Serine 331 and Tyrosine 333 Are Both Involved in the Interaction between the Cytosolic Domain of TGN38 and the \( \mu_2 \) Subunit of the AP2 Clathrin Adaptor Complex*

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TGN38 is a type I integral membrane protein that cycles between the trans-Golgi network and the plasma membrane. Internalization at the cell surface and targeting back to the trans-Golgi network is dependent on a hexapeptide motif, SDYQRL, in the cytosolic tail of the protein. It was recently demonstrated that this motif specifically interacts with the \( \mu_2 \) subunit of the AP2 adaptor complex. We have studied the interaction between the entire cytosolic domain of TGN38 and \( \mu_2 \) using the yeast two hybrid system, in vitro binding of recombinant fusion proteins and IAsys optical biosensor technology. A specific interaction has been demonstrated in each of the systems we have employed. We have shown an absolute requirement for Tyr-333 of TGN38 in binding to \( \mu_2 \). In addition we found that mutation of Ser-331 to alanine reduces the affinity of the interaction. By measuring tryptophan fluorescence at equilibrium, we have also determined the dissociation constant for the interaction between the entire cytosolic tail of TGN38 and \( \mu_2 \) as 58 nM. In contrast to previously published work, our data suggest that not only Tyr-333 but also its context is important in determining the specificity of binding of TGN38 to \( \mu_2 \).

Efficient cell surface internalization of many proteins is mediated by their sequestration into endocytic vesicles. The hallmark feature of the process is the localization of endocytic proteins to clathrin-coated pits at the plasma membrane and their subsequent internalization in coated vesicles. Formation of the coated vesicle depends on recruitment of the vesicle coat to the plasma membrane. The coat of clathrin-coated vesicles involves binding of adaptor complexes onto the membrane and their subsequent binding of soluble clathrin. The adaptor complexes are also believed to be involved in the selective concentration of cargo proteins into coated pits.

The high efficiency internalization of many different proteins at the cell surface has been shown to be mediated through targeting signals within the cytosolic domain. Many proteins including the transferrin receptor, mannose-6-phosphate receptor, and TGN38 have tyrosine-based internalization motifs conforming to the consensus, YXX\( \Phi \), where \( \Phi \) represents a hydrophobic amino acid within their cytosolic domains (1). TGN38 is a type I transmembrane glycoprotein that cycles between the trans-Golgi network (TGN) and the plasma membrane (2–6). The tyrosine-based motif of TGN38 is located within the hexapeptide SDYQRL. This sequence has been shown to be responsible for the high efficiency internalization of the protein at the cell surface and its subsequent targeting back to the trans-Golgi network (3–5). Various roles have been proposed for TGN38 (7), but its precise function remains to be elucidated.

The best characterized of the adaptor complexes are AP1 and AP2, which bind to TGN membranes and the plasma membrane, respectively (8, 9). Both are composed of two large (>100 kDa) adaptin subunits (\( \beta \) and \( \gamma \) for AP1 and \( \beta \) and \( \alpha \) for AP2), a medium chain (\( \mu \) subunit, and a small (\( \sigma \) chain subunit. The \( \beta \) subunits of both AP1 and AP2 complexes have been shown to bind to clathrin (10, 11). Several proteins are known to bind directly to the AP2 adaptor complex. For example, epidermal growth factor receptors can be co-immunoprecipitated with AP2 complexes in stoichiometric amounts (12, 13). Purified adaptor complexes have also been shown to bind synthetic peptides corresponding to regions within the cytosolic domains of the mannose-6-phosphate receptor, low density lipoprotein receptor, and lysosomal acid phosphatase, all of which have tyrosine-based sorting signals in their cytosolic domains (14–16). It was recently shown that the tyrosine-based internalization signal of TGN38, SDYQRL (Fig. 1), interacts with the medium chain subunit (\( \mu_2 \)) of the plasma membrane adaptor complex, AP2 (17) which suggests a role for the \( \mu \) subunits in mediating interaction with, at least some, tyrosine-based targeting signals. The tyrosine motif of Lamp-1 (lysosomal associated membrane protein-1, GYQTI) has also recently been shown to bind to both AP1 and AP2 in vitro (18).

The interaction between TGN38 and the \( \mu_2 \) subunit was initially detected by screening a yeast two hybrid library with a tripel repeat of the hexapeptide motif SDYQRL (17). The system was also used to show that the triplet repeat (SDYQRL)$_3$ could interact with the \( \mu_1 \) subunit of AP1, the TGN adaptor complex. A single copy of the repeat sequence as well as the complete cytosolic domain of TGN38 were also shown to interact with \( \mu_2 \). The specificity of interaction was confirmed from binding assays using in vitro translated \( \mu_2 \) and a GST-(SDYQRL)$_3$ fusion protein. The data obtained from these experiments showed an absolute requirement for the tyrosine residue of the motif, in agreement with previously published data (3–5). Binding could also be competed using a synthetic peptide corresponding to the same sequence.

We decided to examine the interaction between the complete cytosolic tail of TGN38 and \( \mu_2 \) in more detail. Apart from demonstrating an interaction between the two proteins in the

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Interaction of TGN38 and \( \mu \)

**Fig. 1. Amino acid sequence of the cytosolic domain of TGN38.**

The sequence of the cytosolic domain of TGN38 is shown. The internalization motif is underlined. Mutations used in this study are shown.

Two hybrid system, Ohno et al. (17) presented no further work using the entire TGN38 tail sequence. As well as confirming the importance of Tyr-333 of TGN38, we have examined the effect of mutating Ser-331 of the SDYQRL motif to alanine with regard to \( \mu \) binding. This residue has previously been shown to be important for both internalization of TGN38 at the cell surface and targeting back to the TGN (5). We have used the two hybrid system, interaction of recombinant fusion proteins, and optical biosensor technology to study the interaction. In addition, the equilibrium dissociation constant for the interaction has been determined by fluorescence quenching. Our data support previous observations (17) with the exception that we show a clear role for the serine residue of the SDYQRL motif in determining the specificity of binding. This suggests that the context of the sequence and not only the presence of the tyrosine motif is important in determining molecular recognition by the adaptor complexes.

**EXPERIMENTAL PROCEDURES**

All reagents were purchased from Sigma (Poole, UK) unless otherwise stated. All plasmid manipulations were carried out according to standard methodologies (19).

**Mutagenesis of TGN38**

Mutations were introduced into TGN38 by PCR using primers incorporating the mutated codons. Mutation S331A was initially incorporated into the full TGN38 coding sequence, which was then used as a PCR template to amplify the cytosolic domain. The mutation Y333A was incorporated into a construct lacking the four carboxyl-terminal residues of TGN38 that were added back during subsequent amplification of the cytosolic domain alone. Primer sequences were as follows: S331A, 5'-AACCTTTAGTTCACACCGTACGCCGCGTCTTGG-3'; Y333A, 5'-CAAGCTTTAGTGTCGGCGCGCGTCTTGG-3'. Each primer was used in conjunction with a sense TGN38 primer incorporating an Asp718 site at the extreme 5’ end of the TGN38 coding region: 5'-GGTACCATGACAGGATGATCGTCCGG-3'. All oligonucleotides were synthesized within the Biotechnology and Biological Sciences Research Council funded Molecular Recognition Center of the University of Bristol.

**Plasmid Manipulation**

All sequences were amplified for subcloning by 10 cycles of PCR using 2 units of Taq DNA polymerase (Boehringer Mannheim, Lewes, UK), 0.2 mM dNTPs, 1.5 mM MgCl₂, and 5 pmol of each primer. PCR products were gel purified and subcloned via the two hybrid activation domain vector, pVP16 (20), creating sequence of each was amplified by PCR and subcloned, in frame, into (i) Dr. M. S. Robinson (University of Cambridge, UK), 0.2 mM dNTPs, 1.5 mM MgCl₂, and 5 pmol of each primer. PCR was performed using 2 units of Taq DNA polymerase (Boehringer Mannheim, Lewes, UK), 0.2 mM dNTPs, 1.5 mM MgCl₂, and 5 pmol of each primer. PCR products were gel purified and subcloned via pGEM-T (Promega, Southampton, UK) to the target vector using standard methods (19). All sequences amplified by PCR were confirmed by automated DNA sequencing.

\( \mu \) and \( \mu 2 \) cDNAs encoding rat \( \mu \) and \( \mu 2 \) were kindly provided by Dr. M. S. Robinson (University of Cambridge, UK). The full coding sequence of each was amplified by PCR and subcloned, in frame, into (i) the two hybrid activation domain vector, pVP16 (20), creating pVP16-\( \mu 1 \) and pVP16-\( \mu 2 \), respectively, and (ii) the plasmid-based prokaryotic expression vector pET32a (Novagen, Abingdon, UK) for bacterial expression. Amino-terminally truncated \( \mu 2 \) (corresponding to amino acids 121–435 of \( \mu 2 \), generating \( \mu 2 \)AN) was also made and subcloned into pET32a.

**TGN38—Wild-type and mutant versions of the cytosolic tail of TGN38 were amplified by PCR and subcloned into (i) pGEX4T (Pharmacia Biotech Inc., St. Albans, UK) for expression as GST fusion proteins, (ii) pET32a (Novagen), for expression as thioredoxin fusion proteins, and (iii) the two hybrid DNA binding domain vector, pBTM116 (20).

**Expression of Recombinant Proteins**

TGN38 cytosolic domains were expressed as GST fusion proteins in *Escherichia coli* strain BL21DE3 and purified on glutathione-agarose according to protocols (Pharmacia). Briefly, cultures were inoculated with 1% (v/v) of saturated overnight culture, grown to Aₚ₀₀ = 0.4 and induced for 1 h with 0.1 mM isopropyl-β-D-thiogalactoside. Following sonication in 50 mM sodium phosphate, pH 7.4, supplemented with protease inhibitors (1 mM leupeptin, 2 mM antipain, 10 mM benzamidine, 10 mM aprotinin, 1 mM chymostatin, 5 mM pepstatin A), purification was achieved using glutathione-agarose with washes in phosphate-buffered saline. Thioredoxin fusions were expressed in an identical manner and purified using Talon² purification resin (CLONTECH) according to the manufacturer's instructions. All proteins were dialyzed into 50 mM sodium phosphate, pH 7.4, and stored at −70 °C.

Full-length \( \mu 2 \) and \( \mu 2 \)AN were also expressed in BL21DE3 as follows. Cultures were inoculated with 10% (v/v) overnight culture, grown for a further 2 h at 37 °C with shaking, and induced for 30 min with 1 mM isopropyl-β-D-thiogalactoside. Cells were pelleted and lysed by sonication; the insoluble pellet was washed twice in 100 mM sodium phosphate containing 1% (v/v) Triton X-100 before solubilization in 50 mM sodium phosphate containing 8 M urea and 10 mM dithiothreitol for 1 h at room temperature. Proteins were refolded by stepwise dialysis into lower concentrations of urea at 4 °C. Buffers for dialysis down to 0.5 M urea contained 2 mM dithiothreitol; for lower urea concentrations (less than 0.5 M), dithiothreitol was omitted and 10% glycerol was included. Finally, \( \mu 2 \)AN and \( \mu 2 \) proteins were dialyzed into 50 mM sodium phosphate containing 10% glycerol and stored at −70 °C.

**In Vitro Binding of Fusion Proteins**

GST-TGN38 tail fusions (30 μg each) and Trx-\( \mu 2 \)AN (10 μg) were incubated together for 1 h at room temperature in a total volume of 0.2 ml of 50 mM sodium phosphate, pH 7.4, containing 10% glycerol and supplemented with protease inhibitors (5 μM leupeptin, 10 μM antipain, 50 μM benzamidine, 50 units/ml aprotinin, 5 μM chymostatin, 5 μM pepstatin A). Complexes were then immunoprecipitated for 1 h with a polyclonal antibody that specifically recognizes thioredoxin that had been preadsorbed to protein G-Sepharose (20 μl bed volume). Complexes were washed twice in binding buffer and separated by SDS-polyacrylamide gel electrophoresis on 12% gels and stained with Coomassie Blue R-250. Band intensities were quantitated following scanning of individual gels on a Apple Macintosh® computer using the public domain NIH Image program (developed at the U. S. National Institutes of Health and available on the Internet).

**IAsys Optical Biosensor Measurements**

Experiments were performed using an IAsys resonant mirror optical biosensor (Affinity Sensors, Cambridge, UK). Thioredoxin fusions of TGN38 cytosolic domains were immobilized on carboxymethyl-dextran cuvette surfaces according to the manufacturer's instructions. Binding experiments were performed in 50 mM sodium phosphate, pH 7.4, containing 10% glycerol. \( \mu 2 \)AN was added to a final concentration of 0.2 μM in a final volume of 200 μl and the change in resonant angle was monitored at 1-s intervals for approximately 300 s. Experiments were performed at 25 °C with a stirrer speed of 100 rpm. For repeated

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² The Internet address is http://rsb.info.nih.gov/nih-image.
FIG. 2. Two hybrid analysis of the interaction between the cytosolic domain of TGN38 and adaptor proteins, μ1 and μ2. Interaction between TGN38 cytosolic domain constructs and μ1 or μ2. Yeast were grown for 3 days before lifting to filter as described. β-galactosidase expression was assayed by overnight incubation in the presence of X-gal. Positive interactions are identified by hydrolysis of the substrate to produce a blue color. The left-hand column shows yeast transformed with pVP16-μ1; on the right are those transformed with pVP16-μ2. Cells were cotransformed with LexA fusion plasmids encoding either wild-type (WT) TGN38, Y333A mutant, S331A, or a negative control, lamin.

Measurements using the same cuvette, surfaces were regenerated by washing for 2 min in 200 mM formic acid. For peptide competition experiments, μ2AN was preincubated with 10 μM peptide (CKAS-DYQRNLKL) for 1 h at room temperature before addition to the cuvette.

Fluorimetry

Fluorescence emission was monitored between 300 and 450 nm following excitation at 290 nm using a Perkin-Elmer fluorimeter. A 2-μl cuvette containing Trx-μ2AN at 50 μM was used. 5-μl aliquots of GST-TGN38 fusion were added sequentially (each addition increasing the concentration of GST fusion in the cuvette by 30 μM), and fluorescence emission was monitored. A base line of fluorescence from the buffer alone was subtracted from each data set prior to analysis. Data were plotted and analyzed using the GraFit package. Data were analyzed according to the following equations describing the addition of B (GST-TGN38 fusion) to A (Trx-μ2AN), and the equilibrium dissociation constant (Kd) was calculated: Fluorescence, F = F0[A] + Ic[B] + Ic[A][B]; where I represents the fluorescence intensity, [A] represents the concentration of unbound A, [B] represents the concentration of unbound B, and [AB] represents the concentration of AB complex; [A] = [A], (1 - α), [A][B] = ([A][B], [B], [A][B]), and α = ([B][A][B] + [A][B])/(Ic[A][B] + [A][B]).

RESULTS

Two Hybrid Analysis—Two hybrid screening (22) of a mouse spleen cDNA library with a triplet sequence of the SDYQRL motif found within the cytosolic domain of TGN38 has recently led to the identification of the μ2 subunit of the AP2 adaptor complex as a binding partner (17). Further analyses confirmed the interaction using a single copy of the motif as well as the cytosolic domain of TGN38. We have used an alternative two hybrid system to test this interaction as well as to assess the strength of interaction with μ1 and μ2 of a number of TGN38 constructs mutated in the serine or tyrosine residues of the SDYQRL motif.

In frame fusions of TGN38 cytosolic domain sequences with LexA and coding sequences of μ1 or μ2 with VP16 were generated and transformed into yeast strain L40. Fig. 2 shows the result of a two hybrid β-galactosidase assay to test for interaction between the cytosolic domain of TGN38 and μ1 or μ2. No activation of transcription was detected with any of the constructs individually nor following co-transformation of yeast with pVP16-μ1 or pVP16-μ2 and with a negative control plasmid encoding a LexA-Lamin fusion. A positive signal was seen following co-transformation of yeast with μ2 and the wild-type cytosolic tail of TGN38 after a 6-h incubation. No interaction was observed between any of the other paired constructs even following incubation in the presence of X-gal for up to 24 h. Mutation of Tyr-333 of TGN38 to Ala (Y333A) abolished the interaction, confirming the requirement for a tyrosine-based signal. Mutation of Ser-331 to Ala (S331A) also abolished the interaction. This indicates a requirement for Ser-331 in mediating a high affinity binding of TGN38 to μ2. No interaction was detected between any of the TGN38 constructs and μ1 (the medium chain of the TGN adaptor complex, AP1).

Interaction of Fusion Proteins—To extend the data from the two hybrid system, we have used an in vitro system involving interaction of recombinant fusion proteins of the two binding partners. The cytosolic domain of TGN38 (and mutants thereof) was expressed as a GST fusion, and μ2 and μ2AN were expressed as thioredoxin fusions. Proteins were incubated together in solution followed by immunoprecipitation using a thioredoxin-specific polyclonal antibody. The strength of interaction was then assessed by Coomassie Blue staining of polyacrylamide gel electrophoresis. Lane 1, molecular mass markers; lane 2, wild-type TGN38; lane 3, Y333A mutant; lane 4, S331A mutant; lane 5, GST. Data from three identical experiments were analyzed using the NIH Image package and averaged. B, quantitation of binding of wild-type (WT) TGN38 (SDYQRL), Y333A (SDAQRL), and S331A (ADYQRL) to Trx-μ2AN. Binding of each TGN38 fusion protein is expressed relative to that of GST alone.

FIG. 3. In vitro interaction to the cytosolic domain of TGN38 and μ2. GST-TGN38 fusion proteins and Trx-μ2AN were incubated together for 1 h followed by immunoprecipitation with anti-Trx. Complexes were then boiled in sample buffer and separated by SDS-polyacrylamide gel electrophoresis. A, representative polycrylamide gel. Lane 1, molecular mass markers; lane 2, wild-type TGN38; lane 3, Y333A mutant; lane 4, S331A mutant; lane 5, GST. Data from three identical experiments were analyzed using the NIH Image package and averaged. B, quantitation of binding of wild-type (WT) TGN38 (SDYQRL), Y333A (SDAQRL), and S331A (ADYQRL) to Trx-μ2AN. Binding of each TGN38 fusion protein is expressed relative to that of GST alone.
cuvettes and binding of TGN38 and μ2ΔN fusion proteins were immobilized onto carboxy-methyl dextran between the two proteins in real time. TGN38-thioredoxin mirror optical biosensor was used to analyze the interaction. Binding was then measured for TGN38 fusions immobilized on carboxy-methyl dextran cuvette surfaces. A, binding of μ2ΔN to immobilized TGN38 fusion proteins. Binding curves are shown for wild-type TGN38, Y333A, S331A, and thioredoxin alone. B, competition of binding with synthetic peptide. Experimental conditions identical to those in A were used to obtain binding curves for wild-type TGN38 (wt 1). The surface was regenerated with 200 mM formic acid and the binding determination repeated (wt 2). Binding was then measured for μ2ΔN that had been preincubated for 1 h with 10 μM competing peptide of sequence CKASDYQRLNLKL (wt + pep). Following regeneration as before, binding of non-competed μ2ΔN was repeated (wt 3).

Preincubation of μ2ΔN with the peptide followed by subsequent addition to the biosensor cuvette reduced binding significantly (Fig. 4B, wt + pep). Binding of μ2ΔN was repeated following regeneration of the surface as before to remove the peptide and any bound μ2ΔN. In this case a reduction in binding was observed (wt 3 in Fig. 4B). The level of binding was still above that seen in the presence of competing peptide but less than that seen initially. The most plausible explanation for this is that the peptide was not completely removed from the dextran matrix by formic acid treatment. The data obtained from the optical biosensor are in complete agreement with that obtained from the other approaches but also show that the effect of the S331A mutation is not as significant as that of Y333A.

Equilibrium Fluorescence—Equilibrium measurement of tryptophan fluorescence was used to determine the dissociation constant for the interaction between TGN38 and μ2ΔN. Sequential addition of GST-TGN38 to Trx-μ2ΔN would lead to linear increase in fluorescence due to the four tryptophan residues of GST-TGN38. Deviation from the linearity of this increase indicates an interaction between the two proteins. Change in fluorescence was plotted against concentration of GST-TGN38 and then subtracted from the linear increase due to sequential addition of TGN38. The resulting fluorescence quench data were curve fitted to the equation shown under “Experimental Procedures” from which the equilibrium dissociation constant ($K_d$) was calculated (Fig. 5). Using this method the $K_d$ for the interaction between wild-type TGN38 tail and μ2ΔN was calculated as 58 nM (standard error ± 7). The $K_d$ for observation from the two hybrid analysis that the S331A mutation disrupts the interaction with μ2ΔN.

IAsys Optical Biosensor Measurements—The IAsys resonant mirror optical biosensor was used to analyze the interaction between the two proteins in real time. TGN38-thioredoxin fusion proteins were immobilized onto carboxymethyl dextran cuvettes and binding of μ2ΔN determined. The thioredoxin fusion of TGN38 was used for this part of the study due to the increased stability of these constructs over GST fusions. All TGN38 constructs as well as the thioredoxin domain alone were immobilized at a concentration of 8 ng·mm⁻² protein. Change in resonant angle was monitored continuously over a 5-min period following the addition of GST-TGN38 to Trx-thioredoxin alone. A, competition of binding with synthetic peptide. Experimental conditions identical to those in A were used to obtain binding curves for wild-type TGN38 (wt 1). The surface was regenerated with 200 mM formic acid and the binding determination repeated (wt 2). Binding was then measured for μ2ΔN that had been preincubated for 1 h with 10 μM competing peptide of sequence CK-ASDYQRLNLKL (wt + pep). Following regeneration as before, binding of non-competed μ2ΔN was repeated (wt 3).

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The interaction of the S331A fusion with μ2ΔN was similarly determined as 103 nM (standard error ± 8). The dissociation constant for the binding of the Y333A mutant was found to be 755 nM (standard error ± 100). These data are in entirely consistent with those obtained from the other approaches described here. In particular, the lower affinity of the S331A mutant compared with the wild-type protein for μ2ΔN reflects the binding curve obtained using the IAsys optical biosensor, suggesting that the mutation S331A does indeed affect the binding of the cytosolic domain of TGN38 to μ2ΔN.

**DISCUSSION**

We have used a number of complementary approaches to study the interaction of a tyrosine-based internalization signal with the medium chain component, μ2, of the adaptor complex, AP2. We have studied the internalization signal of TGN38, SDYQRL, which in isolation has previously been shown to interact with the μ2 protein in a two hybrid system and in an in vitro binding assay (17). The context of critical tyrosine residues within internalization signals is almost certainly important for specificity, for example, the spacing of the tyrosine motif of lamp-1 from the membrane is critical for interaction with the TGN adaptor complex, AP1 (24). The triplet repeats used by Ohno *et al.* (17) would obviously also be very different in structure to the entire cytosolic domain of TGN38, which has been shown by NMR to form a nascent helix in solution (25).

For this reason, we chose to study the interaction in more detail using the entire cytosolic domain of TGN38.

Initial experiments exploited the yeast two hybrid system (22) to study the interaction. Interaction was detected between μ2 and the wild-type TGN38 cytosolic domain but not following mutation of the tyrosine or serine residues of the SDYQRL motif to alanine. No interaction was detected between the cytosolic tail of TGN38 and μ1. This is in conflict with the results of Ohno *et al.* (17), who readily detected an interaction between a triplet repeat of SDYQRL and μ1. This suggests that the interaction, if any, between the entire cytosolic tail of TGN38 and μ1 is too weak to be detected using the sensitive two hybrid system we employed. It can be inferred from this that the context of the critical tyrosine residue is an important determinant of adaptor binding. Indeed, recent surface plasmon resonance data from the same group showed that the cytosolic domain of TGN38 does bind to AP1 with lower affinity than AP2 (26) in agreement with our two hybrid data. Similarly, the endocytic signal of the epidermal growth factor receptor has been shown to bind to AP1 with lower affinity than to AP2 (27).

The two hybrid experiments reported here also show an importance for Ser-331 of the SDYQRL motif in determining binding specificity. This result complements the earlier biological observation that mutation of serine-331 plays a role in determining the intracellular localization of TGN38 (5). Ohno *et al.* demonstrated that mutation of this residue to alanine did not affect binding of an isolated SDYQRL motif to μ2 when assayed for growth on histidine-deficient medium (17). These growth assays are not quantitative, and so it is difficult to determine the relative importance of this mutation. In our hands the same mutation, in the context of the entire cytosolic tail sequence, completely abolished interaction with μ2 as evidenced by lack of activation of transcription of the β-galactosidase reporter gene. More recently, two hybrid screening of a combinatorial cDNA library of constructs ending AAYXXX (26) also failed to show any role for Ser-331 of TGN38 in mediating binding to μ2. Again, this discrepancy with our data is most easily explained by the context of the tyrosine motif. Our constructs have the additional four amino acids (NLKL) of TGN38 present, whereas those used for screening of the combinatorial library did not. Thus, although the tetrapeptide sequence of YXXΦ may be sufficient for directing binding to μ2, it is clear that the exact sequence context of these motifs affects the affinity of binding and therefore presumably the efficiency of sorting into endocytic vesicles.

To complement the two hybrid data, we investigated the interaction between recombinant fusion proteins of the two binding partners. Fusion proteins were incubated together, and complexes were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. Using this approach we were able to detect binding of the wild-type cytosolic domain of TGN38 to μ2ΔN (a μ2 construct deleted in the first 120 amino acids, a modification that has been shown not to alter binding to the cytosolic domain of TGN38; Ref. 17). In addition, this result confirmed the observation apparent from our two hybrid results that both Ser-331 and Tyr-333 of the cytosolic domain of TGN38 are important in mediating a high affinity interaction with μ2ΔN.

In addition to the above approaches, we have utilized the IAsys resonant mirror optical biosensor to investigate the interaction between TGN38 and μ2ΔN. The biosensor measures changes in resonant angle that occur on binding of, in this case, μ2ΔN to TGN38 cytosolic domains immobilized on a cuvette surface in real time. No binding of the thioredoxin fusion domain alone was observed, indicating that any interaction ob-
served is due to the $\mu^2\Delta N$ domain. We were able to confirm the absolute requirement of Tyr-333 in the interaction because mutation to alanine (Y333A) abolished the interaction. In addition, binding was also competent using a synthetic peptide containing the SDYQRL motif. The inhibition of binding was significant but not complete, probably due to insufficiently high peptide concentrations (10 $\mu M$). Indeed Heilker et al. (28) found that 20 $\mu M$ peptide did not significantly affect binding of internalization motifs to adaptor complexes as assessed by surface plasmon resonance. Very high concentrations of competing peptide were also required for competition of binding of lamp-1 to adaptor complexes (18). This may be a feature of the different affinities of the free peptide and immobilized fusion protein for $\mu^2$ resulting from the context or presentation of the tyrosine motif. The peptide used in this study had only four residues upstream of the SDYQRL motif, and it is possible that this greatly reduced the affinity of interaction with $\mu^2$ compared with the entire cytosolic domain of TGN38. The fact that binding was at least to some extent competent suggests specificity of interaction.

Due to the exquisite sensitivity of the system, the IAsys experiments showed an effect of Ser-331 in determining efficiency of binding not apparent from the two hybrid analyses or immunoprecipitation. Mutation of this residue to alanine (S331A) significantly reduced the amount of binding detected as well as the initial rate of the interaction but unlike the Y333A mutation caused only a partial disruption of binding. This suggests that in the context of the complete TGN38 tail sequence, this upstream residue is important in determining the affinity of interaction between TGN38 and the adaptor complex and is consistent with previously published data implicating Ser-331 in internalization and return of TGN38 to the TGN (5). Furthermore, overexpression of S331A TGN38 in COS-7 cells does lead to enhanced expression of TGN38 at the cell surface compared with similar levels of expression of wild-type TGN38.  

To determine the equilibrium dissociation constant for the interaction between TGN38 and $\mu^2$, we studied quenching of tryptophan fluorescence on binding. On addition of TGN38 fusion proteins, a nonlinear increase of fluorescence was observed, and from this dissociation constants for the interactions were calculated. Strong binding of the wild-type cytosolic domain of TGN38 to $\mu^2$ was observed ($K_d = 58$ nM) with somewhat weaker binding for the S331A mutant ($K_d = 10^3$ nM). Binding of the Y333A mutant was significantly lower ($K_d > 700$ nM). Interaction was barely detectable with this mutant as evidenced by the low confidence limits of the calculated dissociation constant (standard error = $\pm 100$ nM). This quantitative approach confirms the observations from the other techniques described here. The data confirm that the tyrosine residue within the SDYQRL motif of TGN38 is essential for binding to the $\mu^2$ chain of the AP2 complex and in addition indicate that the serine residue within this motif is also important in mediating high affinity binding to the complex.

Our data are somewhat in conflict with previously published results (17) because they indicate an important role for residues other than the tyrosine and bulky hydrophobic residue of the YXX\(\omega\) consensus in determining specificity of binding of tyrosine-based targeting motifs to adaptors. This additional specificity may have a role in determining the subcellular compartment to which proteins are ultimately targeted. This may be of particular relevance to TGN38 given the demonstration of a role for Ser-331 in return of the protein from the cell surface to the TGN (5). The greater affinity of interaction shown here between TGN38 and $\mu^2$ compared with $\mu_1$ might account for the observed traffic of TGN38 to the cell surface and its subsequent internalization as opposed to $\mu_1$-mediated transport direct from the TGN to lysosomal compartments. Similar differences in binding affinity of TGN38 to adaptor complexes at the level of the sorting endosome could mediate transport to the TGN as opposed to entering a recycling pathway such as that seen for the transferrin receptor.

In summary, we have shown using three complementary approaches that the complete cytosolic tail of TGN38 interacts with the $\mu^2$ subunit of the AP2 adaptor complex. The interaction has been shown to occur in free solution, intracellularly (using the two hybrid system) and also by recruitment of $\mu^2$N to immobilized TGN38 tails (IAsys measurements). The equilibrium dissociation constant ($K_d$) for the interaction is 58 nM. The interaction has been shown to be dependent not only on the tyrosine residue of the internalization motif of TGN38 but also on the serine residue upstream of this YXX\(\omega\) motif.

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