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 antiparallel 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 β1 integrins. The positive cooperation between CD31 and β1 integrins can occur in heterologous primate cells (COS cells). The interaction is specific to both CD31 and β1 integrins. Neither intercellular adhesion molecule-I (ICAM-I)/leukocyte function-associated antigen-I (LFA-I) nor neural cell adhesion molecule (NCAM)/NCAM adhesion leads to recruitment of β1 integrin adhesion pathways. Establishment of CD31 contacts has 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-I (ICAM-I) and vascular cell adhesion molecule-I (VCAM-I), play key roles in mediating the adhesion 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 antiendoCAM 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-I [PECAM-I]) 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...
The expression of CD31 on lymphocytes is not a prerequisite has also been demonstrated for murine CD31 (Piali et al., 1992; Zehnder et al., 1992). 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; Simone et al., 1993). 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+ T cells to matrix components (Tanaka et al., 1992). This novel mechanism, involving "cross-talk" between CD31 and β1, and to a lesser extent β2 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 FcγRII leads to induction of proadhesive cytokines (TNF-α, IL-1β, 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 apposed 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 β1 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 American Type Culture Collection (Rockville, MD) and the International Cell Line Resource (Spain). The human endothelial cell line HUVEC was established from umbilical cord veins. All antibodies were raised against human CD31 that cross-react with rat CD31 (Jaffe et al., 1973). For LFA-I expression, COS cells were transfected with 50 µg of both CDIla and CDI8. 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). For cytofluorometric analysis, COS transfectants were washed with PBS/0.1% BSA/0.02% azide and fixed in 70% ethanol. The cell lines U937, H82, COS-1, and COS-7 were obtained from the American Type Culture Collection (Rockville, MD) and the International Cell Line Resource (Spain). The human endothelial cell line HUVEC was established from umbilical cord veins. All antibodies were raised against human CD31 that cross-react with rat CD31 (Jaffe et al., 1973). For LFA-I expression, COS cells were transfected with 50 µg of both CDIla 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/0.2% BSA/0.2% azide and 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) isoform was 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′-GATCAGATCTACTGGT; domain 2, AAC GAG ACT GGG CAT TCC; domain 2, AGA GAA GGA TTC GGT CAG GGT; domain 3, GAA GGA AGA TTC CAG TTG GGG; domain 4, AAT

The Journal of Cell Biology, Volume 128, 1995 1230
CCT GGG CTG GGA GAG CAT; domain 5, CTG GAC CTC ATC CAC

Products were cut with HindIII and BglII and cloned into the pig vector (Fawcett et al., 1992; Simmons, 1993) cut with HindIII plus BamHI.

Neuronal cell adhesion molecule (NCAM) was isolated by transient expression of a brain cDNA library. CD18 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 CD3(D1-D2)Fc (Simmons, 1993), MUC18-Fc (Fawcett et al., 1992), and ICAM-1(D1-D3)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 μg/10² 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 3.0, neutralized immediately in 10% vol/vol 1 M Tris base, buffer exchanged, and concentrated by centrifugal dialysis (Centricon 10; Amicon, Beverly, MA). For production of labeled proteins, COS transfectedants were grown in methionine-free medium containing 5% dialyzed FCS and 50 μCi/ml [35S]methionine/cysteine (Translabel; ICN, Costa Mesa, CA). 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°C.

Generation of Anti-CD31 Polyclonal Antiserum

Young adult New Zealand white rabbits were injected three times subcutaneously with 100 μg 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 for activity against HUVEC using ELISA. Results (not shown) revealed that there was significant binding to HUVEC in the immun serum at 1/10,000 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® analysis of CD31(D1-D6)TM-COS and immunoprecipitation from 125I-surface-labeled HUVEC. HUVEC were labeled using 125I 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-Bijerland, 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 μg/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 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 [125I]thyminude (Amersham) (10 μCi/2 x 10⁸ cells). Transfection efficiency was checked by cytofluorography and was usually 15-30%. Cells were washed three times in RPMI-1640, and resuspended in assay buffer (RPMI 1640 20 mM Hepes/0.2% BSA) at 3-4 x 10⁶ cells/ml when the effect of antibodies was being tested, mAbs or Fab fragments at 10-20 μg/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 μg/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 MKKALL), cells were labeled for 48 h with [125I]thyminude at 10 μCi/10² cells. Cells were washed three times in assay buffer and 2 x 10⁵ cells added per well in 96-well plates. Assays were performed in the absence and presence of heparin at 100 μg/ml to block heterotypic adhesion.

96-well adhesion assay plates (Immuno 3; Dynatech Research Laboratories, Chantilly, VA) were prepared by precoating wells with 1 μg/well of affinity-purified goat anti-human Fc antibody (Sigma) overnight at 4°C. Plates were washed three times in PBS, and remaining unbound sites were blocked with PBS/0.4% BSA (Fraction V; Sigma) for ≥2 h at room temperature. 50 μl of recombinant proteins at 10 μg/ml were then bound, and plates were washed three more times in PBS.

For LPA-1 assays, LPA-1 COS cells were stimulated with PMA (Sigma) by incubating cells in 50 ng/ml (80 nM), PMA for 30 min at 37°C and the PMA was removed by washing in assay buffer.

Cells were allowed to adhere for 60 min (or 3 h in some assays) in assay buffer at 37°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

Immunol-3, 96-well ELISA plates, were prepared as adhesion plates. Plates were precoated with 1 μg/well goat anti-human-Fc Ig (Sigma) overnight at 4°C, blocked with PBS/2% BSA (Fraction V, Sigma) for 2 h at room temperature, and then coated with chimeric proteins (5 μg/ml) in PBS for ≥2 h at room temperature. Antibodies were added at 10 μg/ml or as neat tissue culture supernatants, followed by horseradish peroxidase-conjugated goat anti-mouse Ig (1:1,000 dilution; Dako, London, UK). Each layer was incubated for 60 min at room temperature and followed by six washes with PBS/0.2% 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 Chimera

A pilot experiment was undertaken to determine the dose response of chimeric Fc proteins on the growth of HUVEC. 24-well Primera dishes (Falcon, Becton Dickinson, Oxford, UK) dishes were coated with fibronectin at 10 μg/ml and varying concentrations of CD31(D1-D6)Fc or CD31(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 10⁴ 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 μg/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 + CD31(D1-D2)Fc. For the time course experiment, HUVEC were seeded at 5 x 10³ per well in Falcon Primera 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. 35S-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.

10⁵ HUVEC were seeded in the presence of preimmune or CD31 antisera or Fab fragments into Transwell tissue culture inserts (diameter = 6.5 mm) containing 30-μm pore polycarbonate membrane bases (Costar UK) that had been fibronectin coated (50 μg/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 μg/ml.

The next day, the integrity of the monolayers was assessed using [14C]-mannitol, as described in Bird et al. (1993). Briefly, 4.4 x 10⁴ dpm of [14C]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) x 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

To map the homotypic binding site in CD31, a series of truncated forms of CD31 consisting of the NH₂-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 pIgl, consisting of pCDM8 containing the genomic DNA sequences encoding the hinge, CH2 and CH3 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).

Chimeric 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.

Table I. ELISA Profiles of Eight Anti-CD31 mAbs

| Antibody | D1   | D1-D2 | D1-D3 | D1-D4 | D1-D5 | D1-D6 | MUC18 control |
|----------|------|-------|-------|-------|-------|-------|----------------|
| 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          |
| HCl/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.

HUVEC Culture on Transwell Cell Culture Inserts and Monolayer Integrity Testing

The Journal of Cell Biology, Volume 128, 1995 1232
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+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+ 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+COS transfectants. The deletion series adhesion assay was repeated on a CD31+ 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+COS and MIKALL assays were repeated in the presence of 100 µg/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)Fc 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+ 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 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+COS to CD31(D1-D6)Fc. Cells were incubated with mAbs at 10 µg/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+COS to MUC18-Fc. (b) CD31-Fc proteins as competitive inhibitors. CD31(D1-D6)TM COS cells were incubated with the CD31-Fc chimeras at 250 µg/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+COS to MUC18-Fc.

Figure 4. Cross-talk between CD31 and β1 integrin adhesion. (a) Time course of adhesion of CD31(D1-D6)TM+COS to CD31(D1-D6)Fc and effects of β1 integrin-blocking mAbs. Anti-CD31 mAbs were added at 30, 60, and 100 min at a final concentration of 10 µg/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.

Inhibitors of the binding of CD31(D1-D6)+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 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...
Lack of involvement of β3 integrins in homotypic CD31 adhesion. CD31(D1-D6)TM+COS cells were allowed to adhere for 2 h to CD31(D1-D6)Fc in the absence or presence of β1, β2, or β3 integrin-blocking mAbs. CD31(D1-D6)TM+COS cells bind minimally to MUC18-Fc. (d) Lack of involvement of β1 integrins in heterotypic LFA-1/ICAM-1 adhesion. LFA-I+TM+COS cells, activated with PMA, were allowed to adhere for 2 h to ICAM-I(D1-D5)Fc in the absence or presence of β1 or β2 integrin-blocking mAbs. PMA-activated LFA-I+TM+COS cells bind minimally to MUC18-Fc. (e) Lack of involvement of β1 integrins in homotypic NCAM-NCAM adhesion. NCAM+COS were allowed to adhere for 2 h to NCAM-Fc in the absence or presence of β1 or β2 integrin-blocking mAbs. NCAM*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 β1 Integrins

There have been two reports describing an interaction or "cross-talk" between CD31 and β1 integrins (Tanaka et al., 1992; Piali et al., 1993). For both CD8+ T cells and natural killer cells, cross-linking CD31 by single mAbs leads to increased adhesion mediated by β1 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-β1 integrin mAbs (mAbs 13 and 4B4) known to block β1 integrin–mediated adhesion. These mAbs cross-react with primate β1 integrin on COS cells; 95% of COS cells were positive for β1 integrin by cytofluorometry (data not shown). Both mAbs reduced the overall adhesion by 50–70% (Fig. 4 a). A control β2 integrin blocking mAb had no effect on the course of adhesion. Background binding of CD31(D1-D6)TM*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 <60 min into the assay for the β1 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 of β1 integrin–mediated pathways of cell adhesion after an initial lag period of 60 min. The recruitment of integrin adhesion is specific for the β1 class; an mAb known to block β3 integrin adhesion (RUU-PL 7F12) had no effect on the CD31-recruited adhesion (Fig. 4 c), even though COS cells express high levels of β3 integrins (data not shown).
The interaction between CD31 and β1 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 β1 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-β2 integrin mAb MHM23, but not by either of the β1 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 β1 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 β1 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 μg/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 μg/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 μg/ml of chimeric protein together with fibronectin at 10 μg/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 was established in two ways. Firstly, the affinity-purified IgG fraction from these sera specifically recognized CD31(D1-D6)TM+COS transfectants (Fig. 6 a), and secondly, they precipitated a single 130-kD protein from 125I surface-labeled endothelial cells (Fig. 6 b).

The effect of the polyclonal anti-CD31 antisera was tested to determine whether it blocked homotypic binding of CD31. To avoid the confounding effects of cross-bridging 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+COS transfectants with 50 μg/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.
The first quantitative assay measured the equilibration of $[^{14}C]$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 $[^{14}C]$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
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. 8b, arrow). Other cells were not flattened and appeared raised from the surface of the filter (Fig. 8c). In other areas, cells formed disordered multilayer structures rather than monolayers (Fig. 8d). 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 7. Effect of anti-CD31 antisera and Fab fragments on HUVEC monolayer permeability. (a) Typical [14C]mannitol equilibration results from HUVEC monolayers formed on Transwells in the presence of CD31 and preimmune antisera or Fab fragments (50 µg/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 [14C]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).
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 β1, and to a lesser extent, β2 integrin-mediated adhesion (Tanaka et al., 1992; Piali et al., 1993). Using CD8+CD31+ T cells, Tanaka et al. (1992) showed that cross-linking CD31 using single mAbs could upregulate β1 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 of the key roles of CD31 is to act as an adhesion amplifier. Moreover, COS cells do not establish strong adhesion per se, but rather as elements of the key roles of CD31 is to act as an adhesion amplifier. Thus, in the case of VCAM and ICAMs-1 and -3, a large surface encompassing many Ig domains is involved in ligand binding.

The role of CD31 in the initial formation of interendothelial contacts was also demonstrated by the effects of CDs31(D1-D6)Fc chimera on endothelial cell growth. Premature presentation of CDs31(D1-D6)Fc to preconfluent endothelial monolayers caused significant inhibition of cell growth. It is possible that CDs31-CD31 contacts play a key role in sensing the density of endothelial cells. 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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The molecular basis of this apparent cross-talk is not yet defined. The interaction between CD31 and β1 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 αβ1 integrin. Anti-IAP mAbs can modulate αβ1 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-β1 antiserum and GRGDS peptide inhibited this adhesion. The ability of embigin-expressing L cells to adhere to BSA coated plastic in a β1 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+L cells bind to fibronectin as efficiently as do the parental L cells, implying that the effect of the IgSF/β1 integrin interaction is evident only when the adhesion ligand is limiting.
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Fawcett et al. Homotypic Binding by CD31 1241