Anchoring antibodies to membranes using a diphtheria toxin T domain-ZZ fusion protein as a pH sensitive membrane anchor

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Abstract We have constructed a fusion protein, T-ZZ, in which the IgG-Fc binding protein ZZ was fused to the C-terminus of the diphtheria toxin transmembrane domain (T domain). While soluble at neutral pH, T-ZZ retained the capacity of the T domain to bind to phospholipid membranes at acidic pH. Once anchored to the membrane, the ZZ part of the protein was capable of binding mouse monoclonal or rabbit polyclonal IgG. Our results show that the T-ZZ protein can function as a pH sensitive membrane anchor for the linkage of IgG to the membrane of lipid vesicles, adherent and non-adherent cells.

Key words: Diphtheria toxin; Membrane anchor; Membrane insertion; Protein A; Targeting; Transmembrane domain

1. Introduction

Attachment of antibodies to natural or synthetic membranes may have application in basic science, biotechnology, biosensor technology, diagnostics and biotherapeutics. In the past 15 years the linkage of antibodies to membranes has been developed, mostly for the design of cell-targeted immunoliposomes. The linkage is generally performed by chemical coupling either to a derivatized phospholipid initially incorporated into the membrane or to an acylated chain which will further be incorporated into the lipid bilayer, often with the help of detergents. Recently, it was shown that acylation of a recombinant scFv can also be performed biosynthetically, but its insertion into membranes still requires the use of detergents [1]. The drawback is that detergents must be removed through time consuming steps, and are not compatible with some applications. Moreover, they may not be used for the linkage of antibodies to the membrane of living cells.

Our objective was to design a membrane anchor which would allow for the attachment of antibodies to synthetic or natural phospholipid bilayers as well, without the need for detergents or chemical cross linking reagents which interfere with membranes or cell surface proteins. Anchoring should be fast and triggered when desired by a simple mechanism.

The diphtheria toxin transmembrane domain (T domain) has the remarkable property of being water soluble at neutral pH, whereas it inserts into membranes at acidic pH [2–6]. In the whole toxin, this property enables the T domain to assist the translocation of the catalytic domain from the acidic endocytic compartment to the cytoplasm, during the intoxication of a cell [2,3,7–9]. We have studied whether the T domain could be used as a membrane anchor for the antibody binding protein ZZ. ZZ was generated by duplication of a mutated B domain from the staphylococcal protein A [10] and was shown to bind to the Fc region of IgG [11]. Here we describe a T-ZZ fusion protein and its use as a membrane anchor for mouse and rabbit IgG.

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Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; lipo-ELIFA, liposome enzyme linked immunofiltration assay; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; T domain, diphtheria toxin transmembrane domain

2. Materials and methods

2.1. Bacterial strains, antibodies and cell culture reagents

Escherichia coli strain NovaBlue (Novagen, Madison, WI, USA) was used for plasmid propagation and cloning, and strain BL21(DE3) (Novagen) was used as the host for fusion protein production. Polyclonal horse anti-diphtheria toxin antibody was obtained from Mérieux (Marcy-l'Etoile, France), rabbit anti-horse IgG-, goat anti-mouse IgG- and goat anti-rabbit IgG-F(ab′)$_2$-peroxidase conjugates were purchased from Immunotech (Marseilles, France) and FITC-labeled mouse IgG2a from Sigma. Cell culture media were from Biological Industries (Kibbutz Beit Haemek, Israel).

2.2. Construction of vector pCD2-ZZ

The pET11d derived parental plasmid pETJV127 [12] encoding the diphtheria toxin-interleukin-2 fusion toxin DAB$_{389}$-IL-2 was digested with *Nco*I to introduce a DNA fragment made by hybridization of the following oligonucleotides: 5′-CATGGGTCATCACCATCACCATCACGATGACGATGACAAATG-3′ and 5′-CATGCATTTGTCATCGTCATCGTGATGGTGATGGTGATGACC-3′. As a result, the N-terminal methionine of the encoded DAB$_{389}$-IL-2 protein is preceded by the following sequence: Met-Gly-His-His-His-His-His-His-Asp-Asp-Asp-Asp-Lys-Cys, and an *Nsi*I restriction site encompasses the Cys codon. The resulting plasmid pCD1 was digested with *Nsi*I to remove the region coding the diphtheria toxin catalytic domain, leading to plasmid pCD2-hIL-2. pCD2-hIL-2 was digested with *Sph*I and *Hin*dIII to remove the IL-2 coding region at the 3′ end of the sequence coding for the T domain. The coding sequence for ZZ [10,11] was PCR amplified from plasmid pCP [13] using primers designed to introduce an *Sph*I and a *Hin*dIII restriction site at the 5′ and 3′ ends of the fragment, respectively: 5′-CCACGGACCCGCATGCTGCGCAACACGATGAAGCCGT-3′ and 5′- CCGGTATGCCAAGCTTTTACTAAGAATTCGCGTCTACTTTCGGC-3′. After digestion with *Sph*I and *Hin*dIII, the ZZ amplified sequence was ligated in plasmid pCD2-hIL-2 to give plasmid pCD2-ZZ.

2.3. Expression and purification of the fusion protein T-ZZ

Protein expression was done at 37°C in Terrific Broth (12 g/l tryptone, 24 g/l yeast extract (Difco, Detroit, MI, USA), 0.4% glycerol (v/v), 17 mM KH_2PO_4, 72 mM K_2HPO_4) supplemented with 0.2 mg/l ampicillin from BL21(DE3) cells transfected with plasmid pCD2-ZZ. Induction was started at $OD_{600} = 0.6$ by the addition of 0.1 mM IPTG (Fluka, Buchs, Switzerland) and carried on for 2 h. Cells were harvested by centrifugation and resuspended in 20 ml of 50 mM Tris-HCl pH 8, 10 mM EDTA, 8% sucrose (w/v), 5% Triton X-100 (v/v) supplemented with 0.1 ml of 0.1 M PMSF (Sigma) in ethanol and 1 ml of a 10 mg/ml solution of lysozyme (Sigma). After 1 h incubation on ice, the cells were lysed by sonication for 5 min (power setting 50%, pulse 1 s, rest 1 s) and the soluble cytoplasmic fraction containing T-ZZ was

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recovered by centrifugation at 14 000×*g* for 30 min at 4°C and treated with 1 μg/ml of DNAse and RNAse for 1 h at 20°C.

Purification was done by affinity chromatography on an IgG Sepharose 6 Fast Flow column according to the manufacturer's protocol (Pharmacia, Uppsala, Sweden). The pH of the eluted protein sample was immediately brought back from 3.4 to 7.4 with 2 M Tris-HCl pH 9. Because the activity of the T domain was affected by the rather strong acidic pH of the elution buffer, the protein was treated with 6 M guanidine hydrochloride and 1 mM DTT and refolded by dialysis against 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5% glycerol and stored at −20°C.

Some aggregation of T-ZZ could be observed by quasi elastic light scattering. This problem was eliminated by keeping the protein at a concentration below 0.5 mg/ml in the presence of 5% glycerol. Few remaining aggregates were removed by centrifugation 20 min at 14 000 rpm.

2.4. Liposome perforation assay

Large unilamellar vesicles (LUV) were prepared by reverse phase evaporation as described [14] using a mixture of egg phosphatidylcholine and egg phosphatidic acid (Avanti Polar Lipids, Alabaster, AL, USA) at a molar ratio of 9/1. The buffer used for preparation was 25 mM KH_2PO_4, 50 mM K_2SO_4, 50 mM Na_2SO_4, 25 mM MES pH 7.2 supplemented with 0.2 mM pyranine (Molecular Probes, Eugene, OR, USA). The preparation was extruded through 0.4 and 0.2 μm polycarbonate filters (Millipore, Bedford, MA, USA) to obtain LUV of a uniform size distribution of about 120 nm. The final phospholipid concentration was about 16 mg/ml (20 mM). LUV were separated from non-entrapped pyranine by size exclusion chromatography on a G50 sephadex column.

50 μl of LUV suspension was added to 950 μl of the buffer used for the vesicle preparation in a quartz cuvette in a spectrofluorimeter LS 50 (Perkin-Elmer) under constant magnetic stirring at 37°C. Excitation was performed at 460 nm and emission was recorded at 510 nm. In a typical experiment of 200 s, the following reagents were added sequentially: at $t=0$ s, 2 μl of 0.1 mM valinomycin (Sigma) in ethanol; at $t=15$ s, 38 μl of 1 M H_2SO_4; at $t=40$ s, protein samples; at $t=140$ s, 2 μl of 0.1 mM carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) (Sigma) in ethanol; at $t=180$ s, 38 μl of 1 M KOH. When present, the pyranine quencher DPX (Molecular Probes) was added before starting recording at a final concentration of 20 mM. Diphtheria toxin (Sigma) used as a control was nicked with trypsin [15] and reduced with 20 mM DTT. The amounts of proteins used were 12 μg for T-ZZ (3×10^{-7} M) and 10 μg for diphtheria toxin (10^{-7} M) to get maximum effect. Preincubation of the proteins with antibodies was done by mixing an excess of purified antibodies with the protein sample 5 min before adjunction to the quartz cuvette.

2.5. Liposome floatation experiments

The method was adapted from [16]. LUV containing pyranine were prepared as described above. 0.8 mg of LUV were incubated at 20°C with 20 μg of T-ZZ in 350 μl of 20 mM Tris, 150 mM NaCl pH 7.4 and pH was dropped to 5 or maintained at 7.4 by the adjunction of 50 μl of 250 mM MES at pH 3.4 or 7.4. At this step, concentrations were 2.5 mM for lipids and 1.3×10^{-6} M for T-ZZ. After incubation overnight, samples were then mixed to a 60% sucrose, 20 mM Tris, 150 mM NaCl solution to reach 40% sucrose. A 40%, 30%, 20%, 10% and 5% discontinuous sucrose gradient was made for each sample in a 8 ml Beckman polycarbonate ultracentrifuge tube. Gradients were centrifuged for 1 h at 110 000×*g* in a fixed angle rotor. The top 5% sucrose fraction containing the LUV visible by their turbidity, and the bottom 40% sucrose fraction were harvested. The presence of pyranine was analyzed by fluorescence detection (excitation at 465 nm and emission at 530 nm) after dilution 1/4 in 1 M Tris pH 8, 0.1% SDS. The presence of proteins was analyzed by SDS-PAGE, electrotransfer onto a nylon membrane and Western detection using anti-diphtheria toxin antibodies and an anti-antibody-peroxidase conjugate.

2.6. Liposome enzyme linked immunofiltration assay (lipo-ELIFA)

Small unilamellar vesicles (SUV) were prepared by sonication of a dispersion of 50 mg egg phosphatidylcholine and 5 mg egg phosphatidic acid in 3 ml of PBS pH 7.4 (Sigma) for 5 min. The final phospholipid concentration of the preparation was 18.3 mg/ml (22.9 mM). The assay was performed on 96 well Multiscreen-HA 0.45 μm mixed esters of cellulose filter plates (Millipore). At each step, 200 μl/well of each solution was applied to the plate and vacuum-filtered using a Millipore MultiScreen device. All dilutions and washes were done with PBS pH 7.4 unless otherwise stated. SUV diluted 1/8 were first adsorbed on the filters. Filters were then saturated with PBS containing 0.3% BSA (Sigma) (w/v) and washed once. Ten μg of T-ZZ (2.5×10^{-7} M) was added in PBS at pH 7.4 or at pH 5 followed by a wash. Filters were saturated again with BSA and washed three times. Mouse monoclonal IgG2a or rabbit serum diluted 1/1000 in PBS supplemented with 0.1% BSA were added, plates were washed three times, goat anti-mouse IgG- or goat anti-rabbit IgG-F(ab′)$_2$-peroxidase conjugate at a 1/5000 dilution in PBS supplemented with 0.1% BSA were added, and plates were washed three times. 25 μl of ABTS substrate solution (Pierce, Rockford, Il, USA) prepared according to the manufacturer's instructions was added, the filtrates were harvested in a 96 well ELISA plate placed inside the Millipore MultiScreen device, and wells were rinsed with 200 μl of PBS to ensure good recovery of the substrate transformed by the peroxidase. The ELISA plate was read in a Titertek Multiscan MCC 340 spectrophotometer at 414 nm.

2.7. Cell surface binding immunodetection assay

10^5 adherent fibroblastic mouse L cells per well were seeded on 96 well plates (Nunc) in Dulbecco-MEM supplemented with 10% fetal calf serum and 2 mM glutamine and grown overnight to confluence. Cells were washed three times with PBS at pH 7.4. T-ZZ was diluted in PBS at pH 5 or at pH 7.4, from 5 μg to 0.04 μg per 50 μl (2.6×10^{-6} M to 2×10^{-8} M). 50 μl per well of each dilution was incubated with the cells at 20°C for 15 min. The cells were washed three times with PBS at pH 7.4. 100 μl of mouse monoclonal IgG2a or rabbit serum at a 1/1000 dilution in PBS supplemented with 0.1% BSA was added to the cells at 4°C for 1 h. After three washes with PBS, the cells were incubated with 100 μl of the corresponding goat anti-mouse IgG- or goat anti-rabbit IgG-F(ab′)$_2$-peroxidase conjugate at a 1/5000 dilution in PBS supplemented with 0.1% BSA at 4°C for 1 h. After three washes with PBS, plates were developed with 200 μl of ABTS (Pierce) according to the manufacturer's instructions and absorbency at 414 nm was read in a Titertek 340nm spectrophotometer.

Viability of cells treated with the highest concentration of T-ZZ as above was assayed with the fluorescent metabolic marker AlamarBlue (Biosource International) according to the manufacturer's instructions.

For FACS studies on the non-adherent cell line FDC-P1, the protocol was similar. 2.5 μg of T-ZZ in 50 μl (1.2×10^{-6} M) were incubated with 2.5×10^5 cells 15 min at 20°C. The presence of T-ZZ was detected using a FITC-labeled mouse IgG2a at a 1/30 dilution. Cells were washed by centrifugation in conical bottom 96 well minisorp plates (Nunc).

3. Results

3.1. Construction, expression and purification of the T-ZZ fusion protein

The construction of the plasmid pCD2-ZZ for the expression of the fusion protein T-ZZ is described in the materials and methods section. T-ZZ contains successively: Met-Gly, a six His tag, an enterokinase cleavage site, the T domain from Cys-201 to Thr-386 (numbering from native diphtheria toxin), His-Ala corresponding to the *Sph*I restriction site, Ala-Gln-His-Asp-Glu-Ala, two Z sequences [10,11], and finally Val-Asp-Ala-Asn-Ser.

T-ZZ was expressed in *E. coli* and about 9 mg of protein was purified from 1 l of bacterial culture. Coomassie blue staining of the fusion protein following SDS-PAGE shows that it has been purified nearly to homogeneity (Fig. 1). Its apparent molecular mass is consistent with the value deduced from its amino acid sequence (38.6 kDa).

3.2. pH sensitive interaction of T-ZZ with the membrane of vesicles

The pH sensitive interaction of T-ZZ with membranes was

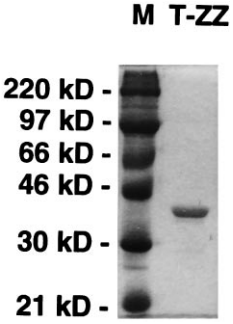

Fig. 1. SDS-PAGE analysis of 2 μg of purified T-ZZ by Coomassie blue staining.

first monitored by measurements of the permeabilization of LUV to small molecules. Fig. 2A shows that the pH sensitive fluorescent probe pyranine, entrapped into LUV, was protected from H^+ added to the medium when pH was brought down from 7.4 to 5. The adjunction of FCCP, a ionophore carrying H^+ across the membranes, provoked extinction of pyranine fluorescence due to its titration by H^+. A similar drop of fluorescence was observed when T-ZZ or diphtheria toxin were added to the vesicles after acidification (Fig. 2A), showing permeabilization of the LUV. Compare to diphtheria toxin on a molar basis, 3-fold more T-ZZ were necessary to obtain a maximum effect. Fig. 2B shows that when the same experiment was done in the presence of DPX, a quencher of pyranine, fluorescence was not recovered after returning the pH to 7.4 at the end of the experiment. This demonstrated mixing of pyranine and DPX after contact with T-ZZ at pH 5. Thus, at acidic pH, T-ZZ permeabilized LUV not only to H^+, but also to small organic compounds. T-ZZ did not perforate LUV at pH 7.4 as DPX did not quench pyranine fluorescence in these conditions (Fig. 2B). Similar observations were made with diphtheria toxin (not shown).

Interestingly, preincubation of T-ZZ with an excess of anti-diphtheria toxin antibodies, but not mouse monoclonal IgG or rabbit polyclonal antibodies, prevented membrane permeabilization (Fig. 2C). Antibodies alone had no effect on membrane permeability in the same conditions of acidic pH (Fig. 2D).

Taken together, these results show that T-ZZ is able to

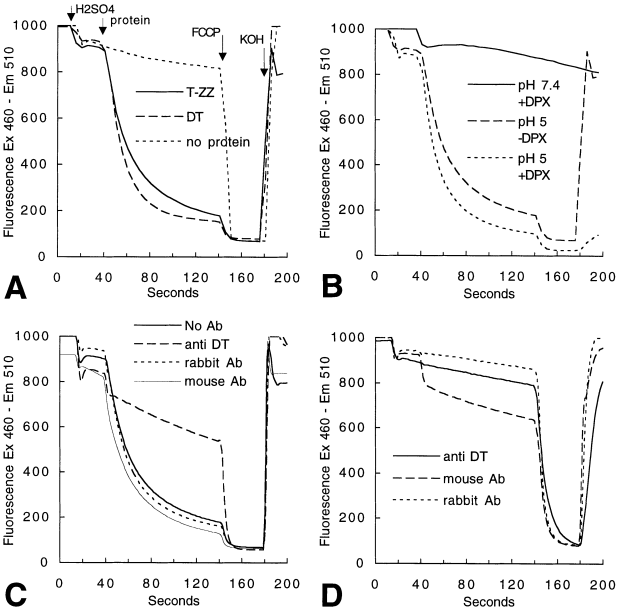

Fig. 2. Permeabilization of LUV by T-ZZ at acidic pH monitored by the pH sensitive fluorescent probe pyranine entrapped inside the LUV. A: Comparison of the effects of T-ZZ and diphtheria toxin (DT). B: Comparison of the effect of T-ZZ at pH 7.4 or 5, in the presence or absence of the pyranine quencher DPX. C: Comparison of the effects of T-ZZ alone or preincubated with horse anti-diphtheria toxin antibodies (anti DT), or mouse or rabbit non specific antibodies. D: Effect of the antibodies in the absence of T-ZZ.

interact with membranes at acidic pH by its T domain. Antibodies bound to ZZ do not prevent this interaction.

3.3. pH dependent permeabilization of LUV by T-ZZ correlates with binding of the protein to the vesicles

T-ZZ was incubated with LUV containing pyranine, at pH 7.4 or 5. The samples were then incorporated in the high concentration sucrose fraction (40%) of discontinuous sucrose gradients. The gradients were ultracentrifuged in order to allow the LUV to float up to the low concentration sucrose fraction (5%) at the top of the gradients where they were visually detectable by their turbidity. Fig. 3A shows that pyranine remained associated with the LUV when incubations were done at pH 7.4. At acidic pH, pyranine remained in

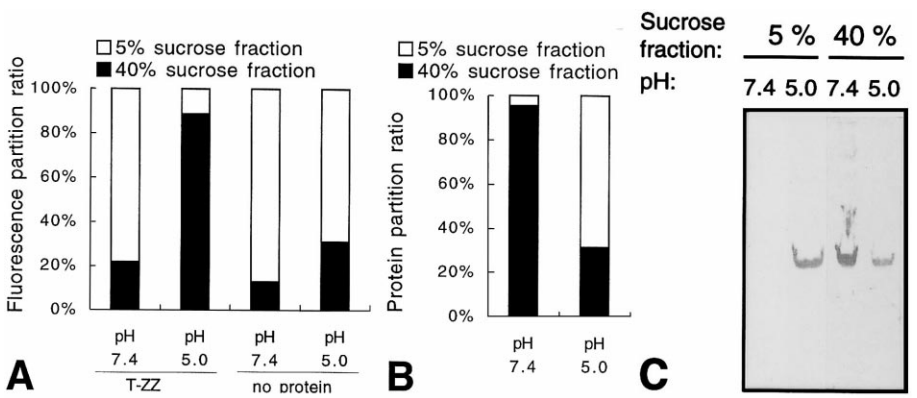

Fig. 3. Floatation of LUV containing pyranine in discontinuous sucrose gradients, following incubation with T-ZZ at neutral or acidic pH. LUV loaded in the 40% sucrose fraction of each gradient were found in the 5% sucrose fraction after centrifugation. A: Partition ratios of pyranine in the 40% and 5% sucrose fractions of each gradient. No fluorescence was detected in intermediary sucrose fractions. B: Partition ratios of T-ZZ in the 40% and 5% sucrose fractions of each gradient analyzed by SDS-PAGE and Western detection (C) and calculated from the densitometry measurements of the bands on the Western blot in C.

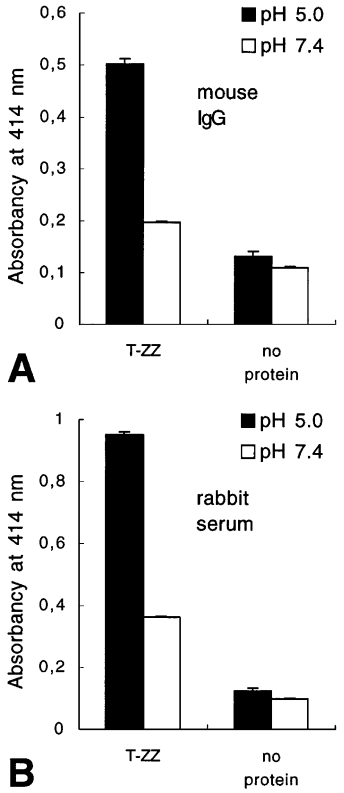

Fig. 4. Binding of a mouse monoclonal IgG2a (A) or rabbit polyclonal IgG (B) to SUV immobilized on filters and treated with or without T-ZZ at neutral or acidic pH. Measures were done in duplicate.

the 40% sucrose fraction, indicating leakage out of the LUV. This confirmed the data from the perforation assay. Leakage of pyranine was not observed at either pH in the absence of T-ZZ.

Fig. 3B,C shows that at pH 5, about 2/3 of the molecules of T-ZZ migrated from the 40% sucrose fraction to the 5% sucrose fraction of the gradient during centrifugation, together with the LUV. The explanation is that these molecules were bound to the LUV. This was not the case at pH 7.4 at which almost all the molecules remained in the 40% sucrose fraction.

Altogether these results show that T-ZZ associates with the membrane of LUV at acidic pH and that membrane permeabilization is most likely a consequence of this association.

3.4. Binding of IgG to T-ZZ anchored to the surface of vesicles

We have studied the capacity of IgG to bind to T-ZZ anchored to the membrane of SUV by lipo-ELIFA. SUV were adsorbed onto filters and put in contact with T-ZZ at pH 7.4 or 5, by filtration. Mouse monoclonal IgG2a or rabbit serum were then incubated by filtration and the presence of IgG was revealed with a goat anti-mouse IgG- or a goat anti-rabbit IgG-F(ab′)$_2$-peroxidase conjugate. Fig. 4 shows that IgG were retained at the surface of the SUV when the SUV were incubated first with T-ZZ at acidic pH. If incubation of T-ZZ was done at neutral pH, IgG bound to the vesicles at a much lower extent. Binding was negligible in the absence of T-ZZ. Incorporation of a fluorescent labeled phospholipid (NBD-phosphatidyl ethanolamine) in the SUV allowed to check that the amount of vesicles was the same on each filter at each step of the experiment whatever the experimental conditions (not shown).

3.5. Binding of IgG to the surface of cells treated with T-ZZ

Adherent fibroblastic L cells grown to confluence were incubated with varying amounts of T-ZZ at pH 5 or pH 7.4. After washing, mouse monoclonal IgG2a or rabbit serum were incubated with the cells and the presence of IgG was detected with a goat anti-mouse IgG- or a goat anti-rabbit IgG-F(ab′)$_2$-peroxidase conjugate (Fig. 5A,B). The results show that IgG attached to cells preincubated with T-ZZ at pH 5 but not at pH 7.4, in a dose dependent manner with respect to T-ZZ. Similar results were obtained with the adherent breast carcinoma cell line SKBR3 (not shown). Interestingly, viability of the cells was not affected by the treatment (Fig. 5C).

Similar experiments were done on the non-adherent cell line FDC-P1. The cells were incubated with T-ZZ at neutral or acidic pH. The cells were washed, incubated with an FITC labeled mouse IgG2a, and washed again. The presence or absence of the antibody at the surface of the cells was analyzed by FACS (Fig. 6). Most of the cells were clearly labeled when T-ZZ was incubated at acidic pH (Fig. 6B). In contrast, labeling was very weak when T-ZZ was incubated at pH 7.4 (Fig. 6A). Binding of the mouse IgG2a could be inhibited if

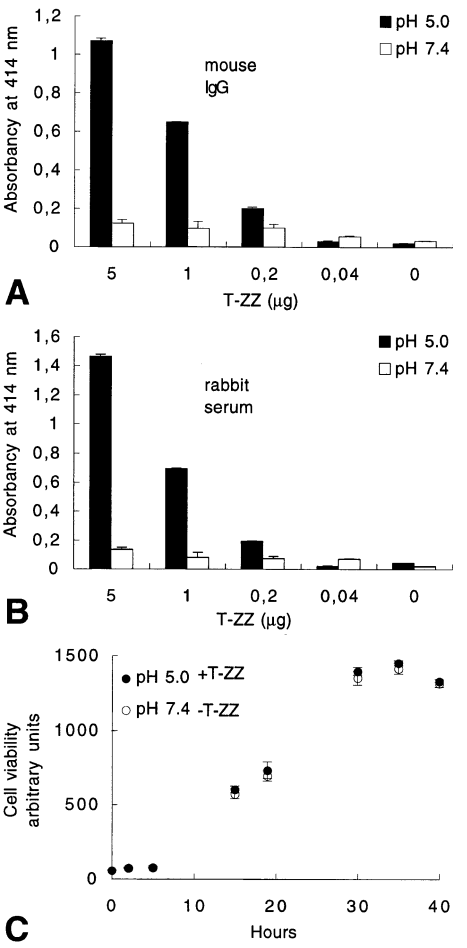

Fig. 5. Binding of a mouse monoclonal IgG2a (A) or rabbit polyclonal IgG (B) to the surface of adherent L cells treated with varying amounts of T-ZZ at neutral or acidic pH. C: Effect of treatment on cell viability. All measures were done in duplicate.

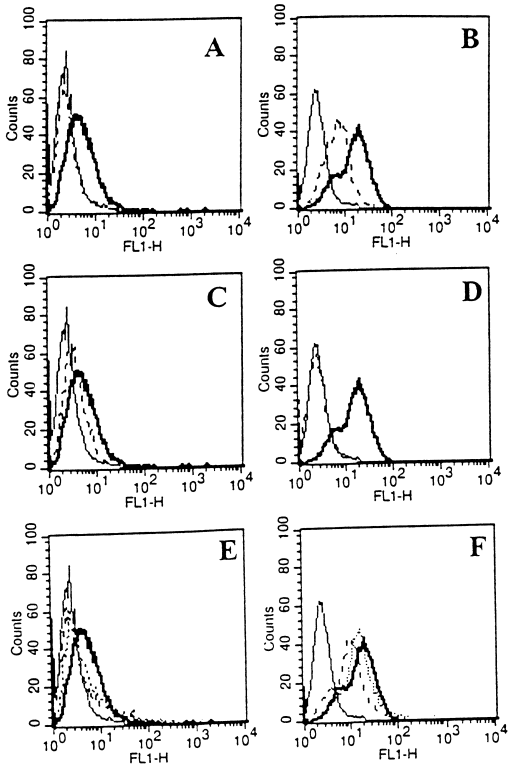

Fig. 6. Binding of an FITC-IgG2a to the surface of FDC-P1 cells treated with T-ZZ at neutral (A,C,E) or acidic pH (B,D,F). In all graphs, the thin line corresponds to T-ZZ treated cells without FITC-IgG2a and the thick line to T-ZZ treated cells with FITC-IgG2a. In A and B, rabbit serum was added before FITC-IgG2a (dashed line). In C and D, T-ZZ was preincubated with anti-diphtheria toxin antibodies before treatment of cells (dashed line). In E and F, controls in which rabbit serum (dashed line) or anti-diphtheria toxin antibodies (dotted line) were added after incubation with FITC-IgG2a.

once treated with T-ZZ at pH 5, the cells were first incubated with rabbit serum (Fig. 6B). Preincubation of T-ZZ with anti-diphtheria toxin antibodies also prevented labeling (Fig. 6D). Labeling was not significantly reduced when rabbit serum or anti-diphtheria toxin antibodies were added after incubation with the FITC labeled mouse IgG2a (Fig. 6F).

These results indicate that T-ZZ attaches to adherent and non-adherent cells at acidic pH by its T domain, and then may bind IgG by its ZZ domain.

4. Discussion

In this work, we have shown that a fusion protein made of the T domain of diphtheria toxin and the IgG-Fc binding protein ZZ combines the properties of both fusion partners and may function as a pH sensitive membrane anchor for IgG. Once bound to the surface of vesicles or cells by the T domain, the ZZ domain of T-ZZ retains its capacity to bind IgG from mouse, rabbit (this study), or hamster (not shown). If IgG are allowed to bind to soluble T-ZZ in the first place, T is still capable of interacting with the membrane of vesicles. Although we did not study this situation in detail, we suppose that it is also possible to anchor T-ZZ/IgG complexes to membranes. Indeed, interaction of IgG with ZZ is stable at pH 5 and is disrupted only below pH 3.5.

Previous reports have described the insertion into membranes of the T domain, isolated [4–6] or in the whole toxin [17–23]. Some of these studies have shown a relation between insertion and permeabilization to small molecules [5,17,18]. Our data are consistent with these reports and suggest that the T domain of T-ZZ penetrates into artificial lipid bilayers. The interaction of the T domain with the cell surface seems to be different. Cell viability is not affected by the binding of the protein which remains detectable on the cell surface for at least 4 h (not shown). This suggests that the cell membranes are not permeabilized and questions whether or not the T domain penetrates the lipid bilayer. Indeed, two types of interactions with cell membranes have been described for diphtheria toxin [24]. When the toxin is first allowed to bind to its receptor it inserts into the membrane leading to the formation of a channel [24–26]. In the absence of receptor, as this is the case in our study, cells are not permeabilized and the interaction is superficial [24]. Thus, different types of interactions with membranes may be observed for the T domain depending on the complexity and on the composition of the membranes [6].

The most interesting feature of T-ZZ is the moderate drop of pH needed for triggering its binding to the membrane. This is well tolerated by many synthetic membrane systems and living cells. Membrane attachment is rapid. Vesicle permeabilization occurs within seconds at protein concentrations around 5×10^{-7} M. If proteins are added before the pH drop (not shown), the kinetic of insertion is even faster, suggesting that the rate limiting step in the process is the diffusion of the protein leading to membrane encounter. The kinetic of binding to cells is also fast, most of the effect occurring within minutes (not shown).

One drawback might be discussed. The modification of membrane permeability is a problem if one wants to use the system for cell receptor targeting of vesicles containing small compounds. Whether the lipid composition of the membrane might influence this phenomenon must be investigated. Anyway, the development of new lipid vehicles such as multilamellar vesicles or vesosomes (a few vesicles entrapped in a larger one) [27] may solve this problem.

Various applications of our system may be found including the targeting of vesicles to cell receptors or the tagging of cells for identification or separation. More broadly, we propose to use the T domain as a membrane anchor for any water soluble protein fused at its C-terminus. In another work (submitted) we have anchored cytokines to the membrane of vesicles and cells by this mean.

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