

# Mapping the Homotypic Binding Sites in CD31 and the Role of CD31 Adhesion in the Formation of Interendothelial Cell Contacts

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**Abstract.** CD31 is a member of the immunoglobulin superfamily consisting of six Ig-related domains. It is constitutively expressed by platelets, monocytes, and some lymphocytes, but at tenfold higher levels on vascular endothelial cells. CD31 has both homotypic and heterotypic adhesive properties. We have mapped the homotypic binding sites using a deletion series of CD31-Fc chimeras and a panel of anti-CD31 monoclonal antibodies. An extensive surface of CD31 is involved in homotypic binding with domains 2 and 3 and domains 5 and 6 playing key roles. A model consistent with the experimental data is that CD31 on one cell binds to CD31 on an apposing cell in an anti-parallel interdigitating mode requiring full alignment of the six domains of each molecule. In addition to establishing intercellular homotypic contacts, CD31 binding leads to augmented adhesion via  $\beta 1$  integrins. The positive cooperation between CD31 and  $\beta 1$  inte-

grins can occur in heterologous primate cells (COS cells). The interaction is specific to both CD31 and  $\beta 1$  integrins. Neither intercellular adhesion molecule-1 (ICAM-1)/leukocyte function-associated antigen-1 (LCAM-1) nor neural cell adhesion molecule (NCAM)/NCAM adhesion leads to recruitment of  $\beta 1$  integrin adhesion pathways. Establishment of CD31 contacts have effects on the growth and morphology of endothelial cells. CD31(D1-D6)Fc inhibits the growth of endothelial cells in culture. In addition, papain fragments of anti-CD31 antibodies (Fab fragments) disrupt interendothelial contact formation and monolayer integrity when intercellular contacts are being formed. The same reagents are without effect once these contacts have been established, suggesting that CD31-CD31 interactions are critically important only in the initial phases of intercellular adhesion.

**A** key function of endothelial cells lining the vasculature is their ability to sustain an extensive network of intercellular adhesive contacts to maintain the integrity of the circulatory system. A number of cell adhesion molecules contribute to this function, including integrins and cadherins (Lampugnani et al., 1991, 1992). Members of the immunoglobulin superfamily, such as intercellular adhesion molecule-1 (ICAM-1)<sup>1</sup> and vascular cell adhesion molecule-1 (VCAM-1), play key roles in mediating the adhe-

sion of circulating leukocytes to endothelial cells (Bevilacqua, 1993). The only report of the role of immunoglobulin superfamily (IgSF) members in maintaining interendothelial cell contacts, comes from work on endoCAM, the bovine homologue of CD31 (Albelda et al., 1990). Polyclonal anti-endoCAM antibodies disrupt bovine adrenal capillary endothelial contacts. Electron microscopy studies have shown that CD31 is present in interendothelial contact areas, but it is absent from the very tight junction zones (Leach et al., 1993; Ayalon et al., 1994).

CD31 (also known as platelet endothelial cell adhesion molecule-1 [PECAM-1]) is a type I integral membrane protein, and its extracellular domain consists of six Ig C2-related domains (Newman et al., 1990; Simmons et al., 1990; Stockinger et al., 1990). Murine CD31 shares the same domain organization, and the amino acid similarity is 79% overall (Xie et al., 1993). CD31 is a constitutively and abundantly expressed surface glycoprotein on vascular endothelium, with up to 1 million molecules per cell (Newman

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1. *Abbreviations used in this paper:* CEA, carcinoembryonic antigen; Fab, papain fragment of antibody; HUVEC, human umbilical endothelial cells; ICAM, intercellular adhesion molecule; IgSF, immunoglobulin superfamily; LFA-1, leukocyte function-associated antigen-1; NCAM, neural cell adhesion molecule; VCAM-1, vascular cell adhesion molecule-1.

and Albelda, 1992; Muller et al., 1989). It is also expressed on platelets (5–8,000 per cell), monocytes, and neutrophils (100,000 per cell). Approximately 50% of T cells also express CD31, but at much lower levels than on myeloid cells (Stockinger et al., 1992; Bird et al., 1993).

CD31 has both homo- and heterotypic adhesive properties (Albelda et al., 1991; Muller et al., 1992; DeLisser et al., 1993; Watt et al., 1993). The heterotypic binding site is located in domain 2, which contains a consensus motif for the recognition of heparan sulphate and has been recently shown to bind glycosaminoglycans via this domain. The domains mediating homotypic adhesion have not been identified.

The high expression levels of CD31 on endothelial cells and its sequestration at sites of intercellular contact strongly suggest that it might play a role in the maintenance of the integrity of the vascular monolayer (Muller et al., 1989; Schimmenti et al., 1992). In addition, the distribution of CD31 in the cell membrane changes as endothelial cells differentiate into capillary-like structures. Moreover, the cytoplasmic tail of CD31 is phosphorylated *de novo* on serine and threonine residues as cells are activated, further pointing to a role in endothelial cell adhesion (Newman et al., 1992; Zehnder et al., 1992). DeLisser et al. (1994) have recently demonstrated a direct role for the cytoplasmic tail in heterotypic binding; deletions of the tail resulted in a change from predominantly heterotypic to homotypic binding.

CD31 is also involved in apparently amplifying the integrin-mediated adhesion of CD8<sup>+</sup> T cells to matrix components (Tanaka et al., 1992). This novel mechanism, involving “cross-talk” between CD31 and  $\beta$ 1, and to a lesser extent  $\beta$ 2 integrins, requires that CD31 is merely dimerized in the cell membrane by single monoclonal antibodies. This effect has also been demonstrated for murine CD31 (Piali et al., 1993). In addition, CD31 may act as a signaling molecule in monocytes since coligation of CD31 with FcR $\gamma$ II leads to induction of proadhesive cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IL-8) (Chen et al., 1994).

CD31 has been shown to play a role in the transendothelial migration of monocytes and neutrophils (Muller et al., 1993). A monoclonal antibody to CD31 inhibited the random migration of monocytes and neutrophils across an endothelial monolayer, as did a recombinant form of CD31 consisting of domains 1–5 and a half of domain 6. However, the expression of CD31 on lymphocytes is not a prerequisite for transendothelial migration (Bird et al., 1993). Two animal model studies have shown that CD31 is important for neutrophil extravasation *in vivo*. Firstly, rabbit polyclonal antibodies raised against human CD31 that cross-react with rat CD31 block accumulation of rat neutrophils into the peritoneal cavity and the alveolar compartment of the lung (Vaporciyan et al., 1993). In addition, this reagent inhibited neutrophil accumulation in human skin grafts in immunodeficient mice. Secondly, in a murine model of acute peritonitis, an antimurine CD31 mAb inhibited leukocyte and particularly neutrophil emigration (Bogen et al., 1994).

To analyze directly the role of CD31 in mediating interendothelial cell interactions, we have generated soluble recombinant forms of CD31 as fusion proteins with human IgG1Fc, and we have used these in a number of structural and functional studies. We confirm that CD31 can mediate homotypic adhesion and extend these studies by showing that homotypic binding involves extensive interdigitation of ap-

posed CD31 molecules and specific engagement of binding sites located in domains 2 and 3 and domains 5 and 6. Furthermore, we have demonstrated positive cooperative interaction between CD31 and  $\beta$ 1 integrin adhesion pathways in heterologous primate cells. Significantly, presentation of CD31-Fc to endothelial cells inhibits cell proliferation. In addition, both CD31-Fc and anti-CD31 polyclonal antibodies dramatically impair the ability of endothelial cells to establish normal intercellular contacts and to form an integral monolayer, but they have no effect once monolayers have formed.

## Materials and Methods

### Cell Culture and Antibodies

Human umbilical vein endothelial cells (HUVEC) were established from freshly isolated full-term umbilical cords and cultured using standard methods (Jaffe et al., 1973). The cell lines U937, H82, COS-1, and COS-7 were obtained from the Imperial Cancer Research Fund (ICRF) cell bank. MIKALL was a gift from Dr. Sue Watt (Medical Research Council, Molecular Haematology Unit, Institute of Molecular Medicine, Oxford). All cells were grown in RPMI/10% FCS or DME/10% FCS. mAbs were as follows: anti-CD31 mAbs 9G11 and 10B8 both IgG1 (Simmons et al., 1990); and British Biotechnology, Oxford, UK); JC70A IgG1 (Parums et al., 1990); HCl/6 IgG1 (Serotec, Kidlington, UK); 5.6E IgG1 (Immunotech, The Binding Site, Birmingham, UK); L133.1 IgG1 (Becton Dickinson, Oxford, UK); WM59 IgG1 (TCS Biologicals, Bucks, UK); CLB/CD31 IgG1 (Monosan, Bucks, UK); anti- $\beta$ 1 integrin mAbs were clone 13 rat IgG2a (Becton Dickinson) and clone 4B4 IgG1 (Coulter Clone, Bucks, UK); anti- $\beta$ 3 integrin mAb was clone RUU-PL 7F12 IgG1 (Becton Dickinson); anti-LFA-1/CD11a integrin mAb MHM24 IgG1 was a gift from Prof. Andrew McMichael (Institute of Molecular Medicine, Oxford). Polyclonal rabbit anti-NCAM antiserum was a gift from Dr. Patrick Doherty (Department of Pathology, United Medical and Dental Schools of Guys' and St. Thomas's Hospitals, London).

### Transient Expression and Immunofluorescence Staining

Individual constructs (10–20  $\mu$ g DNA) were introduced into 10<sup>7</sup> COS cells incubated for 4 h at 37°C with 400  $\mu$ g/ml DEAE-dextran and 100  $\mu$ M chloroquine in serum-free DME followed by a 2-min hypotonic shock in PBS/10% DMSO (Simmons, 1993). For LFA-1 expression, COS cells were transfected with 50  $\mu$ g of both CD11a and CD18. After 48–72 h in culture, the transfected cells were incubated with mAbs for 20 min at 4°C followed by staining with FITC-conjugated goat anti-mouse IgG or IgM antibodies (Sigma Chemical Co., Poole, Dorset, UK) as appropriate. For cytofluorometric analysis, COS transfectants were lifted 48–72 h after transfection in PBS/2 mM EDTA, washed in PBS/0.2% BSA/0.02% azide at 4°C, stained with primary mAbs, washed three times, stained with a 1:200 dilution of FITC-conjugated goat anti-mouse (Sigma), washed three times, fixed in PBS/2% glutaraldehyde, and analyzed on a Becton Dickinson FACScan®. For all assays involving transiently expressed adhesion molecules, the percentage of positive cells was defined by positioning the marker gate such that >2% of the negative control population were deemed positive. All adhesion assays using COS transfectants were normalized with respect to the percentage of COS cells expressing specific constructs.

### Construction of CD31 Deletion Plasmids

A full-length CD31 cDNA clone (CD31(D1-D6) transmembrane [TM]) isolated by transient expression screens of HUVEC cDNA libraries was used as a template for all polymerase chain reaction constructs (Simmons et al., 1990). The PCR strategy for construction of soluble forms was as follows. PCR conditions: four cycles of 94°C, 1 min; 45°C, 2 min; 72°C, 2 min; followed by 30 cycles of 94°C, 30 s; 45°C, 30 s; 72°C, 1.5 min. The primers used were: pCDM8 forward amplification primer plus reverse amplification primers containing the common adaptor sequence 5' GATCAGATCTACT-TACCTGT plus domain specific sequences as follows: domain 1, CAC CCT GGG ACT GGG CAC TCC; domain 2, AGA GAA GGA TTC CGT CAC GGT; domain 3, GAA GGA AGA TTC CAG TTC GGG; domain 4, AAT

CCT GGG CTG GGA GAG CAT; domain 5, CTG GAC CTC ATC CAC CGG GGC; domain 6, TTT CTT CCA TGG GGC AAG AAT 3'. PCR products were cut with HindIII and BglIII and cloned into the pIg vector (Fawcett et al., 1992; Simmons, 1993) cut with HindIII plus BamHI.

Neural cell adhesion molecule (NCAM) was isolated by transient expression/panning of a fetal brain cDNA library. CD11a was a gift of Dr. Clive Landis (Leukocyte Adhesion Laboratory, ICRF). CD18 was isolated by hybridization screening from a U937 cDNA library. Negative control chimeric Fc proteins were CD33(D1-D2)Fc (Simmons, 1993), MUC18-Fc (Fawcett et al., 1992), and ICAM-1(D1-D5)Fc, a gift from Dr. Alastair Craig (Institute of Molecular Medicine, Oxford, UK). All constructions were checked by restriction digests and DNA sequencing.

### Generation of Chimeric Fusion Proteins and Immunoprecipitations

CD31-Fc fusion plasmids were transfected into COS cells ( $10 \mu\text{g}/10^7$  COS cells) using DEAE-dextran as a facilitator (Simmons, 1993). The medium was changed at 24 h to DME/0.5% FCS, and the supernatants were harvested at 7 d. Fusion proteins were affinity isolated on protein A-Sepharose (Pharmacia, Milton Keynes, UK); columns were washed with 0.1 M glycine, pH 5.0 (to remove bovine IgG), and Fc chimeras were eluted with 0.1 M glycine, pH 3.0, neutralized immediately in 10% vol/vol 1 M Tris base, buffer exchanged, and concentrated by centrifugal dialysis (Centricon 10 U; Amicon, Beverly, MA). For production of labeled proteins, COS transfectants were grown in methionine-free medium containing 5% dialyzed FCS and  $50 \mu\text{Ci}/\text{ml}$  [ $^{35}\text{S}$ ]methionine/cysteine (Translabel; ICN, Bucks, UK) for 18 h. Supernatants were harvested, and labeled secreted proteins were isolated by affinity purification with protein A-Sepharose. Bound proteins were eluted by boiling in Laemmli sample buffer under reducing conditions and resolved on 10% SDS-PAGE. Gels were fixed, impregnated with Amplify (Amersham, Bucks, UK), dried, and exposed to x-ray film for 12 h at  $-80^\circ\text{C}$ .

### Generation of Anti-CD31 Polyclonal Antiserum

Young adult New Zealand white rabbits were injected three times subcutaneously with  $100 \mu\text{g}$  of CD31(D1-D6)Fc recombinant protein, once in complete and twice in incomplete Freund's adjuvant, during a course of 8 wk. The preimmune serum and serum from immunized rabbits were tested in an ELISA to determine specificity of binding to HUVEC. Results (not shown) revealed that there was significant binding to HUVEC in the immune serum at 1/100,000 dilution, whereas binding of the preimmune sera was negligible at 1/10,000. All subsequent bleeds were tested for activity against HUVEC using ELISA. The specificity of the sera were checked by FACscan<sup>®</sup> analysis of CD31(D1-D6)TM<sup>+</sup>COS and immunoprecipitation from  $^{125}\text{I}$  surface-labeled HUVEC. HUVEC were labeled using  $^{125}\text{I}$  and lactoperoxidase as Enzymobeads (Bio Rad Laboratories, Richmond, CA) according to the vendor's instructions. In this study, sera were used from two different rabbits, and similar results were seen with both.

### Isolation of IgG and Preparation of Fab Fragments

Final anti-CD31 sera were pooled, and IgG was affinity isolated on protein A-Sepharose. The IgG was desalted on PD10 columns (Pharmacia) and concentrated by centrifugal dialysis (Centricon 10; Amicon). Fab fragments were generated using immobilized papain (Pierce Europe b.v., oud-Beijerland, Holland) according to the manufacturer's instructions. Fab fragments were dialyzed against PBS or RPMI 1640 overnight. Successful generation of fragments was confirmed using SDS-PAGE. In most cases, Fab fragments were used at 50–100  $\mu\text{g}/\text{ml}$ .

### Electron Microscopy

Because of the opacity of the polycarbonate membranes in the Transwell cell culture inserts (Costar UK, High Wycombe, Bucks, UK), HUVEC grown in the presence of anti-CD31 or preimmune Fab fragments were observed by electron microscopy. HUVEC were washed twice in serum-free RPMI 1640 and fixed in 2.5% glutaraldehyde in PBS. The base of each Transwell was carefully cut out, and the samples were dehydrated and processed into Epon Resin. "Gold" (90–120 nm) sections were cut, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips 200 electron microscope. Filters were flat embedded and cut so that cross-sections of endothelial cells on the filter were obtained.

### Adhesion Assays

COS cells were labeled for 24–48 h with [ $^3\text{H}$ ]thymidine (Amersham) ( $10 \mu\text{Ci}/2 \times 10^7$  cells). Transfection efficiency was checked by cytofluorography and was usually 15–30%. Cells were lifted with PBS/2 mM EDTA, washed three times in RPMI-1640, and resuspended in assay buffer (RPMI 1640 20 mM Hepes/0.2% BSA) at  $3\text{--}4 \times 10^4$  cells/well. When the effect of antibodies was being tested, mAbs or Fab fragments at 10–50  $\mu\text{g}/\text{ml}$  were added to the cell suspensions at room temperature for 10 min before the assay and included during the assay, unless otherwise stated. When the effect of CD31Fc fusion proteins as direct competitors was being tested, proteins at 250  $\mu\text{g}/\text{ml}$  were added to COS CD31(D1-D6)TM transfectants at room temperature for 10 min before the assay, and they were included during the assay.

For adhesion assays with cell lines (U937, H82, and MIKALL), cells were labeled for 48 h with [ $^3\text{H}$ ]thymidine at  $10 \mu\text{Ci}/10^7$  cells. Cells were washed three times in assay buffer and  $2 \times 10^5$  cells added per well in 96-well plates. Assays were performed in the absence and presence of heparin at 100  $\mu\text{g}/\text{ml}$  to block heterotypic adhesion.

96-well adhesion assay plates (Immulon 3; Dynatech Research Laboratories, Chantilly, VA) were prepared by precoating wells with 1  $\mu\text{g}/\text{well}$  of affinity-purified goat anti-human Fc antibody (Sigma) overnight at  $4^\circ\text{C}$ . Plates were washed three times in PBS, and remaining unbound sites were blocked with PBS/0.4% BSA (Fraction V; Sigma) for  $\geq 2$  h at room temperature. 50  $\mu\text{l}$  of recombinant proteins at 10  $\mu\text{g}/\text{ml}$  were then bound, and plates were washed three more times in PBS.

For LFA-1 assays, LFA-1<sup>+</sup>COS cells were stimulated with PMA (Sigma) by incubating cells in 50 ng/ml (80 nM), PMA for 30 min at  $37^\circ\text{C}$  and the PMA was removed by washing in assay buffer.

Cells were allowed to adhere for 60 min (or  $\leq 3$  h in some assays) in assay buffer at  $37^\circ\text{C}$ . At the conclusion of the incubations, the wells were washed two times with prewarmed assay buffer using a continuous flow of assay buffer at a head of pressure of 30 cm of water using a siphon arrangement. Between washes, the wells were emptied by inversion. Cells that remained bound after two washes were lysed in 1% SDS, scintillant was added (Ready Safe; Beckman, High Wycombe, UK), and incorporated radioactivity was counted using a Beckman LS 5000 CE counter.

All adhesion assays were performed on at least three independent occasions, and representative experiments are shown. Each data point represents the mean of six replicates.

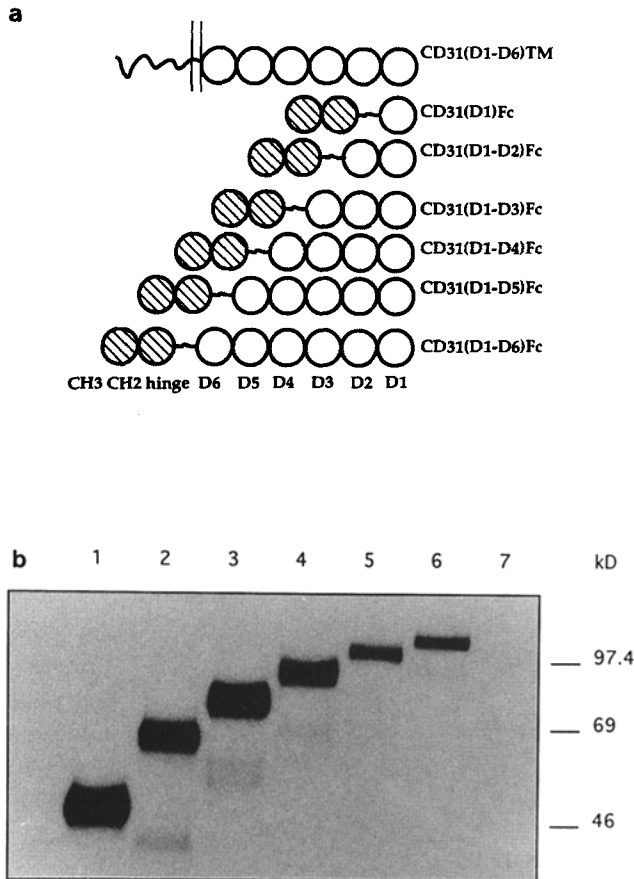
### ELISA of CD31-Fc Proteins

Immulon-3, 96-well ELISA plates, were prepared as adhesion plates. Plates were precoated with 1  $\mu\text{g}/\text{well}$  goat anti-human-Fc Ig (Sigma) overnight at  $4^\circ\text{C}$ , blocked with PBS/2% BSA (Fraction V; Sigma) for 2 h at room temperature, and then coated with chimeric proteins (5  $\mu\text{g}/\text{ml}$ ) in PBS for  $\geq 2$  h at room temperature. Antibodies were added at 10  $\mu\text{g}/\text{ml}$  or as neat tissue culture supernatants, followed by horseradish peroxidase-conjugated goat anti-mouse Ig (1:1,000 dilution; Amersham). Each layer was incubated for 60 min at room temperature and followed by six washes with PBS/0.2% BSA/0.02% azide. The assay was visualized with *O*-phenylenediamine dihydrochloride (Sigma), and absorbance was read at 450 nm.

### Growth of HUVEC in the Presence of Fc Chimeras

A pilot experiment was undertaken to determine the dose response of chimeric Fc proteins on the growth of HUVEC. 24-well Primaria (Falcon, Becton Dickinson, Oxford, UK) dishes were coated with fibronectin at 10  $\mu\text{g}/\text{ml}$  and varying concentrations of CD31(D1-D6)Fc or CD33(D1-D2)Fc (CD33 is a myeloid surface protein with no known ligand on endothelial cells and was chosen as a negative control for IgSF-Fc chimeras; Simmons and Seed, 1988). For all these experiments, early passage HUVEC (p2 or p3) were used. Cells were seeded at a subconfluent initial density of  $10^4$  cells/well. The medium in each well was changed daily, and at 5 d, the total number of cells was counted in each well. Duplicate wells for each concentration of Fc protein coating were set up, and the results are shown as the mean number of cells in duplicate wells. In control wells, there was an approximate six- to sevenfold increase in total cell numbers during the course of the experiment.

The dose response analysis indicated that CD31(D1-D6)Fc was effective at 50  $\mu\text{g}/\text{ml}$ , so this coating concentration was used for the second set of experiments involving a time course of growth over a period of 7 d in the presence of fibronectin (FN) alone, FN + CD31(D1-D6)Fc, or FN + CD33(D1-D2)Fc. For the time course experiment, HUVEC were seeded at  $5 \times 10^3$  per well in Falcon Primaria 24-well cluster plates coated with



**Figure 1.** Characterization of CD31-Fc chimeric proteins. (a) Diagrammatic representation of full-length membrane expressed CD31 (CD31(D1-D6)TM) and deletion series of CD31Fc chimeras: CD31(D1)Fc, CD31(D1-D2)Fc, CD31(D1-D3)Fc, CD31(D1-D4)Fc, CD31(D1-D5)Fc, and CD31(D1-D6)Fc. (b) SDS-PAGE analysis of CD31-Fc domain deletion series. Lane 1, CD31(D1)Fc; lane 2, CD31(D1-D2)Fc; lane 3, CD31(D1-D3)Fc; lane 4, CD31(D1-D4)Fc; lane 5, CD31(D1-D5)Fc; lane 6, CD31(D1-D6)Fc; lane 7, molecular weight markers. <sup>35</sup>S-labeled CD31-Fc proteins were affinity isolated on protein A-Sepharose, eluted with Laemmli sample buffer, and resolved on 10% SDS-PAGE. Molecular masses are indicated in kilodaltons.

fibronectin at 10  $\mu$ g/ml alone or in combination with either CD31(D1-D6)Fc or CD31(D1-D2)Fc at 100  $\mu$ g/ml. Cells were fed daily with DME/10% FCS supplemented with basic FGF at 10 ng/ml. Cell numbers were measured by counting, and results are expressed as the mean number  $\pm$  1 SD ( $n = 4$ ).

## HUVEC Culture on Transwell Cell Culture Inserts and Monolayer Integrity Testing

$10^5$  HUVEC were seeded in the presence of preimmune or CD31 antisera or Fab fragments into Transwell tissue culture inserts (diameter = 6.5 mm) containing 3.0- $\mu$ m pore polycarbonate membrane bases (Costar UK) that had been fibronectin coated (50  $\mu$ g/ml; Sigma). Cells were then left overnight to form a monolayer. In some experiments, HUVEC were seeded in the absence of antisera or Fab fragments. Dilutions of antisera used were in the range 1/50–1/500. Fab fragments were used at 50–100  $\mu$ g/ml.

The next day, the integrity of the monolayers was assessed using [<sup>14</sup>C]-mannitol, as described in Bird et al. (1993). Briefly,  $4.4 \times 10^4$  dpm of [<sup>14</sup>C]mannitol (Amersham) was added to each well insert. After incubation at 37°C for 30 min, proportionally equal samples of tissue culture media were removed from the insert and the well, and were counted on a liquid scintillation counter. The counts from the well were expressed as a percentage of those remaining in the insert. The results from inserts incubated with preimmune or CD31 antisera or Fab fragments were compared for differences in percentage equilibration values (counts in well)/(counts in insert)  $\times$  100. In some experiments, HUVEC monolayers that had been set up on filters alone were tested and then incubated with preimmune or immune antisera or Fab fragments, and were then retested at a later time. Experiments were performed on at least three independent occasions with no less than four replicates in each experiment.

## Results

### Mapping the Homotypic Binding Sites in CD31

**Molecular Tools.** To map the homotypic binding site in CD31, a series of truncated forms of CD31 consisting of the NH<sub>2</sub>-terminal 1, 1–2, 1–3, 1–4, 1–5, and 1–6 IgC2 domains of CD31 fused to the Fc region of human IgG1 were made: CD31(D1)Fc, CD31(D1-2)Fc, CD31(D1-3)Fc, CD31(D1-4)Fc, CD31(D1-5)Fc, and CD31(D1-6)Fc (Fig. 1 a). The chimeric genes were constructed by PCR amplification of CD31 to generate a 3' consensus splice donor sequence and restriction site; the PCR product was ligated into a vector pIg1, consisting of pCDM8 containing the genomic DNA sequences encoding the hinge, CH<sub>2</sub> and CH<sub>3</sub> exons, and associated intronic sequences of human IgG1 3' to the cytomegalovirus promoter/enhancer sequences. When the chimeras were expressed in metabolically labeled COS-1 cells, the affinity-isolated proteins all ran at the predicted sizes (Fig. 1 b). The conformational integrity of the deletion chimeras was assessed by solid-phase ELISA using a panel of eight anti-CD31 mAbs (Table I). This also allowed assignment of mAbs to individual domains. mAbs 9G11 and JC70A recognize all six proteins and thus map to domain 1; mAbs L133.1, CLB/CD31, WM59, and 5.6E map to domain 2; mAb HC1/6 maps to domains 4 and 5 so it could recognize an inter-

**Table I.** ELISA Profiles of Eight Anti-CD31 mAbs

Antibody	CD31 FC protein						MUC18 control
	D1	D1-D2	D1-D3	D1-D4	D1-D5	D1-D6	
9G11	0.235	0.276	0.292	0.291	0.292	0.292	0.050
JC70A	0.29	0.317	0.311	0.317	0.283	0.297	0.052
L133.1	0.054	0.187	0.208	0.207	0.246	0.224	0.049
WM59	0.062	0.233	0.233	0.268	0.306	0.300	0.070
CLB/CD31	0.059	0.230	0.242	0.237	0.293	0.249	0.051
5.6E	0.063	0.204	0.224	0.184	0.229	0.207	0.050
HC1/6	0.066	0.069	0.071	0.140	0.243	0.254	0.060
10B8	0.071	0.056	0.074	0.088	0.260	0.272	0.060

Chimeric CD31-Fc proteins were immobilized via goat anti-human IgG1Fc and screened with anti-CD31 mAbs. MUC18-Fc was included as a negative control IgSF-Fc chimera. Results are means of duplicates and are representative of two separate experiments.

domain junctional epitope; mAb 10B8 maps solely to domain 5.

**Homotypic Adhesion.** The standard assay used to analyze CD31 homotypic adhesion throughout this study involved the binding of COS cells transiently expressing full-length CD31 or CD31 truncation mutants, to immobilized CD31-Fc chimeric proteins. This allowed us to dissect the roles of heterotypic versus homotypic adhesion, and it also allowed for the normalization of the amount of chimeric protein presented to the input cells. Surfaces coated with the full-length CD31(D1-D6)Fc chimera supported the adhesion of CD31(D1-D6)TM<sup>+</sup>COS transfectants, but not sham transfectants (Fig. 2 *a*). To demonstrate that this system was not an artefact of CD31 expressed in COS cells, adhesion assays were performed with CD31<sup>+</sup> cell lines, U937 and MIKALL. Both these cell lines bound to immobilized CD31(D1-D6)Fc (Fig. 2, *a* and *c*), but a small cell lung carcinoma line, H82, which is CD31 negative, did not (Fig. 2 *a*).

**Mapping Using Deletion Mutants.** To define the domain or domains responsible for mediating homotypic binding, COS cells expressing full-length CD31 were allowed to adhere to surfaces coated with the series of CD31-Fc proteins (Fig. 2 *b*). The results show that the presence of domain 6 was necessary to support significant homotypic adhesion, though CD31(D1-D5)Fc allowed partial adhesion (50% of "full-length" CD31(D1-D6)Fc adhesion). No other CD31-Fc chimera was able to bind CD31(D1-D6)TM<sup>+</sup>COS transfectants. The deletion series adhesion assay was repeated on a CD31<sup>+</sup> lymphoid cell line, MIKALL, with similar results (Fig. 2 *c*); only CD31(D1-D6)Fc supported significant homotypic adhesion. To rule out the potential of heterotypic interactions via domain 2, both the CD31(D1-D6)TM<sup>+</sup>COS and MIKALL assays were repeated in the presence of 100  $\mu$ g/ml heparin. This concentration of heparin has been shown to block the heterotypic binding of domain 2 to heparan sulphate-decorated proteoglycans. In the presence of heparin, the adhesion profiles were unaffected (data not shown). Moreover, mock-transfected COS cells do not bind to CD31(D1-D6)Fc (Fig. 2 *a*).

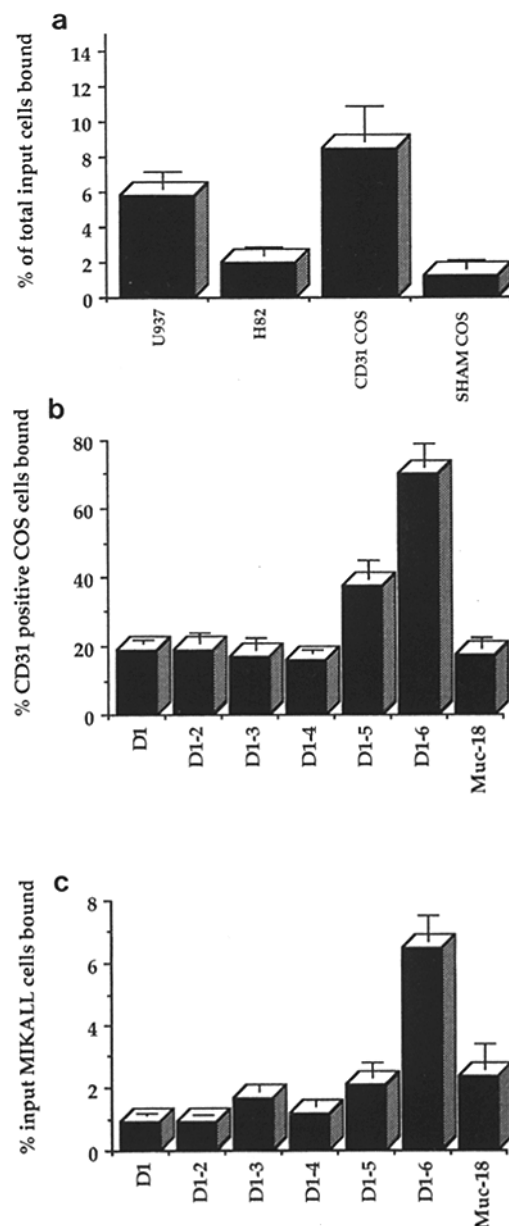
Although domain 6 is a necessary component of CD31 homotypic interactions, these experiments did not indicate whether domain 6 alone was sufficient to support adhesion. Alternatively, binding might require several individually weak adhesion points, which amounted overall to measurable adhesion in this assay system.

### Mapping by Adhesion Blockade

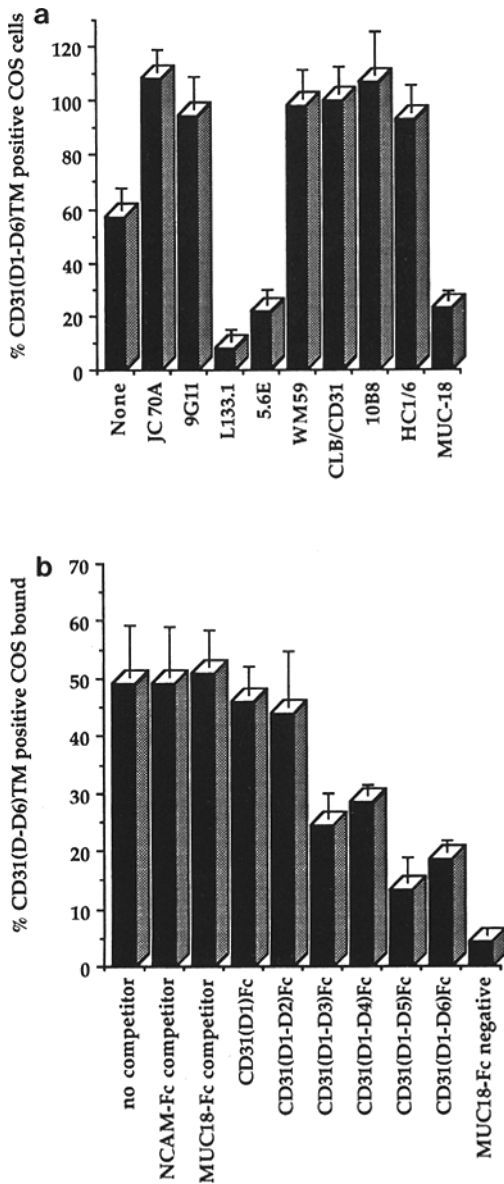
**Blocking with Domain-specific mAbs.** To further define the binding site, the panel of eight anti-CD31 mAbs was screened for their ability to block adhesion. Two of the four mAbs (L133.1 and 5.6E) that map to domain 2 block binding of CD31(D1-D6)<sup>+</sup>COS to CD31(D1-D6)Fc (Fig. 3 *a*). None of the other mAbs blocked in this assay. In fact, since all the mAbs were used as whole antibodies, there was an actual increase in adhesion probably because of cross-bridging of CD31 on the COS cells to CD31 on the assay surface.

The blocking assay was repeated on a CD31<sup>+</sup> lymphoid cell line MIKALL with similar results; again, the only two blocking mAbs were 5.6E and L133.1 (data not shown).

**Blocking with CD31Fc Fusion Proteins.** The series of CD31-Fc fusion proteins was used as direct competitive in-

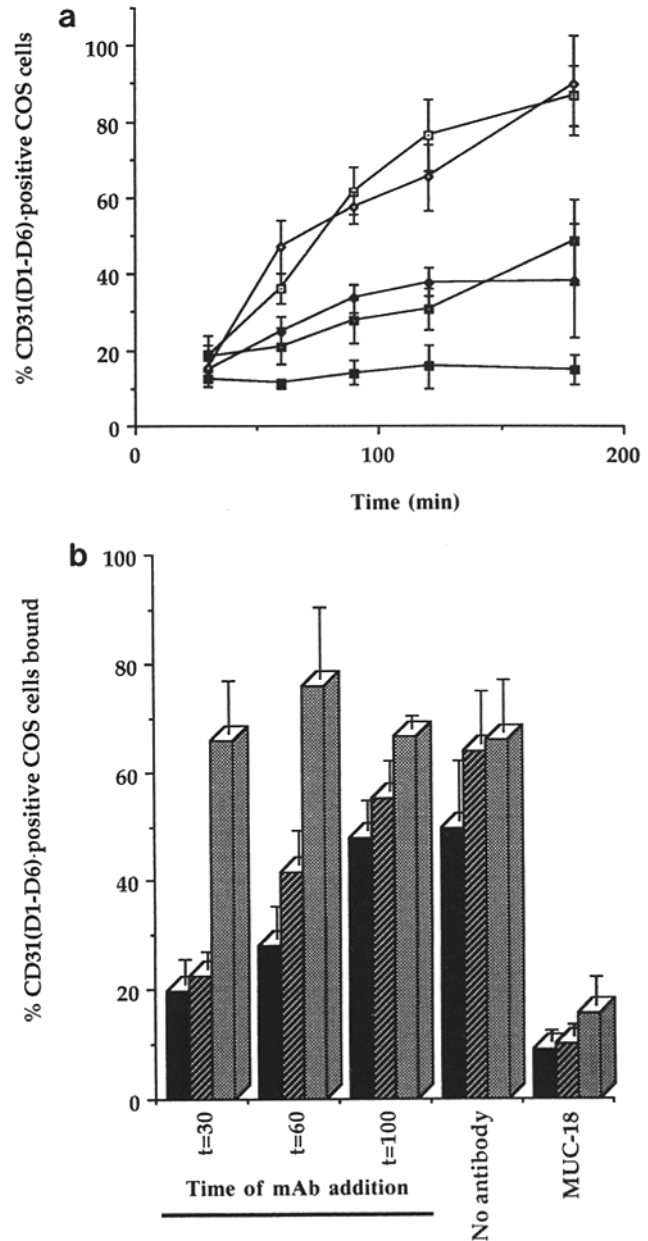


**Figure 2.** CD31<sup>+</sup>COS/CD31-Fc homotypic adhesion assays; mapping the binding site. (a) Adhesion of COS cells transfected with CD31(D1-D6)TM, sham (pCDM8)-transfected COS cells, U937 (promonocytic, CD31<sup>+</sup>), and H82 (small cell lung carcinoma, CD31<sup>-</sup>) to CD31(D1-D6)Fc immobilized on plastic. Assays are expressed as mean percentage of total input cells binding  $\pm$ 1 SD ( $n = 6$ ). (b) Adhesion of CD31(D1-D6)TM<sup>+</sup>COS transfectants to plastic coated with COOH-terminal domain deletion series of CD31-Fc proteins. The series used was CD31(D1)Fc, CD31(D1-D2)Fc, CD31(D1-D3)Fc, CD31(D1-4)Fc, CD31(D1-D5)Fc, and CD31(D1-D6)Fc. Adhesion to a control protein, MUC18-Fc, is included as a negative IgSF-Fc control. Results are expressed as the means of the percentage of CD31(D1-D6)TM<sup>+</sup>COS cells binding  $\pm$ 1 SD ( $n = 6$ ), i.e., corrected for transfection efficiency. (c) Adhesion profile of MIKALL to CD31 deletion chimeras. Results are expressed as the means of the percentage of total input cells binding  $\pm$ 1 SD ( $n = 6$ ). All assays are normalized with respect to the percentage of transfection efficiency.



**Figure 3.** Mapping the homotypic binding site by adhesion blockade. (a) mAb blocking. Screen of eight anti-CD31 mAbs for blockade of adhesion of CD31(D1-D6)TM<sup>+</sup>COS to CD31(D1-D6)Fc. Cells were incubated with mAbs at 10  $\mu$ g/ml for 10 min at room temperature before the assay. mAbs were present during the assay. MUC18-Fc is a negative control. The background level of binding in this assay is indicated by adhesion of CD31(D1-D6)TM<sup>+</sup>COS to MUC18-Fc. (b) CD31Fc proteins as competitive inhibitors. CD31(D1-D6)TM<sup>+</sup>COS cells were incubated with the CD31Fc chimeras at 250  $\mu$ g/ml for 10 min at room temperature before the assay. Cells were allowed to adhere for 1 h to CD31(D1-D6)Fc immobilized on plastic. Chimeras were present during the assay. Two negative control competitors (MUC18-Fc and NCAM-Fc) were also used. The background level of binding in this assay is indicated by adhesion of CD31(D1-D6)TM<sup>+</sup>COS to MUC18-Fc.

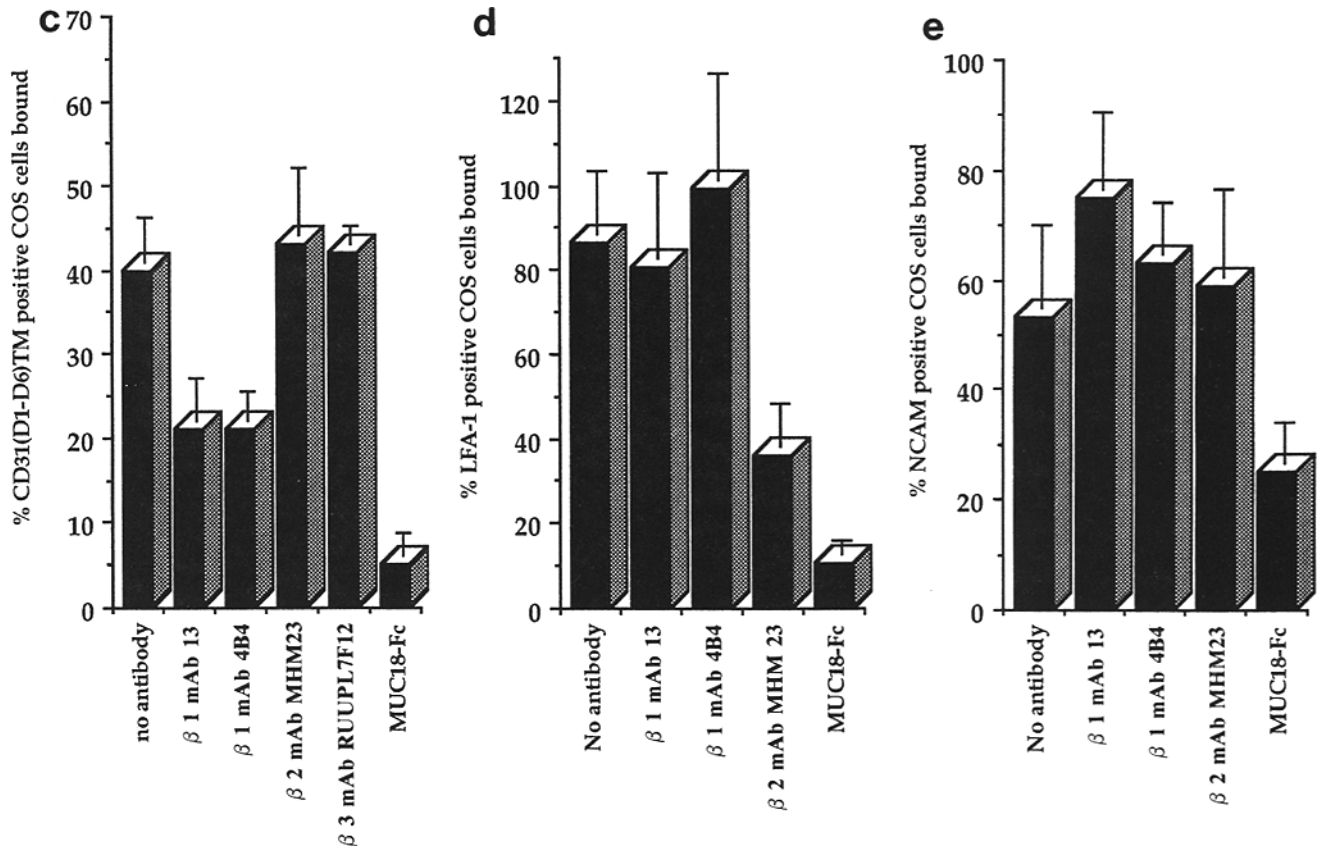
inhibitors of the binding of CD31(D1-D6)<sup>+</sup>COS to CD31(D1-D6)Fc immobilized on plastic. Neither CD31(D1)Fc nor CD31(D1-D2)Fc were effective competitors. However, CD31(D1-D3)Fc reduced binding by 50%. No further inhibition



**Figure 4.** Cross-talk between CD31 and  $\beta$ 1 integrin adhesion. (a) Time course of adhesion of CD31(D1-D6)TM<sup>+</sup>COS to CD31(D1-D6)Fc and effects of  $\beta$ 1 integrin-blocking mAbs. The x axis is total time in minutes. ◆,  $\beta$ 2 MHM23; □, no antibody; ◊,  $\beta$ 1 4B4; ◇,  $\beta$ 1 13; ■, MUC18-Fc. (b) Effect of anti-CD31 blocking mAbs (L133.1 and 5.6E) added at various times on CD31-CD31 adhesion and  $\beta$ 1 integrin adhesion. Anti-CD31 mAbs were added at 30, 60, and 100 min at a final concentration of 10  $\mu$ g/ml, and the assay continued for a total of 2 h, when all sample wells were washed and bound cells were counted. The “no antibody” data set is the maximal level of adhesion at 2 h with no mAbs added. ■, D2 blocker L133.1; ▒, D2 blocker 5.6E; □, D1 non-blocker 9G11.

was seen with CD31(D1-D4)Fc, but a significant reduction in binding was seen with CD31(D1-D5)Fc and CD31(D1-D6)Fc.

Thus, a combination of direct adhesion assays with CD31 deletion mutants and blocking assays with mAbs and



(c) Lack of involvement of  $\beta$ 3 integrins in homotypic CD31 adhesion. CD31(D1-D6)TM<sup>+</sup>COS cells were allowed to adhere for 2 h to CD31(D1-D6)Fc in the absence or presence of  $\beta$ 1,  $\beta$ 2, or  $\beta$ 3 integrin-blocking mAbs. CD31(D1-D6)TM<sup>+</sup>COS cells bind minimally to MUC18-Fc. (d) Lack of involvement of  $\beta$ 1 integrins in heterotypic LFA-1/ICAM-1 adhesion. LFA-1<sup>+</sup>COS cells, activated with PMA, were allowed to adhere for 2 h to ICAM-1(D1-D5)Fc in the absence or presence of  $\beta$ 1 or  $\beta$ 2 integrin-blocking mAbs. PMA-activated LFA-1<sup>+</sup>COS cells bind minimally to MUC18-Fc. (e) Lack of involvement of  $\beta$ 1 integrins in homotypic NCAM-NCAM adhesion. NCAM<sup>+</sup>COS cells were allowed to adhere for 2 h to NCAM-Fc in the absence or presence of  $\beta$ 1 or  $\beta$ 2 integrin-blocking mAbs. NCAM<sup>+</sup>COS cells bind minimally to MUC18-Fc.

CD31Fc fusion proteins reveal that a very extensive surface of CD31 is involved in mediating homotypic adhesion. Multiple domains play a part in the binding with key sites in domains 2 and 3, as well as domains 5 and 6.

#### "Cross-talk" between CD31 and $\beta$ 1 Integrins

There have been two reports describing an interaction or "cross-talk" between CD31 and  $\beta$ 1 integrins (Tanaka et al., 1992; Piali et al., 1993). For both CD8<sup>+</sup> T cells and natural killer cells, cross-linking CD31 by single mAbs leads to increased adhesion mediated by  $\beta$ 1 integrins. In preliminary experiments with the COS/CD31-Fc assay system, after the initial CD31-CD31 contacts had been formed during 30–60 min, we observed a steady increase in cell binding over the course of the next 2–3 h. To investigate the possible involvement of integrin adhesion, we used two anti- $\beta$ 1 integrin mAbs (mAbs 13 and 4B4) known to block  $\beta$ 1 integrin-mediated adhesion. These mAbs cross-react with primate  $\beta$ 1 integrin on COS cells; 95% of COS cells were positive for  $\beta$ 1 integrin by cytofluorometry (data not shown). Both mAbs reduced the overall adhesion by 50–70% (Fig. 4 a). A control  $\beta$ 2 integrin blocking mAb had no effect on the course of

adhesion. Background binding of CD31(D1-D6)TM<sup>+</sup>COS to MUC18-Fc, a control IgSF-Fc chimera, was minimal (10% of CD31(D1-D6)Fc binding) and constant throughout the entire assay period, showing that COS cells do not intrinsically bind to the assay surface during the period of the experiment.

There was a continuing need for primary CD31-CD31 homotypic contacts  $\leq$ 60 min into the assay for the  $\beta$ 1 integrin pathway to be active. If these contacts were blocked with either of the CD31 domain 2-blocking mAbs (5.6E and L133.1) at 30 or 60 min into a homotypic assay, there was no significant adhesion (Fig. 4 b). However, if the mAbs were added at 90 min into the assay, then a significant amount of CD31 independent adhesion remained.

These results strongly suggest that the establishment of initial CD31-CD31 contacts leads to the upregulation or enhancement of  $\beta$ 1 integrin-mediated pathways of cell adhesion after an initial lag period of 60 min. The recruitment of integrin adhesion is specific for the  $\beta$ 1 class; an mAb known to block  $\beta$ 3 integrin adhesion (RUU-PL 7F12) had no effect on the CD31-recruited adhesion (Fig. 4 c), even though COS cells express high levels of  $\beta$ 3 integrins (data not shown).

The interaction between CD31 and  $\beta 1$  integrins seems to be specific to these molecules. Other IgSF adhesion molecules and other integrins do not positively interact to produce increased adhesion. Two different receptor ligand pairs (ICAM-1/LFA-1 and NCAM/NCAM) were used to see if the establishment of other primary contacts between the COS cells and the chimeric protein on the assay surface led to recruitment of  $\beta 1$  integrin adhesion (Fig. 4, *d* and *e*). COS cells transiently expressing CD11a/CD18 were activated with phorbol esters and were allowed to adhere to ICAM-1(D1-D5)Fc. The adhesion was blocked by the anti- $\beta 2$  integrin mAb MHM23, but not by either of the  $\beta 1$  integrin mAbs (Fig. 4 *d*). Similarly, COS cells expressing NCAM bound to NCAM-Fc in a homotypic manner that did not lead to involvement of  $\beta 1$  integrin adhesion (Fig. 4 *e*). In both of these cases, there are comparable or even larger numbers of primary contacts formed between the COS transfectants and the immobilized chimera on the plate, and yet there is no recruitment of  $\beta 1$  integrin adhesion to the system.

### CD31(D1-D6)Fc Inhibits the Growth of Endothelial Cells

Having established that CD31(D1-D6)Fc was capable of mediating homotypic adhesion, the functional effects of this reagent on endothelial cells were investigated. A pilot experiment was undertaken to determine whether CD31(D1-D6)Fc coated onto plastic together with the normal substrate (fibronectin) would affect the growth rate of endothelial cells. Early passage HUVEC (p2 or p3) were seeded at subconfluent densities in the presence of a range of concentrations of CD31(D1-D6)Fc (0.1, 1.0, 10, and 100  $\mu\text{g}/\text{ml}$ ) or a control IgSF member CD33(D1-D2)Fc (Fig. 5 *a*). Cell proliferation was quantitated over a period of 5 d. CD33(D1-D2)-Fc had no inhibitory effect; HUVEC numbers increased sixfold over 5 d. However, CD31(D1-D6)Fc caused significant inhibition of endothelial cell growth at 50  $\mu\text{g}/\text{ml}$ ; HUVEC numbers increased only two- to threefold (Fig. 5 *a*).

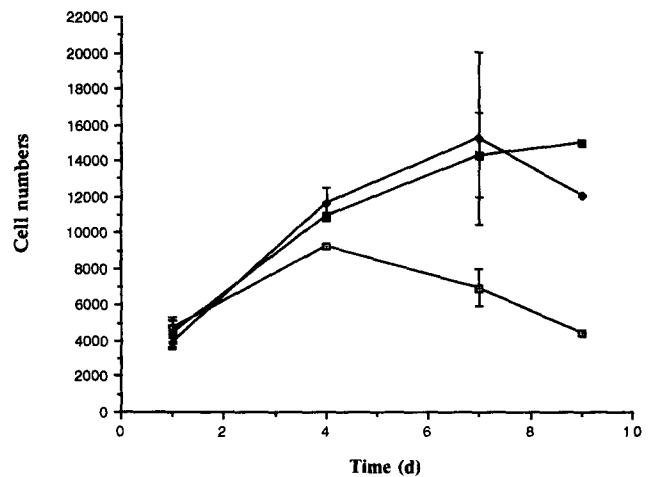
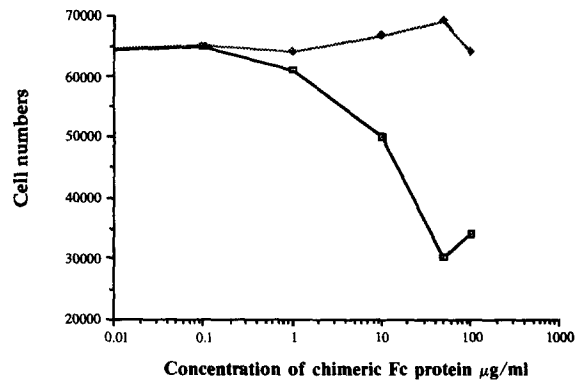
On the basis of this dose response study, a time course experiment was undertaken using a concentration of 50  $\mu\text{g}/\text{ml}$  of chimeric protein together with fibronectin at 10  $\mu\text{g}/\text{ml}$ . During a course of 8 d, the proliferation of HUVECs in wells coated with the CD31(D1-D6)Fc was retarded compared with CD33(D1-D2)Fc-coated wells (Fig. 5 *b*).

In both cases, CD31(D1-D6)Fc retarded the proliferation of HUVEC, but did not completely prevent it or lead to a decrease in cell numbers compared to the initial input. These data suggest that CD31(D1-D6)Fc is cytostatic and not cytotoxic.

### Generation of Anti-CD31 Blocking Reagent

To further explore the functional role of CD31 in intercellular adhesion, polyclonal CD31 antisera were raised in rabbits using CD31(D1-D6)Fc as an immunogen. The specificity of these reagents were established in two ways. Firstly, the affinity-purified IgG fraction from these sera specifically recognized CD31(D1-D6)TM<sup>+</sup>COS transfectants (Fig. 6 *a*), and secondly, they precipitated a single 130-kD protein from <sup>125</sup>I surface-labeled endothelial cells (Fig. 6 *b*).

The effect of the polyclonal anti-CD31 antisera were tested to determine whether it blocked homotypic binding of CD31. To avoid the confounding effects of cross-bridging



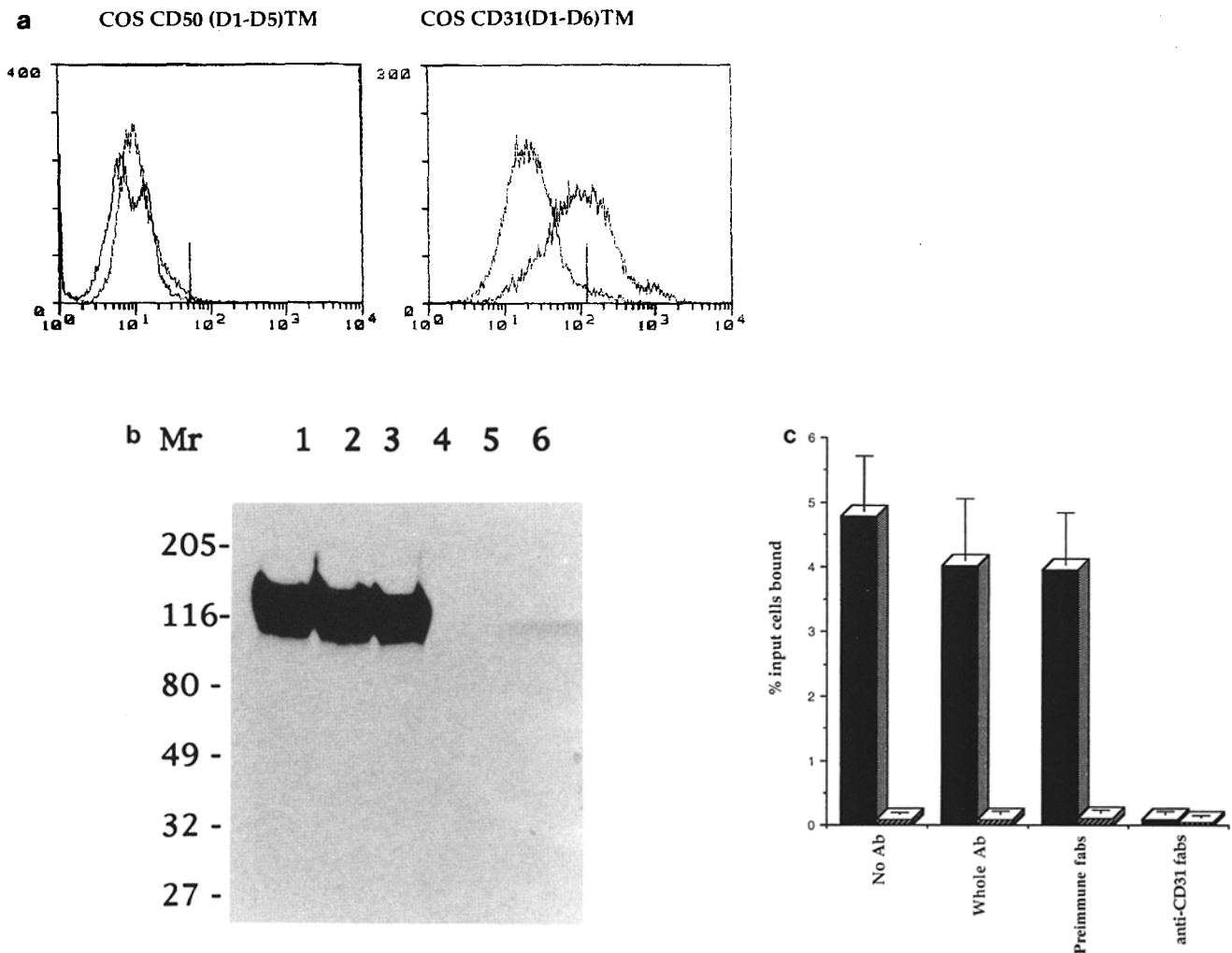
**Figure 5.** CD31(D1-D6)Fc inhibits the growth of endothelial cells. (a) Dose response of CD31(D1-D6)Fc. HUVEC were seeded at a subconfluent density on surfaces precoated with CD31(D1-D6)Fc or CD33(D1-D2)Fc over a range of concentrations. Cells were seeded on day 0 at  $2 \times 10^4$  cells/well. Total cell numbers were counted on day 5. Results are presented as means of duplicate wells.  $\square$ , CD31(D1-D6)Fc;  $\diamond$ , CD33(D1-D2)Fc. (b) Growth rates of HUVEC on surfaces coated with fibronectin alone, or fibronectin plus 100  $\mu\text{g}/\text{ml}$  of CD33(D1-D2)Fc or CD31(D1-D6)Fc. Cells were seeded on day 0 at  $5 \times 10^3$  cells/well, and cell numbers measured by counting. Results are expressed as mean  $\pm 1$  SD ( $n = 4$ ).  $\square$ , CD31(D1-D6)Fc;  $\diamond$ , CD33(D1-D2)Fc;  $\blacksquare$ , fibronectin.

of CD31(D1-D6)TM molecules expressed on the COS cells to CD31(D1-D6)Fc bound to the plate, Fab fragments were prepared. Preincubation of CD31<sup>+</sup>COS transfectants with 50  $\mu\text{g}/\text{ml}$  Fab fragments of the anti-CD31 antibodies resulted in reduction of CD31-CD31 adhesion to background levels (Fig. 6 *c*). Thus anti-CD31 Fab fragments can block homotypic CD31-CD31 adhesion.

### CD31 Antisera and Anti-CD31 Fab Fragments Disrupt Endothelial Monolayer Integrity

The role of CD31 in the formation and maintenance of interendothelial contacts was assessed in two ways.



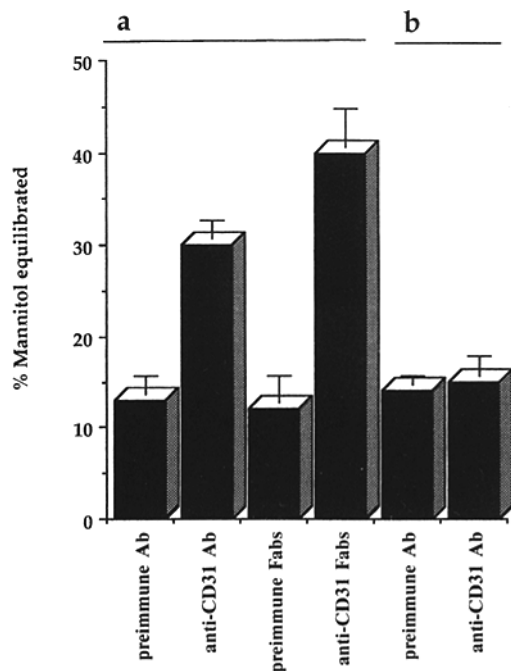


**Figure 6.** Anti-CD31 Fabs block homotypic adhesion. (a) Characterization of polyclonal anti-CD31 antibodies by FACSscan® analysis of CD31(D1-D6)TM<sup>+</sup>COS and CD50 (ICAM-3)-transfected COS using IgG fraction from pooled serum at 1:1,000 dilution. Control background staining of preimmune rabbit IgG is shown in each panel. (b) Characterization of polyclonal anti-CD31 antibodies by immunoprecipitation from surface <sup>125</sup>I-labeled HUVEC. Lane 1, polyclonal anti-CD31 whole serum; lane 2, polyclonal anti-CD31 IgG fraction; lane 3, anti-CD31 mAb 9G11; lane 4, prebleed serum; lane 5, prebleed IgG; lane 6, anti-ICAM-3 (CD50) mAb CH3.3. Molecular masses are indicated to the left in kilodaltons. (c) Anti-CD31 Fabs block homotypic adhesion. Adhesion of CD31(D1-D6)TM<sup>+</sup>COS or mock-transfected COS to CD31(D1-D6)Fc immobilized on plastic in the absence or presence of anti-CD31 polyclonal antibodies (whole antibody or Fabs). All antibodies were present throughout the adhesion assay and used at 50 μg/ml. Results are expressed as means ± 1 SD (*n* = 8). ■, CD31(D1-D6) COS; ◻, sham COS.

The first quantitative assay measured the equilibration of [<sup>14</sup>C]mannitol (molecular mass = 186 D) across HUVEC as a measure of monolayer integrity. HUVEC were allowed to form a confluent monolayer overnight in the presence of either antisera or Fab fragments prepared from anti-CD31 immune sera or preimmune control serum. There was significantly greater equilibration of the [<sup>14</sup>C]mannitol after a fixed 30-min time period in the presence of the anti-CD31 antibodies, both as whole Ig and as Fab fragments, compared with preimmune controls (Fig. 7 a). However, when confluent preformed HUVEC monolayers were incubated with CD31 antisera overnight, no significant difference in monolayer integrity could be detected, compared with preimmune controls (Fig. 7 b). Preformed monolayers tested after 6 h incubation with antisera gave a similar result (data not

shown). Antibodies and Fab fragments appear only to affect monolayer integrity when present during monolayer formation and have no effects on HUVEC monolayer integrity once it is formed.

Secondly, the integrity of the monolayers formed in the presence of preimmune and anti-CD31 Fab fragments was qualitatively assessed by electron microscopy (Fig. 8). The monolayers formed in the presence of preimmune Fab fragments (Fig. 8 a) were uniform and the endothelial cells were in close contact and exhibited a flattened morphology on the filter surface with some cells forming overlapping junctions with adjacent cells. These monolayers resembled previously published studies on HUVEC grown on such permeable filters. Both immunofluorescent and immunoelectron microscopy confirmed that CD31 was localized to cell-cell



**Figure 7.** Effect of anti-CD31 antisera and Fab fragments on HUVEC monolayer permeability. (a) Typical [ $^{14}$ C]mannitol equilibration results from HUVEC monolayers formed on Transwells in the presence of CD31 and preimmune antisera or Fab fragments (50  $\mu$ g/ml). Significant increases in monolayer permeability were observed when HUVEC were incubated with either CD31 antisera or Fab fragments during monolayer formation ( $P < 0.02$ ). (b) Typical [ $^{14}$ C]mannitol equilibration results from preformed HUVEC monolayers incubated overnight with preimmune or CD31 antisera. No significant difference in equilibration values could be detected ( $P > 0.24$ ). All monolayers were tested before the incubation and gave percentage equilibration values within the normal range (data not shown).

junctions (data not shown), in agreement with previous studies (Leach et al., 1993; Ayalon et al., 1994).

Incubation of anti-CD31 Fab fragments with HUVEC during monolayer formation had dramatic effects on endothelial cell shape and monolayer integrity. Several different cell morphologies were observed. In some areas, endothelial cells were elongated and they formed abnormally extended thin processes (Fig. 8 b, arrow). Other cells were not flattened and appeared raised from the surface of the filter (Fig. 8 c). In other areas, cells formed disordered multilayer structures rather than monolayers (Fig. 8 d). Also, there were small areas of filter devoid of cells. There was no evidence of any cytotoxic effects caused by anti-CD31 Fab fragments, since there were no observable differences in cell ultrastructure between control and anti-CD31 treated HUVEC samples. Thus, anti-CD31 Fab fragment incubation with HUVEC resulted in the formation of a disrupted structure rather than the normal cell monolayer.

## Discussion

CD31 is a constitutively and abundantly expressed glycoprotein on endothelial cells. Because of its highly localized expression at sites of intercellular contacts, it has been suggested that CD31 may play a role in the maintenance of the

integrity of the endothelial monolayers lining the vasculature (Muller et al., 1989; Bevilacqua, 1993).

It has already been established that CD31 exhibits both homotypic and heterotypic adhesive properties. While the focus of this study has been to dissect the homotypic adhesion mechanism, it should be noted that CD31 can also mediate heterotypic adhesion (Muller et al., 1992). The heterotypic site has been mapped to domain 2, which contains a consensus motif for the recognition of heparan sulphate (DeLisser et al., 1993). The homotypic binding mechanism has not yet been defined.

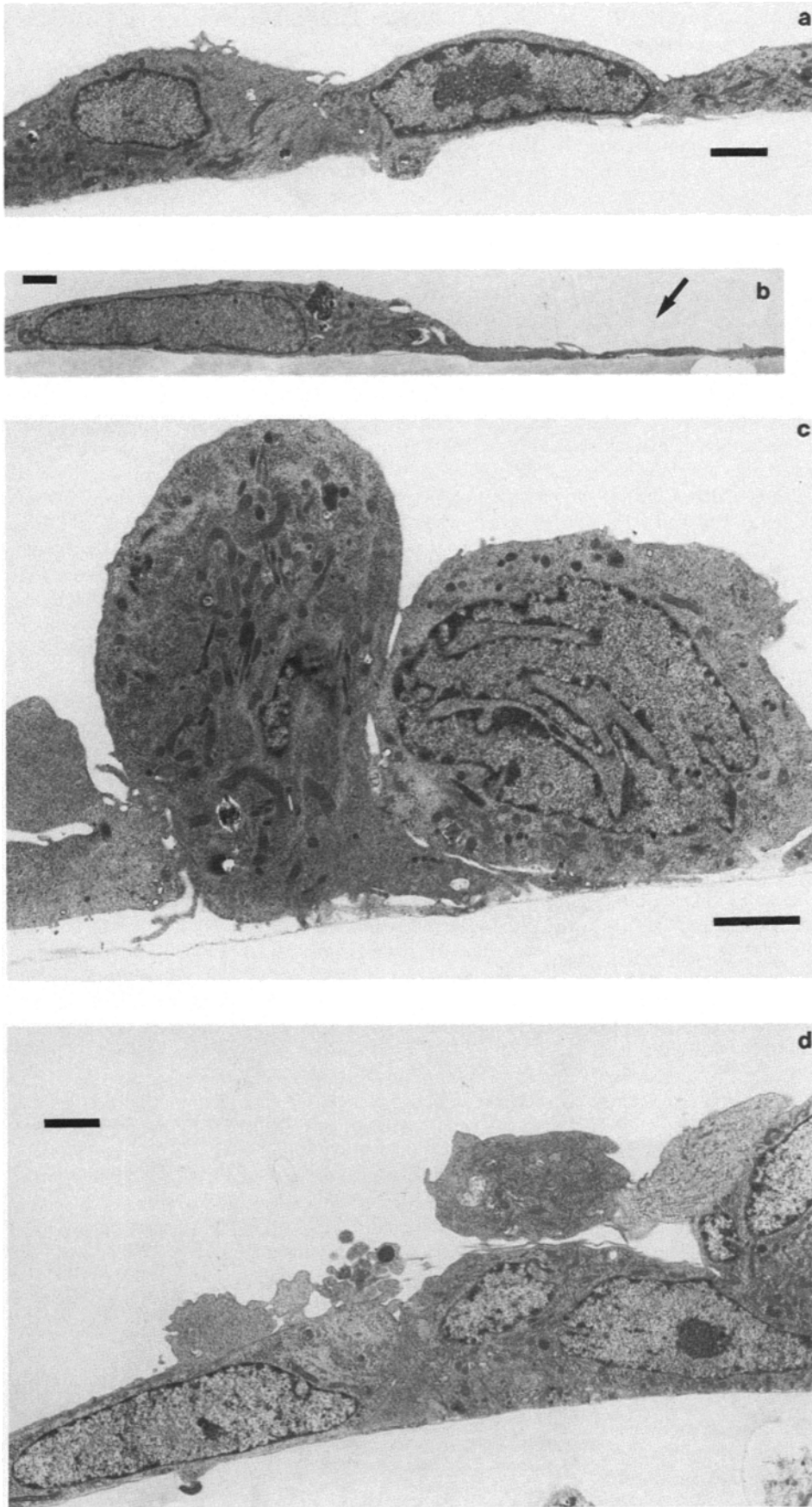
To study the homotypic binding mechanism, the approach we have adopted relies on the presentation of recombinant chimeric forms of CD31-Fc to CD31 $^{+}$  cell lines or COS cells expressing wild-type or truncated forms of CD31. We adopted this strategy for two reasons. First, it is possible to control for homotypic versus heterotypic pathways by use of mock transfectants, irrelevant chimeric proteins, and inclusion of heparin in the binding assays. Secondly, the adhesion assays are quantitative and highly reproducible (Fawcett et al., 1992; Simmons, 1993).

Using a combination of direct adhesion assays with chimeric CD31-Fc deletion proteins and blocking assays with mAbs and the chimeric CD31-Fc deletion proteins, we found that the homotypic binding mechanism involves an extensive surface over multiple domains. Key sites are contained within domains 2 and 3, as well as within domains 5 and 6.

A model consistent with our data is that CD31 on one cell may interact with CD31 on another cell in an antiparallel and fully interdigitating mode. Binding could involve a two-stage process of initial docking or alignment of the two molecules along their length, followed by specific engagement of binding sites in domains 2 and 3, as well as domains 5 and 6. Although we have not shown which domains recognize D2 and D6, it is plausible that in an antiparallel interaction, D2-3 bind to D5-6 and vice versa.

A similar mechanism has recently been proposed to explain the mode of interaction of another homotypic adhesion carcinoembryonic antigen (CEA) (Zhou et al., 1993). The extracellular domain of CEA consists of seven Ig domains, and by constructing CEA/NCAM chimeras and deletion mutants, the data was consistent with a model based on double reciprocal interactions between antiparallel CEA molecules aligned in *trans*.

From the initial studies, mapping the binding sites in IgSF members, particularly the mutagenesis carried out on CD2 (Peterson and Seed, 1987) and CD4 (Peterson and Seed, 1988), a model emerged which suggested that key residues located in single Ig domains were important for mediating binding. In addition, it seemed that the additional Ig domains merely acted as a stalk, projecting the dominant binding domain away from the cell membrane above the cellular glycocalyx. There are many IgSF adhesion molecules where more than one domain in the molecule contributes to the binding site. VCAM-1 has two binding sites, one in domain 1 and one in domain 4, for its ligand, very late activation antigen-4 (VLA-4) (Osborn et al., 1992). In this case, the domains seem to be able to work independently, but they have different properties. Another example is ICAM-1, which uses domain 1 to bind to one of its ligands, LFA-1, and uses domain 3 to bind to another leukocyte integrin, Mac-1 (Staunton et al., 1990; Diamond et al., 1991). We have



**Figure 8.** Anti-CD31 Fab fragments disrupt HUVEC monolayer morphology. Electron micrographs of HUVEC monolayers formed in the presence of either preimmune (A) or anti-CD31 Fab fragments at 50 µg/ml (B-D). In B, the arrow indicates the abnormally extended cell processes. Bar, 2 µm.

shown recently that ICAM-3, a very close relative of ICAM-1, uses domains 1 and 2 to bind to LFA-1 (Holness et al., 1995). Thus, in the case of VCAM and ICAMs-1 and -3, a large surface encompassing many Ig domains is involved in ligand binding.

From this recent work and our present study, a more complex picture is beginning to emerge, where several Ig domains in an adhesion molecule may actively contribute to engagement of ligand and not act merely as a passive stalk.

In addition to the homotypic contact formation, CD31 can lead to the recruitment or amplification of  $\beta 1$ , and to a lesser extent,  $\beta 2$  integrin-mediated adhesion (Tanaka et al., 1992; Piali et al., 1993). Using CD8<sup>+</sup>CD31<sup>+</sup> T cells, Tanaka et al. (1992) showed that cross-linking CD31 using single mAbs could upregulate  $\beta 1$  adhesion, and they postulated that one of the key roles of CD31 is to act as an adhesion amplifier. Thus, the primary role of CD31-CD31 contacts may not be to establish strong adhesion per se, but rather as elements in cell-cell recognition, to allow other adhesive paths to be recruited or upregulated. We have shown that CD31(D1-D6)TM expressed in a heterologous cell (COS) can recruit or amplify  $\beta 1$  integrin adhesion.  $\beta 3$  integrins do not seem to be involved. Recruitment of  $\beta 1$  adhesion is not a general feature of COS transfectants binding to chimeric Fc proteins; neither LFA-1<sup>+</sup>COS cells binding to ICAM-1(D1-D5)Fc nor NCAM<sup>+</sup>COS cells binding to NCAM-Fc led to the recruitment of  $\beta 1$  integrin adhesion. Moreover, COS cells do not bind intrinsically to an irrelevant IgSF-Fc chimera (MUC18-Fc) even during 3 h. Establishment of direct CD31-CD31 homotypic contacts for  $\geq 30$  min and  $\leq 60$  min is needed for  $\beta 1$  pathways to be amplified. After that homotypic contact period, the  $\beta 1$  integrin pathway seems to be engaged and adhesion then occurs without the need for continued CD31-CD31 contacts.

The molecular basis of this apparent cross-talk is not yet defined. The interaction between CD31 and  $\beta 1$  integrins may occur via direct intermolecular contacts or indirectly via an adaptor molecule or via intracellular signaling events. There are many other cases of cooperative interactions between adhesion molecules and other molecules in the same cell. For example, NCAM and L1 are thought to pair, probably via glycan/lectin interactions, to produce "assisted homophilic binding" (Kadmon et al., 1990), i.e., *cis* association of NCAM-L1 yields increased L1-L1 adhesion (Horstkorte et al., 1993). The integrin-associated protein IAP, which is a unique member of the IgSF comprising a single Ig domain attached to a transmembrane domain of five spanning regions, binds to the  $\alpha_v\beta_3$  integrin. Anti-IAP mAbs can modulate  $\alpha_v\beta_3$  integrin function (Linberg et al., 1993; Schwartz et al., 1993). Embigin, an IgSF member containing two Ig C2 domains, which is expressed early during mouse embryogenesis (Huang et al., 1993), shows remarkably similar properties to those found here for CD31. L cells expressing embigin adhered to BSA-coated tissue culture plastic, whereas parental L cells did not. Anti- $\beta 1$  antiserum and GRGDS peptide inhibited this adhesion. The ability of embigin-expressing L cells to adhere to BSA coated plastic in a  $\beta 1$  integrin-dependent manner parallels our system, where CD31 expressing COS cells adhere to an identical surface. The ligand used by either of these cells has not been defined. Interestingly, embigin<sup>+</sup>L cells bind to fibronectin as efficiently as do the parental L cells, implying that the

effect of the IgSF/ $\beta 1$  integrin interaction is evident only when the adhesion ligand is limiting.

There is much circumstantial evidence implicating CD31 in intercellular adhesion of endothelial cells. This evidence derives largely from observations that CD31 is often localized at sites of cell-cell contact. There is some experimental evidence that the bovine homologue of CD31, endoCAM, is involved in interendothelial cell adhesion, since polyclonal anti-endoCAM antibodies disrupt the formation of bovine adrenal capillary endothelial monolayers (Albelda et al., 1990). However, there have been no direct demonstrations or quantitative analyses of a role for human CD31 in the formation or maintenance of interendothelial contacts.

To directly test the role of CD31 in human endothelial cell adhesion, we generated a polyclonal anti-CD31 antiserum that blocks the homotypic adhesion process. Anti-CD31 Fab fragments had dramatic effects on the formation of an integral endothelial monolayer, judged both quantitatively by low molecular mass solute equilibration, and qualitatively by electron microscopy. The anti-CD31 reagents were only effective when presented to the endothelial cells during monolayer formation, i.e., during the time when interendothelial cell-cell contacts were forming. The antisera were completely without effect when presented to an already established monolayer, even though the endothelial cells in these layers were still expressing very high constitutive levels of CD31.

There are two possible explanations for this difference. The anti-CD31 Fabs may be unable to compete with established CD31-CD31 contacts and break the monolayer once formed. Alternatively, CD31-CD31 interactions could mediate growth controlling signals to endothelial cells that are crucial during the process of monolayer formation, but become irrelevant once formed. We favor the latter model because the Fab fragments are clearly capable of inhibiting homotypic adhesion of CD31<sup>+</sup>COS cells to CD31(D1-D6)Fc. Given this, the Fab fragments should be able to compete for binding to CD31 and disrupt monolayers if the main function of CD31 is to function as an adhesion molecule.

The role of CD31 in the initial formation of interendothelial contacts was also demonstrated by the effects of CD31(D1-D6)Fc chimera on endothelial cell growth. Premature presentation of CD31(D1-D6)Fc to preconfluent endothelial monolayers caused significant inhibition of cell growth. It is possible that CD31-CD31 contacts play a key role in sensing the density of endothelial cells; as endothelial cells begin to contact each other, CD31-CD31 binding in *trans* between two neighboring cells may deliver negative signals back into those cells, taking them out of the division cycle and thus maintaining a quiescent state once the monolayer has formed.

Thus, current evidence suggests that CD31 may have at least two distinct functions: first as a cell-cell recognition molecule involved in the formation of initial intercellular contacts before handing over to other CAMs, and secondly, as a mediator of contact-dependent growth inhibition.

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