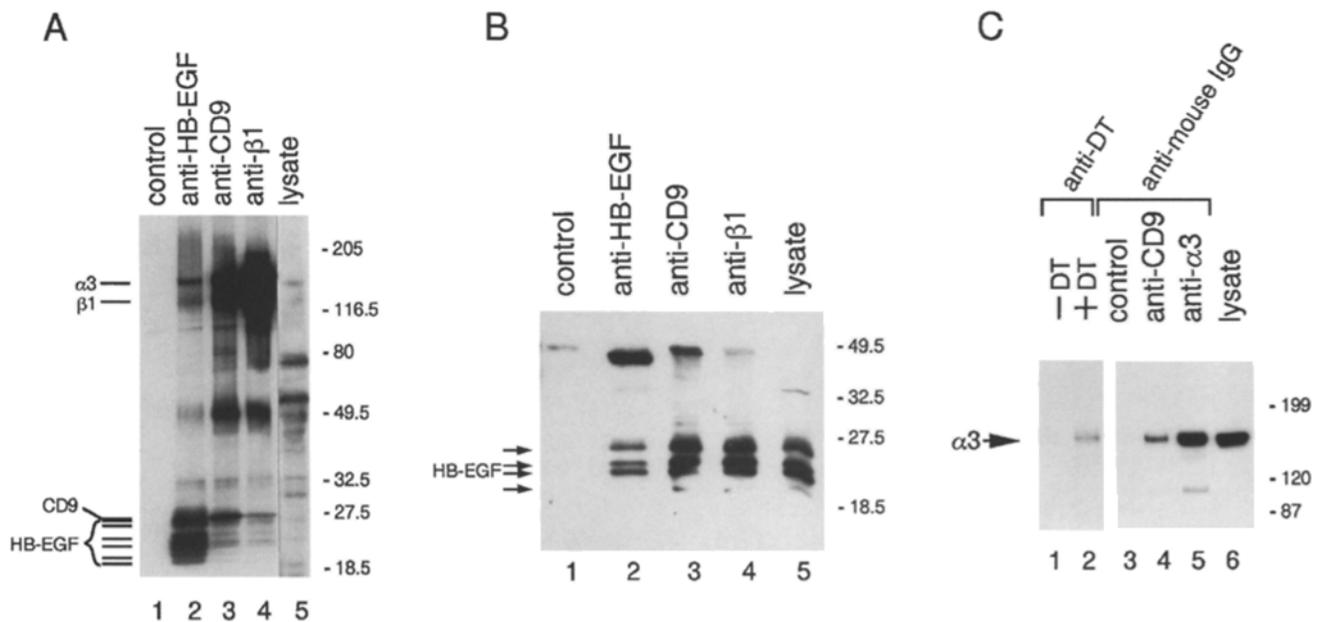


**Figure 3.** Cross-linking of DRAP27/CD9 and integrin  $\alpha 3\beta 1$ . Radioiodinated Vero cells were treated with the homobifunctional cleavable cross-linking reagent DSP (lanes 4–6) or control solution (lanes 1–3). Then cells were labeled with  $^{125}\text{I}$ Na, lysed with solution containing 1% Triton X-100, and the lysate was immunoprecipitated with irrelevant mAb (lanes 1 and 4), anti-CD9 mAb 007 (lanes 2, 5, and 6) or rabbit anti- $\beta 1$  (lane 3). Precipitated materials were analyzed by SDS-PAGE under nonreducing (lane 5) or reducing conditions (lanes 1–4, 6).

DRAP27/CD9 using anti-DRAP27/CD9 antibody (Fig. 2 C, lane 7), and 27-kD polypeptide coprecipitated with integrin  $\alpha 3$  or  $\beta 1$  mAb (Fig. 2 C, lanes 3 and 6). Reprecipitation experiments confirmed that the 150- and 115-kD bands were integrin  $\alpha 3$  and  $\beta 1$ , respectively (data not shown). Thus, association of DRAP27/CD9 and integrin  $\alpha 3\beta 1$  was observed in all three cell lines tested. Trace amounts of  $\alpha 2$  and  $\alpha 5$  integrin were detected in the reprecipitation experiment with the material precipitated with anti-DRAP27/CD9 mAb from MG63 cell lysate; thus,  $\alpha 2\beta 1$  and  $\alpha 5\beta 1$  may also associate with DRAP27/CD9 in this cell line to a lesser degree.

#### **Association of Integrin $\alpha 3\beta 1$ with proHB-EGF-DRAP27/CD9 Complex**

We have recently demonstrated that DRAP27/CD9 forms a complex with DTR/HB-EGF precursor (Iwamoto et al., 1994). Therefore, we examined whether integrin  $\alpha 3\beta 1$  is associated with only DRAP27/CD9, or also with the proHB-EGF-DRAP27/CD9 complex. Because it is difficult to demonstrate coprecipitation of proHB-EGF and DRAP27/CD9 from Vero cell lysate due to the large difference in the abundance of DRAP27/CD9 and proHB-EGF, we used cell lysates of Vero-H cells, stable transformants of Vero cells expressing  $\sim 20$  times more proHB-EGF on the cell surface (Goishi, K., S. Higashiyama, M. Klagsbrun, N. Nakano, T. Umata, M. Ishikawa,



**Figure 4.** Coprecipitation of proHB-EGF, DRAP27/CD9 and integrin  $\alpha 3 \beta 1$ . (A) Biotinylated Vero-H cells were lysed with 10 mM CHAPS solution. Lysates were immunoprecipitated with irrelevant mouse IgG (lane 1), rabbit anti-HB-EGF antibody M6 (lane 2), anti-DRAP27/CD9 mAb 007 (lane 3), or anti- $\beta 1$  K20 antibody (lane 4) followed by protein A-Sepharose 4B. Precipitated materials and the total cell lysate (lane 5) were subjected to SDS-PAGE and Western blotting. Protein bands were detected with streptavidin-HRP. Bars on the right margin represent the molecular mass markers in kilodaltons. (B) Vero-H cell lysate was immunoprecipitated with irrelevant mAb (lane 1), anti-HB-EGF M6 (lane 2), anti-CD9 BU16 (lane 3), or anti- $\beta 1$  K20 antibody (lane 4). Precipitated material and total cell lysate (lane 5) were subjected to SDS-PAGE (15% gel), and polypeptides were transferred to a membrane. The membrane was probed with rabbit anti-HB-EGF antiserum H6 and HRP-conjugated donkey anti-rabbit IgG. (C) Vero-H cell lysate was precipitated with (lane 2) or without DT (lane 1), followed by Sepharose 4B-conjugated anti-DT mAb. The same cell lysate was immunoprecipitated with irrelevant mouse antibody (lane 3), mouse anti-CD9 mAb BU16 (lane 4), or mouse anti- $\alpha 3$  mAb (lane 5) followed by anti-mouse IgG-Sepharose 4B. Precipitated material and total lysate (lane 6) were subjected to SDS-PAGE, Western blotting, and probed with rabbit anti- $\alpha 3$  antibody and HRP-conjugated donkey anti-rabbit IgG.

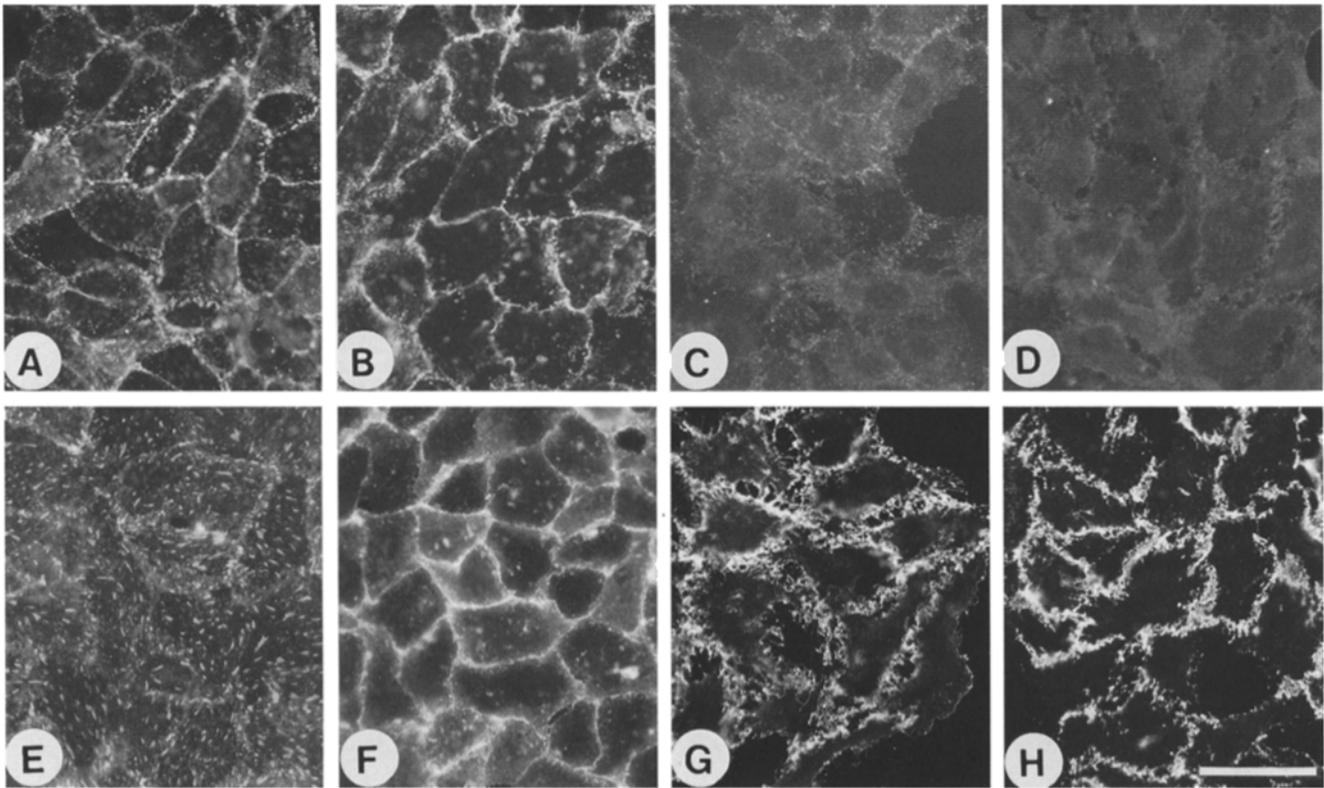
E. Mekada, and N. Taniguchi, manuscript in preparation). Since HB-EGF is much more efficiently labeled by the biotinylation method than by iodination, lysate from surface-biotinylated Vero-H cells was precipitated with anti-HB-EGF antibody, anti-DRAP27/CD9 mAb, or anti-integrin  $\beta 1$  mAb, and protein A-Sepharose 4B.

Immunoprecipitates were analyzed by SDS-PAGE and Western blotting. When cell lysate was precipitated with anti-DRAP27/CD9 mAb, several bands of 150, 115, 50, 27, and 27–20 kD were observed (Fig. 4 A, lane 3). The 27-kD band was confirmed as DRAP27/CD9 itself by Western blotting (data not shown). The 150- and 115-kD bands were integrin  $\alpha 3 \beta 1$ , and the bands ranging from 20 to 27 kD were proHB-EGF. The 50-kD band has not been identified. When cell lysate was precipitated with anti-HB-EGF antibody, the 150- and 115-kD integrin bands were also coprecipitated in addition to DRAP27/CD9 (27 kD) and proHB-EGF itself (Fig. 4 A, lane 2). Immunoblotting confirmed that the 115-kD band is  $\beta 1$  (data not shown). Conversely, when cell lysate was precipitated with anti-integrin  $\beta 1$  antibody, 180-, 150-, 115-, 50-, 27-, and 20–27-kD bands were observed (Fig. 4 A, lane 4). The 180-, 150-, and 115-kD bands were confirmed to be integrin  $\alpha$  and  $\beta$  subunits, and the faint but significant 20–27-kD bands proHB-EGF by immunoblotting (Fig. 4 B). As proHB-EGF is known to serve as a receptor for DT, we performed immunoprecipitation of Vero-H cell lysate with DT and immo-

bilized anti-DT antibodies and Western blotting studies with anti-HB-EGF and anti-integrin  $\alpha 3$ . As a result, DT precipitated a 150-kD band of integrin  $\alpha 3$  (Fig. 4 C, lane 2) as well as 20–27-kD bands of proHB-EGF (data not shown), while no bands are observed in the absence of DT (Fig. 4, lane 1). These results indicate that at least some of the integrin  $\alpha 3 \beta 1$  is in a complex with proHB-EGF, possibly mediated by association with DRAP27/CD9.

#### Colocalization of DRAP27/CD9 with Integrin $\alpha 3 \beta 1$ at Cell-Cell Contact Sites

We next examined where in the cell integrin  $\alpha 3 \beta 1$  associates with DRAP27/CD9 and proHB-EGF. First, localization of DRAP27/CD9 and integrins in Vero cells was analyzed by indirect immunofluorescence staining. Fig. 5 shows a typical immunofluorescence pattern from Vero cells stained with anti-DRAP27/CD9 antibody or one of the antibodies to the integrin subunit  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha v$ , or  $\beta 1$  from a conventional fluorescence microscope. Anti-DRAP27/CD9 produced intense staining at cell-cell boundaries (Fig. 5 A). Weak punctate fluorescence was also observed at the apical area. With sparsely grown cells, fine needlelike processes attaching neighboring cells were stained with anti-DRAP27/CD9 (see Fig. 6 A). These needlelike processes at cell-cell contact sites were especially prominent when nonfixed cells were stained (Fig. 5 G).



**Figure 5.** Localization of DRAP27/CD9 and integrins. Vero cells were fixed, stained with anti-CD9 BU16 (A), anti- $\alpha 3$  (B), anti- $\alpha 2$  (C), anti- $\alpha 5$  (D), anti- $\alpha v$  (E), or anti- $\beta 1$  4B4 antibody (F), and observed under a fluorescence microscope. Exposure times for photographs were adjusted depending on the intensity of fluorescence. G and H show patterns from staining of nonfixed cells in semiconfluent culture with anti-DRAP27/CD9 and anti- $\alpha 3$  antibodies, respectively. Bar, 50  $\mu\text{m}$ .

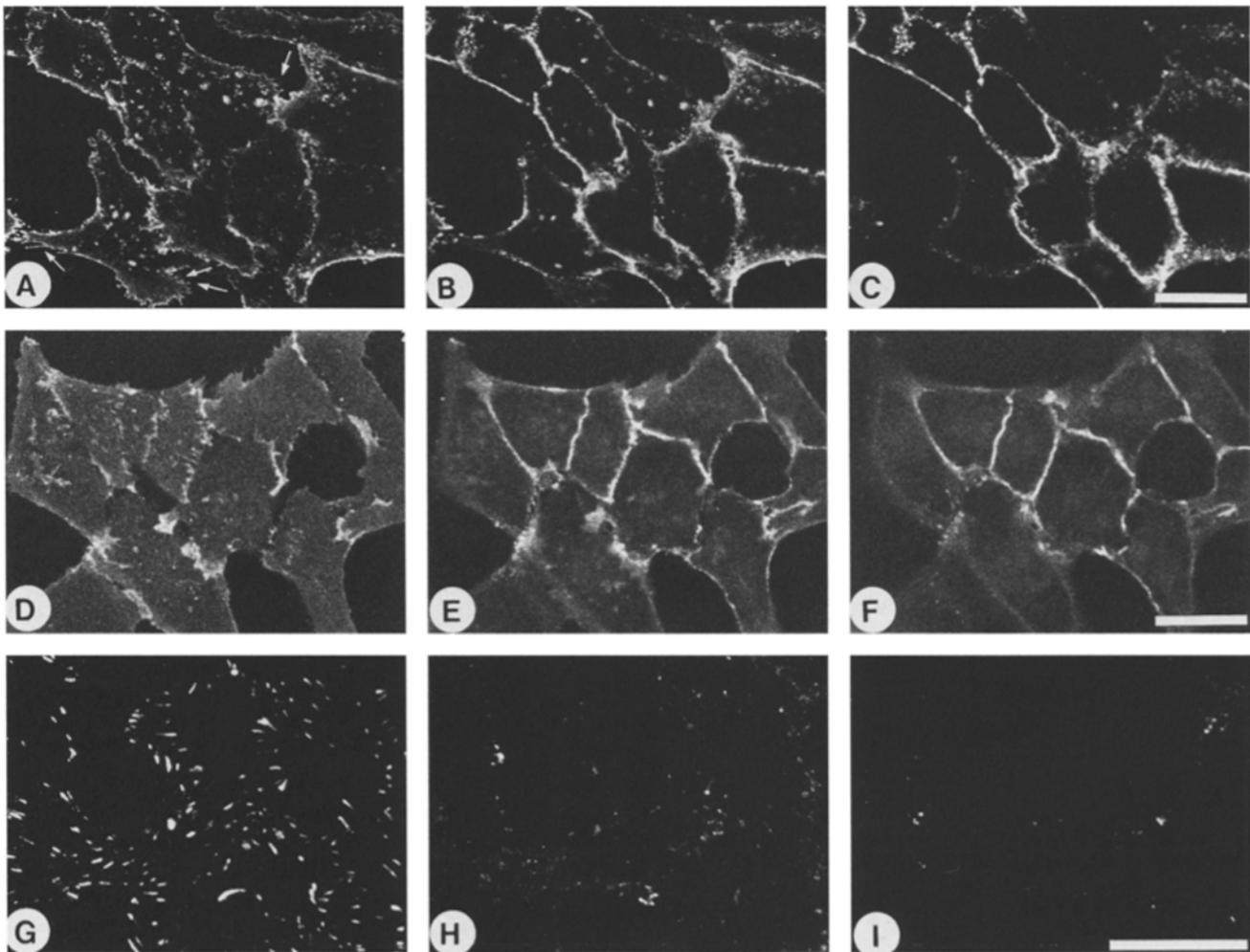
DRAP27/CD9 was also observed at cell surfaces not in contact with other cells and especially at microvilli-like structures projecting from the cell surface (see Fig. 6 A, arrows).

Integrin  $\alpha 3$  was also localized at cell-cell boundaries of Vero cells (Fig. 5, B and H), consistent with previous studies for other types of cells (Larjava et al., 1990; Symington et al., 1993). The staining pattern is quite similar to that of DRAP27/CD9. Integrin  $\alpha 3$  was also observed in the microvilli-like structures. The only difference in staining patterns of DRAP27/CD9 and integrin  $\alpha 3$  was integrin  $\alpha 3$  was not observed at free surfaces (see Fig. 6 D). Integrin  $\alpha 2$ ,  $\alpha 5$ , and  $\alpha v$  were observed mainly at the basement membrane and could be scarcely detected at cell-cell boundaries on account of their weak fluorescence (Fig. 5, C–E). Weak fluorescence was observed at the basement membrane after staining for DRAP27/CD9, but the pattern was quite different from those of integrin  $\alpha 2$ ,  $\alpha 5$ , and  $\alpha v$ . Integrin  $\beta 1$  forms heterodimers with  $\alpha 2$ ,  $\alpha 3$ , or  $\alpha 5$ , and in fact this integrin was observed both at cell-cell boundaries and at the basement membrane (Fig. 5 F).

Observation with a confocal laser microscope showed that it is  $\sim 10 \mu\text{m}$  from the basement membrane to the apical tip of Vero cells. We obtained sectioned images of the same field by changing the focus from the basement membrane to apical tip at 1.2  $\mu\text{m}$  intervals. Fig. 6 displays optically sectioned images of Vero cells. When the focus was adjusted at basement membrane ( $z = 1$ ), DRAP27/CD9 was

observed mainly at the cell-cell boundary and free surfaces (Fig. 6 A). Where one cell appears to overlap another cell, DRAP27/CD9 is present in planar cell processes. At this height,  $\alpha 3$  is also localized at cell-cell boundaries with a pattern similar to DRAP27/CD9, except that there is little or no staining at free surfaces (Fig. 6 D). When the focus was adjusted to 1.2 ( $z = 2$ ) or 2.4 ( $z = 3$ )  $\mu\text{m}$  above the basement membrane, both DRAP27/CD9 (Fig. 6, B and C) and integrin  $\alpha 3$  (Fig. 6, E and F) were observed in continuous lines at cell-cell boundaries. Similar patterns at positions  $z = 1$  through  $z = 3$  were seen in cells stained with anti-integrin  $\beta 1$  (data not shown). In contrast to integrin  $\alpha 3$  and DRAP27/CD9, integrin  $\alpha v$  is observed almost exclusively in arrowhead-like structures at the level of the basement membrane ( $z = 1$ ) (Fig. 6, G–I).

The staining patterns for DRAP27/CD9 and integrin  $\alpha 3$  indicate that these molecules colocalize at cell-cell boundaries. To verify the colocalization, double staining of DRAP27/CD9 and integrin  $\alpha 3$  was performed. Fig. 7, G–J shows the specificity of the double staining. When anti- $\alpha 3$  mAb was omitted in the double staining procedure, only DRAP27/CD9 (red) was observed, while when anti-CD9 mAb was omitted, only integrin  $\alpha 3$  protein (green) was observed. Fig. 7, A and B, show DRAP27/CD9 and  $\alpha 3$  at a lower height (between  $z = 1$  and  $z = 2$ ). Superposition of the two images (Fig. 7 C) shows that the broader areas of staining of cell processes are well overlapped (arrowheads). The cell-cell boundary seen at position  $z = 3$  also largely overlapped (Fig.



**Figure 6.** Confocal images of cells stained for DRAP27/CD9, integrin  $\alpha 3$  and  $\alpha v$ . Vero cells were fixed and stained with anti-CD9 mAb BU16 (A–C), anti- $\alpha 3$  mAb (D–F), or anti- $\alpha v$  mAb (G–I) and observed with a confocal laser microscope. The same field of cells was observed from basement membrane to apical tip by adjusting different foci: basement membrane (A, D, and G); 1.2  $\mu\text{m}$  above basement membrane (B, E, and H); or 2.4  $\mu\text{m}$  above basement membrane (C, F, and I). Arrows show microvilli-like structures projecting from cell surface. Bars, 100  $\mu\text{m}$ .

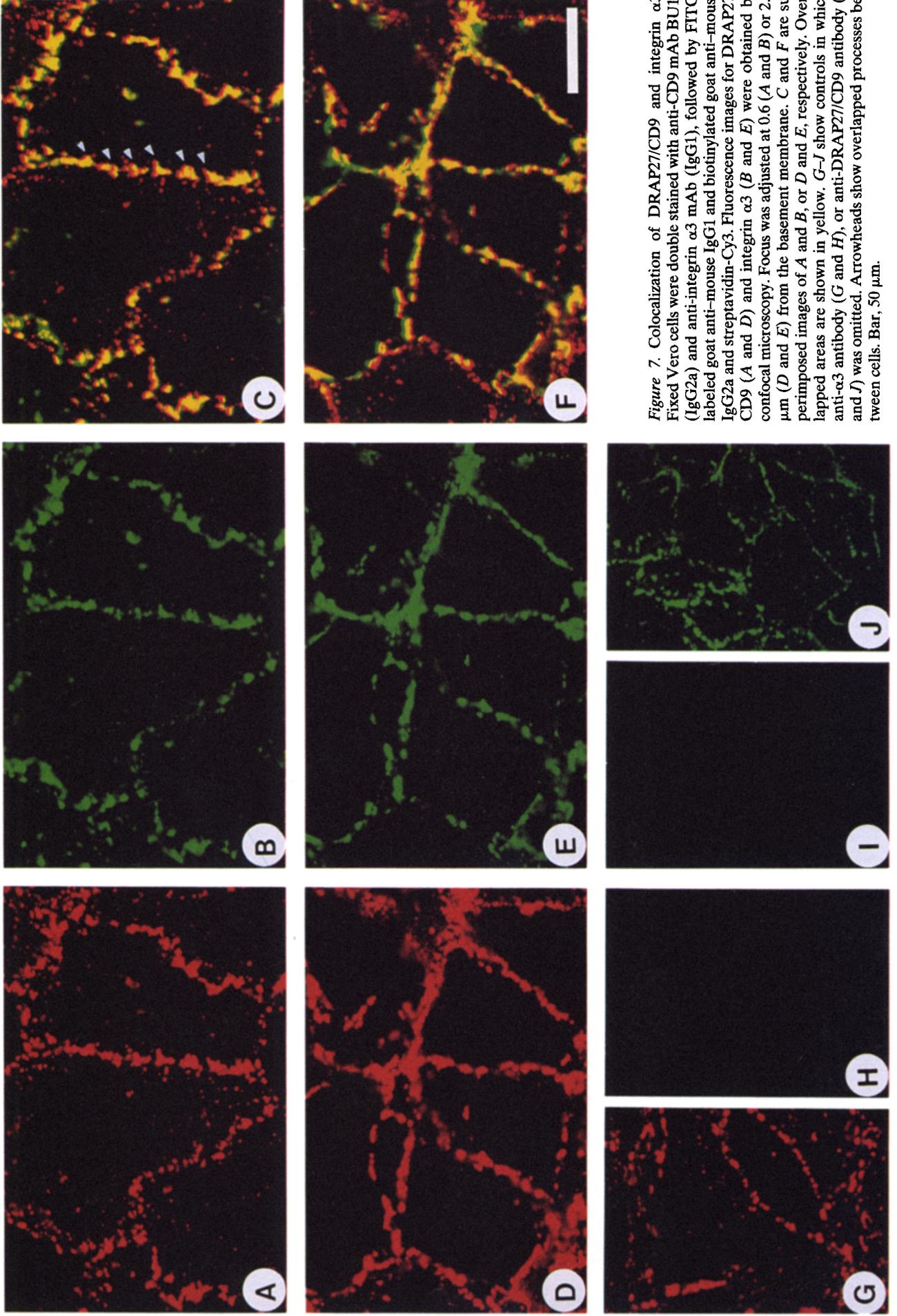
7, D–F). These results indicate that DRAP27/CD9 colocalizes with integrin  $\alpha 3\beta 1$  at cell–cell contact sites.

#### **Colocalization of proHB-EGF and Integrin $\alpha 3\beta 1$**

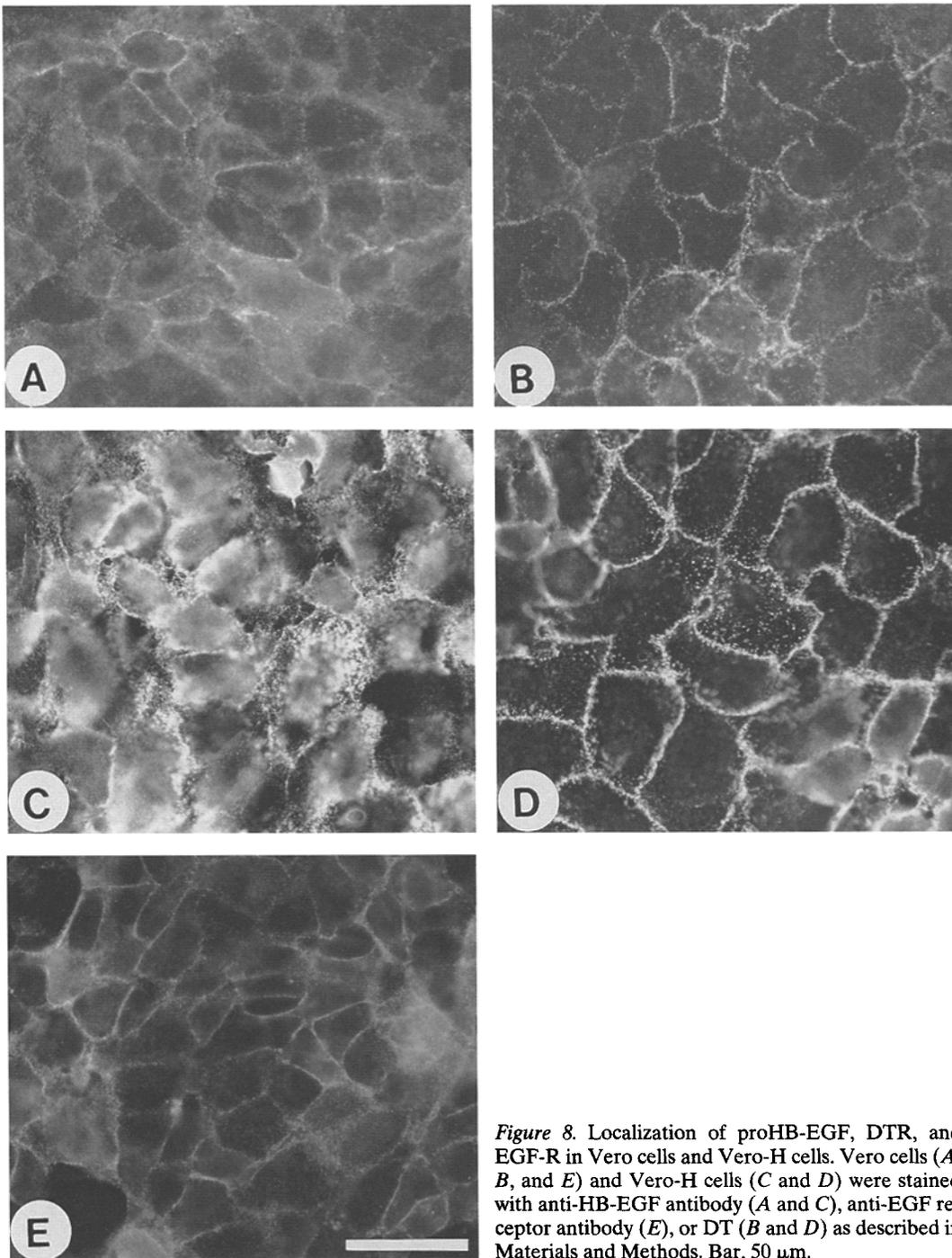
Localization of proHB-EGF was also analyzed by immunofluorescence. Fig. 8 A shows the distribution of proHB-EGF in Vero cells stained with anti-HB-EGF antibody H6. Although the fluorescence was very faint, specific fluorescence of proHB-EGF was observed mainly at cell–cell boundaries as expected. Under similar staining conditions, however, Vero-H cells showed intense, punctate fluorescence all around the cell surface in addition to intense fluorescence at cell–cell contact sites (Fig. 8 C). Observation with a confocal microscope showed that proHB-EGF in Vero-H cells is present from the basement membrane to the apical tip (data not shown). This broad distribution of proHB-EGF may be due to the overexpression of the protein.

We also examined the distribution of proHB-EGF by

staining with DT and anti-DT antibody. A recent study shows that DT binds to the EGF domain of HB-EGF but does not bind to other EGF family growth factors (Mitsumura et al., 1995). Furthermore, a series of experiments on the interaction of proHB-EGF and DRAP27/CD9 suggested that DT preferentially binds to the proHB-EGF–DRAP27/CD9 complex (Iwamoto et al., 1991, 1994; Mitsumura et al., 1992). Fig. 8 B shows that the staining pattern with DT is almost identical to that seen with anti-HB-EGF antibody, indicating localization of the proHB-EGF–DRAP27/CD9 complex at cell–cell boundaries. When CRM107, a mutant protein of DT without receptor-binding activity, was used as a control for nonspecific staining, no fluorescence was observed (data not shown). Compared with anti-HB-EGF, DT stained Vero-H cells preferentially at cell–cell contact sites (Fig. 8 D). These results indicate that the distribution of the proHB-EGF–DRAP27/CD9 complex in Vero-H cells is more restricted than that of proHB-EGF itself in these cells. To examine where the receptor for HB-EGF, that is, EGF receptor, is localized,



**Figure 7.** Colocalization of DRAP27/CD9 and integrin  $\alpha 3$ . Fixed Vero cells were double stained with anti-CD9 mAb BUI6 (IgG2a) and anti-integrin  $\alpha 3$  mAb (IgG1), followed by FITC-labeled goat anti-mouse IgG1 and biotinylated goat anti-mouse IgG2a and streptavidin-Cy3. Fluorescence images for DRAP27/CD9 (A and D) and integrin  $\alpha 3$  (B and E) were obtained by confocal microscopy. Focus was adjusted at 0.6 (A and B) or 2.4  $\mu\text{m}$  (D and E) from the basement membrane. C and F are superimposed images of A and B, or D and E, respectively. Overlapped areas are shown in yellow. G-J show controls in which anti- $\alpha 3$  antibody (G and H), or anti-DRAP27/CD9 antibody (I and J) was omitted. Arrowheads show overlapped processes between cells. Bar, 50  $\mu\text{m}$ .



**Figure 8.** Localization of proHB-EGF, DTR, and EGF-R in Vero cells and Vero-H cells. Vero cells (A, B, and E) and Vero-H cells (C and D) were stained with anti-HB-EGF antibody (A and C), anti-EGF receptor antibody (E), or DT (B and D) as described in Materials and Methods. Bar, 50  $\mu$ m.

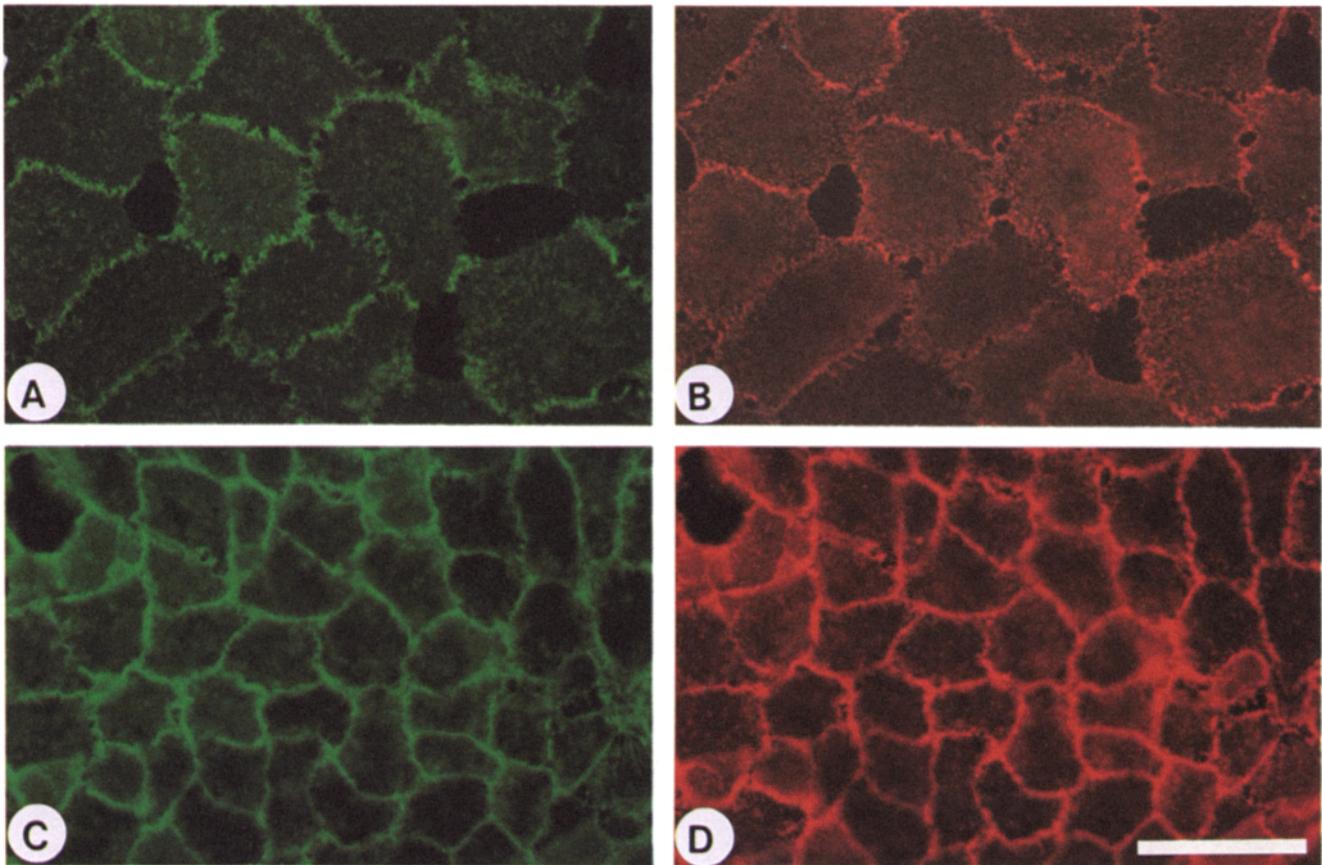
we stained Vero cells with anti-EGF receptor antibody. It was also predominantly localized at cell-cell contact sites at least when cells were confluent (Fig. 8 E), indicating that membrane anchored HB-EGF can interact with EGF receptor of adjacent cells.

Double staining of Vero-H cells with DT and anti-integrin  $\alpha 3$  mAb is shown in Fig. 9. The patterns are quite similar for proHB-EGF and integrin  $\alpha 3$ . Although staining of intercellular process-like structures is more prominent in Vero-H cells than Vero cells when focus was adjusted at the basement membrane, the patterns stained with DT and anti- $\alpha 3$  mAb overlapped well (Fig. 9, A and B). Cell-

cell borders stained with anti- $\alpha 3$  as a continuous line (Fig. 9 C) were also stained with DT (Fig. 9 D). As discussed above, DT preferentially labels proHB-EGF complexed with DRAP27/CD9. Thus, we conclude that proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$  are colocalized at cell-cell contact sites.

#### ***Colocalization of proHB-EGF, DRAP27/CD9, and Integrin $\alpha 3$ with $\alpha$ -Catenin and Vinculin***

To investigate the significance of the association and colocalization of proHB-EGF, DRAP27/CD9, and integrin



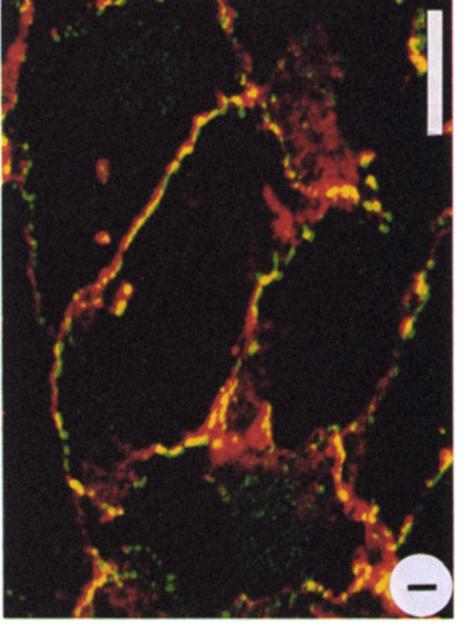
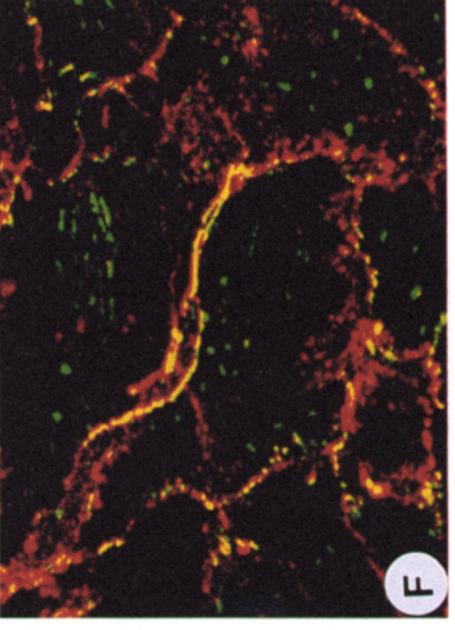
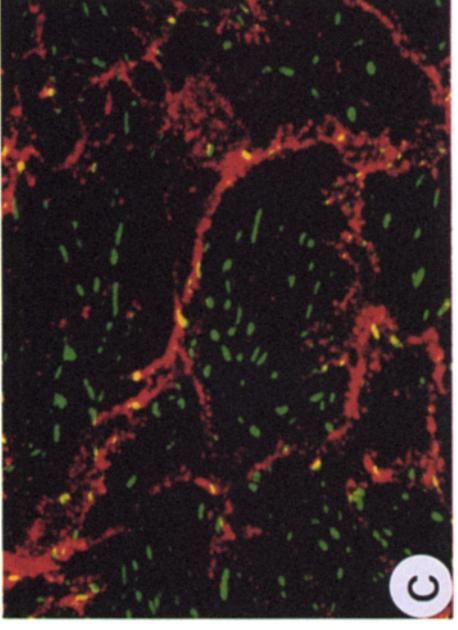
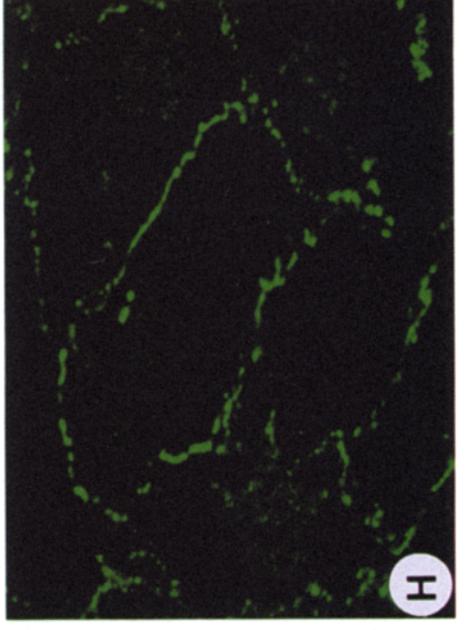
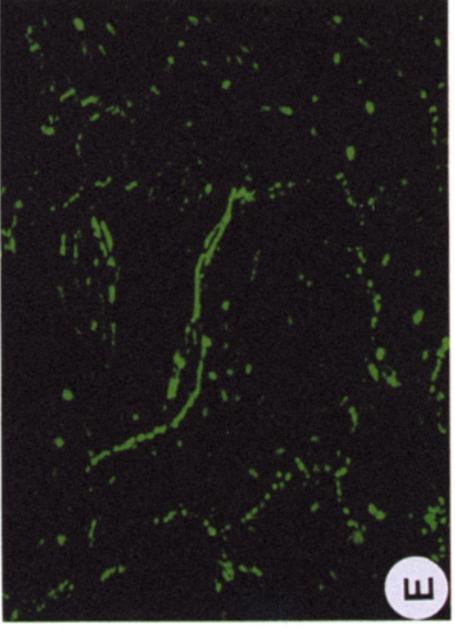
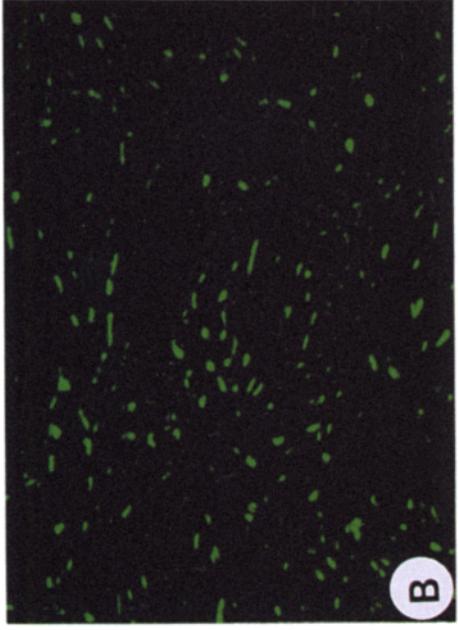
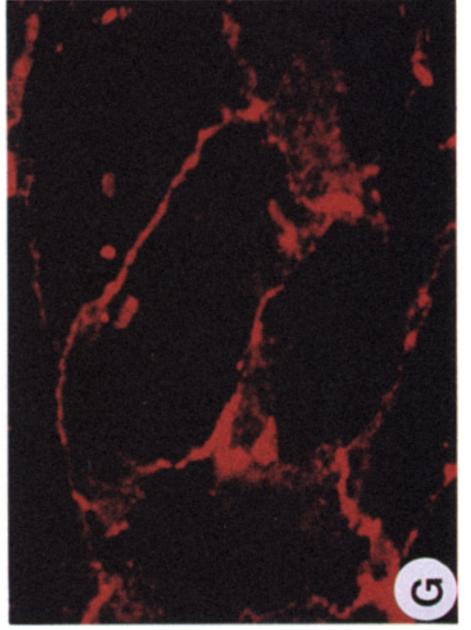
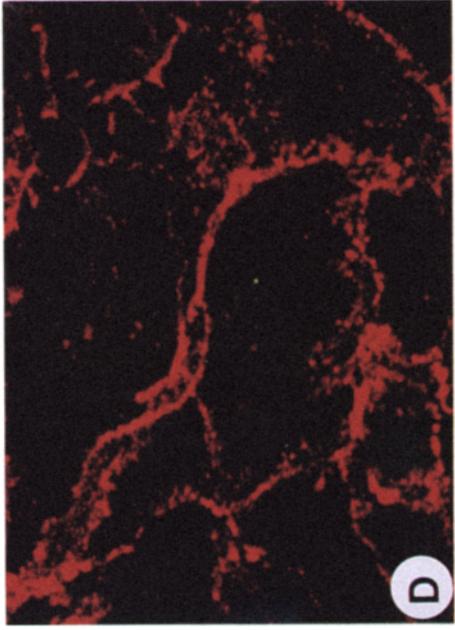
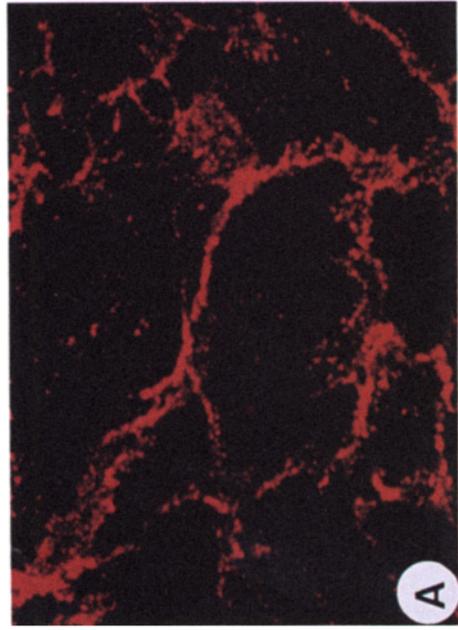
**Figure 9.** Colocalization of integrin  $\alpha 3\beta 1$  and proHB-EGF stained with DT. Vero-H cells were double stained with DT and anti-integrin  $\alpha 3$  antibody and observed under a fluorescence microscope. *A* and *C* show integrin  $\alpha 3$  and *B* and *D* show proHB-EGF stained with DT. Focus was adjusted at basement membrane (*A* and *B*), or lateral cell-cell contact sites (*C* and *D*). Bar, 50  $\mu\text{m}$ .

$\alpha 3\beta 1$ , we compared the location of these proteins with other proteins known to be involved in cell-cell and cell-substratum adhesions. Fig. 10 shows images from double staining for DRAP27/CD9 and either vinculin or  $\alpha$ -catenin. Vinculin is known to be localized at actin-concentrated focal contact sites, termed focal plaque and adherence junction in epithelial cells (Geiger, 1979; Volberg et al., 1986). At the basement membrane ( $z = 1$ ), vinculin was concentrated at focal plaques, which is a pattern quite different from that of DRAP27/CD9 (Fig. 10, *A-C*). However, at the position  $z = 2$ , vinculin was localized mainly at cell-cell boundaries, where it largely overlaps with DRAP27/CD9 (Fig. 10, *D-F*).  $\alpha$ -Catenin associates with cytoplasmic tail of cadherins and is localized at adherence junctions (Takeichi, 1991; Ozawa et al., 1989). In Vero cells,  $\alpha$ -catenin was mainly observed at positions  $z = 2$  to  $z = 4$ , and its pattern overlapped that of DRAP27/CD9, as shown in Fig. 10, *G-I*. Similar colocalization was observed with integrin  $\alpha 3$  and  $\alpha$ -catenin (data not shown). Although we have not directly shown the colocalization of proHB-EGF with either  $\alpha$ -catenin or vinculin, proHB-EGF was colocalized with integrin  $\alpha 3$  and DRAP27/CD9 as shown in Fig. 9. These results indicate that proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$  are localized at cell-cell contact sites where  $\alpha$ -catenin and vinculin are localized. Although it is not clear whether Vero cells form

typical junctional structures, results suggest that proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$  colocalize at cell-cell contact sites around adherence junctions. These results support the idea that the proHB-EGF complex, including CD9 and integrin  $\alpha 3\beta 1$ , interacts with EGF receptors of adjacent cells at cell-cell contact sites.

### Discussion

From the coprecipitation, cross-linking, and immunofluorescence results described here, we conclude that (a) DRAP27/CD9 associates with integrin  $\alpha 3\beta 1$  in Vero cells and other human cell lines; (b) proHB-EGF associates with DRAP27/CD9 and integrin  $\alpha 3\beta 1$  in Vero-H cells; and (c) all of these proteins, that is, proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$ , are localized at cell-cell contact sites. However, the number of proHB-EGF molecules on the surface of Vero cells is much lower than the number of DRAP27/CD9 molecules, while the amount of integrin  $\alpha 3\beta 1$  appears to be similar to or greater than that of DRAP27/CD9. While a large fraction of  $\alpha 3\beta 1$  associates with DRAP27/CD9, much smaller fractions of  $\alpha 3\beta 1$  and DRAP27/CD9 are likely to be associated with proHB-EGF in Vero cells. In Vero-H cells, which express at least 20 times more proHB-EGF molecules on the cell surface, some of the proHB-EGF molecules on the cell surface are apparently



not associated with DRAP27/CD9 and  $\alpha 3\beta 1$ . Staining with anti-HB-EGF antibody revealed that proHB-EGF is present all around the cell surface in Vero-H cells, while DRAP27/CD9 and integrin  $\alpha 3\beta 1$  were localized mainly at cell-cell boundaries. Since proHB-EGF is localized at cell-cell contact sites in Vero cells, the broader distribution of proHB-EGF seen in Vero-H cells may be due to the overexpression of this protein. However, it is also possible that some of the proHB-EGF molecules in Vero cells also have a more dispersed distribution, but the amount is below the limit of detection.

Does DRAP27/CD9 mediate the association of proHB-EGF with integrin  $\alpha 3\beta 1$ ? These studies do not rule out the possibility that other factors are responsible for the association of proHB-EGF and  $\beta 1$  integrin. For example, proHB-EGF possibly binds heparan sulfate proteoglycans with its heparin-binding domain. Fibronectin is known to bind heparan sulphate proteoglycan and possibly integrin  $\alpha 3\beta 1$  by different domains (Ruoslahti, 1988). Therefore, fibronectin or fibronectin-like molecules may indirectly cross-link proHB-EGF and  $\alpha 3\beta 1$ . However, immunoprecipitation experiments showed that integrin  $\alpha 3\beta 1$  is coprecipitated much more efficiently by anti-DRAP27 antibody than by anti-HB-EGF, so the simplest explanation is that association of proHB-EGF with integrin  $\alpha 3\beta 1$  is mediated by DRAP27/CD9.

#### **Significance of the Association of Integrin $\alpha 3\beta 1$ with DRAP27/CD9**

The existence of membrane proteins that associate with integrins has been suggested because the cytoplasmic domains of most integrins may be too short to transduce outside adhesion signals to the inside of cells (Juliano and Haskill, 1993). However, until now only one membrane protein had been characterized as an integrin-associating protein. IAP, a 50-kD membrane protein with five membrane-spanning domains and an immunoglobulin-like domain, is known to associate with leukocyte response integrin and integrin  $\alpha v\beta 3$  (Brown et al., 1990; Lindberg et al., 1993). We demonstrated here that DRAP27/CD9 associates with integrin  $\alpha 3\beta 1$ , the first example of a  $\beta 1$  integrin-associated membrane protein.

An explanation of the biological significance of the association of DRAP27/CD9 and integrin  $\alpha 3\beta 1$  should take into account the observation that DRAP27/CD9 and integrin  $\alpha 3\beta 1$  colocalize at cell-cell contact sites. Integrin  $\alpha 3\beta 1$  possibly functions as a receptor for a variety of ECM proteins including collagen, laminin, fibronectin (Takada et al., 1991), and the newly identified ECM proteins epiligrin (Carter et al., 1991) and kalinin (Rousselle and Aumailley, 1994). A feature that distinguishes  $\alpha 3\beta 1$  from other  $\beta 1$ -containing integrins is that it is localized at cell-cell contact sites in epithelial cells (Carter et al., 1990). There have been a number of reports of the involvement of  $\beta 1$  integrin in intercellular adhesion. For example, anti-

$\alpha 3$  and anti- $\beta 1$  antibodies inhibit intercellular adhesion and disrupt cell-cell contact (Carter et al., 1990; Larjava et al., 1990). Another anti- $\alpha 3$  mAb, P1B5, stimulated intercellular adhesion of a human keratinocyte-derived cell line and also induced temperature-dependent homotypic cell aggregation of  $\alpha 3$ -transfected cell lines (Symington et al., 1993; Weitzman et al., 1993). Homotypic interaction of integrin  $\alpha 3\beta 1$  (Sriramarao et al., 1993) and interaction of  $\alpha 3\beta 1$  with  $\alpha 2\beta 1$  (Symington et al., 1993) suggest that  $\alpha 3\beta 1$  might mediate intercellular adhesion. Thus we suggest that CD9, by its association with  $\alpha 3\beta 1$ , is implicated in cell-cell interaction.

Interestingly,  $\alpha 3\beta 1$  expression has been shown to increase on oncogenic transformation, suggesting that  $\alpha 3\beta 1$  may contribute to tumorigenicity or metastatic cell motility. Analysis of clinical specimens showed that expression levels of integrin  $\alpha 3\beta 1$  are related to invasiveness of malignant melanoma (Natali et al., 1993). CD9 is also linked to tumorigenicity and cell motility; motility-related protein (MRP-1) is identical to CD9 (Miyake et al., 1991). Anti-MRP-1 mAb inhibited cell motility of several cancer cell lines. Other studies showed that CD9 expression is inversely related to metastatic potential of melanoma (Si and Hersey, 1993), and transfection of CD9 cDNA causes suppression of cell motility and metastasis of cancer cell lines (Ikeyama et al., 1993). Thus, both integrin  $\alpha 3\beta 1$  and CD9 are implicated in cell motility and metastasis of tumor cells. We suggest that CD9 and  $\alpha 3\beta 1$  are included in an effector complex that is involved in intercellular adhesion and cell motility, and that CD9 may stimulate or suppress the function of integrin  $\alpha 3\beta 1$ .

Although we could not detect the association of DRAP27/CD9 with integrins other than  $\alpha 3\beta 1$  in Vero cell and A431 cell lysates, slight coprecipitation of  $\alpha 2$  and  $\alpha 5$  was observed from MG63 cell lysates. Previous reports also suggested interaction of CD9 and other kinds of integrins: (a) incubation of platelets with anti-CD9 antibodies induces the association of CD9 with platelet integrin gpIIb/IIIa (Slupsky et al., 1989); (b) anti-CD9 antibody and anti-integrin  $\alpha 4$  antibody induce homotypic cellular aggregation by similar effector mechanisms in acute lymphocytic leukemia cell lines not expressing the  $\alpha 3$  subunit (Letarte et al., 1993); and (c) anti-CD9 antibody promotes the integrin-mediated adhesion of pre-B cell to bone marrow stroma cells (Masellis-Smith and Shaw, 1994). Therefore, CD9 may interact with other types of integrins in other systems.

#### **Significance of the Association of Integrin $\alpha 3\beta 1$ with proHB-EGF-DRAP27/CD9 Complex**

Membrane-anchored growth factors can exert mitogenic activity by two modes: the paracrine mode, in which a cleaved soluble form is released, and the juxtacrine mode, in which the membrane-anchored form is active (Massigné and Pandiella, 1993). The latter can provide mito-

**Figure 10.** Colocalization of DRAP27/CD9 with vinculin or  $\alpha$ -catenin. Fixed Vero cells were double stained with anti-CD9 mAb BU16 (IgG2a) and anti-vinculin mAb (IgG1) or rat anti- $\alpha$ -catenin, followed by FITC-labeled goat anti-mouse IgG2a and biotinylated goat anti-mouse IgG1 or goat anti-rat IgG and streptavidin-Cy3. Fluorescent images for DRAP27/CD9 (A, D, and G), vinculin (B and E) and  $\alpha$ -catenin (H) were obtained using a confocal microscope. Overlapping areas between A and B, D and E, or G and H are shown in yellow in C, F, and I, respectively. Focus was adjusted at the basement membrane (A-C), or at 1.2  $\mu$ m above the basement membrane (D-I). Bar, 50  $\mu$ m.

genic stimulation specifically to cells in direct contact even if other nearby cells are expressing the same receptor molecules. We have recently shown that membrane-anchored HB-EGF stimulates cell growth in juxtacrine manner to acceptor cells expressing EGF receptor, and that DRAP27/CD9 greatly upregulates the juxtacrine mitogenic activity of proHB-EGF (Higashiyama et al., 1995). DRAP27 has no stimulatory effect on the mitogenic activity of soluble HB-EGF, indicating that DRAP27/CD9 must play a role in the regulation of juxtacrine activity of membrane-anchored HB-EGF. However, the acceptor cells used for the experiments were suspension cells, not adherent cells; thus, the conditions in which the juxtacrine mitogenic activity was assayed might be somewhat artificial. In this study we showed that proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$  are localized at cell-cell contact sites near adherence junctions. The EGF receptor is also known to be localized around adherence junctions (Fukuyama and Shimizu, 1991), and we confirmed the localization of EGF receptors at the cell-cell boundary in Vero cells. Thus, all components of the juxtacrine signal mechanism are located in the same region, suggesting that membrane-anchored HB-EGF on one adherent cell and EGF receptor on a neighboring adherent cell can interact.

While it is not certain whether  $\alpha 3\beta 1$ -associated proHB-EGF-DRAP27/CD9 complex is mitogenically active, several possible roles for the complex can be considered: (a)  $\alpha 3\beta 1$  may sustain the interaction of proHB-EGF-DRAP27/CD9 complex with EGF receptor. Since membrane-anchored growth factors require direct contact with the receptors to transmit a signal, the involvement of cell adhesion molecules in this process would be reasonable. (b) DRAP27/CD9 forms a complex with proHB-EGF and upregulates its juxtacrine mitogenic activity. Integrin  $\alpha 3\beta 1$  may regulate the mitogenic activity of proHB-EGF through its interaction with DRAP27/CD9, rather than as an adhesion molecule. In this context it should be noted that colocalization of proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$  at cell-cell boundaries was observed in confluent cell cultures, in which cell growth of Vero cells and Vero-H cells has ceased or slowed greatly compared with the semiconfluent stage. Therefore,  $\alpha 3\beta 1$  may suppress the mitogenic activity of membrane-anchored HB-EGF rather than stimulate it.

We should also consider that proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$  are colocalized with  $\alpha$ -catenin and vinculin at cell-cell contact sites.  $\alpha$ -Catenin and vinculin are known to be localized at adherence junctions, one of the junctional structures where cadherin plays a central role in cell-cell adhesion (Takeichi, 1991). A recent report showed that activation of the E-cadherin system in PC9 lung carcinoma cells retarded cell growth, suggesting that the cadherin system is involved in regulation of cell proliferation as well (Watabe et al., 1994). Although it is not clear whether Vero cells form typical junctional structures, our results indicate that proHB-EGF, DRAP27/CD9, and integrin  $\alpha 3\beta 1$  are colocalized with adherence junction proteins. It appears that cell adhesion molecules and some growth control factors are concentrated around adherence junctions, and these molecules form a network that controls cell proliferation and cell motility in a cell adhesion-dependent process (Tsukita et al., 1991; Behrens et al.,

1993; Hamaguchi et al., 1993). This process would be dynamically controlled by the adhesion molecules and growth factors. Our results indicate the possibility of direct cooperation of membrane-anchored growth factors and adhesion molecules in cell-cell interactions between adjacent cells, and that DRAP27/CD9 may play a role in this cooperation.

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