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# Creation of intercellular bonds by anchoring protein ligands to membranes using the diphtheria toxin T domain

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**Abstract** **We describe the creation of cell adhesion mediated by cell surface engineering. The Flt3-ligand was fused to a membrane anchor made of the diphtheria toxin translocation domain. The fusion protein was attached to the surface of a cell by an acid pulse. Contact with another cell expressing the receptor Flt3 lead to its activation. This activity involved direct cell–cell contact. A mean force of 20 nN was needed to separate functionalized cells after 5 min of contact. Overall, we showed that it is possible to promote specific cell–cell adhesion by attaching protein ligands at the surface of cells.**


*Keywords:* Diphtheria toxin T domain; Cell surface engineering; Membrane anchor; Flt3-ligand

## 1. Introduction

Cell surface engineering is of great potential in the development of cell therapies. This enables for instance the manipulation of cell adhesion, recognition, signalling, and stimulation. The display of novel proteins on the surface of cells is generally obtained by introducing genes into them. The expression of a protein with a signal sequence and a transmembrane sequence ensures proper targeting to the cell membrane. However, transfection efficiency and the degree of expression vary greatly from one cell type to another. In addition, the genetic manipulation of cells for therapeutic applications is not devoid of risk [1].

The diphtheria toxin translocation (T) domain can be used as a low-pH-activated membrane anchor to attach proteins to the cell surface [2,3]. The T domain, which is soluble at neutral pH, inserts itself irreversibly in cell membranes at acid pH. If a soluble protein is fused to its N- or C-terminal end, and incubated with cells at acid pH, it will attach to the plasma membrane. In the present work, we demonstrate the possibility of creating bonds between cells using this technique. We created adhesion mediated by specific interaction between a ligand anchored on the surface of a cell and its receptor expressed on the surface of another cell.

We fused the T domain of diphtheria toxin to Flt3-ligand (FL), a cytokine active in its homodimeric form [4,5] and synthesized by numerous cell types in biologically active soluble and membrane forms [6,7]. FL is involved in the proliferation of hematopoietic stem cells, the only cells that have the Flt3 receptor, boosting the number of myeloid and lymphoid dendritic cells [8], and natural killer cells [9].

We anchored active FL to the surface of tumor cells and tested the recognition of FL-bearing cells by cells expressing the Flt3 receptor. We showed that FL remains active after fusion to the T domain, and anchoring to the cell surface. We demonstrated direct adhesion between FL-carrying cells and target cells bearing the Flt3 receptor and measured the interaction force engaged in the cell–cell interaction.

## 2. Materials and methods

### 2.1. Expression and purification of proteins FL and FLT

The cDNA of FL was from the Core Facility Lab of the University of Michigan. The sequence encoding the T domain was from the plasmid pA/T [2]. The cDNA of FL and of the T domain were amplified by PCR and fused behind the T7 promoter in a vector of the pET series (Novagen), to give the plasmid pFLT. The vector pFL allowing expression of isolated FL was derived from pFLT by deletion of the sequence coding for the T domain. The coding sequence of these two proteins is preceded by a polyhistidine sequence ($MH_6G$). A flexible and hydrophilic arm ($H_6ANS_3GSG_2SG_2SGSGI_2YPG$) separates the two domains of FLT. This sequence contains a 6 His Tag, which comes from the initial sequence of the T domain from plasmid pA/T used to construct the plasmid pFLT.

Proteins were expressed in *Escherichia coli* Rosetta (Novagen) in culture medium containing 0.2 mg/ml ampicillin and 0.025 mg/ml chloramphenicol and purified as described previously for T-IL3 [10]. Before purification, the protein extract was sulfonated by addition of sodium sulfite over 20 min to a final concentration of 0.3 M, and then addition of 50 mM disodium 2-nitro-5-thiosulfobenzoate for 1 h to reduce the disulfide bridges and block free cysteine residues [11]. The protein was purified as a monomer by ion metal affinity chromatography under denaturing condition using its N-terminal 6 His tag.

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*Abbreviations:* FL, Flt3-ligand; T, translocation

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*2.2. Protein refolding*

Purified proteins (0.5 mg/mL) were dialyzed against 6 M GdnHCl, 0.1 M Tris–HCl, pH 8, and then four times (12 h each time, 4 °C, protected from light) against 0.9 M GdnHCl, 4 mM EDTA, 0.1 M Tris–HCl, pH 8.3, 10% glycerol, 8 mM cysteine, 1 mM cystine. The dialyzed extract was centrifuged for 1 h at 4500 rpm to remove precipitate. The protein solution was dialyzed against 2.5 l of PBS-10% glycerol, pH 7.4, centrifuged again, and concentrated to an approximate concentration of $10^{-5}$ M, using an Amicon cell (Millipore) fitted with a membrane of molecular cut-off 10 kDa. Proteins were stored at −20 °C.

*2.3. Cell lines and proliferation tests*

RMA T lymphoma and Ba/F3 4G1 cells were grown in RPMI 1640 containing 10% fetal calf serum and 2 mM glutamine in the presence of murine IL-3 for Ba/F3 4G1 cells.

Proliferation tests were done on Ba/F3 4G1 cells as described previously [10,12].

*2.4. Anchoring of FLT to the surface of RMA cells*

RMA cells ($10^7$) washed in PBS were incubated for 1 h at room temperature with increasing concentrations of FLT, with shaking in 1.5 mL of PBS-citrate at pH 4.7. The cells were washed 4 times in 10 mL of PBS pH 7.4 and stored at 4 °C. If necessary, and to block their proliferation before anchoring, the RMA cells were treated overnight with 3 μg/mL mitomycin C (Sigma–Aldrich) in the culture medium, and washed 4 times in PBS.

*2.5. Flow cytometry detection of FLT anchoring to the membrane*

For each test, $3 \times 10^5$ cells were washed, resuspended in 100 μL of PBS, and incubated for 30 min at 4 °C with goat anti-FL polyclonal antibodies (R&D Systems) diluted 1/50. The cells were washed in PBS and incubated for 30 min on ice and protected from light with a secondary phycoerythrin-coupled antibody (Jackson ImmunoResearch) diluted 1/50. The cells were then washed and resuspended in PBS. Samples were analyzed in a FACSCalibur flow cytometer (Becton–Dickinson) using CellQuest Pro software.

*2.6. Dual pipette aspiration technique to test cell–cell interaction*

We used dual pipette assay to quantify the strength of cell–cell adhesion. The experiments were performed on the stage of a Leica inverted microscope, equipped with ×10 and ×63 objectives and positioned on an anti-vibration platform. The incubation chamber consisted of the bottom of a 90-mm Petri dish coated with BSA and filled with complete RPMI medium. RMA cells loaded with FLT at a concentration of $10^{-6}$ M and Ba/F3 4G1 cells were each gently deposited at two different places of the experimental chamber in order they did not mix. Micropipettes were obtained by pulling (with a Sutter instrument, model P-2000), cutting and fire-polishing glass capillaries with a homemade microforge. Micropipettes were coated with BSA. Cells were manipulated with two such micropipettes of 4–6 μm internal diameter, each held in its own micromanipulator and connected to a combined hydraulic/pneumatic system that provided the necessary control of the aspiration force applied to the cells. The protocol we used is very similar to that of Chien and co-workers [13]. An RMA cell and a Ba/F3 4G1 cell, collected by gentle aspiration onto the tip of each pipette, were brought into contact through the use of the micromanipulators and allowed to remain in contact for 5 min. To separate the cells, aspiration in the right pipette was maintained at a level sufficiently high to hold the RMA cell tightly, while the aspiration in the left pipette was increased in steps measured with a pressure sensor (Validyne: model DP103–38; ranging from 0 to 50000 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 the left pipette by the right pipette was moved back to the left pipette orifice, the aspiration in this 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 the left pipette was sufficient to pull one cell apart from the other. The aspiration employed in each cycle was monitored continuously. In most cases, cell deformation and contact area variation during the separation process were very limited (less than 20% for the contact area), and the separation took place suddenly, in less than a tenth of a second. 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, $F_s$ with equation $F_s = (\Delta P_{n-1} + \Delta P_n)\pi r_p^2/2$, where $\Delta P_{n-1}$ and $\Delta P_n$ are respectively the aspiration in the left pipette at cycles $n-1$ and $n$, and $r_p$, its internal radius. This relation assumes that the pressure inside the cell is the same as that in the chamber; valid in our case since the tension of the cell is essentially zero.

## 3. Results

*3.1. FL activity of recombinant proteins FL and FLT*

Two recombinant proteins called FL and FLT were produced. Human FL was used as control. FLT was human FL fused to the N-terminal end of the diphtheria toxin T domain. The N-terminal end was chosen to reproduce the organization of the natural membrane form of FL, its transmembrane sequence being at the C-terminal end [5].

To assess the structural and functional integrity of FL in the fusion protein, we tested the capacity of the recombinant proteins FL and FLT to recognize and activate the Flt3 receptor. Ba/F3 4G1 cells transfected with the Flt3 receptor gene were incubated with increasing concentrations of proteins. Cell growth was measured by incorporation of tritiated thymidine. Fig. 1A shows that FL (●) and FLT (▲) concentration-dependently induced proliferation of Ba/F3 4G1 cells. The $EC_{50}$ val-

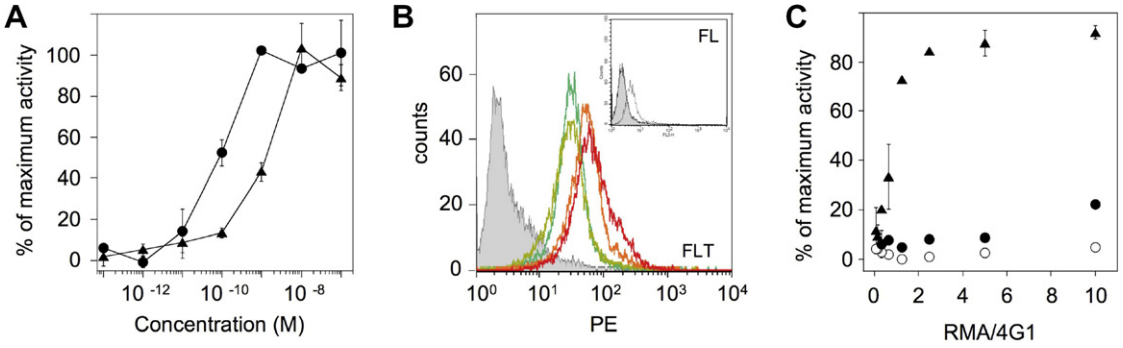


Fig. 1. (A) FL activity of the recombinant proteins FL (●) and FLT (▲). Proliferation of the Ba/F3 4G1 cells was measured by [$^3$H] Thymidine incorporation. (B) Detection by flow cytometry of the binding of FLT to the surface of RMA cells as a function of protein concentration: red, $3 \times 10^{-6}$ M; orange $10^{-6}$ M; green, $3 \times 10^{-7}$ M; light green, $10^{-7}$ M. Inset: detection of FL to the surface of RMA cells at a protein concentration of $10^{-6}$ M. (C) Proliferation of Ba/F3 4G1 cells in the presence of RMA cells carrying FLT. RMA cells were treated overnight with 3 μg/mL mitomycin C and incubated for 1 h at pH 4.7 with the recombinant proteins FLT (▲) and FL (●) at a protein concentration of $10^{-6}$ M or no proteins (○). Proliferation of the Ba/F3 4G1 cells was measured by [$^3$H] Thymidine incorporation.

ues (effective concentration required to give 50% of peak activity) of FL and FLT were, respectively, $10^{-10}$ M and $2 \times 10^{-9}$ M. FLT was therefore 20 times less active than FL. The proliferation-inducing activity of FL and FLT was concentration-dependently inhibited by an antibody directed against FL (data not shown). These results indicate that recombinant FL and FLT bind to and specifically activate the Flt3 receptor. They are therefore correctly folded as a homodimer, which is the active form of FL [5]. Steric hindrance related to the presence of the T domain and the N-terminal 6 His tag probably explains why FLT is less active than FL.

As the fusion protein is anchored to cell membranes at acid pH, we tested the effect of acid treatment on its FL activity. FL activity of FLT was preserved after 1 h treatment at pH 4.7 (not shown).

### 3.2. Anchoring of FLT to the surface of RMA cells

RMA cells were incubated for one hour at pH 4.7 with increasing concentrations of protein FLT. Binding of FLT to the cell surface was studied by flow cytometry (Fig. 1B). The results show that FLT binds to RMA cells at acid pH, in amounts that depend on the FLT concentration used in the incubation. Since FL incubated with the cells in the same conditions is weakly detected at their surface (inset) membrane binding is due to the presence of the T domain.

### 3.3. FL activity of FLT-carrying RMA cells

We tested whether RMA cells loaded with FLT at acidic pH would stimulate the proliferation of Ba/F3 4G1 cells (Fig. 1C). RMA cells were treated with mitomycin C to block their proliferation. The results show that RMA cells carrying FLT induced proliferation of Ba/F3 4G1 cells (▲). This induction intensified with an increase in number of RMA cells, compared with Ba/F3 4G1 cells, and with an increase in FLT concentration used in membrane anchoring (not shown). In contrast, RMA cells incubated with FL (●) did not stimulate Ba/F3 4G1 cells.

The supernatant of cells carrying FLT was tested on Ba/F3 4G1 cells for the presence of soluble FL activity. Some activity was found (not shown), indicated that some of the FLT molecules bound to the RMA cells were shed in the medium. But the majority of the FL activity carried by the Ba/F3 4G1 cells was due to molecules attached to their surface. This suggested that direct cell–cell contacts were involved in the stimulation.

### 3.4. Detection of a cell–cell interaction

We used a dual micropipette assay to investigate the occurrence of intercellular contacts. For that, the force required to separate a FLT-bearing RMA cell and a Ba/F3 4G1 cell was measured and compared to that measured as a control with a T-IL2-bearing RMA cell and a Ba/F3 4G1 cell. Anchoring of the cytokine IL2 to the surface of cells using the T domain fused to its N-terminal end has been described previously [12]. For each pair of cells, the two cells held by gentle aspiration at the tips of two micropipettes were first brought into contact for 5 min. Fig. 2A illustrates an example of a cell doublet obtained after 5 min of contact, the right pipette withdrawn to visualize the resulting adhesion. Such a doublet was cyclically brought back into contact with the left pipette and then withdrawn to the right, each time after a step-wise increase in the strength

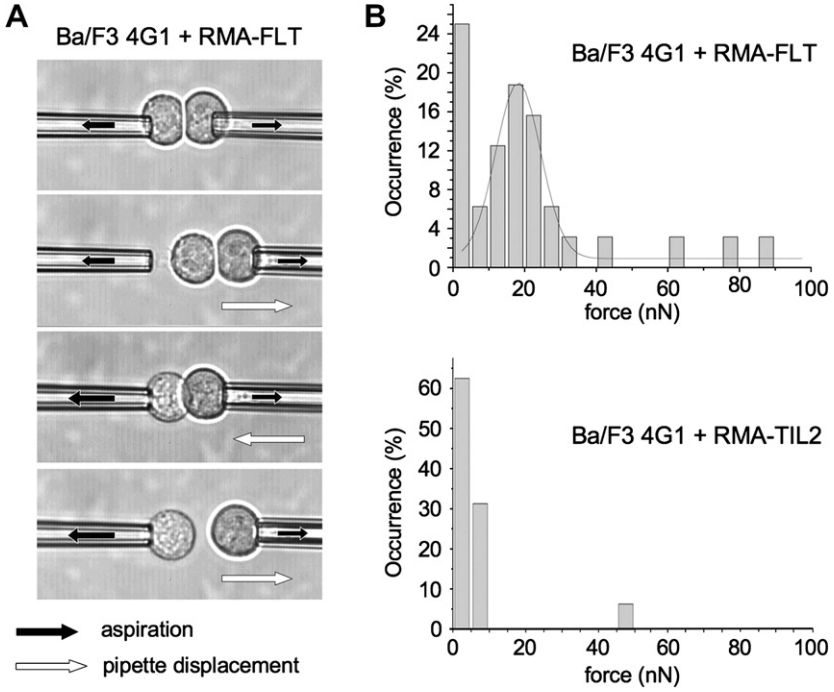


Fig. 2. Measurement of the force needed to separate RMA cells carrying the recombinant proteins FLT or T-IL2 from Ba/F3 4G1 cells expressing the Flt3 receptor. (A) Aspiration in the right pipette was maintained at a level sufficiently high to hold the RMA cell tightly while the aspiration in the left pipette (Ba/F3 4G1) was increased in steps. At each step, the right pipette was displaced in order to try to separate the cells. If the suction force in the left pipette is weaker than the adhesive force between the two cells, the doublet remains intact and Ba/F3 4G1 cell detaches from the micropipette. This procedure was repeated with increasing suction in the left pipette until the two cells separated. The separation force was deduced from the last two suction forces. (B) Probability density of the force needed to separate RMA cells carrying FLT or T-IL2 and Ba/F3 4G1 cells.

of aspiration by the left pipette, until the cells were separated (see Section 2). The separation force $F_s$ was defined as the aspiration force required in the left pipette to separate the doublet, such that one cell remained in each pipette when the right pipette was withdrawn. $F_s$ was considered to be zero for pairs of cells that did not form adherent doublets in this assay.

Fig. 2B shows the histogram of separation forces obtained for 32 FLT-bearing RMA and Ba/F3 4G1 paired cells. Regarding their adhesive properties, two populations of doublets could be observed. One minority (25%) of the paired cells could be separated immediately upon withdrawal of the right pipette therefore no separation force could be measured. But the majority (75%) of the paired cells formed adherent doublets. The mean force required to separate them after 5 min of contact was 20 nN. By contrast, interaction between T-IL2-bearing RMA and Ba/F3 4G1 cells was almost never detected (Fig. 2B).

## 4. Discussion

A fusion protein (FLT) formed by joining FL to the N-terminal end of the diphtheria toxin T domain has the biological properties of both proteins. Thus FLT binds to cell membranes at acid pH through the T domain (Fig. 1B), in a concentration-dependent manner. This membrane form of FL is still able to stimulate target cells carrying the Flt3 receptor (Fig. 1C). It therefore mimics the natural membrane form of FL. The properties of FLT are comparable to those of proteins T-IL2 and T-IL3 [10,12]. In the case of FL, the fusion of the T domain and membrane anchoring still allow dimerization of FL, which is only active as a homodimer [5].

We used a dual pipette assay to quantify intercellular adhesion in terms of mechanical forces at the cellular level and to investigate a potential adhesion that would specifically be supported by FLT and its Flt3 receptor. The force required to separate FLT-bearing RMA and Flt3-expressing Ba/F3 4G1 paired cells was measured as an index of an interaction specifically regulated by a membrane form of FL and its Flt3 receptor. Comparison with T-IL2-bearing RMA and Flt3-expressing Ba/F3 4G1 paired cells were done. T-IL2-bearing RMA cells is a good control since it provides similar mechanical and steric conditions as FLT-bearing RMA cells. For both types of cells, non-specific interactions are therefore expected to be the same. Moreover, any difference in adhesive behaviour of T-IL2-bearing RMA and Ba/F3 4G1 paired cells and FLT-bearing RMA and Ba/F3 4G1 paired cells should come from FL/Flt3 specific effects. Results obtained with the dual pipette assay (Fig. 2) showed that most of the FLT-bearing RMA and Ba/F3 4G1 paired cells were adherent while the T-IL2-bearing RMA and Ba/F3 4G1 doublets in the same conditions were not. The latter observation indicates that non-specific interactions between RMA and Ba/F3 4G1 cells are negligible. The comparison between both types of doublets provides a direct experimental evidence of the ability for membrane anchored FLT to interact with Flt3 receptors through cellular contacts. The mean force required to separate FLT-anchoring RMA and Flt3-expressing Ba/F3 4G1 paired cells after 5 min of contact is about 20 nN. Qualitatively, this separation force is comparable to forces needed to separate cells expressing (by transfection) either natural adhesion proteins such as E-cadherins [14,15], or some chemokines that function as adhesion molecules [16]. More accurate comparison would be hazardous because each of these studies involve different cell lines that can give rise to different non-specific interactions and also because the number of proteins that control cell adhesion (i.e. proteins in the contact area) is unknown in all cases. However, such a level of adhesion means that soluble protein ligands attached at the membrane of cells through the low-pH-activated T domain membrane anchor can engage with their receptor present at the surface of another cell thereby generating significant cellular adhesion.

In this cellular context, the dual pipette assay has not only proved that membrane anchored FLT can interact with Flt3 receptors through cellular contacts but to our knowledge it is the first time that cell/cell adhesion generated by membrane anchored proteins have been established and quantified.

The anchoring of receptor ligands to the surface of cells opens the way to the engineering of cell surfaces, with a view to manipulating cell interactions and communication. This approach has the advantage that it does not require the transfer of genetic material, and so does not alter the cell's genetic make-up.

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