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**FUNCTIONAL ADHESIVENESS OF THE CX3CL1 CHEMOKINE REQUIRES ITS
AGGREGATION: ROLE OF THE TRANSMEMBRANE DOMAIN***

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In its native form, the chemokine CX3CL1 is a firmly adhesive molecule promoting leukocyte adhesion and migration and hence involved, with its unique receptor CX3CR1, in various inflammatory processes. We investigated here the role of molecular aggregation in the CX3CL1 adhesiveness. Assays of bioluminescence resonance energy transfer (BRET) and homogeneous time-resolved fluorescence (HTRF) in transfected cell lines and in primary cells showed specific signals indicative of CX3CL1 clustering. Truncation experiments showed that the transmembrane domain (TM) played a central role in this aggregation. A chimera with mutations of the 12 central TM residues had significantly reduced BRET signals and characteristics of a non-clustering molecule. This mutant was weakly adhesive, according to flow and dual pipette adhesion assays and was less glycosylated than CX3CL1, although, as we demonstrated, loss of glycosylation did not affect the CX3CL1 adhesive potency. We postulate that cell surfaces express CX3CL1 as a constitutive oligomer and that this oligomerization is essential for its adhesive potency. Inhibition of CX3CL1 self-assembly could limit the recruitment of

CX3CR1 positive cells and may be a new pathway for anti-inflammatory therapies.

Migration of circulating leukocytes to injury sites is the first step of the inflammation process, which involves a sequence of coordinated interactions between leukocytes and endothelial cells (1). Central to this physiological and pathological event are chemokines, a family of low molecular weight soluble proteins that function to attract leukocytes bearing the appropriate receptors (2). Chemokines trigger leukocyte activation and their firm adhesion to inflamed endothelium, mainly through the mediation of integrins and their cognate ligands (3).

Among chemokines, two are exceptions: CXCL16 and CX3CL1, are type-I membranous proteins: in addition to their chemokine domain (called CD), they are composed of a long mucin-like stalk, a transmembrane domain and a cytoplasmic tail (4,5) (Figure 1). The CX3CL1 molecule, with its unique CX3CR1 receptor (6), has been shown to be central in defenses against neurodegenerative disorders (7,8) and against several cancers in murine models (9,10). The CX3CL1/CX3CR1 axis is also involved in various inflammatory diseases (2), including renal inflammation (11) and atherosclerosis

(12,13). Understanding the structure of this pair of molecules is necessary for exploring pharmacological methods to regulate their activity. The structure of CX3CR1, a receptor of the GPCR family, is relatively well known, but that of CX3CL1 much less so.

The CX3CL1-CD (76 residues) - a globular protein domain 3 nm in diameter (14) maintained by two disulfide bridges - is structurally similar to other chemokines. It is composed of a disordered N-terminus up to the first cysteine (Cys8), a long loop followed by a 3-stranded antiparallel β -sheet (residues 24-51) and a C-terminal α -helix (residues 56-67) packed against the β -sheet (15). The stalk (241 residues) is 26 nm in length (14) and highly glycosylated with 17 degenerate mucin-like repeats (6,16). This native form can be cleaved by various metalloproteinases (17,18) to release the soluble form, which, like other chemokines, is chemoattractant. In contrast, membrane-associated CX3CL1, with CX3CR1, mediates stable adhesion of leukocytes such as integrins (16,19). The mechanism of this adhesive property is poorly understood. While we know that the potent adhesiveness of CX3CR1 under flow requires that CX3CL1-CD have a high affinity for it (19), nothing is known about the ligand's quaternary structure.

Most of the soluble chemokines bind sulphated glycoaminoglycans of the extracellular matrix and endothelial cell surfaces. This immobilization leads to chemokine clustering and is essential for establishing the chemokine gradient that leads to directed migration. Moreover, numerous chemokines tend to self-associate at high concentration in solution and this clustering sometimes appears to be essential to their *in vivo* activity (20). For CX3CL1, however, the CD itself remains monomeric in solution, even at high concentration (15). The CD structure has been studied in crystals (21), where it includes four monomers, arranged as two asymmetric dimers. The potential aggregation

of the complete CX3CL1 molecule has not yet been investigated, however. We reasoned that CX3CL1 clustering could be important for its adhesive properties, as it is for integrins (22).

Among the many methods for studying aggregation of cellular proteins, resonance energy transfer techniques have emerged as useful tools for analyzing the proximity of membrane molecules in living cells (23). We used these techniques here to study the aggregation status of CX3CL1 in its native membranous state. We report that CX3CL1 is indeed clustered and that this clustering takes place primarily through its transmembrane domain. Moreover, we show that this aggregation is associated with the mature glycosylation of the protein and, most importantly, is required for its adhesive potency in flow conditions (flow adhesion assay) and under normal pulling (dual pipette assay).

EXPERIMENTAL PROCEDURES

Chemicals, proteins and monoclonals- Benzyl-2-acetamido-2-deoxy- α -D-galactopyranoside was purchased from Calbiochem (VWR International, Fontenay-sous-Bois, France), O-sialoglycoprotein endopeptidase from Cedarlane Laboratories (Tebu-Bio, Le Perray en Yvelines, France). Neuraminidase (acylneuraminydase) and O-glycosidase (endo-D-galactosyl-N-acetyl- α -galactosaminohydrolase) came from Roche Diagnostics (Meylan France). Monoclonal antibodies against CX3CL1-CD (clone 51637) and full-length CX3CL1 tagged with His₆ were purchased from R&D Systems (Lille, France), and CX3CL1-CD from Peprotech (Levallois-Perret, France).

Plasmid constructs- The CX3CL1 constructs in pcDNA3 (Invitrogen, Cergy-Pontoise, France), in pEYFP-N1 (Clontech, Ozyme, St Quentin-en-Yvelines, France) and pRluc-N2 (BioSignal Packard, PerkinElmer, Courtaboeuf, France) were made with CX3CL1-pBLAST (Invivogen Cayla,

Toulouse, France) as template and primers containing a Hind III restriction site in 5' position and a BamH I restriction site in 3' position for the PCR amplification, as explained in Supplementary Table S1. The HindIII/BamHI fragment was then cloned in the different plasmids. The truncated and mutated constructs were generated with the QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, Amsterdam, Netherlands). Briefly, 10 ng of various plasmid constructions was used as a template, with mutated nucleotide primers as described in Supplementary Table S1. PCR conditions were as follows: pre-denaturing at 95°C for 3 min, followed by 18 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 50 s and extension at 68°C for 7 min. After digestion with DpnI, 2 µl of PCR product was used to transform the XL10-Gold Ultracompetent cells provided with the kit. Appropriate clones were identified by sequencing.

Cell culture- HEK293 cells were maintained in Dulbecco modified Eagle's medium (DMEM) plus GlutaMax I (Invitrogen, Cergy-Pontoise, France) supplemented with 10% foetal bovine serum, and 1 mM sodium pyruvate. To obtain HEK clones expressing CX3CL1 or ALA12, CX3CL1-pEYFP and ALA12-pEYFP constructs were transfected into HEK cells with the cationic polymer transfection reagent, jetPEI (Polyplus transfection, Ozyme, St Quentin-en-Yvelines, France). Stable transformant resistant to 0.5 mg/ml G418 (Invitrogen, Cergy Pontoise, France) was selected with a FACS Aria cell sorter (Becton Dickinson, Le Pont de Claix, France). HUVEC were cultured in endothelial cell medium (PAA Laboratories, Les Mureaux, France), supplemented with 10% fetal bovine serum. HUVEC were incubated for 20 hours with TNF- α at 20 ng/ml and interferon γ at 500 U/ml (Peprotech; Levallois-Perret, France) to induce CX3CL1 expression.

Expression of CX3CL1 on HUVEC and HEK cells and clones was tested with phycoerythrin-labelled murine anti-CX3CL1 mAb (mAb clone 51637, R&D Systems Lille, France) and analysed by flow cytometry with a FACScan (Becton Dickinson, Le Pont-de-Claix, France).

BRET- Cells were seeded at a density of 100 000 cells per well in 12-well dishes 24 h before transfection. Transient transfections were performed with the cationic polymer transfection reagent, JetPEI (Polyplus transfection, Ozyme, St Quentin-en-Yvelines, France) in NaCl 150mM. In most experiments, 0.2 µg of various CX3CL1-pRLuc constructs was transfected alone or with increasing quantities of various CX3CL1-pEYFP constructs. The total amount of DNA transfected in each well was completed to 2.2 µg with empty pcDNA3 vector. After overnight incubation, the transfected cells were detached with phosphate buffer saline (PBS) and washed with HBSS buffer supplemented with 10 mM Hepes, 1 mM CaCl₂ and 0.5 mM MgCl₂. They were then seeded in 96-well black plates (Packard Bioscience, PerkinElmer) in 100 µl supplemented HBSS. Coelenterazine H (Interchim, Montluçon, France) was added to reach a final concentration of 5µM. Readings were collected with a microplate analyzer (Fusion Packard Bioscience PerkinElmer) that allowed the sequential integration of signals detected in the 485 \pm 20 nm window for luciferase and the 540 \pm 20 nm window for YFP light emissions. The BRET signal was determined by calculating the ratio of the light intensity emitted by the CX3CL1-YFP over the light intensity emitted by the CX3CL1-Luc. The values were corrected by subtracting the background BRET signal detected when the CX3CL1-Luc construct was expressed alone. The values are the mean over 15 measurements. To relate the BRET measurements to the actual CX3CL1 concentration, we measured the luminescence

or fluorescence of HEK transfected either with CX3CL1-Luc or CX3CL1-YFP constructs and quantified their CX3CL1 content in western blot experiments with CX3CL1-His₆-Tag (R&D Systems, Lille, France) as standards (Figure 2B).

HTRF- The monoclonal anti-CX3CR1 (clone 51637) was labeled with the different fluorescent donors or acceptors as described previously (24,25). The donor was a pyridine–bipyridine (PBP) europium cryptate (europium cryptate-PBP). The number of europium cryptate-PBP per antibody (molar ratio) was determined spectrophotometrically by measuring their absorbance at 280 nm and 317 nm and inserting these values into the equation

$$\text{Molar ratio} = (\text{OD}_{317 \text{ nm}} / \epsilon_{\text{europium cryptate-PBP}}) / - [(\text{OD}_{280 \text{ nm}} - (\text{OD}_{317 \text{ nm}} / A)) / \epsilon_{\text{antibody}}]$$

where the molar extinction coefficient of the europium cryptate-PBP ($\epsilon_{\text{europium cryptate-PBP}}$) was taken as 19,800 M⁻¹ cm⁻¹ and that of the antibodies as 210,000 M⁻¹ cm⁻¹. The factor A expressed the ratio ($\text{OD}_{317 \text{ nm}} / \text{OD}_{280 \text{ nm}}$) for europium cryptate-PBP and was determined to be 2. The final molar ratio was determined to be 1.1.

The anti-CX3CR1 monoclonal was labeled with the NHS ester derivatives D2 acceptor as previously described (26). The final number of dyes per antibody was determined spectrophotometrically. By using the maximum absorption for D2 at 650 nm and its molar extinction coefficient value (240,000 M⁻¹ cm⁻¹), the final ratio was determined to be 1.3. HEK clone cells and HUVEC (activated or not) were harvested and resuspended in KREBS buffer (Tris/HCL 20 mM pH 7.4, NaCl 118 mM, glucose 5.6 mM, KH₂PO₄, MgSO₄ 1.2 mM, KCl 4.7 mM, CaCl₂ 1.8 mM, and BSA 0.1%). Fifty thousand cells in 100 µl were distributed in 96-well black plates (Packard Bioscience, PerkinElmer) where 4 nM europium cryptate-labeled donor anti-CX3CL1 and 4 nM D2-labelled acceptor antibody (anti-CX3CL1 or

anti-Flag as control) were already present, diluted in the same buffer. After incubation at 37°C for 4 h, fluorescence emissions were measured, at both 620 nm and 665 nm, on a multidetection microplate reader (PHERAstar, BMG Labtech).

The HTRF ratio was calculated as $R = (\text{fluorescence at 665 nm} / \text{fluorescence at 620 nm}) \times 10^4$. The specific signal over background, noted as Delta F (ΔF), was calculated with the following formula:

$$\Delta F = (R_{\text{pos}} - R_{\text{neg}}) / R_{\text{neg}}$$

where R_{pos} corresponds to the ratio with anti-CX3CL1 mAb as the acceptor and R_{neg} to the ratio with anti-Flag mAb as the acceptor.

Static adhesion assay- Two different assays were performed. In the cell-cell model, performed as previously described (27), CHO-CX3CR1 and parental CHO were grown in supplemented DMEM in 96-well plate (5.10⁴ cells per well) and washed in HBSS buffer. Then 10⁵ HEK cells expressing variants of CX3CL1 were added for 45 min at room temperature. In the cell to protein adhesion model, a monoclonal anti-polyhistidine antibody (MAB050, R&D Systems, Lille, France) (250 ng in 25 mM Tris, pH 8, 150 mM NaCl) was adsorbed for 2 h at room temperature to flat-bottom 96-well microtiter plates (Nunc A/S, Roskilde, Denmark). Then CX3CL1-H₆, diluted in the same buffer, was incubated overnight at 4°C at a concentration of 1.8 µg/ml (50 µl/well in triplicate). Adhesion of HEK-CX3CR1 cells was performed as previously described (27) with an incubation time of 45 min at room temperature in HBSS buffer with or without enzymatic treatment.

In both cases, before the adhesion assay, the cells were labeled with 1 mM 5(6)carboxyfluorescein diacetate, succinimidyl ester (Interchim, Montluçon, France) and the microplate was read at 535 nm with a microplate analyzer (Fusion Packard Bioscience PerkinElmer).

Deglycosylation treatment- The adsorbed protein was digested with 4 mU neuraminidase in 40 mM Tris pH8 and 4 mM CaCl₂ for 2 h at 37°C before incubation with 1 mU O-glycosidase in PBS for 4 h at 37°C. Western blotting using an anti-CX3CL1 antibody analyzed the digested protein.

Parallel Plate Laminar Flow Chamber Adhesion Assay- The technique we used has previously been thoroughly described (28). Briefly, the coverslips were cultured with adherent clone HEK cells expressing either CX3CL1 or ALA12 mutant. The coverslip was mounted in a chamber set on the stage of an inverted microscope (TE300, Nikon, France) equipped with a phase contrast 10x objective (Nikon, n.a. 0.25) and a cooled CCD camera (Sensicam, PCO, Kelheim, Germany). The entire apparatus was kept at 37°C by a thermostatic chamber (Life Imaging Services, Reinach, Switzerland). HEK-CX3CR1 clone cells were suspended in PBS, incubated for 30 min at 37°C with 5 µM carboxy-SNARF-1 (SemiNaphthoRhodaFluor-1-acetoxymethylester, Molecular Probes, Invitrogen, Cergy-Pontoise, France) for labeling and resuspended in flow buffer (HBSS supplemented with 2.5 mM EDTA, 2.5 mM EGTA, 10 mM HEPES and 2 mg/ml BSA) at 10⁶ cells per ml. A syringe pump (PHD 2000; Harvard Apparatus, Les Ulis, France) drove 0.5 ml of cell suspension through the chamber at a wall shear stress of 1.5 dynes.cm⁻². After a 10-min wash at 1.5 dynes.cm⁻², fluorescent images of three separate 0.5 mm² fields were recorded to count the adherent cells (excitation 450-500 nm, emission 510-560 nm, dichroic filter Q505lp, Chroma, Brattleboro, VT, USA). The number of non-specific adhering cells was obtained in the same manner, except that the HEK-CX3CR1 clone cells were preincubated for 2 min with 100 nM soluble CX3CL1-CD (chemokine domain alone, R&D Systems, Lille, France)

Dual Pipette Aspiration Technique- The dual pipette adhesion assay was performed as already described (28). Briefly, two cells, collected by gentle aspiration onto the tip of each pipette (cell number 1 in pipette A, cell 2 in pipette B), were brought into contact with the micromanipulators and allowed to remain in contact for 4 minutes. To separate the cells, aspiration in pipette B was maintained at a level sufficiently high to hold cell number 2 tightly, while the aspiration in pipette A was increased in steps measured with a pressure sensor (Validyne, model DP103-38; ranging from 0 to 50 000 Pa). After each step, the pipettes were moved apart in an effort to detach the adherent cells from one another. A pair pulled intact from pipette A was moved back to the pipette orifice, the aspiration in the pipette was increased, and another attempt was made to detach the cells from each other. The cycle was repeated until the level of aspiration in pipette A was sufficient to pull one cell apart from the other. The aspiration employed in each cycle was monitored continuously. The values recorded for each of the last two cycles in the series (P_{n-1} and P_n) were used to calculate the separation force F for the pair tested, with the equation:

$$F = \pi (d/2)^2 (P_{n-1} + P_n) / 2 \quad (1)$$

with d the internal diameter of pipette A. The results were expressed as the mean ± SEM for 14 measurements.

RESULTS

BRET of CX3CL1 in the HEK cell line- To investigate by BRET the aggregation state of the chemokine CX3CL1 in its native membranous form, we developed various constructs of CX3CL1 chimera that expressed luciferase (Luc) or yellow fluorescence protein (YFP) on the cytoplasmic C-terminal side. As expected, the tagged CX3CL1 was expressed at the plasma membrane in transiently transfected HEK cells (Figure 2A insert). The membrane-targeted CX3CL1-YFP or CX3CL1-Luc accounted for about 50% of the total cellular CX3CL1, as already shown

for CX3CL1-GFP chimera (29). Finally, our CX3CL1 chimera also functioned as native CX3CL1 according to adhesion tests (data not shown), as the GFP chimera did (29).

Using such chimera with a ratio of 1/10 YFP (acceptor) over Luc (donor), we measured a high BRET ratio (Figure 2A right), higher than that obtained when the CX3CL1-YFP plasmid was replaced by the empty pEYFP construct (Figure 2A left), by CXCL16-YFP (Figure 2A middle) or by ICAM-4-YFP (not shown). These data suggest the specificity of the homotypic CX3CL1 BRET and show for the first time the probable self-assembly of CX3CL1 proteins in a cellular context. After transfecting HEK cells with various amounts of CX3CL1-YFP and a constant amount of CX3CL1-Luc (yielding 1.5 fmole CX3CL1-Luc per mg total protein, see Figure 2B), we plotted the BRET data versus the CX3CL1-YFP/CX3CL1-Luc ratio and observed a saturation curve (Figure 2C, filled squares), as expected for specific clustering (30,31). In contrast, the same curve plotted with CXCL16-YFP replacing CX3CL1-YFP remained at low amplitude (empty squares). Working with a constant CX3CL1-YFP/CX3CL1-Luc ratio, here equal to 5 (Figure 2D), we verified that the BRET level was constant when plotted against the total amount of tagged CX3CL1 (donor plus acceptor) (Figure 2D) and against the CX3CL1-YFP concentration (acceptor) (Figure 2E). Indeed, when BRET is specific, its amplitude should not depend on the total amount of interacting proteins (30-34) or on the concentration of the donor moiety (30). Finally, the significant BRET decrease in the presence of an excess of an untagged unit (Figure 2F) showed that the BRET was specific and not due to random collision. We concluded that CX3CL1 appears to aggregate specifically, at least in the HEK cell line.

HTRF measurements with cellular CX3CL1- To test whether native CX3CL1 aggregates similarly in primary cells, we used

the method called homogeneous time-resolved fluorescence (HTRF) (35), which has been applied to detect biomolecular interactions (36), including ligand-enzyme relations (37) and protein clustering (24). It consists here of measuring the resonance energy transfer between labeled antibodies specific against CX3CL1. Using both stably CX3CL1-expressing HEK cells (data not shown) and cytokine-activated HUVEC, also expressing CX3CL1 (16,38) (Figure 3A), we observed a marked level of energy transfer (Figure 3B, right, solid bar), substantially higher than the level observed with irrelevant monoclonals (Figure 3B, right, empty bar). This result was confirmed by plotting HTRF signals as the difference between specific and non-specific data (ΔF , see Materials and Methods) (Figure 3B, insert). In contrast, measurements of resting HUVEC, which do not express CX3CL1 (Figure 3A), showed no specific HTRF (Figure 3B left and insert). Accordingly, CX3CL1 appears natively aggregated when it is expressed at the external membrane of primary cells.

CX3CL1 clustering is mainly due to its transmembrane domain- We next sought to identify the domain of CX3CL1 involved in clustering, by progressively deleting the external - first the CD and then the mucin stalk - and intracellular domains of the molecule (Figure 4, top). We first checked that all these constructs, when transfected in HEK, produced proteins that effectively targeted the membrane (Figure 4A-C) and were expressed at levels similar to that of whole CX3CL1 (Figure 2A insert). After transfection with these constructs, the HEK still exhibited BRET. Each construct produced BRET with saturation curves (BRET versus [YFP]/[Luc] at a constant Luc concentration) (Figure 4D). Moreover, the BRET of these truncated CX3CL1 constructs met the criteria for specificity described above (Figure 2D), even the TM alone (Figure 4E). Yet, the Muc and TM constructs gave a $BRET_{max}$ lower than

that of CX3CL1 (Figure 2C), while the TM&cyto construct gave a higher level (Figures 4D and 4E). One should here recall that the BRET was not only dependent of the proximity of the donor and acceptor moieties, but also of their relative orientation (39). Hence little conformation change could dramatically affect the BRET level. Nevertheless, all the constructs we tested gave specific BRET (Figures 4D and 4E). So our data strongly suggest that the TM domain alone still aggregates and that this domain is the primary site involved in CX3CL1 clustering.

The ALA12 CX3CL1 mutant is not fully glycosylated and does not cluster- For additional evidence that CX3CL1 clusters through its TM, we looked for the TM residues whose mutations could suppress the specific BRET observed with the entire protein. We mutated sequentially in alanine the residues thought to be important for self-association of transmembrane helices (Figure 5 upper panel), such as glycine (40), leucine (41) and phenylalanine (42), and observed no significant decrease of BRET (Figure 5 lower panel).

We therefore decided to proceed to larger modifications. The 321-325 and 326-332 residues were replaced by their alanine counterparts, for the ALA5 and ALA7 mutants (Figure 6, upper panel). We also mutated the 321-332 residues together, for an ALA12 mutant (Figure 6, upper panel). Protein expression of ALA5 and ALA7 at the membrane was assessed by fluorescence imaging (Figures 6A and 6B) and flow cytometry (data not shown) and was substantial, similar to that of the native CX3CL1 (Figure 2A insert). The ALA12 mutant, however, was mainly intracellular (Figure 6C). Flow cytometry assays revealed that it was expressed overall at the same level as the other mutants (data not shown), but that the expression of the ALA12 protein at the membrane level was actually half that of that

of CX3CL1 and of the ALA5 and ALA7 constructs.

Moreover, western blot analysis revealed that each construct gave rise to two CX3CL1-YFP species (Figure 6D), one mature and complete, with a molecular weight of 120 kDa (90 kDa for CX3CL1 plus 30 kDa for YFP) and a smaller one (60 kDa plus 30 kDa for YFP). The latter did not seem to be a truncated fragment of the mature form, since it contained both the CD and YFP moieties, as assayed by western blot (data not shown). It most probably corresponds to the 60-kDa form pointed out in an earlier study (17) as an immature, intracellular form of CX3CL1. Interestingly, this shorter form, which accounted for only a small proportion of the expression of the ALA5 and ALA7 mutants, was the major form in the ALA12 mutant (Figure 5D). This is consistent with its intracellular location (Figure 5C).

The ALA5 and ALA7 mutants exhibited BRET saturation curves (Figure 6E) similar to those of CX3CL1 (Figure 2C). Figure 6F shows that the BRET data for the ALA5 and ALA7 mutants was characteristic of specific BRET (constant versus acceptor plus donor). The ALA12 mutant, in contrast, had a very low BRET amplitude (Figure 6E), which was not constant when plotted against the total amount of tagged protein (Figure 6G): the BRET data were clearly linear and going to near zero, that is, they corresponded to a non-specific aggregation (30,33). The BRET curve versus the acceptor concentration behaved similarly (data not shown) and thus confirmed that the BRET of ALA12 was mainly unspecific. Mutation of the 12 central residues of the TM domain of CX3CL1 led to less protein maturation (Figure 6D) and, according to BRET assays, to reduced ability to aggregate (Figures 6E and 6G).

Role of clustering in the adhesive potency of CX3CL1- To test the non-clustering CX3CL1 mutant functionally, we derived HEK clones that stably expressed either the

CX3CL1-YFP or the ALA12-YFP chimera. We obtained two clones with similar YFP fluorescence and expressing the same level of CX3CL1 at the cell membrane, according to flow cytometry (Figure 7A) and confocal microscopy (Figure 7B). Both clones expressed the two forms of CX3CL1 (90 and 120 kDa, Figure 7B), both of which were full-length peptides, since they were recognized by both anti-CX3CL1-CD (Figure 7C left) and anti-YFP (Figure 7C middle). The complete, mature form is the major form in the CX3CL1-YFP clone, either in the whole cell (Figure 7C left) or at the external membrane level, as shown by immunoprecipitation of the CX3CL1 protein accessible from outside (Figure 7C right). Conversely, in confirmation of the data obtained with transient transfectants (Figure 6D), the smaller form is the main component of the ALA12-YFP clone (Figure 7C left) and is dominantly expressed at the clone's surface membrane (Figure 7C right). Finally, both clones released a similar amount of the 80 kDa soluble form of CX3CL1 (data not shown), probably after cleavage by ADAM10 and ADAM17 (17,43-46).

Both clones were assayed for their adhesiveness to a CX3CR1-expressing HEK or CHO clone. When assayed in static conditions, both clones specifically adhered to CX3CR1 positive CHO cells (Figure 7D). Despite a similar CX3CL1 surface expression (Figure 7A), the number of adhering ALA12-YFP cells was significantly lower than the number of adhering CX3CL1-YFP clone cells. Besides, in flow conditions at 1.5 dyne.cm^{-2} , the number of CX3CR1-positive HEK clone cells adhering to ALA12 cells was less than 25% of the number adhering to standard CX3CL1 cells (Figure 7E). In the dual pipette assay, quantifying the strength required to dissociate an adhesive cell pair (28), a dissociation force of approximately 1 nN (Figure 7F) was sufficient to tear apart the ALA12-CX3CR1 paired cells. This level was

also obtained with parental HEK (28) and was considered as non specific adhesion. In contrast, a dissociation force of 4 nN was required for CX3CL1-CX3CR1 pairs. The latter value is similar to that already found for adhesion of HEK cells expressing CX3CR1 and CX3CL1 without the YFP extension (28). Taken together, our data indicate that the primary TM domain of CX3CL1 is required for the shear-resistant adhesiveness of the CX3CL1/CX3CR1 pair.

Role of glycosylation in CX3CL1 adhesive potency- The preceding data show that the ALA12 mutant - mainly containing a non-clustering and light form of CX3CL1 - was hardly adhesive. Moreover, this form was lighter, probably because it was not fully glycosylated (Figure 7B). To determine whether either clustering or glycosylation was required for adhesion, our HEK clone that expressed CX3CL1-YFP was treated with benzyl-2-acetamido-2-deoxy- α -D-galactopyranoside, which inhibits glycosylation of the mucin family proteins (47). Alternatively, it was incubated with neuraminidase (acylneuraminyl hydrolase) plus O-glycosidase (endo-D-galactosyl-N-acetyl- α -galactosamino hydrolase), which deglycosylates cellular CXCL16 (45). Unfortunately, the apparent molecular weight of CX3CL1 was not altered by the application of any of these treatments to our CX3CL1-YFP clone (either on intact cells or on cell lysate). We therefore decided to work with the commercially available external moiety of the CX3CL1 molecule, containing the CD domain and most of the mucin stalk fused to a His₆ tag (48). This purified protein had an apparent molecular weight of 90 kDa (Figure 8A, lane a) and showed adhesive properties under both static (Figure 8B, lane a) and flow conditions (Figure 8C, lane a). After treatment with neuraminidase, the molecular weight of CX3CL1-His₆ was reduced to 65-70 kDa (Figure 8A, lane b). If O-glycosidase was included in the treatment, the CX3CL1-His₆

was found around 60 kDa (Figure 8A, lane c), that is equivalent to that of the lighter form of CX3CL1 detected in our CX3CL1 and ALA12 HEK clones (Figure 7B). However, these lighter forms of CX3CL1 displayed the same adhesion features than the mature form (Figures 8B and 8C, lanes b and c). The failure of the deglycosylation treatment to affect either static adhesion (Figure 8B) or adhesion under flow (Figure 8C) suggests that mature glycosylation is not involved in the adhesive potency of CX3CR1.

DISCUSSION

The CX3CL1/CX3CR1 axis is a highly adhesive pair involved in the firm adhesion of monocytes or lymphocytes on activated endothelium (4). CX3CL1 adhesion potency is completely independent of that of selectin or integrin (16) but is similar in strength to that of integrins (16,19). The adhesive potency of integrins relies on molecular activation and aggregation, due in part to the fact that the molecule is a flexible heterodimer forming what have been called “compliant legs” (22). In contrast, the relation between the structural characteristics of CX3CL1 and its adhesive features remains largely unknown. Earlier studies showed that the mucin stalk contributes to the ligand’s ability to act as an adhesion molecule in flow conditions (48) and that the nature of the stalk is unimportant, since it can be replaced by the rod-like segment of E-selectin (14). Nonetheless, the potential self-aggregation of CX3CL1 has not yet been investigated.

The BRET method is now widely used as a molecular proximity assay to study the clustering of membrane proteins including circadian clock proteins (49), integrins (50), growth factor receptors (51) and GPCR (52,53). Working with CX3CL1, we found a specific and large BRET level in the HEK cell line (Figure 2A) and the CHO cell line (data not shown). Several criteria are used to assess BRET and ensure that its results are not due merely to random protein (bystander)

interactions, but to constitutive clustering (30-33,53). BRET of CX3CL1 in the HEK cell line met all of them: (i) Replacing one of the monomers with an irrelevant protein produced no transfer (Figure 2A); (ii) expression of an excess of a non-tagged unit along with the Luc- and YFP-chimeras reduced BRET (Figure 2F); (iii) BRET signals for a given acceptor/donor ratio (at constant amount of donor) reached saturation: at a sufficient acceptor concentration, each donor molecule was engaged in a cluster and the BRET signal reached a maximum (Figure 2C). If BRET were due to random collision, BRET amplitude would increase continuously with the acceptor/donor ratio; (iv) BRET versus the total amount of tagged protein (donor plus acceptor) at a given acceptor/donor ratio should be constant or vary only slightly: this curve should not go to zero at low protein density, as recently pointed out (32,33), and it clearly did not for CX3CL1 (Figure 2D). The same criterion can be checked by directly plotting the BRET against the acceptor concentrations (30) (Figure 2E). These data, obtained with transfected HEK cells were supplemented with data from HTRF of primary cells (Figure 3B). All the data sets provided convergent evidence that CX3CL1 behaves as an aggregating protein, both as a transfected protein in cell lines and as a native protein expressed in HUVEC cells.

Moreover, flow cytometry and fluorescence imaging of our transfected HEK showed that approximately half of the CX3CL1 was located at the membrane (see Figure 2A), consistent with recent findings (29). The HTRF experiment (Figure 3B) showed that the membrane functional CX3CL1 molecules were definitely clustered. Because the BRET assay did not discriminate between intracellular and membranous proteins, we cannot assert the aggregation status of the intracellular CX3CL1 pool.

We found that the major content of the ALA12 mutant was a 60-kDa protein (90 kDa

with the YFP tag), while the ALA5 and ALA7 mutants were, like the native CX3CL1 (90 kDa, 120 kDa with the YFP tag, Figures 6D and 7C) composed of the mature molecule. Our data indicate that the 60-kDa species cannot be a shorter fragment of CX3CL1 and is thus likely to represent immature, not fully glycosylated, species. First, we found that this small form exists in cells expressing the native CX3CL1 (Figures 6D left and 7B left), as previously observed (17,45). It also has the same terminals as the native CX3CL1 (Figure 7B left and centre). Moreover, it is preferentially located intracellularly (Figure 6C), as is the immature form of CX3CL1 (17). Finally, enzymatic deglycosylation of the purified CX3CL1 gives rise to a 60-kDa form (Figure 8A). Hence, our data suggest that the same conformational characteristic of the TM is essential for CX3CL1 clustering, mature glycosylation and targeting the mature form to the external membrane. Nonetheless, we isolated a HEK clone that expressed an immature, non-aggregating CX3CL1 molecule that is well inserted in the external membrane (ALA12, Figures 7A, 7B and 7C right).

To identify the domain primarily involved in the self-association, we conducted numerous mutation experiments (Figures 4, 5 and 6), which demonstrated the essential role of the TM. This was confirmed by our observation that the BRET of CX3CL1 was completely abolished after treatment with a concentration of Triton X100 as low as 0.1% (data not shown). We failed however to pinpoint the precise sequence motif involved in the TM helix association. Self-association of transmembrane helices may involve a glycine motif, as in glycoporphin A (40), or leucine zipper (41) or a phenylalanine in a “ball and socket” dimer interface (42). Our work shows that it is not the glycine or leucine or phenylalanine residues alone that are involved (Figure 5). In addition, replacement of the 321-325 residues (ALA5) left the specific BRET unchanged and replacement of

the 326-332 residues (ALA7) increased it (Figure 6). The higher BRET amplitude for some mutants, e.g. TM&cyto (Figure 4D) and ALA7 (Figure 6E), compared with the whole CX3CL1 molecule, is probably due to slight modifications of relative orientations of the dipole moments of the acceptor and donor, which can lead to more efficient BRET (39). Indeed, relatively small conformational changes could dramatically affect the BRET level (54-57), included conformational changes due to genetic variations (58)

Finally, the simultaneous replacement of 12 TM residues (ALA12) dramatically decreased BRET and made it non-specific (Figures 6E and 6G). Thus, a long stretch of the TM domain appears to be involved in aggregation but no special motif has a particular role; other examples of this type of structure have already been observed (59). One might think that CX3CL1 is clustered in some membrane microdomains, such as “lipid rafts”, although recent work showed that CX3CL1 in renal tubular epithelial cells is found outside lipid rafts (60). The CX3CL1 aggregation may also be due to external constraints imposed by companion molecules. However, the observation of CX3CL1 aggregation in very different cellular contexts, such as HUVEC, HEK and CHO cell lines, suggests that it is most likely due to intrinsic properties of the CX3CL1-TM domain. Since this aggregation is functional and takes place in various cellular environments, we propose that it corresponds to oligomerization. Definitive proof of this oligomerization of CX3CL1, however, must await reconstitution in membrane models, and many questions must be answered before we can understand how it takes place. How can such small (0.5 nm in diameter) TM domains interact despite the large diameter of mucin-like glycosylated moieties (14)? How many monomers are involved in this aggregation/oligomerization?

The unique adhesive features of CX3CL1 under flow depend on its CD. They do not

change after replacement of the mucin stalk by the rod-like segment of E-selectin (14). On the other hand, the chimeras that replaced the CD of CX3CL1 with those of CCL2, CCL4, CCL5, CCL21 or CXCL8 did not act as adhesion molecules with the cognate receptors (48). The ability of CX3CL1 to mediate adhesion may therefore depend on its unique slow receptor off-rate. Our data here (Figure 7) complement these findings in showing that self-assembly is also required for the adhesive potency of CX3CL1. The ALA12 molecule behaves similarly to the CX3CL1 chimera presenting CCL2 or CXCL8 as a CD (48): it adheres to CX3CR1 in a mild static assay (Figure 7D), but is not resistant to shear stress (Figures 7E-F).

Our results with the purified CX3CL1-H₆ external domain (Figure 8) show that glycosylation is not involved in adhesive potency. The immobilized protein, however, is not representative of the complete protein embedded in a cell membrane. Unfortunately, we could not test that point directly: all of our trials to deglycosylate cellular CX3CL1 were unsuccessful (see Results). Nonetheless, the molecular weight loss of the CX3CL1-H₆ molecule induced by our deglycosylation treatment was equivalent to the difference in molecular weight between the mature CX3CL1 form and the immature form (Figures 6 and 7) and there is evidence that the major form of the ALA12 mutant is the deglycosylated and immature form of CX3CL1. These results suggest that, while glycosylation has no direct role in the adhesive CX3CL1-CX3CR1 interaction, it facilitates the emergence of the CX3CL1-CD moiety from the glycocalyx layer and therefore enables the ligand to target the receptor.

We can conclude that the adhesiveness of CX3CL1 requires at least three simultaneous conditions: (i) the unique features of the CX3CL1-CD, probably related to its slow receptor off-rate (48); (ii) presentation of the CX3CL1-CD atop a glycosylated stalk (48), the nature of which is unimportant (14); and (iii) a TM domain capable of aggregation (this work). Another chemokine - CXCL16 - has a similar primary structure (5), but seems to be less adhesive in flow conditions than CX3CL1 (61) (our unpublished data). It is therefore essential to investigate its structure-function relations, to compare its function to that of CX3CL1.

That clustering is crucial for the adhesive capacity of CX3CL1 expands its similarity with others adhesion molecules. Integrin clustering increases its avidity (62) and is required for efficient leukocyte adhesion (63). Similarly, the dimerization of P-selectin glycoprotein ligand-1 (PSGL-1) is required for optimal recognition of P-selectin (64). Moreover, as shown here for CX3CL1, mutational changes in the TM domain of PSGL-1 affect its self-assembly (65).

Our data open up the possibility of inhibiting CX3CL1 self-assembly by using competing peptides analogous to the TM domain, as already done for integrins (66). This could lead to the specific inhibition of the adhesive function of CX3CL1 and thus help to delineate the different roles of the two forms of this chemokine. More importantly, it also points out a new way to antagonize the CX3CL1 function specifically, without interfering with the CX3CL1-CX3CR1 interaction. This type of pharmacology may be helpful in treating the ever-growing number of diseases in which CX3CL1 is involved (3), especially atherogenesis (13), inflammation diseases (2) and degenerative disorders (7,8).

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FOOTNOTES

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The abbreviations used are: BRET, Bioluminescence Resonance Energy Transfer; HTRF, homogeneous time-resolved fluorescence; HUVEC, Human Umbilical Vein Endothelial Cells; TM, transmembrane domain of CX3CL1; CD, chemokine domain; Muc, CX3CL1 without chemokine domain; TM&cyto, CX3CL1 without chemokine domain and mucin stalk; ALA5, CX3CL1 mutant with alanines replacing the 321-325 residues; ALA7, CX3CL1 mutant with alanines replacing the 326-332 residues; ALA12, CX3CL1 mutant with alanines replacing the 321-332 residues.

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FIGURE LEGENDS

Figure 1. Schematic drawing of the structure of the CX3CL1 molecule. The domains of CX3CL1 are drawn according to the available structural data (6,14).

Figure 2. Characteristics of BRET of CX3CL1 in HEK cells. **A.** HEK-293 cells were transfected with 0.05 µg CX3CL1-Luc and 0.5 µg of various YFP constructs: empty pEYFP (left), CX3CL1-YFP (middle), CXCL16-YFP (right). The values are the mean of 15 measurements (\pm SD). Insert. Fluorescence microscopy imaging of HEK cells transfected with CX3CL1-YFP (bar = 50 µm). **B.** HEK cells were transfected with 1, 2 or 3 µg of CX3CL1-Luc or of CX3CL1-YFP constructs. The luminescence of fluorescence of 50000, 100000 and 200000 cells was then measured. Besides, the CX3CL1 content of each transfectants was analyzed by SDS-PAGE and western blot and evaluated with CX3CL1-H6 as standard. **C.** BRET variation using HEK cells transfected with a constant amount of donor (CX3CL1-Luc) and increasing amount of acceptor (CX3CL1-YFP) (■). CX3CL1-Luc and CX3CL1-YFP contents in each sample were quantified with the calibration curve in Figure 2B. Here the CX3CL1-Luc was kept constant at 1.5 fmole/mg total protein. The same measurements were taken with CXCL16-YFP as acceptor (□). The data were fitted with GraphPad Prism4 with one-site binding hyperbola. The resulting BRET_{max} was 0.230 for CX3CL1-Luc/CX3CL1-YFP and 0.027 for CX3CL1-Luc/CXCL16-YFP. **D.** BRET variation using HEK cells co-transfected with CX3CL1-Luc and CX3CL1-YFP at a constant ratio ([YFP]/[Luc] = 5). The linear regression is given and indicates that BRET is constant in this range. **E.** BRET variation versus CX3CL1-YFP concentration using HEK cells transfected with various amounts of CX3CL1-Luc and CX3CL1-YFP. **F.** HEK-293 cells were transfected with 0.1 µg CX3CL1-Luc and 0.2 µg CX3CL1-YFP supplemented with 2 µg of empty pcDNA3 (left) or of CX3CL1-pcDNA3 (right). The values are the mean of 15 measurements (\pm SEM). The difference between the CX3CL1 BRET ratio with or without native CX3CL1 was significant (***: $p < 0.0001$). The CX3CL1 content of each sample was analyzed by western blot to check that the untagged CX3CL1 expression did not change expression of CX3CL1-Luc and CX3CL1-YFP (data not shown).

Figure 3. HTRF measurements of CX3CL1 in HUVEC. *A.* Resting HUVEC (dotted line) and activated HUVEC (continuous line) were stained with phycoerythrin murine mAb anti-CX3CL1 and analyzed by flow cytometry. *B.* Resting HUVEC (left), activated HUVEC (right) were stained with anti-CX3CL1 mAb labeled with cryptate (donor) and with D2-labelled anti-CX3CL1 mAb (acceptor) (filled bars) as described in Methods. Control measurements were made with anti-Flag D2-labelled mAb as acceptor (empty bars). The FRET signals were plotted as HTRF ratio R ($R = (\text{fluorescence at } 665 \text{ nm} / \text{fluorescence at } 620 \text{ nm}) \times 10^4$). The HTRF difference between HUVEC and activated HUVEC was significant (** $p < 0.0001$). *Insert.* The specific signal over background called ΔF was calculated with the following formula: $\Delta F = (R_{\text{pos}} - R_{\text{neg}}) / R_{\text{neg}}$ where R_{pos} corresponds to the HTRF ratio with anti-CX3CL1 mAb as acceptor and R_{neg} to the ratio with anti-Flag mAb as acceptor.

Figure 4. BRET of CX3CL1 deletion mutants. *A, B, C.* HEK cells were co-transfected with YFP-tagged constructs containing the various truncated CX3CL1-YFP constructs and were observed with fluorescence microscopy (bar = 50 μm). *D.* BRET variation using HEK cells co-transfected with a constant amount of Luc-tagged truncated CX3CL1 constructs (4, 4 and 7 fmol/mg total protein for respectively Muc, TM&cyto and TM mutants) and increasing amount of YFP-tagged constructs, as indicated. The data were calibrated (Figure 2B) and fitted using GraphPad Prism4 with one-site binding hyperbola. *E.* BRET variation using HEK cells co-transfected with Rluc- and YFP-tagged constructs containing truncated CX3CL1 at a constant [YFP]/[Luc] ratio. Muc (circles, [YFP]/[Luc] = 20). TM&cyto (triangles, [YFP]/[Luc] = 40) and TM (diamonds, [YFP]/[Luc] = 12).

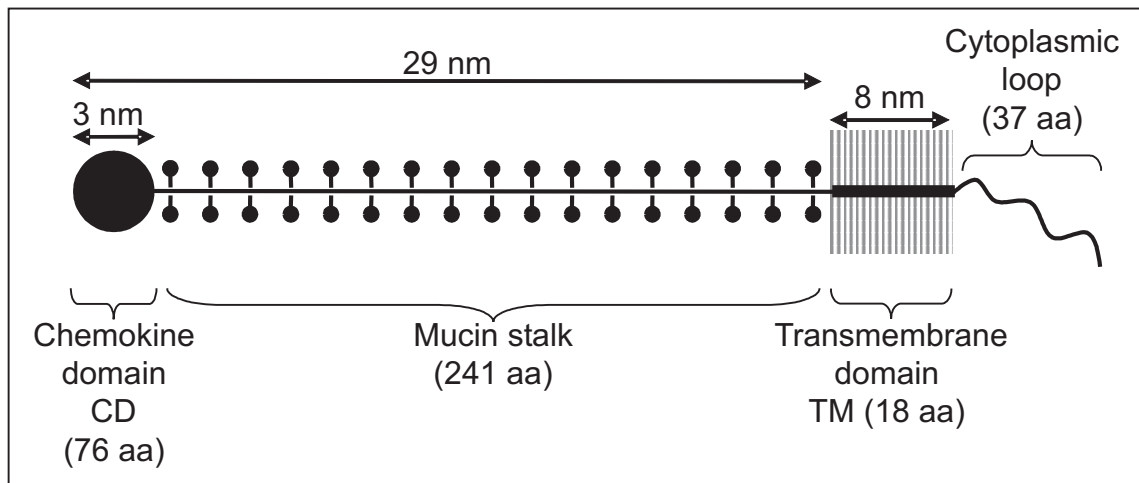
Figure 5. BRET of the CX3CL1 mutants with modifications in the TM. HEK cells were co-transfected with a constant amount of CX3CL1-Luc-tagged constructs and increasing amounts of CX3CL1-YFP constructs with various mutations in the TM domain, as indicated in the top of the figure. In each case, BRET_{max} ($\pm \text{SE}$) was analyzed as done in Figure 2C.

Figure 6. BRET of the CX3CL1 mutants with modifications in the TM. *A, B, C.* The various CX3CL1-YFP constructs were transiently expressed in the HEK cell line and observed with fluorescence microscopy (bar = 50 μm). *D.* Lysates of HEK transiently transfected with the various CX3CL1-YFP constructs were analyzed by SDS-PAGE and western blot with a goat anti-CX3CL1 antibody. *E.* HEK cells were co-transfected with a constant amount of Luc-tagged constructs (giving 1.5 fmol/mg total protein) and increasing amount of YFP-tagged constructs, as indicated. In each case, data were calibrated (Figure 2B) and fitted with GraphPad Prism4 with one-site binding hyperbola. *F.* BRET variation using HEK cells co-transfected with various amounts of Luc-tagged constructs and YFP-tagged constructs at a constant ratio (ALA7 triangles, [YFP]/[Luc] = 5; ALA5 diamonds [YFP]/[Luc] = 9). *G.* BRET variation using HEK cells co-transfected with various amounts of ALA12 Luc-tagged constructs and ALA12 YFP-tagged constructs at a constant ratio, with [YFP]/[Luc] = 9.

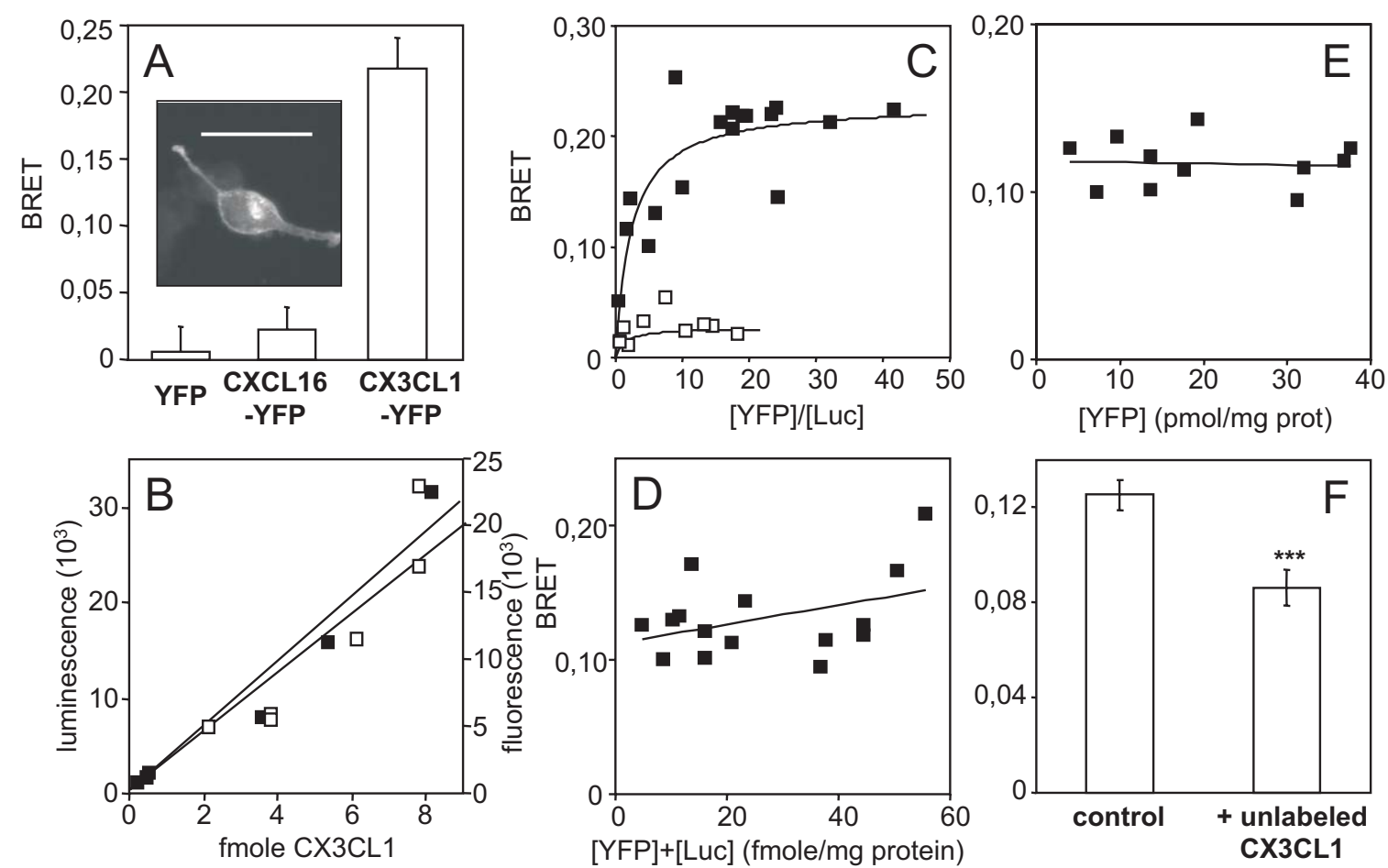
Figure 7. Adhesive properties of HEK clone cells expressing CX3CL1-YFP or ALA12-YFP. *A.* Parental HEK cells (continuous line), stable HEK clone expressing CX3CL1-YFP (fine dotted line) or ALA 12-YFP (large dotted line) were stained with phycoerythrin-labeled murine anti-CX3CL1 mAb and analyzed by flow cytometry. *B.* The two clones were observed with confocal microscopy (Leica SP2 AOBs) (bar = 50 μm) *C.* The two clones were analyzed by SDS-PAGE

and western blot using antibodies against CX3CL1 (left) or against YFP (middle). For immunoprecipitation of antigen located at the cell surface (right), 2×10^6 cells of both clones were incubated in suspension for 1 h at 4°C with 5 µg of murine mAb anti-human CX3CL1 in PBS plus 0,2% BSA. After two washings in cold PBS, the cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1% Nonidet P40 (Sigma, Saint Quentin-Fallavier, France), Complete (Roche Diagnostics, Meylan, France). The immune complex was separated with Protein G-agarose (Sigma, Saint Quentin-Fallavier, France) and analyzed by western blotting, with a goat anti-human CX3CL1. *D.* HEK-CX3CL1 or HEK-ALA12 clone cells (10^5 cells/well) labelled with CFSE were deposited onto confluent CX3CR1-CHO cell or parental CHO cell monolayers. At the end of incubation period and after washings, the plate was read at 535nm as described in Experimental Procedures. The adherent cells are expressed as percentage of total cells minus the mean background corresponding to the number of HEK-CX3CL1 or ALA12 cells adhering on parental CHO cells. *E.* HEK clone cells expressing CX3CR1 were suspended and assayed for adhesion in a parallel plate laminar flow chamber with coverslips coated with adherent HEK-CX3CL1 or HEK-ALA12 clones as described in Methods section. Adhering cells were counted after 10 minutes over four fields (mean \pm SEM). The unspecifically adhering cell numbers were obtained in the same conditions, after addition of 100 nM FKN in HEK-CX3CR1 cells before injection in the flow chamber. The difference between the number of cells specifically adhering either on CX3CL1 or on ALA12 was significant (** $p = 0.002$). This result was characteristic of three independent experiments. *F.* HEK-CX3CL1 or HEK-ALA12 clones were assayed for adhesion by the dual pipette aspiration technique with HEK-CX3CR1 clone cells as described in Methods section. The dissociation force was evaluated after 4 minutes of adhesion (mean over 14 cell pair measurements \pm SEM). The difference between adhesion of the CX3CL1/CX3CR1 and ALA12/CX3CR1 pairs was significant (** $p = 0.008$).

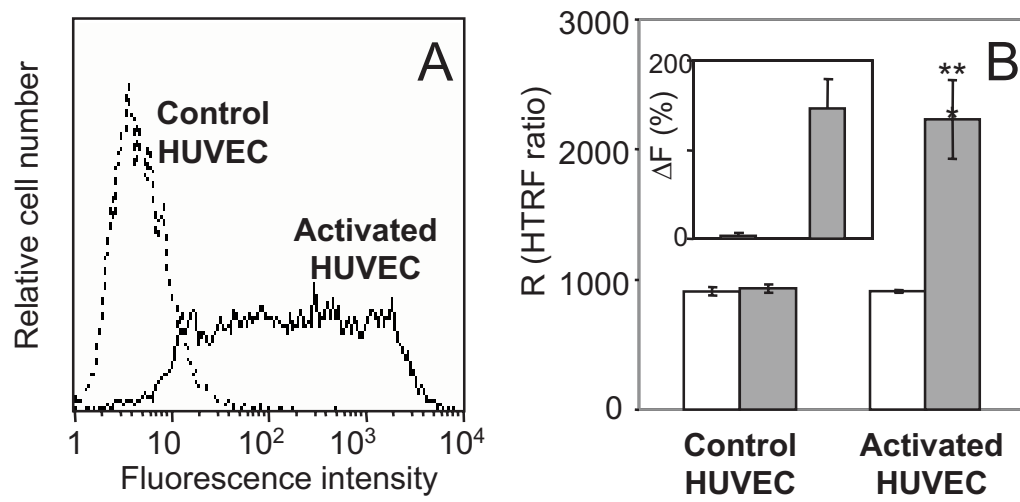
Figure 8. Functional analysis of the deglycosylated CX3CL1. *A.* 90 ng of CX3CL1-H₆ was treated or not (a) with neuraminidase alone (b) or with neuraminidase and O-glycosidase (c). Then the proteins were analyzed by SDS-PAGE and western blot. *B.* 90 ng of CX3CL1-H₆ was absorbed onto wells of flat-bottom 96-well microtiter plates and treated or not (a) with neuraminidase alone (b) or with neuraminidase and O-glycosidase (c). Then 10^5 HEK-CX3CR1 cells were added and incubated for 45 minutes before washing. The results are expressed as percentage of total cells (mean of triplicates \pm SD). The control corresponds to a well without CX3CL1-H₆. *C.* HEK clone cells expressing CX3CR1 were suspended and assayed for adhesion in the presence (filled bars) or absence (empty bars) of 100nM CX3CL1 in a parallel plate laminar flow chamber with coverslips coated with 180 ng CX3CL1-H₆ treated or not (a) with neuraminidase alone (b) or neuraminidase and with O-glycosidase (c). The results are expressed as the mean of triplicate experiments (\pm SEM). The data is representative of three experiments.



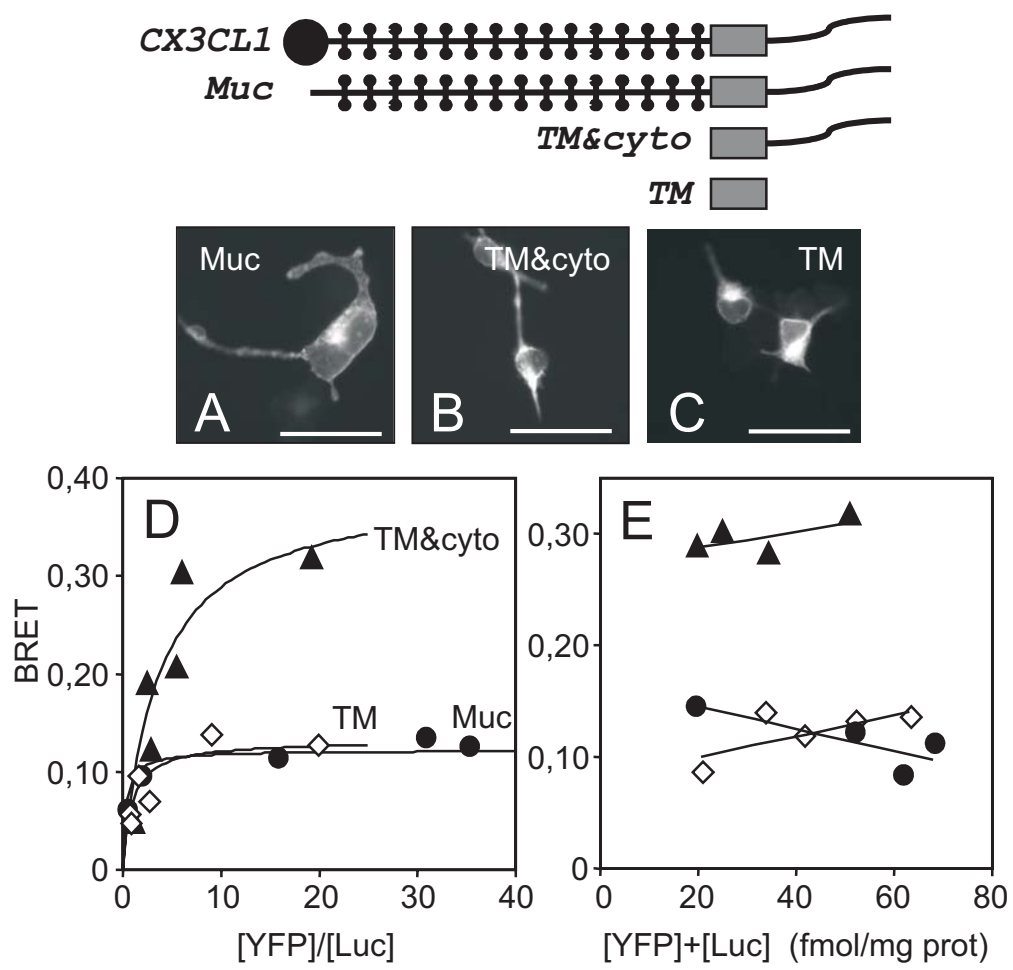
Hermant et al. **Figure 1**



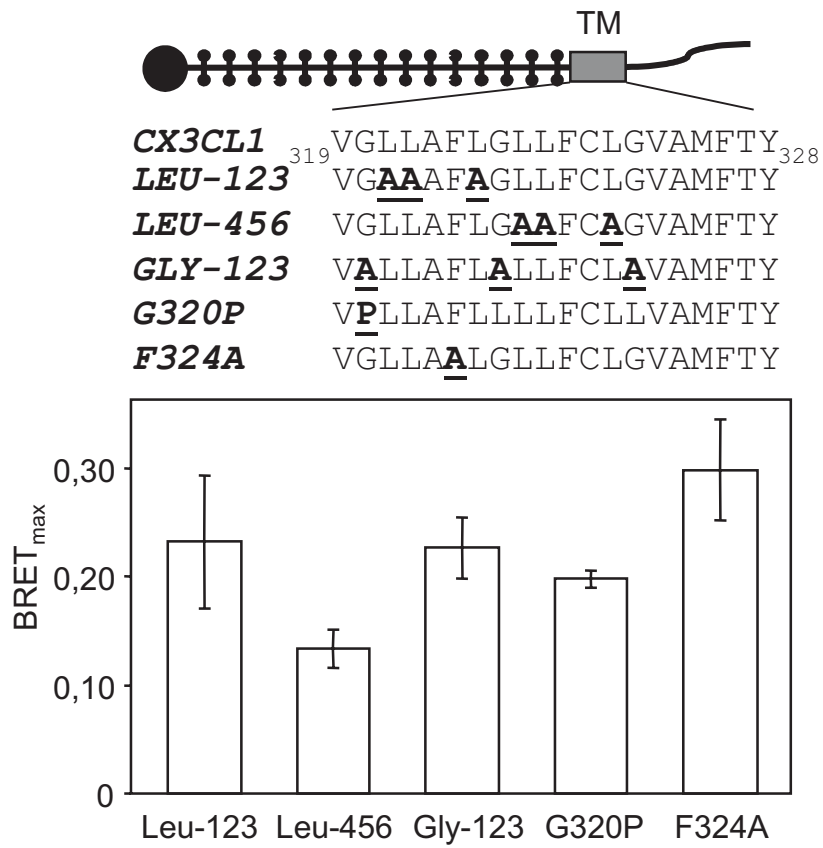
Hermand et al. **Figure 2**



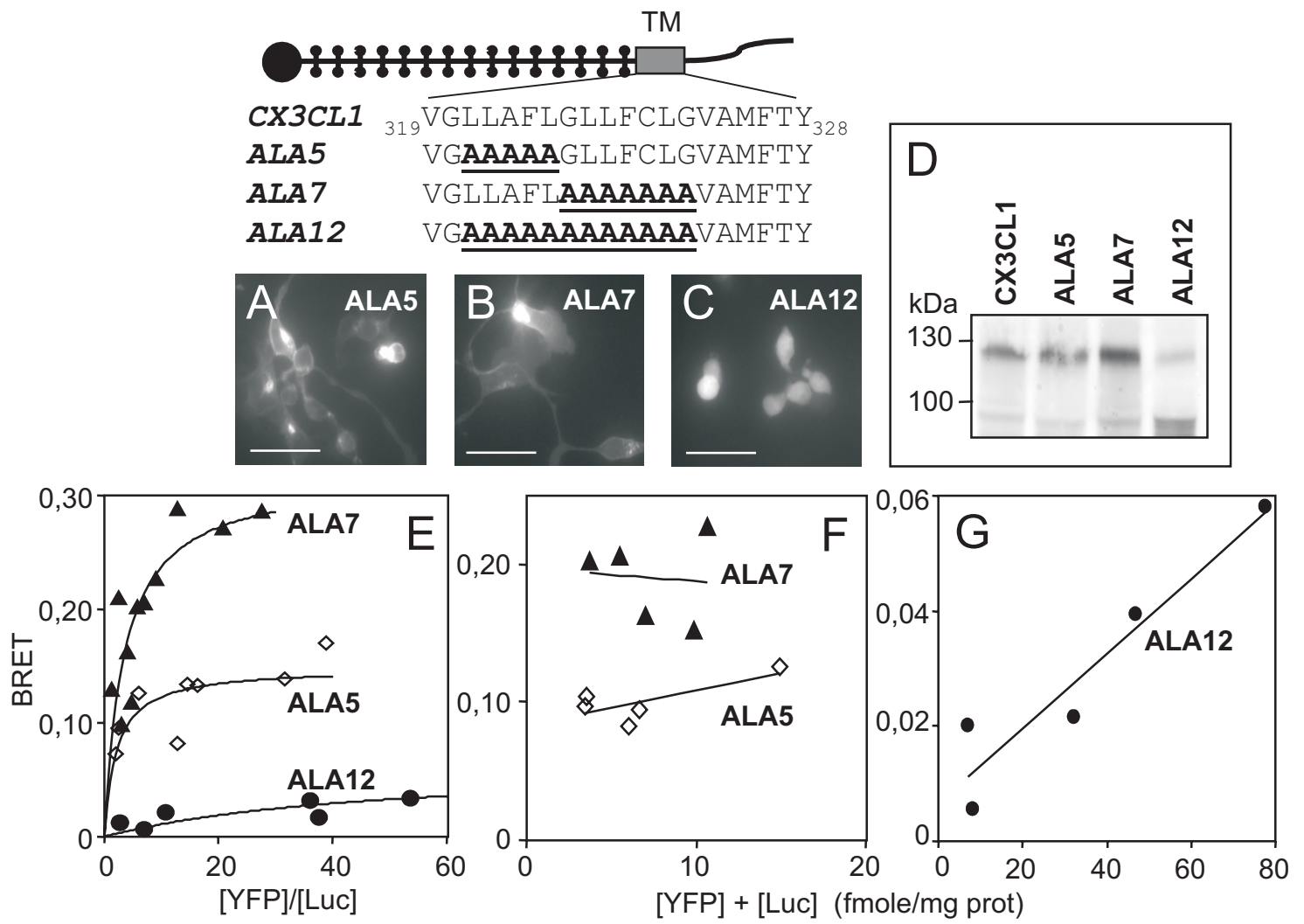
Hermand et al. **Figure 3**



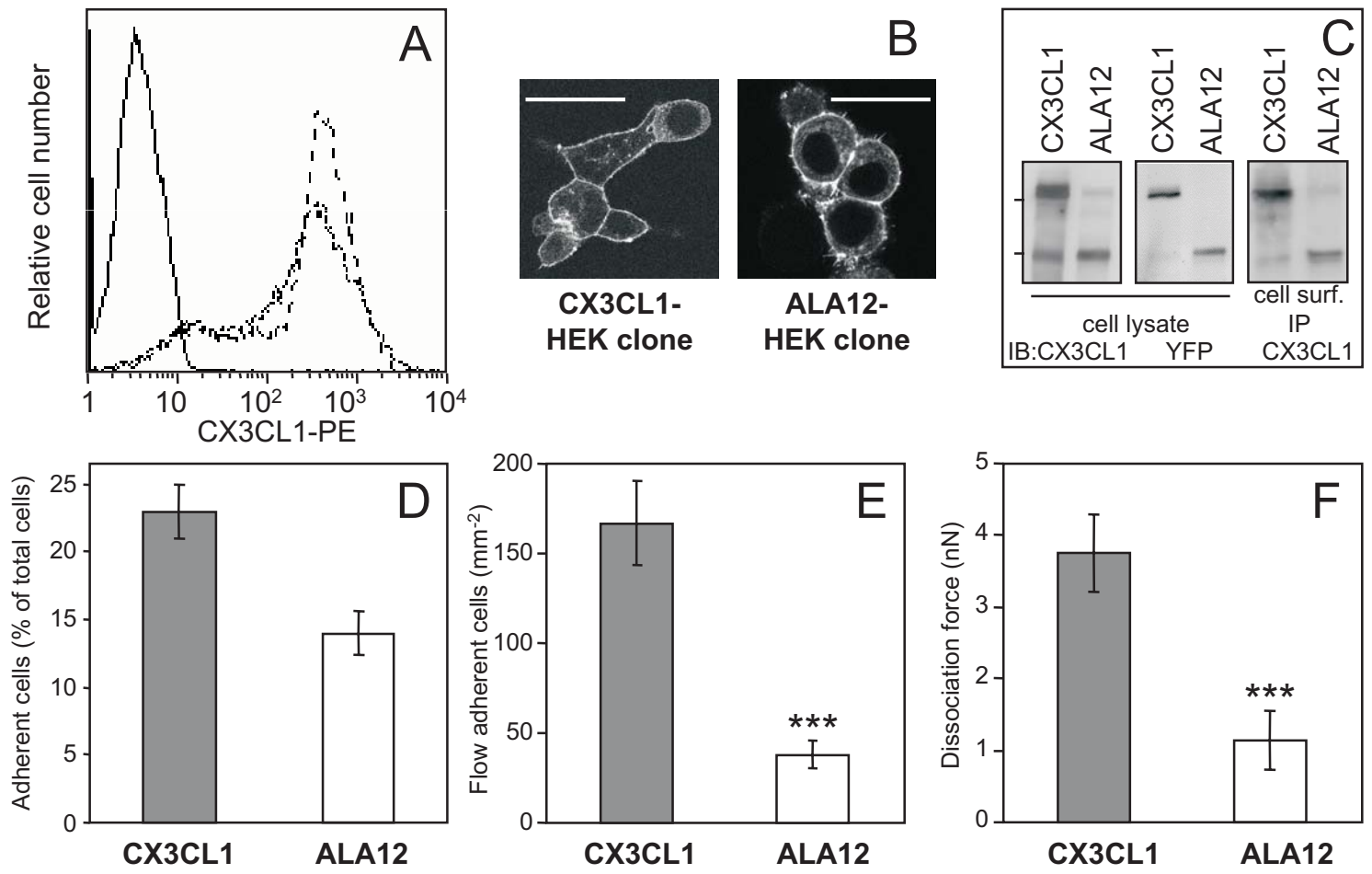
Hermand et al. **Figure 4**



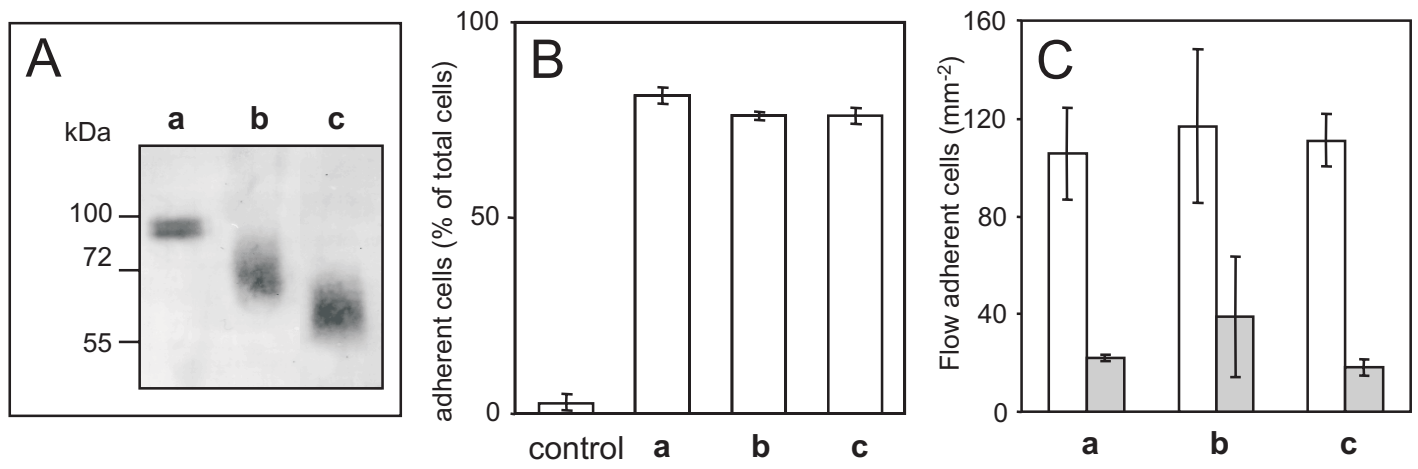
Hermand et al. **Figure 5**



Hermand et al. **Figure 6**



Hermand et al. **Figure 7**



Hermand et al. **Figure 8**