

## Residues on Both Faces of the First Immunoglobulin Fold Contribute to Homophilic Binding Sites of PECAM-1/CD31\*

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Justin P. Newton, Christopher D. Buckley, E. Yvonne Jones‡, and David L. Simmons§

From the Imperial Cancer Research Fund Cell Adhesion Laboratory, Imperial Cancer Research Fund Laboratories, University of Oxford, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom and the ‡Laboratory of Molecular Biophysics and the Oxford Centre for Molecular Sciences, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

**CD31 (PECAM-1) is a member of the immunoglobulin superfamily whose extracellular domain is comprised of six immunoglobulin-like domains. It is widely expressed on endothelium, platelets, around 50% of lymphocytes, and cells of myeloid lineage. CD31 has been shown to be involved in interendothelial adhesion and leukocyte-endothelial interactions, particularly during transmigration. CD31-mediated adhesion is complex, because CD31 is capable of mediating both homophilic and multiple heterophilic adhesive interactions. Here we show that the NH<sub>2</sub>-terminal (membrane-distal) immunoglobulin domain of CD31 is necessary but not sufficient to support stable homophilic adhesion. Key residues forming the binding site within this domain have been identified by analysis of 26 single point mutations, representing the most systematic analysis of a fully homophilic interaction between immunoglobulin superfamily family members to date. This revealed five mutations that affect homophilic binding. Uniquely, the residues involved are exposed on both faces of the immunoglobulin fold, leading us to propose a novel mechanism for CD31 homophilic adhesion.**

CD31 (also known as PECAM-1, platelet-endothelial cell adhesion molecule) is a heavily glycosylated transmembrane protein of approximately 130 kDa (1, 2). It is widely expressed on circulating platelets, vascular endothelium, myeloid cells, around 50% of circulating lymphocytes (primarily CD8<sup>+</sup>, CD45RA naive lymphocytes), and CD34<sup>+</sup> hemopoietic progenitor cells in human bone marrow. Molecular cloning identified CD31 as a member of the immunoglobulin superfamily (3–5), whose extracellular domain of 574 amino acids is comprised of six immunoglobulin-like domains, each encoded by a single exon (6), a membrane-spanning hydrophobic region and a cytoplasmic tail of 118 amino acids. Unusually among adhesion molecules, the cytoplasmic tail is comprised of eight exons from which it is possible to derive a number of splice variants (6). These splice variants have been identified as being expressed in a developmentally specific manner (7) and also in regulating

CD31-mediated adhesion (8).

There is now significant evidence from both *in vivo* and *in vitro* studies to show that CD31 is involved in the extravasation of monocytes and neutrophils at inflammatory sites (9–13). These studies indicate that CD31 is a potential target for therapeutic intervention in both acute and chronic inflammatory conditions.

CD31-mediated adhesion is complex, because in common with other members of the immunoglobulin superfamily, such as neural cell adhesion molecule (14) and L1 (15, 16), it is capable of binding both to itself (homophilic adhesion) and to non-CD31 ligands (heterophilic adhesion). A number of potential heterophilic ligands have been identified, including the integrin  $\alpha_v\beta_3$  (17, 18), a 120-kDa ligand on T-cells involved in down-regulation of T-cell responses (19) and an as yet uncharacterized glycosaminoglycan decorated ligand on L-cells (20). In addition, it has also been shown that CD31 is capable of up-regulating both  $\beta_1$ - and  $\beta_2$ -mediated adhesion following homophilic engagement (22–24).

Given the complexity of CD31 interactions and the wide distribution of potential ligands, it has proved necessary to study CD31-mediated adhesion in the context of heterologous systems. To define the domain or domains responsible for mediating homophilic binding, we have previously used chimeric fusion proteins comprised of the NH<sub>2</sub>-terminal 1, 1–2, 1–3, 1–4, 1–5, and 1–6 Ig domains of CD31 fused to the Fc portion of human IgG1 to form a nested series of deletions (24). COS cells expressing full-length CD31 allowed to adhere to surfaces coated with this domain deletion series of CD31-Fc proteins showed that domains 5 and 6 were necessary to support homophilic adhesion but did not exclude the possibility that other more NH<sub>2</sub>-terminal domains are also required. To address the roles of domains 1 to 4 in homophilic adhesion, we assessed the ability of antibodies to block homophilic adhesion. It was found that antibodies mapping to domain 1–2 of CD31 were able to inhibit the binding of CD31(D1-D6)<sup>+</sup>COS to CD31(D1-D6)Fc. We subsequently proposed a model in which the NH<sub>2</sub>-terminal domains of CD31 on the surface of one cell bind to the membrane proximal domains of CD31 expressed on the surface of an apposing cell and *vice versa*, in a fully interdigitating anti-parallel mode of adhesion. This double reciprocal model of adhesion has also been proposed for homophilic interactions mediated by other members of the immunoglobulin superfamily, for example carcinoembryonic antigen (25), neural cell adhesion molecule (26), and L1 (27). In the present study we set out to test this model and to identify specific residues involved in mediating CD31 homophilic adhesion.

### EXPERIMENTAL PROCEDURES

**Materials**—Unless specified otherwise, all reagents and chemicals were purchased from Sigma. Protein A-Sepharose was purchased from Pharmacia Biotech Inc., and Immulon-3 microtitre plates were from

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§ To whom correspondence should be addressed: Cell Adhesion Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK. Tel.: 44-1865-222355; Fax: 44-1865-222431; E-mail: dsimmons@molbiol.ox.ac.uk.

Dynatech Laboratories Inc. (Chantilly, VA). COS-1 cells were provided by the Imperial Cancer Research Fund Cell Bank (Clare Hall, UK) and grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin-streptomycin.

**Antibodies**—The following monoclonal antibodies (mAbs)<sup>1</sup> were used. Anti-CD31 mAbs were: 9G11 (3); L133.1 (Becton Dickinson, Oxford, UK); hec 7.2 (Endogen, TCS Biologicals Ltd., Bucks, UK); 5.6E (Immunotech, Marseilles, France); 10B8 (R & D Systems, Oxford, UK); CLB-486 and CLB/CD31 (Monosan, Bucks, UK); VM64 (Monosan, Bucks, UK); WM59 (TCS Biologicals Ltd., Bucks, UK); IG118 (Bender Medsystems, Vienna, Austria); P2B1 (Chemicon International Ltd., Harrow, UK). Anti-ICAM-3 mAbs were CH3.2, CAL 3.10, CAL 3.24 (28), and KS128 (30). Anti-CD14 mAb UCHM1 was obtained from the Imperial Cancer Research Fund hybridoma unit (Clare Hall, UK).

Polyclonal anti-sera specific for the cytoplasmic tail of CD31 were raised in rabbits against the peptide sequence ETVYSEVRKAVP-DAVESR (Imperial Cancer Research Fund Peptide Synthesis Laboratory) coupled to keyhole limpet hemocyanin as immunogen according to the method of Hancock and Evan (29), and the IgG fraction from sera isolated by protein A-Sepharose chromatography. Immunofluorescence of COS cells expressing CD31 with either pre- or post-immune sera showed a specific anti-CD31 response following immunization. Monospecificity was demonstrated by blocking staining with the addition of a 200-fold molar excess of the immunizing peptide, but a second peptide (sequence NHAMKPIINDNKE) also derived from the cytoplasmic tail of CD31 had no effect (data not shown).

**Generation of Chimeras and Mutants**—Recombinant soluble adhesins, comprised of the extracellular domain of adhesion molecules fused to the constant (Fc) portion of human IgG1 have been described previously: CD31 (D1-D6)Fc (24); ICAM-3 (D1-D5)Fc; and MUC-18 (D1-D5)Fc (31).

Chimeras between CD31 and ICAM-3 were generated as follows. ICAM-3 (D1)/CD31 (D2-D6)TM, in which the NH<sub>2</sub>-terminal Ig domain of human full-length CD31 is replaced by the equivalent domain from human ICAM-3, and ICAM-3 (D1-D2)/CD31 (D3-D6)TM, in which the two NH<sub>2</sub>-terminal Ig domains of human full-length CD31 are replaced by the equivalent domains from human ICAM-3, were generated by a two-step recombinant PCR method (31, 32). All PCR amplifications were performed using *Pwo* DNA Polymerase (Boehringer Mannheim), using CD31 in pCDM8 (3) or ICAM-3 in pCDM8 (30) as templates, and using a maximum of 10 cycles to reduce the rate of adventitious mutation. For ICAM-3 (D1)/CD31 (D2-D6)TM, primers used were: ICAM-3 ATG forward amplification primer (5'-ATATAAGCTTATGGTACCATCGTGTGTGGCCC-3'), ICAM-3 D1 reverse amplification primer with CD31 overhanging sequence (5'-CACCTGGGACTGGGCACTCCACGCTCCGGAGCCCGGTACAC-3'), CD31 D2 forward amplification primer with ICAM-3 overhanging sequence (5'-GTGTACGGGCTCCCGGAGCGTGGAGTGCCAGTCCAGGGTG-3') and CD31 reverse amplification primer (5'-TATCTGATGCGGCCGCTAAGTTCCATCAAGGGAGCC-3'). For ICAM-3 (D1-D2)/CD31 (D3-D6)TM, primers were: ICAM-3 forward amplification primer, ICAM-3 D2 reverse primer with CD31 overhanging sequence (5'-GTGGAAGTTGGGTGTA-GAGAAGGGCAGGACAAAGGTTCCGAG-3'), CD31 D3 forward amplification primer with ICAM-3 overhanging sequence (5'-CTCCGAACCTTTGTCTGCCCTTCTACACCCAAGTTCCAC-3') and CD31 reverse amplification primer. PCR products were digested with *Hind*III and *Not*I and cloned into pIg vector digested *Hind*III/*Not*I. ICAM-3 (D1-D3)/CD31 (D4-D6)TM, ICAM-3 (D1-D4)/CD31 (D5-D6)TM, and ICAM-3 (D1-D5)/CD31 (D6)TM were generated by PCR amplification of the required fragments of CD31 and ICAM-3. Primers used were as follows: ICAM-3 forward amplification primer, ICAM-3 D3 reverse amplification primer (5'-ATATGAATCCAAAGACCGTCAAGTTCTCCCG-3'), ICAM-3 D4 reverse amplification primer (5'-ATATGAATCTT-TATCTTTCCATTTCAAGTGCTG-3'), ICAM-3 D5 reverse amplification primer (5'-ATATGAATTCATTTCTATCCTGTCAAGTAAGG-TG-3'), CD31 D4 forward amplification primer (5'-ATATGAATTC-GGGCTTGAAAATAGTTCTGTAT-3'), CD31 D5 forward amplification primer (5'-ATATGGATTCACAGGATTTCTATGATGCCAG-3'), CD31 D6 forward amplification primer (5'-ATATGAATTCATTC-TATCCTGTCAAGTAAGGTG-3'), and CD31 reverse amplification

primer. ICAM-3 PCR products digested with *Hind*III and *Eco*RI and CD31 PCR products digested with *Eco*RI and *Not*I were ligated into pCDM8 digested with *Hind*III and *Not*I.

ICAM-3 (D1)/CD31 (D2-D6)Fc, ICAM-3 (D1-D2)/CD31 (D3-D6)Fc, CD31 (D1-D2)/ICAM-3 (D2-D5)Fc, and CD31 (D1-D3)/ICAM-3 (D3-D5)Fc constructs were produced using the same two-step PCR strategy previously described. ICAM-3 (D1)/CD31 (D2-D6)Fc and ICAM-3 (D1-D2)/CD31 (D3-D6)Fc were made using the same primers as their membrane bound equivalents, substituting CD31 D6 reverse amplification primer (5'-GATCAGATCTACTTACCTGTTTCTTCCATGGGGCAAG-AAT-3') for the CD31 STOP reverse amplification primer. For CD31 (D1-D2)/ICAM-3 (D2-D5)Fc the following primers were used: CD31 ATG forward amplification primer (5'-TCTGAAGCTTCTGCAGTCTCTACTCTCAGGATG-3'), CD31 D2 reverse amplification primer with ICAM-3 overhang (5'-CGACCCACCCTCCACTACAGAGAAGGATTC-CGTAC-3'), ICAM-3 D2 forward amplification primer with CD31 overhang (5'-GTGACGGAATCCTTCTCTGTAGTGGAGGGTGGGTCG-3') and ICAM-3 D5 reverse amplification primer (5'-GATCAGATCTACTTACCTGTGCGACGGGGGGTCCACGGG-3'). For CD31 (D1-D3)/ICAM-3 (D3-D5)Fc the following primers were used: CD31 ATG forward amplification primer, CD31 D3 reverse amplification primer with ICAM-3 overhang (5'-GGGGTCCAGGACCTTGAAATA-GTTCTGT-3'), ICAM-3 D3 forward amplification primer with CD31 overhang (5'-ACAGAAGTATTTTCCAAAGGTCCTGCCGTGACCCCC-3'), and ICAM-3 D5 reverse primer. PCR products were digested *Hind*III/*Bgl*II and cloned into pIg vector digested *Hind*III/*Bam*HI. Constructs were verified by restriction digests and sequencing.

pGPI-CD31, a construct that gives rise to a glycosylphosphatidylinositol (GPI)-linked form of CD31 lacking the transmembrane and cytoplasmic tail of CD31, was generated in stages. To generate pGPI the GPI-anchor signal peptide was amplified from an LFA-3 template in pCDM8 (isolated by expression cloning, sequence identical to that reported in Ref. 35). Primers used were: LFA-3 forward amplification primer (5'-ATACGGATTCAAGCAGCGGCTCATTCAAGA-3') and LFA-3 reverse primer (5'-ATCTATGCGCGCGCAAATGAGAAATCAGATGGCTT-3'). The PCR product was digested with *Bam*HI and *Not*I and ligated into pCDNA3 to produce pGPI. The extracellular domain of CD31 was amplified using CD31 ATG forward amplification primer and CD31 GPI D6 reverse primer (5'-TATAGATCTTTCTCCATGGGGC-AAGAAT-3'), the PCR product digested with *Hind*III and *Bgl*II and cloned into pGPI digested with *Hind*III and *Bam*HI.

Point mutations were introduced into CD31 (D1-D6)Fc chimera or full-length CD31 using a two-step PCR strategy using common forward amplification (5'-TCTGAAGCTTCTGCAGTCTTCACTCTCAGGATG-3') and reverse amplification primers (5'-GATCAGATCTACTACTCTAGAGAAGGATTCGTCACGGT-3'), encompassing the first two NH<sub>2</sub>-terminal domains of CD31, in addition to sequence-specific mutagenic primers (list available on request). PCR products were digested with *Hind*III and *Bgl*II and subcloned into pIg vector digested with *Hind*III and *Bam*HI. Mutants were verified by sequencing of the amplified region, excised with *Hind*III and an endogenous *Bam*HI site at the 3' end of domain two in CD31, and subcloned into CD31(D1-6)Fc chimera in pIg or full-length CD31 in pCDNA3 digested with *Hind*III and *Bam*HI. Subcloning was verified by sequencing of the mutated region.

Recombinant chimeric-Fc plasmids were transiently expressed in COS cells, and secreted proteins were purified from tissue culture supernatants after 7–10 days by protein A-Sepharose chromatography as described previously (33). Eluted proteins were buffer exchanged into 20 mM Tris, pH 8.0, by centrifugal dialysis (Centricon-10 columns, Amicon, Beverly, MA) and checked by SDS-polyacrylamide gel electrophoresis.

**Transfection and Cytofluorometric Analysis**—Individual constructs (10–20 μg/10<sup>7</sup> cells) were transiently expressed in COS-1 cells using DEAE-dextran as a facilitator (33). To assess the efficiency of transfection, COS cells were harvested 48 h post-transfection in PBS/2 mM EDTA, washed in PBS/0.25% bovine serum albumin at 4 °C, stained with primary mAbs (10 μg/ml), washed, stained with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG, washed, fixed in PBS/2% formaldehyde, and analyzed on a Becton Dickinson FACScan. COS cell transfectants were routinely 40–60% positive by FACScan analysis.

**Adhesion Assays**—Immulon-3 96-well plates were precoated overnight at 4 °C with 1 μg/well goat-anti-human-Fc Ig in bicarbonate buffer, pH 9.6, blocked with PBS/0.25% bovine serum albumin (Fraction V) for 2 h at room temperature, and then coated with Fc chimeric proteins in PBS for at least 2 h at room temperature. At 48 h post-transfection, COS cells were harvested in 2 mM EDTA/PBS, labeled

<sup>1</sup> The abbreviations used are: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; ICAM, intercellular adhesion molecule; LFA, leukocyte function associated antigen; GPI, glycosylphosphatidylinositol; VCAM, vascular cell adhesion molecule; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

with the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (Molecular Probes) for 30 min at 37 °C, and washed twice in assay buffer (Dulbecco's modified Eagle's medium), 10 mM HEPES buffer, 0.25% bovine serum albumin). Labeled cells were added to plates at  $5 \times 10^4$  cells/well in a volume of 50  $\mu$ l and allowed to adhere for 1 h at 37 °C. Plates were washed with prewarmed assay buffer until the cells in the negative control wells were sufficiently removed by visual inspection. Typically this was two or three times. Adhesion was quantitated using a Cytofluor II fluorescent plate reader (Millipore, Watford, UK), comparing the total input fluorescence and the bound fluorescence, to calculate the percentage of input cells bound. Where COS cells expressing different chimeric constructs were used in the same assay, the percentage input cells bound was corrected to take into account the transfection efficiency.

**Phosphatidylinositol-specific Phospholipase C Treatment of Cells**—Transfected COS cells were harvested with 2 mM EDTA/PBS, resuspended at  $5 \times 10^6$  cells/ml in PBS/0.2% bovine serum albumin, and treated with 5 units/ml phosphatidylinositol phospholipase C (Boehringer Mannheim) for 30 min at 37 °C. Cells were washed in PBS/0.2% bovine serum albumin and stained for FACScan analysis as described above. CD31 staining was with mAb 9G11, and CD14 staining was with mAb UCHM1.

**Immunofluorescence**—Two color immunofluorescence of COS cells expressing full-length CD31 and GPI-CD31 was performed as follows. Transfected COS cells were plated at a density of  $2.5 \times 10^4$  cells/well in 8-well chamber slides (Nunc and Life Technologies Inc.) and grown overnight. Cells were washed in serum-free Dulbecco's modified Eagle's medium, fixed with ice-cold methanol, and blocked with 20% goat serum in PBS. The extracellular domain of CD31 was detected using 9G11, a mouse anti-CD31 monoclonal, at 2  $\mu$ g/ml and visualized with goat anti-mouse IgG tetramethylrhodamine isothiocyanate conjugated secondary antibody (Sigma). Cells were washed and blocked for a second time with 20% goat serum, and the cytoplasmic tail of CD31 was detected with the rabbit polyclonal serum described above at 20  $\mu$ g/ml. Bound antibody was visualized with goat anti-rabbit IgG (whole molecule) (Fab)<sub>2</sub> fragment fluorescein isothiocyanate-conjugated secondary antibody. The cells were observed using a Zeiss Axiovert photomicroscope with 40 $\times$  objective and photographed on Kodak Ektachrome 200. A combination of automatic and manual exposure times were used to ensure equal exposure times between different CD31 constructs.

**Enzyme-linked Immunosorbent Assay of Fc Proteins**—Immulon-3 96-well plates were precoated with 1  $\mu$ g/well goat-anti human-Fc Ig overnight at 4 °C, blocked with PBS/2% bovine serum albumin for 2 h at room temperature, and then coated with chimeric proteins (0.5  $\mu$ g/well) for at least 2 h at room temperature. Primary antibody was added in saturating amounts, followed by peroxidase-conjugated goat anti-mouse (1:1000 dilution, Amersham Life Sciences, Bucks, UK). Each layer was incubated for 30 min at room temperature and followed by four washes. The assay was visualized with *O*-phenylenediamine dihydrochloride, and absorbance was read at 450 nm.

## RESULTS

**The Extracellular Domain of CD31 Alone Is Competent to Mediate Homophilic Adhesion**—Studies by Sun *et al.* (34) have suggested that exons 9 and 10 (the transmembrane domain and the first 15 amino acids of the cytoplasmic tail) are required for CD31-mediated homophilic adhesion. Clearly, if this were the case, the use of soluble chimeras comprised of the extracellular domain of CD31 fused to human Fc would not be an appropriate approach to dissecting CD31 homophilic adhesion. To test this assertion, we constructed a GPI anchored form of CD31 (CD31-GPI), in which the transmembrane and cytoplasmic domains of CD31 are replaced by the 31 carboxyl-terminal amino acids of LFA-3 to provide a GPI-anchor signal sequence (35). Transient expression of both CD31-GPI and full-length CD31 in COS cells supported comparable binding in a homophilic adhesion assay to CD31 (D1-D6)Fc immobilized on plastic compared with negative control protein MUC18 (D1-D5)Fc (Fig. 1A). The expression of a GPI-anchored form of CD31, was verified by two independent methods. Firstly, dual immunofluorescence with 9G11 (a monoclonal antibody raised against the extracellular domain of CD31) and a rabbit polyclonal antibody raised against a cytoplasmic tail peptide of CD31 (Fig. 1B) demonstrated expression of CD31 extracellular domain but

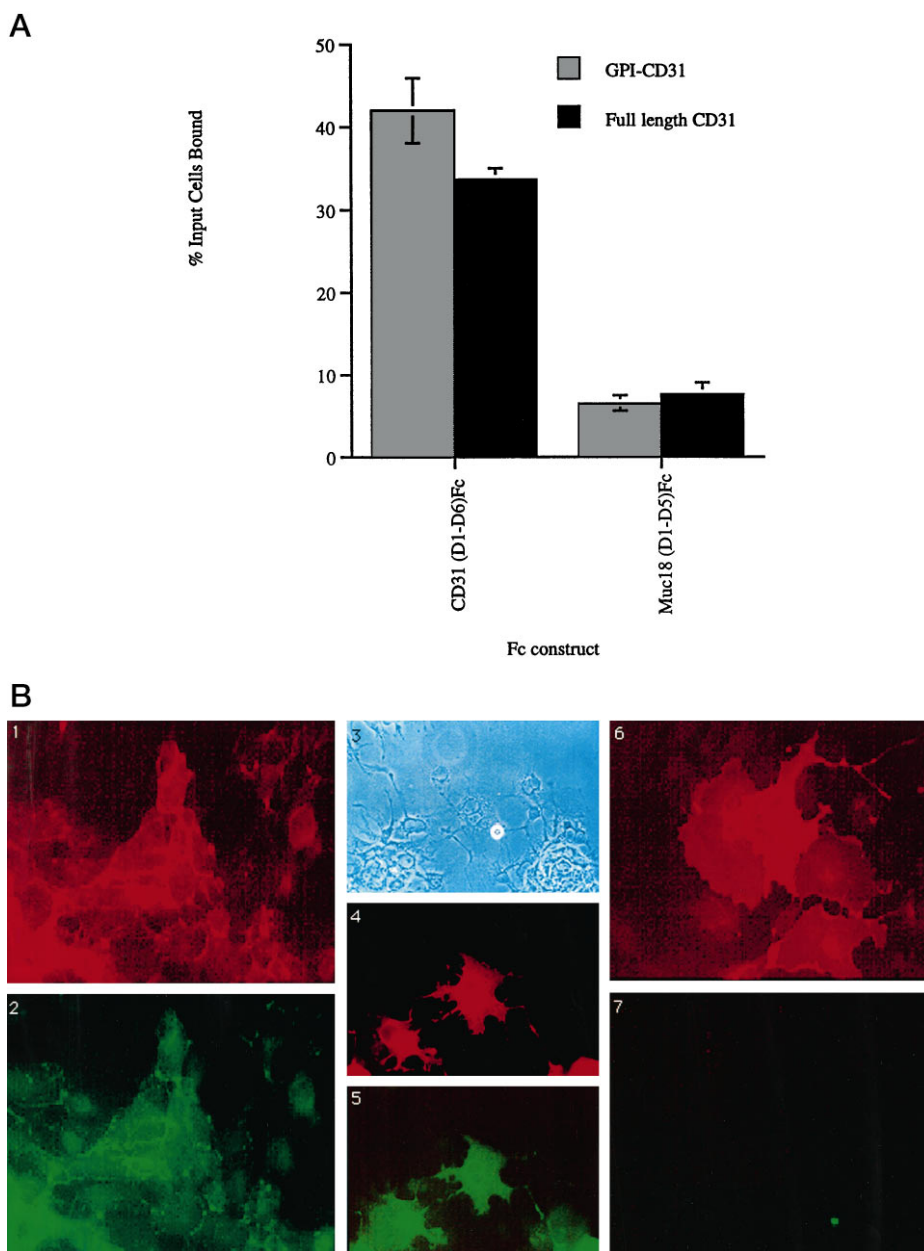
no detectable cytoplasmic tail. Secondly, the presence of a GPI linkage was confirmed by treating COS transfectants with phosphatidylinositol phospholipase C and following the loss of cell surface expression by FACScan. Full-length CD31 expression was not significantly affected by phosphatidylinositol phospholipase C treatment (increase of 5.5%), whereas CD31-GPI expression is reduced by 47.5%, comparable with the 37.8% reduction observed for CD14, which is known to be GPI-linked. These results demonstrate that the extracellular domain of CD31 alone is sufficient to support homophilic adhesion and that adhesion is not dependent on either transmembrane or cytoplasmic sequences.

**The NH<sub>2</sub>-terminal Domain of CD31 Is Necessary but Not Sufficient to Support Homophilic Adhesion**—Previous studies have led us to propose a model for CD31 homophilic adhesion in which the NH<sub>2</sub>-terminal immunoglobulin domains bind to membrane proximal domains of CD31 expressed on the surface of an apposing cell and *vice versa*, in a fully interdigitating anti-parallel mode of adhesion (24). Evidence for the role of the NH<sub>2</sub>-terminal domains in homophilic adhesion was obtained by antibody blockade and by use of truncated soluble recombinant forms of CD31 as inhibitors of adhesion. One limitation of this approach is the paucity of domain-specific blocking antibodies. To directly investigate the role of the NH<sub>2</sub>-terminal domains in homophilic adhesion, we made a series of chimeric ICAM-3/CD31 constructs in which immunoglobulin domains from CD31 are replaced by the equivalent domains from ICAM-3 (CD50) as summarized in Fig. 2A. COS cells expressing each construct were analyzed by FACScan and immunoprecipitation to show expression of chimeras containing the appropriate domains (Table I). Transfectants were then allowed to adhere to plastic coated with CD31 (D1-D6)Fc (Fig. 2B). Replacement of the first NH<sub>2</sub>-terminal domain of CD31 results in complete ablation of adhesion, indicating that domain 1 is necessary for homophilic adhesion.

A significant prediction of a fully interdigitating mode of adhesion for CD31 is that two binding sites exist on each molecule, and as such, removal of one site would be expected to reduce rather than ablate binding. Clearly the results obtained with the ICAM-3/CD31 chimeras binding to wild type CD31 described above are not fully consistent with this model. The identification of domains 5 and 6 as a component of the homophilic binding site was based upon the observation that in a series of nested deletion constructs, only those containing 5 or 6 domains supported homophilic adhesion. One possibility is that the identification of domains 5 and 6 as a component of the homophilic binding site arose because steric hindrance blocked the binding of cell-expressed CD31 to truncated CD31Fc constructs when in proximity to plastic. Previous experience with similar constructs for CD66 (36), ICAM-3 (37), and sialoadhesin (38) would suggest that this is not the case. However, to completely eliminate this possibility we have produced two additional constructs in which NH<sub>2</sub>-terminal domains of CD31 are fused to an irrelevant ICAM-3 stalk, in all cases preserving the overall length of the extracellular portion as six Ig type domains (Fig. 2A and Table II). In an adhesion assay where COS cells expressing full-length CD31 were allowed to adhere to each of these constructs (Fig. 2C), the three NH<sub>2</sub>-terminal domains of CD31 did not support adhesion, even though the CD31 NH<sub>2</sub>-terminal domains were presented in the context of a construct the same length as wild type CD31. The combined results from these constructs indicate that although the NH<sub>2</sub>-terminal domains are necessary for CD31 homophilic adhesion, they are not sufficient. Significant homophilic binding occurs only in the context of a full-length extracellular domain.

**Further Definition of the Homophilic Binding Site in Domain**

FIG. 1. *A*, full-length transmembrane CD31 and CD31-GPI are adhesion competent. COS cells transiently expressing full-length CD31 or GPI-linked CD31 were allowed to adhere to plastic coated with CD31(D1-D6)Fc or negative control MUC18(D1-D5)Fc proteins. Both GPI-linked and full-length CD31 support homophilic adhesion. *B*, CD31-GPI constructs lack a cytoplasmic tail. COS cells expressing full-length CD31 or CD31-GPI and human umbilical vein endothelial cell endothelial monolayers were stained with 9G11, a mouse monoclonal antibody against the extracellular domain of CD31, and a rabbit polyclonal anti-serum against a peptide derived from the cytoplasmic tail of CD31. *Panels 1* and *2* show human umbilical vein endothelial cell staining; *panels 3, 4, and 5* are COS full-length CD31 transfectants; and *panels 6 and 7* show COS CD31-GPI transfectants. *Panels 1, 4, and 6* are stained with 9G11, goat anti-mouse tetramethylrhodamine isothiocyanate secondary; *panels 2, 5, and 7* are stained with anti-CD31 cytoplasmic tail polyclonal, goat anti-rabbit fluorescein isothiocyanate secondary. *Panel 3* is phase contrast. Staining shows specificity of polyclonal reagent in full-length CD31 transfectants and human umbilical vein endothelial cell and shows that no cytoplasmic tail is detectable in CD31-GPI transfectants.



*1 of CD31 by Site-directed Mutagenesis*—Having established that the NH<sub>2</sub>-terminal domain of CD31 plays a genetically dominant role in mediating homophilic binding, a mutagenesis screen of domain 1 was undertaken. We aligned the primary amino acid sequences of CD31 and VCAM-1, and using the co-ordinates for the crystal structure of VCAM-1 domains 1 and 2 (39), produced a homology based model of the first two domains of CD31. As targets for mutagenesis, we selected all of the predicted solvent exposed aspartate, glutamate, arginine, and lysine residues in domain 1 and a subset of charged residues in domain 2 encompassing the putative heparin binding motif LKREKN (22). For the most part, charged residues were substituted with alanine, using a two-step recombinant PCR approach to generate 26 single point mutants in a CD31(D1-D6)Fc backbone. Mutant proteins were produced in COS cells and purified from culture supernatants with protein A-Sepharose.

The structural integrity of the mutants was assessed by enzyme-linked immunosorbent assay profile (Table III) using a panel of 12 anti-CD31 monoclonal antibodies predominantly against epitopes in domains 1 and 2 (including blocking anti-

bodies L133.1, hec 7.2, and 5.6E) but also including antibodies mapping throughout the extracellular domain. Of the mutants examined, only one (K50E) was judged to be grossly misfolded and excluded from further analysis.

To address the effect of mutations of CD31 on homophilic adhesion, assays were performed to determine the adhesion of COS cells transiently expressing full-length CD31 to plastic coated with mutant and wild type Fc proteins at 0.5  $\mu$ g/well (a concentration determined to be saturating). When compared with wild type CD31(D1-D6)Fc, mutations D11A and K89A were found to abolish binding, whereas mutations D33A, K50A, and D51R reduced binding (Fig. 3A). To verify these findings, mutant proteins D11A, D33A, D51R, and K89A were titrated in the range 0.1–2  $\mu$ g/well. There was no increase in cell binding with increasing concentration of protein (Fig. 3B), showing that the assay was indeed saturating.

This was further confirmed by subcloning each mutant that exhibited a functional effect from the CD31(D1-D6)Fc background into full-length transmembrane expressed CD31. In adhesion assays with COS cells expressing mutant CD31 binding to wild type CD31(D1-D6)Fc, significant inhibition of ho-

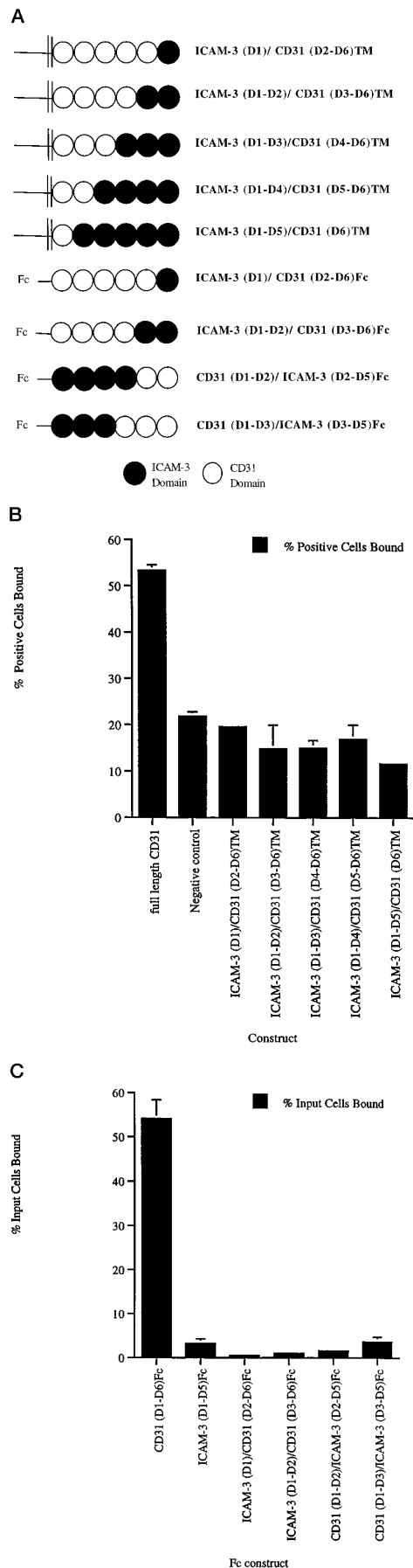


FIG. 2. A, ICAM-3/CD31 chimeric constructs. A series of ICAM-3 (CD50) and CD31 chimeric constructs were produced in which specific immunoglobulin-like domains of CD31 were replaced by equivalent

homophilic adhesion was observed (Fig. 3C). The mutant profile revealed using Fc chimeras was fully confirmed in the context of cell expressed transmembrane bound forms.

Based on the model of CD31, examination of the relative position of the residues identified by site-directed mutagenesis shows both faces of immunoglobulin domain 1 to be involved. Residues D11 and D33 form a discrete acidic cluster on the A and B strands, respectively, located on the ABDE face. K89 is located at the top of the interstrand FG-loop, and K50 and D51 are located in the CD loop, collectively indicating an extensive contact area on the CFG face.

#### DISCUSSION

In the present study we have used a well established and defined system of recombinant IgG chimeric constructs and heterologous COS cell adhesion assays to dissect CD31 homophilic adhesion. The major findings may be summarized as follows. First, the extracellular domain of CD31 is capable of supporting homophilic adhesion, independent of any contribution from either the transmembrane or the cytoplasmic tail sequences. Second, CD31 domain 1 is necessary to mediate homophilic adhesion, but together with domains 2 and 3 it is not sufficient to support adhesion when expressed out of the context of the whole extracellular domain. Thus domain 1 is necessary but not sufficient for homophilic binding. Third, we have identified five residues in domain 1 that are essential to mediate homophilic adhesion.

Others have suggested that the transmembrane and cytoplasmic tail of CD31 are required to mediate homophilic adhesion (34); however, in the present study we show that the extracellular domain alone expressed on a GPI anchor is sufficient to support adhesion. Goldberger *et al.* (40) have identified a soluble form of CD31 present in human plasma at levels of 10–25 ng/ml, resulting from alternative splicing of the transmembrane domain. This form of CD31 may therefore function as a homophilic ligand for cell expressed CD31 and serve as an endogenous regulator of leukocyte transmigration either by restricting leukocyte transmigration under normal circumstances or as a mechanism of down-regulating transmigration following inflammatory stimuli.

The experiments described above were designed to test our previous model of CD31-mediated homophilic adhesion, which envisaged that the NH<sub>2</sub>-terminal, membrane proximal domains of CD31 binding to the membrane proximal domains on an opposing cell surface and *vice versa*. To further explore the role of the NH<sub>2</sub>-terminal domains, we made a series of chimeric ICAM-3/CD31 constructs, in which a nested series of replacements of CD31 domains by the equivalent domains from

domains from ICAM-3. D1 through D6 indicate the immunoglobulin folds of the extracellular domain (numbering from the NH<sub>2</sub> terminus). *TM* indicates that the transmembrane and cytoplasmic tail of CD31 are present. *Fc* indicates that the protein is produced as a soluble recombinant fusion with a human IgG constant region tag. *B*, CD31 domain 1 is necessary to support homophilic adhesion. COS cells transiently expressing the ICAM-3/CD31 TM chimeras described in Fig. 2 were allowed to adhere to plastic coated with CD31 (D1-D6)Fc or negative control ICAM-3 (D1-D5)Fc proteins. Replacement of NH<sub>2</sub>-terminal domain 1 of CD31 with the equivalent domain of ICAM-3 results in complete ablation of CD31 homophilic adhesion, indicating that the homophilic binding site resides at least in part in domain 1 of CD31. *C*, the NH<sub>2</sub>-terminal domains of CD31 alone are not sufficient to support homophilic adhesion. COS cells expressing full-length CD31 were allowed to adhere to plastic coated with CD31/ICAM-3 Fc chimeras, in which either the NH<sub>2</sub>-terminal domains of CD31 are replaced by equivalent domains from ICAM-3 or in which the NH<sub>2</sub>-terminal domains of CD31 were expressed on an ICAM-3 stalk. Removal of NH<sub>2</sub>-terminal domain 1 ablates binding, as noted previously. However, expression of the NH<sub>2</sub>-terminal domains alone without the membrane proximal domains is not competent to support adhesion.

TABLE I  
Expression of ICAM-3/CD31 transmembrane chimeras

COS cells transiently expressing full-length CD31 or ICAM-3/CD31 chimeras were cell surface labelled with biotin, immunoprecipitated (IP) with anti-CD31 or anti-ICAM-3 mAbs, run out on SDS-polyacrylamide gel electrophoresis gels, and protein detected with streptavidin-horseradish peroxidase and Amersham ECL kit. All of the chimeras are recognized by the anti-ICAM-3 domain 1-specific antibody CAL 3.10 as expected and with the exception of ICAM-3 (D1-D5)/CD31 (D6) are recognized by CAL 31.1, an anti-CD31 antibody specific for domain 6 of CD31, which is consistent with the expected chimeric nature of the proteins. The failure of ICAM-3 (D1-D5)/CD31 (D6)/TM to be recognized by CAL31.1 may indicate that this construct is misfolded or that the antibody recognizes an epitope at the boundary between domains 5 and 6. None of the chimeras is recognized by 9G11, an anti-CD31 mAb specific for domain 1. The percentage of COS cells expressing each construct was assessed by FACScan analysis with either mAb 9G11 or mAb CAL 3.10.

Construct	IP result with			COS cells expressing construct %
	9G11 (anti-CD31 D1)	CAL 31.1 (anti-CD31 D6)	CAL 3.10 (anti-ICAM-3 D1)	
full-length CD31	+	+	–	40.6
ICAM-3 (D1)/CD31 (D2-D6)/TM	–	+	+	63.2
ICAM-3 (D1-D2)/CD31 (D3-D6)/TM	–	+	+	43.1
ICAM-3 (D1-D3)/CD31 (D4-D6)/TM	–	+	+	55.1
ICAM-3 (D1-D4)/CD31 (D5-D6)/TM	–	+	+	48.5
ICAM-3 (D1-D5)/CD31 (D6)/TM	–	–	+	38.9

TABLE II  
ELISA profile of ICAM-3/CD31 Fc chimeras

Solid phase ELISAs were performed on each construct using anti-CD31 and anti-ICAM-3 antibodies previously mapped to specific domains (24,28). ELISAs show the expected pattern of cross-reactivity with each construct, with the exception of L133.1 (anti-CD31 domain 2), which fails to recognize ICAM-3 (D1)/CD31 (D2-D6)Fc, indicating that the epitope may reside in the boundary between domains 1 and 2.

Construct	anti-CD31 mAbs				anti-ICAM3 mAbs			
	9G11 (D1)	L133.1 (D2)	10B5 (D5)	CAL31.1 (D6)	CAL3.10 (D1)	CAL3.24 (D1–2)	CH3.2 (D1–2)	KS128 (D2)
CD31 (D1-D6)Fc	+	+	+	+	–	–	–	–
ICAM-3 (D1-D5)Fc	–	–	–	–	+	+	+	+
ICAM-3 (D1)/CD31 (D2-D6)Fc	–	–	+	+	+	+	+	+
ICAM-3 (D1-D2)/CD31 (D3-D6)Fc	–	–	+	+	+	+	+	+
CD31 (D1-D2)/ICAM-3 (D2-D5)Fc	+	+	–	–	–	–	–	–
CD31 (D1-D3)/ICAM-3 (D3-D5)Fc	+	+	–	–	–	–	–	–

TABLE III  
ELISA profile of CD31 (D1-D6)Fc mutants

Solid phase ELISAs were performed on each mutant with a panel of 12 anti-CD31 monoclonal antibodies. Antibodies recognizing epitopes throughout the extracellular domain were used (domain mapping (24) and unpublished observations). +++ indicates greater than 80% of wild type CD31 positive control, ++ indicates 60–80% of positive control, + represents 40–60% positive control, and – indicates less than 40% of positive control. On the basis of this profile K50E was judged to be grossly misfolded and excluded from subsequent analysis. Interestingly none of the known blocking antibodies were affected by the blocking mutations in domain 1.

Mutant (domain)	CLB468 (D2)	VM64 (ND)	P2B1 (ND)	CLB-58 (D5)	5.6E (D2)	WM59 (D2)	CAL 31.1 (D6)	9G11 (D1)	Igi18 (ND)	L133.1 (D2)	10B8 (D5)	hec 7.2 (D2)	Negative
E2A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	–
D11A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
K13A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
D17A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
K24A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
D33A	+++	++	+++	+++	+++	+++	++	++	+++	+++	++	+++	–
K41A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	–
K50A	+++	++	+++	++	++	+++	++	++	++	+	++	++	–
K50E	+	++	–	–	–	+	–	–	–	–	+	–	–
D51R	+++	+++	–	–	++	+++	+++	+++	+++	++	+++	+++	–
D52A	+++	+++	+++	+++	++	+++	++	+++	+++	++	+	++	–
K62A	+++	+++	++	+++	+++	+++	+++	+++	+++	++	++	++	–
E65A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	–
E71A	+++	+++	+++	–	++	++	+++	+++	++	++	+++	+++	–
R73A	+++	++	+++	+++	+++	+++	++	+++	+++	+++	++	+++	–
D76A	+++	+++	–	++	+++	+++	+++	+++	+++	+++	+++	+++	–
K81A	+++	+++	++	+++	+++	+++	++	+++	+++	++	++	++	–
K89A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	–
E90A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	–
K91A	+++	+++	+++	+++	+++	++	++	+++	+++	+++	+++	+++	–
E95A	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	++	+++	–
E101A	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
E139A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	–
E142A	+++	+++	++	+++	+++	+++	++	+++	+++	++	++	++	–
E145A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
E153A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–

ICAM-3 were allowed to adhere to full-length CD31, demonstrating that domain 1, in addition to domains 2 and 3 previously implicated (24), is involved in homophilic adhesion (Fig.

2, B and C). An important prediction of a fully interdigitating anti-parallel model for homophilic binding is that removal of one of the two binding sites on each molecule would be expected

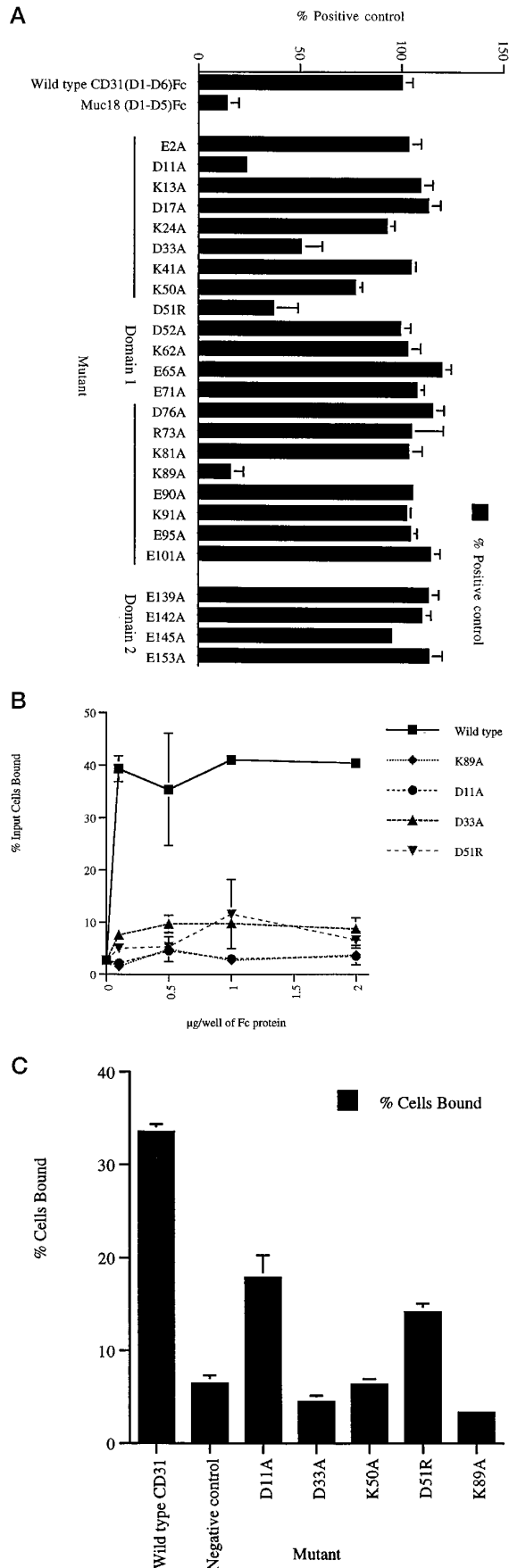


FIG. 3. A, site-directed mutagenesis dissection of the homophilic adhesion face. COS cells transiently expressing wild type full-length CD31 were allowed to adhere to CD31(D1-D6)Fc mutant and wild type proteins or MUC18(D1-D5)Fc as a negative control. The percentage of

to reduce rather than eliminate binding. In our present study we find that replacement of the NH<sub>2</sub>-terminal domain 1 alone completely ablates binding (Fig. 2, B and C). In principle this could arise because removing one binding site in a two-site interaction reduces the affinity of the interaction below the detectable threshold of the assay employed. The obvious alternative interpretation is that homophilic binding results from a direct interaction between NH<sub>2</sub>-terminal domains, excluding a role for the membrane proximal portion of the molecule. This latter interpretation is consistent with the results obtained by Sun *et al.* (34), who used an elegant series of loss-of-function and gain-of-function mutants to demonstrate that homophilic adhesion required the presence of domains 1 and 2 and that it was possible to confer the ability to bind human CD31 on mouse CD31 by replacing its first two domains with the equivalent domains from human CD31. To determine whether the NH<sub>2</sub>-terminal domains alone are homophilically competent in a non-CD31 backbone, we produced constructs in which the NH<sub>2</sub>-terminal domains of CD31 were expressed on a stalk consisting of ICAM-3 Ig domains. These chimeras preserved the overall length of the extracellular domain as compared with wild type CD31, thus removing any steric constraints arising from the use of truncated CD31-Fc chimeric proteins.

Importantly, these reagents revealed that although the NH<sub>2</sub>-terminal domains are necessary, they are not sufficient to support stable homophilic adhesion outside of the context of an accessory function provided by the membrane proximal CD31 domains. We propose a model in which stable homophilic contact between the NH<sub>2</sub>-terminal domains of CD31 requires that these binding domains are held in the correct spatial orientation. We propose that this function is provided by the membrane proximal domains. This would explain the positive gain-of-function results obtained by Sun *et al.* (34) and described above. In this construct the binding functions would be provided by the human NH<sub>2</sub>-terminal domains, whereas the murine domains perform an accessory role in maintaining the human domains in the correct orientation. Interestingly a number of studies have suggested a positive role for domain 6 in CD31-mediated interactions. For example, Fab fragments of the antibody 4G6, which recognizes the epitope CAVNEG in domain 6 of CD31 (41), has been shown to enhance CD31 homophilic adhesion (42). Similarly, in a mixed lymphocyte reaction proliferation assay, an antibody recognizing domain 6 and a peptide derived from this domain were found to exert an inhibitory effect (43).

The mechanism by which the membrane proximal domains modulate the the binding domains is currently under investigation. One attractive possibility is that these domains mediate *cis*-interactions between CD31 on the same cell to dimerize or

input cells bound after two washes was calculated relative to the wild type positive control, and the results were expressed as the means  $\pm$  S.D. ( $n = 3$ ). The results are representative of four experiments on two independently produced batches of protein. B, titration of CD31 mutants. COS cells transiently expressing CD31 were allowed to adhere to a concentration range (0–2  $\mu$ g/well) of CD31(D1-D6)Fc mutant and wild type proteins. The concentration range was achieved by precoating 1  $\mu$ g/well anti-human Fc immunoglobulin and then coating with chimeric protein at the indicated concentration. Adhesion was quantified after two washes, and the results are expressed as the means  $\pm$  S.D. ( $n = 3$ ). C, CD31 mutants expressed as transmembrane bound forms on the surface of COS cells. Mutants identified by screening Fc chimeras were subcloned from an Fc chimera background into a full-length transmembrane form of CD31. COS cells expressing mutant CD31 were allowed to adhere to plastic coated with wild type CD31(D1-D6)Fc or negative control MUC18(D1-D5)Fc protein and adhesion quantified after two washes. The results are expressed as the means  $\pm$  S.D. ( $n = 3$ ) and are corrected for transfection efficiency (20–40% cells positive as determined by FACScan analysis).

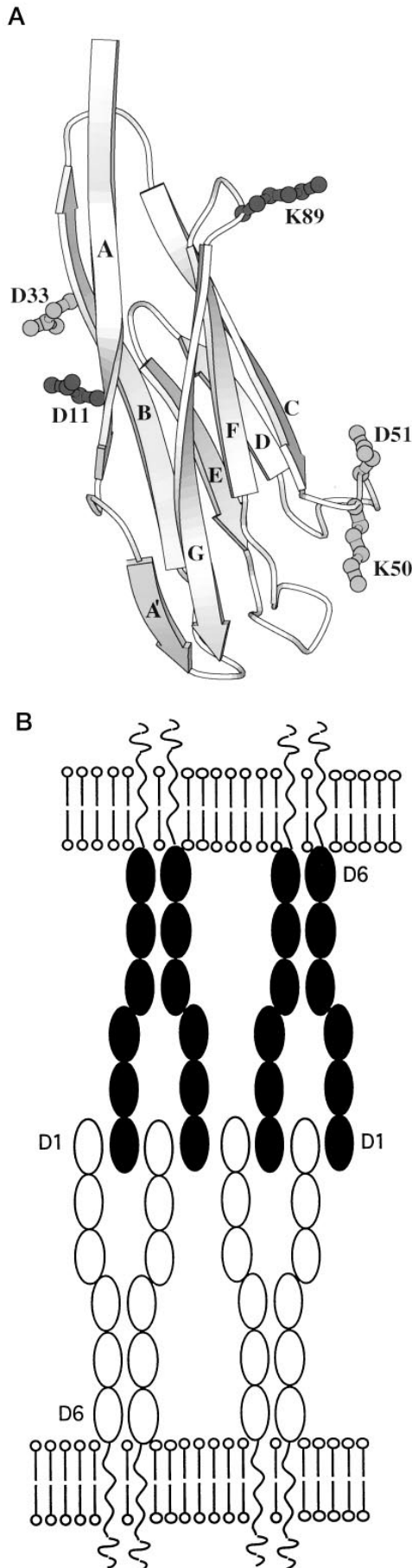


FIG. 4

cluster multiple low affinity binding domains, resulting in an increase in overall avidity, as has been shown for ICAM-1 (44) and cadherins (45, 46). In addition it has also been demonstrated that immunoglobulin domains distinct from the ligand binding domains are capable of mediating homodimerization, for example, the fourth immunoglobulin domain of the stem cell factor receptor (47).

We therefore envisage a model in which CD31 homophilic engagement involves both *cis*- and *trans*-interactions. In our model, low affinity *trans*-interactions mediated by NH<sub>2</sub>-terminal domains are clustered to achieve higher avidity by *cis*-interactions mediated by the membrane proximal domains, forming a zipper-like interaction (Fig. 4B). This model provides interesting regulatory possibilities. A puzzling aspect of CD31 biology is the fact that although CD31 is widely expressed on mature, circulating hemopoietic cells, CD31<sup>+</sup> cells do not undergo spontaneous CD31-mediated homotypic aggregation. This could be explained in terms of regulation of the *cis*-interactions of CD31, for example by cytoskeletal association and cell surface distribution. The recent finding that cytokine treatment of endothelial monolayers induces a redistribution of CD31 from sites of cell-cell contact to the apical surface are particularly suggestive and could also provide a mechanism for switching between homophilic and heterophilic interactions (48). Alternatively, conformational changes in the membrane proximal domains may be directly translated into conformational shifts in the binding domains and consequent changes in affinity in a fashion analogous to integrins.

The immunoglobulin fold has been identified as mediating a variety of interactions, for example antigen-antibody complexes (50), heterophilic interactions with integrins such as ICAM-1,2,3/LFA-1, VCAM/VLA-4, MadCAM/ $\alpha_4\beta_7$ , and CD31/ $\alpha_v\beta_3$ , (reviewed in Ref. 49) pseudo-homophilic interactions, *e.g.* CD2/CD48 (51), and fully homophilic interactions such as CD31-CD31. The key feature of the immunoglobulin fold is a core structure formed by two  $\beta$ -sheets packed face to face. A comparison of structures solved at atomic resolution shows that the regions peripheral to the core, such as the edge strands of the  $\beta$ -sheets, and the loops that link the strands can have quite different conformations. Mirroring this structural diversity, different immunoglobulin-folds utilize different regions to interact with their ligands.

In antigen-antibody complexes, antigen recognition and binding is mediated by loops at the NH<sub>2</sub>-terminal end of the fold (50). A recurring theme among integrin-Ig superfamily interactions is the utilization of acidic residues presented in the NH<sub>2</sub>-terminal portion of the loop joining the CD  $\beta$ -strands on the CFG face of the Ig fold, for example, the LETS motif identified in ICAM-3 (37) and the QIDS motif in VCAM (51). In the case of CD2/CD48, a pseudo-homophilic interaction, mutagenesis data implicate an adhesive surface comprised of the equivalent GFCC'C'' faces of the immunoglobulin fold in both

FIG. 4. A, distribution of the residues implicated in homophilic adhesion on an Ig-like fold. A schematic diagram of a representative adhesive immunoglobulin domain (based on the crystal structure of VCAM domain 1 (39)) is shown. Modelled inserts of five, four, and one additional residue at the NH<sub>2</sub> terminus, the BC loop, and the FG loop, respectively, are included to indicate a putative topology appropriate for residues 3-100 of CD31. The residues implicated in homophilic CD31 adhesion when mapped in this three-dimensional context cluster on two sides of the Ig fold. Inserts were modelled in the interactive graphics program FRODO (55), and the figure was produced in the program MOLSCRIPT (56). B, model of CD31 homophilic engagement. We propose a zipper model of CD31 homophilic adhesion at the cell surface, in which the NH<sub>2</sub>-terminal domains mediate homophilic binding but require lateral clustering mediated by *cis*-interactions, increasing avidity to give stable adhesive contacts.

CD2 and CD48 (52). To date, no systematic site-directed mutagenesis of any fully homophilic Ig superfamily interaction has been undertaken. There is some limited data on the interaction of neural cell adhesion molecule based on the ability of a decapeptide derived from its third immunoglobulin domain to block homophilic adhesion (53). Likewise spontaneous mutations in L1 give rise to neurological disease such as X-linked hydrocephalus (54); however, many of these mutations result in frameshifts, premature truncation, etc., and only 26 result in missense mutations. Of these, 13 are predicted to affect key structural residues or domain boundaries and destabilize the protein, whereas others introduce cysteines and may promote intermolecular disulfides. Hence spontaneous mutations have so far yielded few insights into the nature of homophilic interactions. Our present study therefore represents the most extensive directed analysis of the structures mediating Ig superfamily homophilic adhesion.

Significantly, site-directed mutagenesis of CD31 has identified residues on both faces of the immunoglobulin fold as being involved in the homophilic contact sites. Specifically a cluster of two acidic residues, D11 and D33, which lie on the predicted A and B strands, K89, which lies at the top of the FG loop, and two residues K50 and D51 on the CD-loop. This finding represents a novel mode of interaction between immunoglobulin folds and is consistent with the zipper model proposed above, where each CD31 molecule interacts with two others on an apposing cell surface, thus requiring two distinct binding faces.

CD31-mediated adhesion is complex both in terms of the number of potential ligands, both homophilic and heterophilic, and in terms of the domains involved in each interaction. This study has identified a role for both NH<sub>2</sub>-terminal and membrane proximal domains of CD31 in homophilic adhesion and proposes a zipper model analogous to that seen among cadherins to explain the available data. This model is consistent with mutagenesis data, which indicates that both faces of immunoglobulin domain 1 are involved in the homophilic interaction and provides a framework for biophysical analysis of homophilic adhesion.

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