Specificity of interaction between adaptor-complex medium chains and the tyrosine-based sorting motifs of TGN38 and Igp120

David J. STEPHENS and George BANTING¹

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

Multiple sorting steps within eukaryotic cells are mediated by tyrosine-based sorting motifs. Motifs conforming to the consensus -YXX ϕ - (where ϕ indicates a bulky hydrophobic residue) have been shown to specify high-efficiency internalization from the plasma membrane, targeting from the plasma membrane to the *trans*-Golgi network and targeting to lysosomal compartments as well as being involved in basolateral sorting in polarized cells. These motifs are recognized by the medium-chain subunits of heterotetrameric adaptor complexes. Whereas these motifs have been shown to be sufficient to mediate interaction with the μ -chains, we and others have shown that their context is important in determining the affinity of interaction. In this study we have investigated the interaction between the tyrosine motifs of the type-1 integral membrane proteins TGN38 and Igp120 with medium-chain subunits using the yeast two-hybrid system.

Whereas the wild-type version of the cytosolic domain of TGN38 interacts with highest affinity with μ 2, we show that the cytosolic domain of Igp120 interacts almost exclusively with μ 3A. The specificity of binding of tyrosine-based sorting motifs to μ -chains is shown to be highly sensitive to the context in which the motif lies. For example, the -YQTI- motif of Igp120 is effectively non-functional with regard to μ -chain binding when placed in the context of the TGN38 cytosolic domain. Deletion of four amino acids (NLKL) at the extreme C-terminus of TGN38, leaving the YXX ϕ motif as the C-terminus, greatly enhances the affinity of interaction with μ 2. Furthermore, addition of these same residues to the extreme C-terminus of Igp120 effectively abolishes the interaction of the cytosolic domain of Igp120 with μ -chains. We also show that the newly identified μ -adapting-related protein 2 (μ 4) only interacts weakly with tyrosine-based sorting motifs.

INTRODUCTION

Tyrosine-based sorting motifs mediate the targeting of many integral membrane proteins of the secretory pathway. Perhaps the best characterized of these motifs are those conforming to the consensus -YXX ϕ - (where ϕ indicates a bulky hydrophobic residue) [1,2]. Such motifs have been shown to mediate many different sorting steps within cells, such as high-efficiency endocytosis [1], internalization and targeting to the *trans*-Golgi network (TGN) [3–7], lysosomal sorting of lysosome-associated membrane proteins (such as Igp120, where Igp stands for lysosomal glycoprotein, [8]) and basolateral targeting in polarized cells [9]. Thus, a common core motif can mediate a number of distinct steps, implying that the local environment of this common core motif must impart some specificity to these interactions. The targeting sequences within the cytosolic domains of both TGN38 and Igp120 have been employed extensively in the identification of the pathways and machinery used by these molecules. It has been shown that the cytosolic domain of TGN38 is capable of binding clathrin-adaptor complex subunits with high affinity [10–12] and that the precise context of the motif greatly affects the distribution and trafficking of the protein [12,13]. Experiments using the short (11-amino-acid) tail of Igp120 have shown that, in addition to the critical tyrosine residue within the YQTI motif, the spacing of this motif from the transmembrane domain [14] and the identity of the C-terminal amino acid of the motif [15] have significant implications for the trafficking itinerary of this protein.

It has been demonstrated in recent years using several different protein-interaction systems that a number of tyrosine-based motifs interact with clathrin-associated adaptor-protein complexes (APs) [10–12,16]. These complexes, AP1, involved in

TGN-to-lysosome delivery, and AP2, involved in clathrin-mediated endocytosis at the plasma membrane, are composed of four non-covalently associated subunits and are responsible for the assembly of clathrin on to the relevant membrane [17]. The medium chain subunits of these complexes have been shown to interact with tyrosine motifs of the form -YXX ϕ - [10]. More recently, a further adaptor complex, AP3, has been identified [18]. This complex is highly similar to AP1 and AP2 in subunit composition and indeed the β -3A subunit has been shown to associate with clathrin [19]. The isolated tyrosine-based motif from the cytosolic domain of TGN38 has been shown to interact with the medium chain of AP3 [11]. There are two isoforms of medium-chain subunit of AP3, μ 3A and μ 3B, which show 84 % identity. It is of note that there is discrimination between μ 3A and μ 3B interactions with the YQRL motif from the cytosolic domain of TGN38 [11].

Conceptual translation of the cDNA encoding TGN38 predicts that the mature C-terminus of the protein is SDYQRLNLKL [3]; however, the precise C-terminal residue *in vivo* remains to be identified. Thus, whereas TGN38 is almost certainly synthesized as a protein terminating SDYQRLNLKL, subsequent proteolytic processing may, for example, cleave one or more of these C-terminal residues. A precedent for such processing exists, since the C-terminus of Igp120 isolated from lysosomes has been shown to be SHAGYQ, whereas the protein is synthesized with the C-terminal sequence, SHAGYQTI [8]. Removal of these last four amino acids from the cytosolic domain of TGN38 has been shown to greatly enhance the interaction with both μ 1 and μ 2 [20].

We have shown previously that the exact context of the SDYQRL motif of TGN38 is critical for interaction with the medium-chain subunit μ 2 [12], in particular with regard to

Abbreviations used: TGN, *trans*-Golgi network; Igp, lysosomal glycoprotein; 3-AT, 3-amino-1,2,4-triazole; AP, adaptor-protein complex.

¹ To whom correspondence should be addressed (e-mail g.banting@bristol.ac.uk).

lgp120-AGYQTI* (wild-type):	KRKSHAGYQTI
lgp120-AGYQRL*:	KRKSHAGYQRL
lgp120-SDYQRL*:	KRKSHSDYQRL
lgp120-YQTINLKL*:	KRKSHAGYQTIINLKL
TGN38-SDYQTI*:	KRKIIAFALEGKRSKVTRRPKASDYQTI
TGN38-SDYQTIINLKL*:	KRKIIAFALEGKRSKVTRRPKASDYQTIINLKL
TGN38-SDYQRLNLKL* (wild-type):	KRKIIAFALEGKRSKVTRRPKASDYQRLNLKL
TGN38-SDYQRL*:	KRKIIAFALEGKRSKVTRRPKASDYQRL

Figure 1 Nomenclature of constructs used in this study

Standard single-letter amino acid codes are used throughout. Sequences shown represent the entire coding regions that were expressed as LexA fusions. In this Figure and throughout the text, * indicates a stop codon.

the serine residue within this motif. This is in conflict with results from others [20], which have shown that mutation of this serine residue to alanine has little or no effect on interaction of the tyrosine motif with medium-chain subunits [20]. However, this might be explained by the fact that data presented in the study of Ohno et al. [20] were obtained using constructs lacking the terminal four amino acids of TGN38, namely NLKL. In this current study, we have further assessed the role of this upstream serine residue in specifying medium-chain interaction in the context of the truncated TGN38 cytosolic domain.

In addition, we also set out to determine the specificity of medium-chain interaction with a different tyrosine-based sorting motif, that of the type-1 lysosomal membrane glycoprotein lgp120 (AGYQTI, [8]). Our reasoning for doing so arose from the observation that chimaeric molecules in which the tyrosine motifs of TGN38 (SDYQRL) and lgp120 (AGYQTI) have been swapped were localized to the same place as wild-type molecules [21]. Given these results, one may expect the same array of medium-chain interactions to be seen with the hybrid constructs as with the wild-type cytosolic domains of TGN38 and lgp120. We have generated a number of constructs in which the tyrosine-based motifs of TGN38 and lgp120 have been exchanged and placed in the context of the full-length cytosolic domains of these proteins (see Figure 1). In addition to $\mu 1$, $\mu 2$, $\mu 3A$ and $\mu 3B$, we also analysed interactions with the newly identified μ -adaptin-related protein-2 [22]. This protein shows 31 % identity to $\mu 1$ and $\mu 2$, 24 % identity to $\mu 3A$ and 23 % to $\mu 3B$, and has been suggested to be the medium chain of a novel adaptor complex. Henceforth, we shall refer to this subunit as $\mu 4$.

Removal of the terminal four residues from TGN38 (NLKL) led to greatly altered specificity of interaction, with the Δ NLKL construct (TGN38-SDYQRL) now interacting with $\mu 3A$ as well as $\mu 2$. Mutation of the upstream serine to alanine or aspartate completely abolished these interactions, as we have shown previously for the complete cytosolic domain of TGN38 [12]. We also show that the wild-type cytosolic domain of lgp120 interacts strongly with $\mu 3A$ and that addition of the C-terminal four amino acids from TGN38 (NLKL) to the cytosolic domain of lgp120 completely abolishes interaction with medium chains.

EXPERIMENTAL

All reagents were purchased from Sigma (Poole, Dorset, U.K.) unless otherwise stated. Oligonucleotide primers for PCR (sequences are available on request from the authors) were synthesized and the DNA sequencing was performed within the Department of Biochemistry at the University of Bristol, Bristol,

U.K. All plasmid manipulations were carried out according to standard methodologies [23].

Plasmid manipulation

All constructs were generated using the PCR and cloned via pGEM-T (Promega, Southampton, U.K.) into pBTM116 [24] for two-hybrid analyses. Mutant receptor cDNAs of lgp120-TGN38 hybrids (wild-type lgp120-AGYQTI*, lgp120-AGYQRL*, lgp120-SDYQRL*, TGN38-SDYQTI* and TGN38-SDYQTIINLKL*; * indicates a stop codon) were provided generously by Dr. B. J. Reaves (University of Bath, Somerset, U.K.). lgp120-AGYQTIINLKL was constructed from wild-type lgp120 by PCR. Δ NLKL TGN38 constructs were similarly generated from previously described constructs [12]. Medium-chain cDNAs were kindly provided by Dr. M. S. Robinson (University of Cambridge, Cambs, U.K.). Full coding regions of $\mu 1$, $\mu 2$, $\mu 3A$ and $\mu 3B$ were cloned by PCR into pVP16; $\mu 4$ (see [22]) was amplified by PCR and subcloned from a partial expressed sequence tag, obtained from the IMAGE consortium via Dr. M. S. Robinson, into pVP16. The coding region of this clone includes the entire C-terminal sequence including the regions known to be involved in tyrosine-motif interaction in the other medium chains [25]. All sequences amplified by PCR were confirmed by automated DNA sequencing.

Two-hybrid analyses

Yeast strain L40 [24] was maintained as described [12,24]. Transformation was carried out exactly as described in [26]. Quantitative β -galactosidase assays were performed using chlorophenolred- β -D-galactopyranoside exactly as described in the Clontech Yeast Protocols Handbook (Clontech, Basingstoke, Hants, U.K.). Results are expressed as β -galactosidase units defined thus: β -galactosidase = $1000 \times A_{578} / (t \times V \times A_{600})$, where t is the time in min and V is the volume of culture used. Quantitative growth assays were performed essentially as described in [25]. The A_{600} of triplicates was measured and the concentration of 3-amino-1,2,4-triazole (3-AT) required for 50 % inhibition of cell growth was determined.

RESULTS

Several novel adaptor-complex subunits have been identified recently; mainly from homology searches of expressed sequence tag (EST) databases. Since we have previously characterized the

Figure 2 The last four amino acids of TGN38 modulate its interaction with adaptor medium chains

Yeast cells were transformed with a bait construct (LexA fusion) containing the cytosolic domain of TGN38 in wild-type (SDYQRLNLKL) or mutant form and a prey construct (VP16 fusion) containing one of the adaptor-complex medium (μ) chains as indicated (- denotes an 'empty' pVP16 vector). Plates were grown for 72 h in the presence of histidine (+His) or in the presence of 20 mM 3-AT. Streaked yeast grown in the presence of histidine were subsequently used for filter β -galactosidase assays. The single-letter amino acid sequences shown represent the extreme C-termini of the constructs.

interactions between the cytosolic domain of TGN38 and the μ 1 and μ 2 subunits of the AP1 and AP2 complexes, we decided to investigate the interaction between the cytosolic domain of TGN38 and the medium chains of the AP3 and putative AP4 complexes. Constructs that express mutants of the cytosolic domains of TGN38 or lgp120 as the bait partner in a two-hybrid system [27] were generated and used to analyse the interaction with adaptor-complex medium chains [12].

Plate growth and β -galactosidase assays

The results of plate assays for both histidine biosynthesis and β -galactosidase enzyme activity are shown in Figure 2. Selecting in the absence of histidine alone was not sufficient to eliminate growth of the bait constructs expressing mutant versions of the TGN38 cytosolic domain co-transformed with the negative-control plasmid pVP16. To overcome this problem, yeast were streaked on to medium containing 20 mM 3-AT. 3-AT is a competitive inhibitor of histidine biosynthesis. Thus, growth of the L40 yeast strain in the presence of 3-AT only occurs where a strong interaction is present between the LexA and VP16 fusion proteins. Those yeasts grown in the presence of histidine (Figure 2, first panel; i.e. all transformants) were subsequently assayed for production of β -galactosidase. The results in Figure 2 clearly show that deletion of the four C-terminal amino acids of TGN38 facilitates binding of μ 3A in addition to that of μ 2. We have shown previously that mutation of either tyrosine-333 to alanine (Y333A) or serine-331 to alanine or aspartate (S331A and S331D respectively) eliminates binding of TGN38 to μ 2 [12]. The data presented here show that neither μ 2 nor μ 3A bind to Y333A, S331A or S331D mutants of the cytosolic domain of TGN38 that lacks the four C-terminal amino acids of the wild-type sequence (i.e. NLKL).

This result led us to investigate further binding of tyrosine motifs to μ 3. Reaves et al. [21] have shown that exchanging the tyrosine-based motifs of TGN38 and lgp120 does not significantly alter either the steady-state intracellular location of the proteins or the delivery of internalized anti-TGN38 or anti-lgp120 antibodies to intracellular compartments. Expression of a construct in which the YQTI motif of lgp120 was replaced with the YQRL motif of TGN38 (lgp120-AGYQRL*) did not alter the steady-state late endosomal/lysosomal location of the protein. Equiv-

alent experiments, in which the YQRL motif of TGN38 was replaced with YQTI (TGN38-SDYQTI*), showed similar results, in that the protein remained localized to the TGN and cycled via the plasma membrane with efficient internalization and delivery back to the TGN, rather than late endosomes or lysosomes [21]. In light of these experiments, we decided to test the mutants used in the study by Reaves et al. [21] for binding to specific adaptor medium chains. Hybrid constructs were generated in which the tetrapeptide tyrosine motifs of TGN38 (YQRL) and lgp120 (YQTI) were interchanged. Further to this we assessed the role of the C-terminal four amino acids of TGN38 (NLKL) in defining the specificity of interaction of these hybrid proteins with different medium chains. The constructs used in this study are shown in Figure 1.

The results of the relevant two-hybrid interaction assays are shown in Figure 3. In the case of all constructs used in Figure 3, no activation of transcription was detected using bait constructs alone or in combination with negative-control plasmids. The plate-growth assays were therefore carried out in the absence of 3-AT. The lack of any competitive inhibitor of histidine biosynthesis allows detection of a very weak interaction between the full-length cytosolic domain of TGN38 and μ 1 (which has been reported previously [10,20]). Furthermore, Figure 3 shows that, in addition to the interaction between the truncated cytosolic domain of TGN38 (TGN38-SDYQRL*) and μ 3A, there is also a weak but readily detectable interaction between this truncated TGN38 protein and μ 3B.

The experiments using lgp120 constructs show that the 11-amino-acid, wild-type cytosolic domain of lgp120 is capable of interacting directly with μ 3A (Figure 3, top row). No interaction is detected in these qualitative plate-growth assays with μ 1, μ 2, μ 3B or μ 4. Interestingly, substitution of the YQTI motif of lgp120 with the YQRL motif of TGN38 (generating lgp120-AGYQRL*, see Figure 1) drastically alters the specificity of medium-chain interactions. Instead of a sole interaction with μ 3A, lgp120-AGYQRL* interacts strongly with μ 2 and μ 3A, and weakly with μ 3B (Figure 3, row 2). A very weak interaction is also detectable with μ 1. Substitution of the AGYQTI* motif in lgp120 with the hexapeptide motif SDYQRL* from TGN38 dramatically decreases the affinity of all of these interactions (Figure 3, row 3). As shown in Figure 2, the last four amino acids (NLKL) of TGN38 have a strong negative effect on adaptor

Figure 3 The interaction of the motifs YQRL and YQTI with medium chains is dependent upon their context

Transformants were grown in the presence or absence of histidine as indicated and assayed as described in Figure 2.

Table 1 Quantitative β -galactosidase assays of liquid cultures

Assays were carried out on yeast transformed with combinations of LexA–cytosolic-domain fusions and VP16–medium-chain fusions as described in the Experimental section. Data are shown to 1 decimal place and are expressed as β -galactosidase units as defined in the Experimental section. S.D.s were determined from 3 separate experiments. The table layout reflects that of the yeast patches shown in Figure 3. Values shown as 0 were below the reliable limit of detection in these assays.

Medium chain ...	μ 1	μ 2	μ 3A	μ 3B	μ 4
lgp120–AGYQTI* (wild-type)	0	0	0.8 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
lgp120–AGYQRL*	0	98.0 ± 5.8	5.5 ± 0.1	0.1 ± 0.0	0
lgp120–SDYQRL*	0	23.4 ± 0.8	4.2 ± 0.2	0	0
lgp120–AGYQTINLKL*	0	0	0.1 ± 0.0	0	0
TGN38–SDYQTI*	0	0	0.1 ± 0.0	0	0
TGN38–SDYQTINLKL*	0	0	0	0	0
TGN38–SDYQRLNLKL* (wild-type)	0.1 ± 0.0	2.8 ± 0.1	0.1 ± 0.0	0	0
TGN38–SDYQRL*	0.1 ± 0.0	6.0 ± 0.1	1.5 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

medium-chain binding. A remarkably similar negative effect is seen when these four amino acids are appended to the C-terminus of lgp120 (lgp120–AGYQTINLKL*, Figure 3, row 4). In the qualitative plate assays shown in Figure 3, no interaction at all is detectable between TGN38–SDYQTI* and any medium chain (Figure 3, row 5). Unsurprisingly, in this context, addition of the NLKL sequence to the end of this construct has no effect (Figure 3, row 6).

Quantitative liquid assay for β -galactosidase

To quantify these data, liquid culture assays for β -galactosidase were undertaken (Table 1). This assay quantitatively measures the activity of the β -galactosidase expressed by the reporter gene. Results are expressed as β -galactosidase units, which normalize the data to account for differences in growth rate and cell

number, allowing comparative analysis of the data. Data are presented in such a way as to repeat the layout of yeast co-transformants shown in Figure 3. Values shown as zero were below the reliable limit of detection in these assays. Standard deviations shown are derived from at least three independent experiments. These quantitative assays precisely confirm the apparent hierarchy of interaction affinities suggested by the plate-growth and β -galactosidase assays. Wild-type lgp120 (lgp120–AGYQTI*) interacts with highest affinity with μ 3A (0.8 β -galactosidase unit), moderately with μ 3B (0.2 unit) and a weak but detectable affinity with μ 4 (0.1 unit). The interaction between lgp120 and μ 3A is increased approximately 7-fold and 5-fold following replacement of AGYQTI* with AGYQRL* (5.5 units) or SDYQRL* (4.2 units) respectively. However, these substitutions both generate a very strong interaction with μ 2 (98.0 and 23.4 units respectively). This interaction is remarkably strong for the lgp120–AGYQRL* construct, which in this assay reveals an affinity much greater than that seen with any other combination of constructs used in this study. These assays also underline the effect the C-terminal four amino acids of TGN38 (NLKL) have on interaction with the different medium chains (compare the data for lgp120–YQTI with those for lgp120–YQTINLKL*). The data obtained using the TGN38 constructs are also consistent with those obtained from the plate assays shown in Figures 2 and 3 with the additional observations of a very weak interaction between TGN38–SDYQTI* and μ 3A (0.1 unit) and between TGN38–SDYQRL* and both μ 3B (0.1 unit) and μ 4 (0.1 unit).

Growth-inhibition assays

Since the two-hybrid assays incorporate a measure of transcription of the *HIS3* gene product, one can exploit inhibition of the histidine biosynthetic pathway as an additional measure of interaction affinity. Higher-affinity interactions will lead to increased transcription of the *HIS3* gene, resulting in higher

Table 2 Quantitative growth-inhibition assays

Values shown represent the concentration of 3-AT (mM) required for 50% inhibition of growth in histidine-deficient medium. Results are $\pm$ S.D.s of triplicate determinations. The table layout reflects that of the yeast patches shown in Figure 3. —, not tested.

Medium chain ...	$\mu 1$	$\mu 2$	$\mu 3A$	$\mu 3B$	$\mu 4$
Igp120-AGYQTI* (wild-type)	—	—	0.9 ± 0.2	< 0.1	< 0.1
Igp120-AGYQRL*	—	46 ± 4.3	3.2 ± 0.3	1.0 ± 0.2	—
Igp120-SDYQRL*	—	5.0 ± 0.2	3.0 ± 0.3	—	—
Igp120-AGYQTINLKL*	—	—	1.1 ± 0.5	—	—
TGN38-SDYQTI*	—	—	—	—	—
TGN38-SDYQTINLKL*	—	—	—	—	—
TGN38-SDYQRLNLKL* (wild-type)	< 0.1	15 ± 0.4	—	—	—
TGN38-SDYQRL*	< 0.1	29 ± 2.0	8 ± 1.3	< 0.1	—

concentrations of 3-AT being required for inhibition of growth. Titration of transformant growth against 3-AT concentration again confirmed the observations from the previous experiments (Table 2). A_{600} measurements were taken after 48 h in the presence of increasing concentrations of 3-AT. Data are represented as the millimolar concentration of 3-AT required for 50% inhibition of growth. Growth of the weakest-interacting pairs is inhibited at the lowest 3-AT concentration. The results show that many of the interactions are inhibitable at sub-millimolar concentrations of 3-AT. All of the interactions are completely abolished in 10 mM 3-AT with the exception of those between $\mu 2$ and each of Igp120-AGYQRL* ($IC_{50} = 46$ mM), TGN38-SDYQRL* ($IC_{50} = 29$ mM) and TGN38-SDYQRLNLKL* ($IC_{50} = 15$ mM).

By assessing the data as a whole, one can generate a hierarchy of interaction affinities for each bait construct used. For wild-type Igp120 (Igp120-AGYQTI*), this is $\mu 3A > \mu 4 > \mu 3B$ (we do not detect any interaction with either $\mu 1$ or $\mu 2$); for Igp120-AGYQRL*, $\mu 2 \gg \mu 3A > \mu 3B$ with no interaction with $\mu 1$ or $\mu 4$ detectable; for Igp120-SDYQRL*, $\mu 2 > \mu 3A$, with no interaction with $\mu 1$, $\mu 3B$ or $\mu 4$; Igp120-AGYQTINLKL* only shows a weak interaction with $\mu 3A$; TGN38-SDYQTI* only shows a weak interaction with $\mu 3A$; TGN38-SDYQTINLKL* shows no readily detectable interaction with any medium chain; for wild-type TGN38 (TGN38-SDYQRLNLKL*), $\mu 2 \gg \mu 1 > \mu 3A$ with no detectable interaction with $\mu 3B$ or $\mu 4$. TGN38-SDYQRL* appears to interact with varying affinity with all of the adaptor-complex medium chains tested in this study. The hierarchy of affinities in the case of TGN38-SDYQRL* in these assays is $\mu 2 = \mu 3A > \mu 3B > \mu 1 = \mu 4$.

DISCUSSION

The cytosolic domains of both TGN38 and Igp120 have been used extensively as models to study a variety of intracellular trafficking steps in eukaryotic cells [10–16]. The hexapeptide motif, SDYQRL, from TGN38 has also been used to identify the components of the trafficking machinery with which it interacts, using the yeast two-hybrid system [10]. This approach demonstrated that the SDYQRL motif is capable of interacting with the medium (μ) chain subunits of adaptor complexes, notably $\mu 2$ of the AP2 complex involved in clathrin-mediated endocytosis at the plasma membrane.

Using the yeast two-hybrid system, we engineered bait constructs containing the cytosolic domain of TGN38 in which these last four amino acids had been removed. Interaction analysis using adaptor-complex medium chains of the AP1, AP2, AP3

and putative AP4 complexes showed that this premature termination of the protein coding sequence led to a significant change in medium-chain binding specificity, facilitating interaction with all medium chains tested in our assays. This is entirely consistent with the results of Ohno et al. [20], which showed a great increase in affinity of the TGN38 cytosolic domain for $\mu 2$ following removal of these terminal four residues. However, we cannot reproduce the strong interaction with $\mu 1$ seen in the data of Ohno et al. [10,20], consistent with our previous results using the entire cytosolic domain of TGN38 [12] and the known trafficking itinerary of TGN38 [3–7]. It is interesting to note that in what is effectively the converse experiment, adding the residues NLKL to the C-terminus of the lysosomal membrane protein Igp120 almost completely abolished the normal range of medium-chain interactions seen (Figure 3, row 6, and Tables 1 and 2). It has been shown previously that the tyrosine-based targeting motif of TGN38 lies within a nascent helix [28]. It is likely that the terminal four amino acids of the cytosolic domain stabilize the helical nature of the SDYQRL motif. The structural difference between the wild-type cytosolic domain (SDYQRLNLKL) and the Δ NLKL constructs may underlie the differences in adaptor medium-chain binding.

AP3 has indeed been shown to be involved in the membrane traffic to the yeast vacuole of a number of proteins, including alkaline phosphatase [29,30], and genetic evidence obtained from *Drosophila melanogaster* suggests a role for AP3 in delivery of newly synthesized lysosomal enzymes [18]. In the light of these results we examined the interaction between Igp120 and different adaptor medium chains. Igp120 is a lysosomal-membrane glycoprotein that is delivered to the late endosome/lysosome predominantly by direct traffic from the TGN, although there is also a small but significant pool that traffics to these compartments via the cell surface [31]. The cytosolic domain of Igp120 has recently been shown to be capable of interacting with the adaptor complexes AP1 and AP2 [16], suggesting mechanisms for both lysosomal delivery from the TGN (AP1) and internalization at the cell surface (AP2). In our assays, however, we do not readily detect binding of the cytosolic domain of Igp120 to $\mu 1$ or $\mu 2$. This is likely to indicate some structural requirement of the other adaptor-complex subunits in mediating recognition of the Igp120 sorting motif not provided by the medium chain in isolation. However, it is of note that, in our quantitative solution assays, the isolated medium chain of the AP3 complex binds the cytosolic domain of Igp120 with much greater affinity (at least one order of magnitude) than the medium chain of either AP1 or AP2. It is possible that all mechanisms operate in concert and that AP1 is involved in TGN-to-lysosome delivery with AP2 and AP3 acting sequentially in delivery of the component trafficking via the plasma membrane. The interaction between the cytosolic domain of Igp120 and adaptor complexes, particularly $\mu 3A$, provides an explanation for the observation that targeting of Igp120 to lysosomal compartments is a saturable process [32].

Our experiments in which the tyrosine consensus motifs of TGN38 and Igp120 are switched provide good evidence that the exact context of these motifs is a critical determinant of their function. A significant aspect of the findings shown in Figure 3 is that the YQTI motif of Igp120 is effectively non-functional as a lysosomal targeting signal when located in the context of the cytosolic domain of TGN38. Yet data from Reaves et al. [21] show that the Igp120-AGYQRL* and TGN38-SDYQTI* mutants behave normally with respect to endogenous Igp120 and TGN38 respectively in terms of both steady-state localization and traffic from the plasma membrane. Our data reinforce those of Reaves et al. [21], which show a hierarchy of targeting information within the luminal and transmembrane domains of

lgp120 in addition to that of the cytosolic domain. That we do not see significant interaction between TGN38-SDYQTI* and the adaptor-complex medium chains is rather surprising given that this mutant also behaves in the same manner as endogenous TGN38. This may reflect the reported propensity for TGN38 to dimerize *in vivo* [33].

The observed difference in binding of μ 3B to lgp120-AGYQRL* versus lgp120-SDYQRL* is also of interest in light of the fact that binding of these polypeptides to μ 3A is approximately identical (Figure 3, rows 2 and 3, and Tables 1 and 2). This may reflect the presence of the upstream glycine residue (present in lgp120-AGYQRL* but absent from lgp120-SDYQRL*, see Figure 1), which has been shown previously to influence targeting of lgp120 *in vivo* [8,13,28]. Similarly the marked difference in binding of lgp120-AGYQRL* to μ 2 versus that of lgp120-SDYQRL* may well be a result of the presence of the upstream serine residue of lgp120-SDYQRL*, since we and others have shown that this residue is important in determining trafficking of TGN38 [6,12,13].

What is rather surprising is that TGN38-SDYQRL* interacts with varying affinity with all of the adaptor-complex medium chains tested in this study. It is possible that this is due to the YQRL motif being the best generalized consensus for binding to the medium chains of those that we have tested. Indeed, experiments using other motifs from other transmembrane proteins have shown that the YQRL motif binds with greater affinity than any other motif that we have tested (D. J. Stephens and G. Banting, unpublished work). That the only interactions that remain detectable in the presence of 100 mM 3-AT are all with μ 2 might be a reflection of the need for higher-efficiency sorting in endocytosis than other intracellular transport steps.

In conclusion, our data show that the cytosolic domain of lgp120 interacts with high affinity with μ 3A, further implicating the AP3 adaptor complex as a mediator of lysosomal traffic. We also show that the last four residues (NLKL) of TGN38 have a strong negative effect on engagement of adaptor complexes by this protein, a phenomenon that may have implications for the regulation of trafficking of TGN38 and indeed other proteins by proteolytic modification. It will obviously be of benefit to determine the precise C-terminus of TGN38 *in vivo* since any truncation of the predicted C-terminus of TGN38 would change adaptor interactions and therefore trafficking. It is also of note that the orthologues of TGN38 always have four amino acids following the SDYQRL motif, although there is variation in the sequence [34]. Our data also show that μ 4 is capable of interacting with tyrosine-sorting motifs, albeit with very low affinity. Thus it is evident that the precise context of the polypeptide sequence in which tyrosine-based sorting motifs lie significantly affects the pattern and affinity of recognition by different adaptor medium chains, with post-translational processing of the region flanking the tyrosine-based sorting motif with irreversible proteolytic processing, providing a potential mechanism to regulate such interactions.

We thank Dr. B. J. Reaves and Dr. M. S. Robinson for kindly providing constructs. We also thank Liz Roquemore and Colin Crump for critical reading of the manuscript and the Medical Research Council for financial support.

REFERENCES

- 1 Trowbridge, I. S., Collawn, J. F. and Hopkins, C. R. (1993) *Annu. Rev. Cell Biol.* **9**, 129–161
- 2 Marks, M. S., Ohno, H., Kirchhausen, T. and Bonifacio, J. S. (1997) *Trends Cell Biol.* **7**, 124–128
- 3 Luzio, J. P., Brake, B., Banting, G., Howell, K. E., Braghetta, P. and Stanley, K. K. (1990) *Biochem. J.* **270**, 97–102
- 4 Bos, K., Wright, C. and Stanley, K. K. (1993) *EMBO J.* **12**, 2219–2228
- 5 Humphrey, J. S., Peters, P. J., Yuan, L. C. and Bonifacio, J. S. (1993) *J. Cell Biol.* **120**, 1123–1135
- 6 Wong, S. H. and Hong, W. (1993) *J. Biol. Chem.* **268**, 22853–22862
- 7 Reaves, B., Horn, M. and Banting, G. (1993) *Mol. Biol. Cell* **4**, 93–105
- 8 Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y. and August, J. T. (1993) *J. Biol. Chem.* **268**, 1941–1946
- 9 Keller, P. and Simons, K. (1997) *J. Cell Sci.* **110**, 3001–3009
- 10 Ohno, H., Stewart, J., Fournier, M.-C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T. and Bonifacio, J. S. (1996) *Science* **269**, 1872–1875
- 11 Dell'Angelica, E. C., Ohno, H., Ooi, C. E., Rabinovich, E., Roche, K. W. and Bonifacio, J. S. (1997) *EMBO J.* **16**, 917–928
- 12 Stephens, D. J., Crump, C. M., Clarke, A. R. and Banting, G. (1997) *J. Biol. Chem.* **272**, 14104–14109
- 13 Roquemore, E. R. and Banting, G. (1998) *Mol. Biol. Cell* **9**, 2125–2144
- 14 Rohrer, J., Schweizer, A., Russell, D. and Kornfeld, S. (1996) *J. Cell Biol.* **132**, 565–576
- 15 Gough, N. R. and Famborough, D. M. (1997) *J. Cell Biol.* **137**, 1161–1169
- 16 Honing, S., Griffith, J., Geuze, H. J. and Hunziker, W. (1996) *EMBO J.* **15**, 5230–5239
- 17 Robinson, M. S. (1994) *Curr. Biol.* **6**, 538–544
- 18 Simpson, F., Peden, A. A., Christopoulou, L. and Robinson, M. S. (1997) *J. Cell Biol.* **137**, 835–845
- 19 Dell'Angelica, E. C., Klumperman, J., Stoorvogel, W. and Bonifacio, J. S. (1998) *Science* **280**, 431–434
- 20 Ohno, H., Fournier, M.-C., Poy, G. and Bonifacio, J. S. (1996) *J. Biol. Chem.* **271**, 29009–29015
- 21 Reaves, B. J., Banting, G. and Luzio, J. P. (1998) *Mol. Biol. Cell* **9**, 1107–1122
- 22 Wang, X. and Killiman, M. W. (1997) *FEBS Lett.* **402**, 57–61
- 23 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor
- 24 Vojtek, A. B., Hollenberg, S. M. and Cooper, J. A. (1993) *Cell* **74**, 205–214
- 25 Aguilar, R. C., Ohno, H., Roche, K. W. and Bonifacio, J. S. (1997) *J. Biol. Chem.* **272**, 27160–27166
- 26 Gietz, D., St. Jean, A., Woods, R. A. and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20**, 1425
- 27 Fields, S. and Song, X. (1989) *Nature (London)* **340**, 245–246
- 28 Wilde, A., Dempsey, C. and Banting, G. (1994) *J. Biol. Chem.* **269**, 7131–7136
- 29 Stepp, J. D., Huang, K. and Lemmon, S. K. (1997) *J. Cell. Biol.* **139**, 1761–1774
- 30 Cowles, C. R., Odorizzi, G., Payne, G. S. and Emr, S. D. (1997) *Cell* **91**, 109–118
- 31 Harter, C. and Mellman, I. (1992) *J. Cell Biol.* **117**, 311–325
- 32 Marks, M. S., Woodruff, L., Ohno, H. and Bonifacio, J. S. (1997) *J. Cell Biol.* **135**, 341–354
- 33 Jones, S. M., Crosby, J. R., Salerno, J. and Howell, K. E. (1993) *J. Cell Biol.* **122**, 755–788
- 34 Ponnambalam, S., Girotti, M., Yapso, M.-L., Owen, C. W., Perry, A. C. F., Suganuma, T., Nilsson, T., Fried, M., Banting, G. and Warren, G. (1996) *J. Cell Sci.* **109**, 675–685