Transferrin Receptor Containing the SDYQRL Motif of TGN38 Causes a Reorganization of the Recycling Compartment but Is Not Targeted to the TGN

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Abstract. The SDYQRL motif of the cytoplasmic domain of TGN38 is involved in targeting TGN38 from endosomes to the TGN. To create a system for studying this pathway, we replaced the native transferrin receptor (TR) internalization motif (YTRF) with the SDYQRL TGN-targeting motif. The advantages of using TR as a reporter molecule include the ability to monitor trafficking, in both biochemical and microscopy experiments, using the natural ligand transferrin. When expressed in CHO cells, the SDYQRL-TR construct accumulated in juxtanuclear tubules and vesicles that are in the vicinity of the TGN. The SDYQRL-TR–containing structures, however, do not colocalize with TGN markers (e.g., NBD ceramide), and therefore the SDYQRL motif is not sufficient to target the TR to the TGN. The morphology of the SDYQRL-TR–containing juxtanuclear structures is different from the recycling compartment found in cells expressing the wild-type TR. In addition, the SDYQRL-TR–containing juxtanuclear compartment is more acidic than the recycling compartment in cells expressing the wild-type TR. The juxtanuclear compartment, however, is a bona fide recycling compartment since SDYQRL-TR was recycled back to the cell surface at a rate comparable to the wild-type TR, and sphingomyelin and cellubrevin, both of which label all compartments of the endocytic recycling pathway, colocalize with SDYQRL-TR in the juxtanuclear structures. These findings demonstrate that expression of the SDYQRL-TR construct alters the morphology and pH of endocytic recycling compartments rather than selectively affecting the intracellular trafficking pathway of the SDYQRL-TR construct. Therefore, the SDYQRL trafficking motif is not simply a molecular address that targets proteins to the TGN, but it can play an active role in determining the physical characteristics of endosomal compartments.

TGN38 is a transmembrane glycoprotein believed to be involved in the formation of transport vesicles from the TGN (Stanley and Howell, 1993). At steady-state, >90% of TGN38 is in the TGN (Bos et al., 1993; Stanley and Howell, 1993). This concentration is achieved by a retrieval mechanism in which TGN38 is rapidly internalized from the plasma membrane and targeted from the endosomal system to the TGN (Bos et al., 1993; Humphrey et al., 1993; Luzio and Banting, 1993). Consequently, TGN38, in addition to containing an internalization motif, must also contain a motif that targets it to the TGN (i.e., retrieval motif) and/or a motif that concentrates it in the TGN (i.e., retention motif) (Luzio and Banting, 1993). It has been reported that the cytoplasmic domain of TGN38 contains the sequences required for localization to the TGN (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). Previous studies have demonstrated that the hexapeptide sequence of the cytoplasmic domain of TGN38, SDYQRL, contains at least part of the information required for TGN localization (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). The SDYQRL motif, when transferred to the cytoplasmic domain of heterologous membrane proteins (e.g., LDL receptor or glycophorin A), has been reported to be sufficient for targeting to the TGN (Bos et al., 1993; Wong and Hong, 1993). The transmembrane domain of TGN38 also contains information that can localize chimeras to the TGN (Ponnambalam et al., 1994). Thus, localization of TGN38 to the TGN may require both a cytoplasmic retrieval motif and a transmembrane TGN retention motif (Ponnambalam et al., 1994).

It is known that a number of membrane proteins can be

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trafficked from the plasma membrane to the Golgi complex (Snider, 1991; Green and Kelly, 1992). The half-time for appearance of these proteins in the Golgi is severalfold lower than the half-times for return to the cell surface, indicating that per round of internalization general traffic through the Golgi complex is inefficient. TGN38, a resident TGN protein, may contain information that efficiently targets it to the TGN per round of internalization, although it is possible that TGN38 is trafficked to the TGN with the same efficiency as other non-TGN resident proteins and TGN38 accumulates in the TGN because it is specifically retained.

To develop an experimental tool for studying the pathway from the plasma membrane to the TGN, we transferred the SDYQRL motif and related motifs to the cytoplasmic domain of the human transferrin receptor (TR)[1] (this report). The advantages of using the TR as a reporter molecule are that biochemical and microscopy assays have been developed for studying each aspect of TR trafficking. The compartments through which TR traffics have been characterized and the kinetics of trafficking among these compartments are known. The advantages of using the TR as a reporter molecule for studies of endocytic trafficking motifs have been exploited in a number of recent studies (Garippa et al., 1994; Odorizzi et al., 1994).

In this report, we demonstrate that the SDYQRL sequence is not sufficient to localize the TR to the TGN, suggesting that additional sequences of TGN38 are required for TGN localization. Unexpectedly, expression of the SDYQRL-TR or YQRL-TR constructs resulted in a reorganization of the endosomal recycling compartments. These results demonstrate that membrane protein trafficking motifs can play an active role in determining the physical characteristics of compartments through which membrane proteins traffic.

Materials and Methods

TR Constructs

Overlapping oligonucleotide PCR was used to make the TR constructs (Garippa et al., 1994). The wild-type TR cDNA plasmid, pTM1010, was used as a template (McGraw et al., 1991). Oligonucleotides were purchased from Operon Technologies (Alameda, CA). To create the SDYQRL-TR, the oligonucleotide 5′-GGAGAACCATCGGACTATCAGCGGTTG and its complement were used. To create YQRL-TR, the oligonucleotide 5′-CCATTGTCATATCAGCGGTTGAGCCTGGC and its complement were used. To create YQDL-TR, the oligonucleotide 5′-CCATTGTCATATCAGGATTTGAGCCTGGC and its complement were used. The general scheme of using overlapping oligonucleotides and PCR to create mutations in the TR has been described previously (Garippa et al., 1994). The cytoplasmic and transmembrane domains of the PCR-generated constructs were sequenced by the Columbia University Cancer Center (ABI sequencing).

Cell Lines

Cell lines were carried in Ham's F12 or McCoy's 5A medium containing 5% FBS, penicillin-streptomycin, and 220 mM sodium bicarbonate. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. The TR constructs were transfected into TRVb cells, a CHO cell line lacking endogenous hamster TR (McGraw et al., 1987), using Lipofectin (GIBCO BRL, Gaithersburg, MD). Plasmid pSV3-Neo was used as a dominant selectable marker. Cells were selected in 400 μg/ml G418, and clonal lines were isolated using cloning cylinders. A chimera containing the cytoplasmic and transmembrane domains of TGN38 fused to the extracellular domain of T cell antigen (Humphrey et al., 1993) was cotransfected with the hygromycin resistance plasmid, pMEP. Doubly transfected cells were selected in 420 μg/ml hygromycin and 400 μg/ml G418.

Ligands

Human transferrin (Tf) was purchased from Sigma Chemical Co. (St. Louis, MO) and further purified by Sephacryl S-300 gel filtration. Iron-loaded diferric Tf and ^{125}I-labeled Tf were prepared as described previously (Yamashiro et al., 1984). $^{55}FeCl_2$ was purchased from DuPont New England Nuclear (Cambridge, MA) and $^{55}Fe_2$-labeled Tf was prepared by the nitriloacetic acid method (Klausner et al., 1984). Tf was derivatized with Cy3 according to manufacturer's instructions (Biological Detection Systems, Pittsburgh, PA). C_6-NBD-ceramide was purchased from Molecular Probes Inc. (Eugene, OR). The anti–human TR mAb, B3/25, was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and monoclonal anti-Tac (IL-2 receptor) antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Fluorescent rabbit anti–mouse antibodies were purchased from Cappel (Malvern, PA). The rabbit polyclonal anticellubrevin antibody (MC.16) was a generous gift from Dr. P. de Camilli, the immunopurified anti-γ adaptin (AP1) antibody (AE/1) was a generous gift from Dr. L. Traub, the anti-α-adaptin (AP2) mAb (AP.6) was a generous gift from Dr. F. Brodsky, and the anti–mannose 6-phosphate receptor antibody was a generous gift from Dr. E. Holtzman.

Fluorescence Microscopy

For microscopy experiments, cells were grown in 35-mm coverslip dishes. For Tf uptake studies, cells were incubated in McCoy's 5A salts, penicillin-streptomycin, 220 mM sodium bicarbonate, 20 mM Hepes, pH 7.4 (med 1), containing 10 μg/ml Cy3-Tf and 2 mg/ml ovalbumin. Cells were washed in 150 mM NaCl, 20 mM Hepes, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4 (med 2), and fixed in 3.7% formaldehyde for 30 min. For indirect immunofluorescence, cells were incubated in primary antibody in med 2 containing 100 μg/ml saponin (med 3) for 60 min at 37°C. The cells were washed three times with med 3, 5 min each wash, incubated with secondary antibody in med 3 for 60 min at 37°C, washed, and examined in fluorescence microscopy. The TGN was stained by incubating fixed cells with 5 mM C_6-NBD-ceramide in med 2 for 30 min at room temperature. The cells were washed four times in med 2, 25 mg/ml fatty acid–free BSA, 15 min each wash.

NBD-sphingomyelin (C_6-NBD-SM) labeling of cells was performed as described previously (Mayor et al., 1993). In brief, the cells were incubated in 15 mg/ml Cy3-Tf for 30 min to label early endosomal compartments. The cells were then chilled and incubated at 4°C for 30 min with Cy3-Tf and small unilamellar vesicles made of 2.5–5 mM C_6-NBD-SM and dioleylphosphatidylcholine (1:1). The cells were extensively rinsed and then incubated for 10 min at 37°C in medium containing Cy3-Tf. During this incubation, C_6-NBD-SM in the plasma membrane is internalized. The cells were chilled to 4°C, and C_6-NBD-SM remaining on the cell surface was removed by incubating in 5% fatty acid–free BSA in ice-cold med 2. The cells were rinsed in ice-cold PBS and fixed in 2.5% paraformaldehyde for 10 min.

Cells were imaged using either a Zeiss Axiovert 35 microscope (Thornwood, NY) equipped with standard rhodamine/fluorescein filters or a Leitz Diavert microscope (Weltzar, Germany), as described before (Mayor et al., 1993; Presley et al., 1993). For high-resolution confocal imaging, a confocal attachment (model MRC-600; BioRad Labs, Hercules, CA) on a Zeiss Axiovert microscope was used with a 63× 1.4 NA plan-apochromat objective. C_6-NBD-ceramide and Cy3-conjugated probes were imaged by the confocal microscope using the 488-nm line from an argon ion laser for excitation, and the two emissions were separated using a 560-nm dichroic mirror with the Cy3 fluorescence being selected by a 575-nm-long pass filter and the NBD fluorescence being selected by a 515–545–nm band-pass filter. There was no Cy3 fluorescence leakage into the green channel, but because of NBD's broad emission spectra, significant NBD emission was seen in the red channel. This problem was solved by imaging NBD first and then scanning the image to photobleach all the NBD. The Cy3 image was collected after the photobleaching. The z-axis resolution was determined by obtaining images from multiple focal position using 300 nM fluorescent beads, and the thickness of that optical section is reported as the full width to half-maximal intensity of the beads.

1. *Abbreviations used in this paper*: M6P-R, mannose-6 phosphate receptor; NBD, 7-nitrobenz-2-OXA-1,3-diazol; R/F, rhodamine/fluorescein fluorescence ratio; SM, sphingomyelin; Tf, human transferrin; TR, human transferrin receptor.

TR Trafficking Assays

The kinetic assays have been previously described (Garippa et al., 1994). For biochemical assays, cells were grown in six-well clusters to a density of ~5 × 10^5 per well (~80 confluent). To measure Tf recycling, cells were incubated in med 1 with 3 μg/ml iodinated Tf for 2 h at 37°C in 5% CO_2. The cells were washed with med 2, incubated for 2 min in 0.5 M NaCl, 50 mM MES, pH 5.0, followed by three washes with med 1 supplemented with 3 μg/ml unlabeled Tf. The cells were incubated at 37°C in 5% CO_2 in med 1 with 3 μg/ml unlabeled diferric Tf and 100 μM of the iron chelator desferroxamine. At the indicated times, the medium was collected, the cells were solubilized, and the radioactivity was measured using a gamma counter. Two wells per six-well cluster were used to determine specific Tf binding by including a 100-fold excess of unlabeled Tf in the incubation medium. The data has been corrected for nonspecific binding, which was typically <10% of the total.

Tf internalization was measured by incubating cells in med 1 with 3 μg/ml iodinated Tf at 37°C in 5% CO_2. At the times indicated, the cells were washed once with med 2, placed on ice, washed twice with med 2 (4°C), and incubated for 5 min at 4°C in 0.5 M NaCl, 0.5 M glacial acetic acid, pH 3.0. The cells were washed three times with med 2 (4°C) and solubilized, and radioactivity was measured using a gamma counter. The number of surface Tf-binding sites was determined by incubating cells with iodinated Tf for 2 h at 4°C. A plot of the ratio of internal Tf (cell-associated radioactivity after acid wash) to surface Tf versus time yields a straight line whose slope is the internalization rate constant. The data has been corrected for nonspecific binding as described above. In general, nonspecific binding is <10% of total.

Fe accumulation from diferric-Tf assay was measured as previously described (Garippa et al., 1994). Cells were incubated with 10 μg/ml ^{55}Fe in med 1 at 37°C. At the times indicated, cells were washed and solubilized, and cell-associated ^{55}Fe was measured. Specific ^{55}Fe-labeled Tf accumulation was determined by subtracting the amount of cell-associated radioactivity in the presence of 50-fold excess of unlabeled Tf. The Fe accumulation data is corrected for the amount of surface Tf binding, determined in parallel dishes as described above. By correcting for surface Tf binding, Fe-accumulation rates of different cell lines can be directly compared.

Fluorescence Ratio Imaging pH Determinations

Tf was conjugated with both rhodamine and fluorescein by reacting with succinimidyl esters of both carboxytetramethylrhodamine and carboxyfluorescein according to manufacturer's instructions (Molecular Probes, Inc.). This probe was tested in uptake studies and yielded a pattern of endosome fluorescence characteristic of Tf in CHO cells. The specificity of this probe for the TR was confirmed by inhibition of uptake in the presence of excess unlabeled Tf and wash out of fluorescence during a chase period in the presence of 0.5 mg/ml unlabeled Tf and 100 μM desferroxamine.

The procedure used to measure endosome pH is described in detail elsewhere (Dunn et al., 1994). Cells were plated 2 d before each experiment in 35-mm coverslip dishes. Experimental dishes were preincubated in Medl containing 20 μg/ml F-R-Tf for 40 min at 37°C. Fluorescent labeling was continued for ~10 min on the microscope stage (model Axiovert; Carl Zeiss, Inc.) warmed to 37°C. At this time, fluorescent images of cells were collected by a confocal attachment (model MRC-600; BioRad Labs). A 488-nm line from an argon ion laser was used to stimulate both fluorescein and rhodamine fluorescence, and their emissions were separated by a 560-nm dichroic mirror. Fluorescein emission was selected using a 515–545-nm band-pass emission filter while rhodamine emission was selected using a 600-nm long-pass emission filter. The two emission wavelengths were simultaneously collected by the two photomultiplier tubes of the confocal microscope. The focal depths for both wavelengths were the same, as the same size confocal aperture was used for both. Using image processing techniques developed previously to measure endosome pH (Dunn et al., 1994), the ratio of rhodamine/fluorescein fluorescence (R/F) fluorescence was calculated for each endosome.

Results

Three TR constructs were made in which the native TR internalization motif, YTRF, was replaced with either SDYQRL, YQRL, or YQDL (Fig. 1). In these constructs, the tyrosine is separated from the membrane by 41 amino acids. The SDYQRL-TR and the YQRL-TR constructs were made because there are conflicting reports as to which is the minimum sequence required for targeting to the TGN (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). The YQDL construct was made because mutation of arginine to aspartate uncouples the ability of the YQRL motif to function as an internalization motif from its ability to function as a TGN-targeting motif: this mutation inhibits targeting to the TGN and only partially reduces internalization (Humphrey et al., 1993). The cytoplasmic YTRF motif of the TR promotes rapid internalization through clathrin-coated pits (Collawn et al., 1990; Jing et al., 1990; McGraw and Maxfield, 1990). Recycling of the TR back to the cell surface occurs by a bulk flow mechanism, and therefore unlike internalization, the intracellular itinerary of the TR is not dependent on determinants on its cytoplasmic domain (Jing et al., 1990; Johnson et al., 1993; Mayor et al., 1993). Consequently, the cytoplasmic domain of the TR should not interfere with the intracellular trafficking determined by the SDYQRL motif.

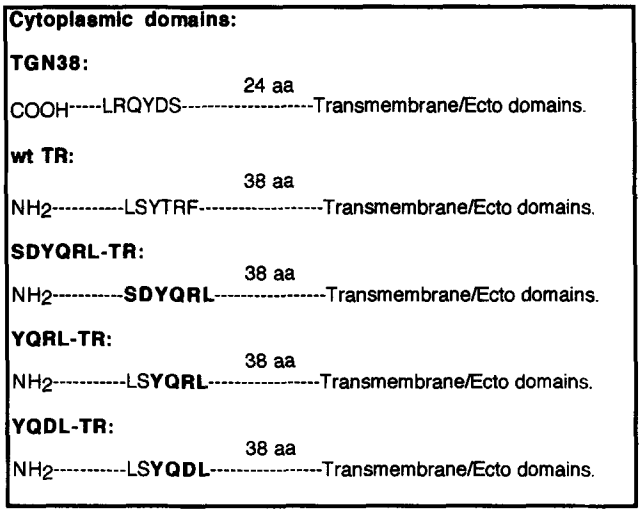

Figure 1. Schematic of TR constructs used in this study. The TR internalization motif, YTRF (position 20 to 23 of the 61-amino acid cytoplasmic domain), was converted to either YQRL, SDYQRL, or YQDL using PCR and overlapping oligonucleotides (Materials and Methods).

The TR constructs were transfected into the TRVb CHO cell line, which does not express functional endogenous TR (McGraw et al., 1987). The advantage of using TRVb cells for these studies is that the behaviors of the transfected TR constructs can be examined in a background free of endogenous TR. The behaviors of the TR constructs were compared to the behavior of the wild-type TR expressed in TRVb cells. For this purpose, a previously isolated and characterized TRVb clonal cell line expressing the wild-type human TR, TRVb-1, was used (McGraw et al., 1987). The results of single clonal cell lines expressing each of the TR constructs are presented. Similar results were observed for other clonal lines expressing each of the constructs. In each of the clonal lines examined, the level of expression of the TR constructs was less than the ~1 × 10^5 surface expression per cell of the wild-type TR expressed in TRVb-1 cells.

The Steady-State Distribution of SDYQRL-TR Is Different from the Wild-Type TR

Two probes were used to determine the intracellular distribution of the TR constructs. Cells were incubated with fluorescent Tf for 45 min at 37°C, washed, fixed, permeabilized, and stained with an mAb to the human TR (B3/25) (Materials and Methods). Monoclonal B3/25 recognizes an epitope on the extracellular domain of the human TR and therefore will recognize all the constructs under study. Fluorescent Tf will label intracellular compartments that are in communication with the surface, whereas the antibody will label all the compartments containing the TR constructs, regardless of whether they are in equilibrium with the cell surface. A 45-min incubation with Tf at 37°C is sufficient to achieve steady-state labeling of the wild-type TR expressed in CHO cells (Johnson et al., 1993). In Fig. 2 *A*, the pattern of Tf uptake in cells expressing the wild-type human TR is shown. TR is internalized from the plasma membrane into peripherally distributed punctate early sorting endosomes, rapidly trafficked to the peri-centriolar endocytic recycling compartment, and then returned to the plasma membrane (Dunn et al., 1989). The peri-centriolar recycling compartment is a tightly clustered accumulation of 50–70-nm-diam tubules and vesicles located in the vicinity of the centrioles (Yamashiro et al., 1984). The peri-centriolar recycling compartment is most frequently found in focal planes above the nucleus. At steady-state, Tf accumulates in the peri-centriolar recycling compartment because the rate-limiting step in recycling is transport from this compartment (Presley et al., 1993). This peri-centriolar compartment is the general endosomal recycling compartment through which other recycling molecules traffic on their way back to the plasma membrane (Mayor et al., 1993; McGraw et al., 1993).

The Tf labeling pattern of cells expressing either SDYQRL-TR or YQRL-TR are shown in Fig. 2, *C* and *E*, respectively. The distributions of YQRL-TR or SDYQRL-TR are indistinguishable from one another. Tf internalized by the YQRL-TR and SDYQRL-TR constructs is localized to juxtanuclear vesicles that are not as tightly clustered as they are in cells expressing the wild-type TR. In general, these vesicles are loosely localized to one side of the nucleus in focal planes near the bottom surface of the cells. Thus, the morphology of the endocytic compartments labeled with Tf in cells expressing SDYQRL-TR or YQRL-TR is different from the morphology of endosomal compartments labeled with Tf in cells expressing the wild-type TR. Tf internalized by SDYQRL-TR or YQRL-TR is rapidly delivered to the juxtanuclear structures, accumulating there within 10 min of internalization (not shown). The distribution of Tf in cells expressing the SDYQRL-TR or YQRL-TR does not change when cells are incubated for extended periods of time in the continued presence of fluorescent Tf (18 h), demonstrating that the steady-state distribution of the SDYQRL-TR or YQRL-TR is achieved within 45 min (not shown).

The distribution of YQDL-TR is similar to the distribution of the wild-type TR, which indicates that the YQDL sequence, unlike the YQRL and SDYQRL sequences, does not divert the TR from the general recycling pathway (Fig. 2 *G*). It is interesting to note that in some cells, YQDL-TR is found in both the peri-centriolar recycling compartment and in juxtanuclear structures like those observed in cells expressing YQRL-TR. This intermediate distribution may indicate that mutation of arginine only partially affects trafficking directed by the YQRL motif. The observation that the morphology of compartments containing YQDL-TR is different from that of the compartments containing the YQRL-TR construct indicates that the arginine residue is required for the intracellular trafficking directed by the YQRL motif. This finding is consistent with previous observations that the arginine of the YQRL sequence is required for TGN targeting (Humphrey et al., 1993).

In Fig. 2, *B*, *D*, *F*, and *H*, the distribution of the TR constructs visualized with B3/25 antibody are shown. In each case, the structures labeled with Tf are also labeled with B3/25. This qualitative analysis demonstrates that the juxtanuclear structures containing SDYQRL-TR and YQRL-TR are in communication with the cell surface and that the pattern observed after a 45-min incubation with Tf reflects the steady-state distribution.

Despite Morphological Similarities, the Juxtanuclear SDYQRL-TR Compartment Is Not the TGN

The morphology of the juxtanuclear SDYQRL-TR compartment is similar to the morphology of the TGN in cultured cells. To determine if SDYQRL-TR is in the TGN, the distribution of the SDYQRL-TR construct was compared to the distribution of the TGN labeled with C_6-NBD-ceramide (Pagano et al., 1989). The juxtanuclear SDYQRL-TR compartment was labeled with fluorescent Tf in a 45-min incubation at 37°C. The cells were washed, fixed, and stained with C_6-NBD-ceramide and examined using scanning laser confocal microscopy (Materials and Methods). In Fig. 3, single 400-nm-thick optical sections are shown. In these pseudocolored images, Tf is red, NBD ceramide is green, and regions of overlap are yellow. There is no significant overlap of the Tf labeled peri-centriolar recycling compartment in cells expressing wild-type TR with the TGN (Fig. 3 *A*). The peri-centriolar recycling compartment is most frequently adjacent to the TGN, and in some cells the TGN forms a ring that surrounds the peri-centriolar recycling compartment. In cells expressing SDYQRL-TR and NBD ceramide, the Tf and C_6-NBD-ceramide are interspersed (Fig. 3 *B*). There is, however, no significant overlap between the SDYQRL-TR compartments and the TGN. Similar results were found when the distribution of YQRL-TR was compared to the TGN labeled with NBD-ceramide (not shown). Thus, although the SDYQRL motif appears to change the intracellular itinerary of the TR, neither the SDYQRL-TR nor YQRL-TR constructs are localized to the TGN labeled by C_6-NBD-ceramide.

TGN38-Tac Chimera Is Targeted to the TGN in Cells Expressing SDYQRL-TR

One explanation for the failure to see SDYQRL-TR localized to the TGN is that exogenous TGN proteins cannot be localized to the TGN in the cells under study. To establish that this is not the case, cells expressing the SDYQRL-TR were cotransfected with TGN38-T cell antigen chi-

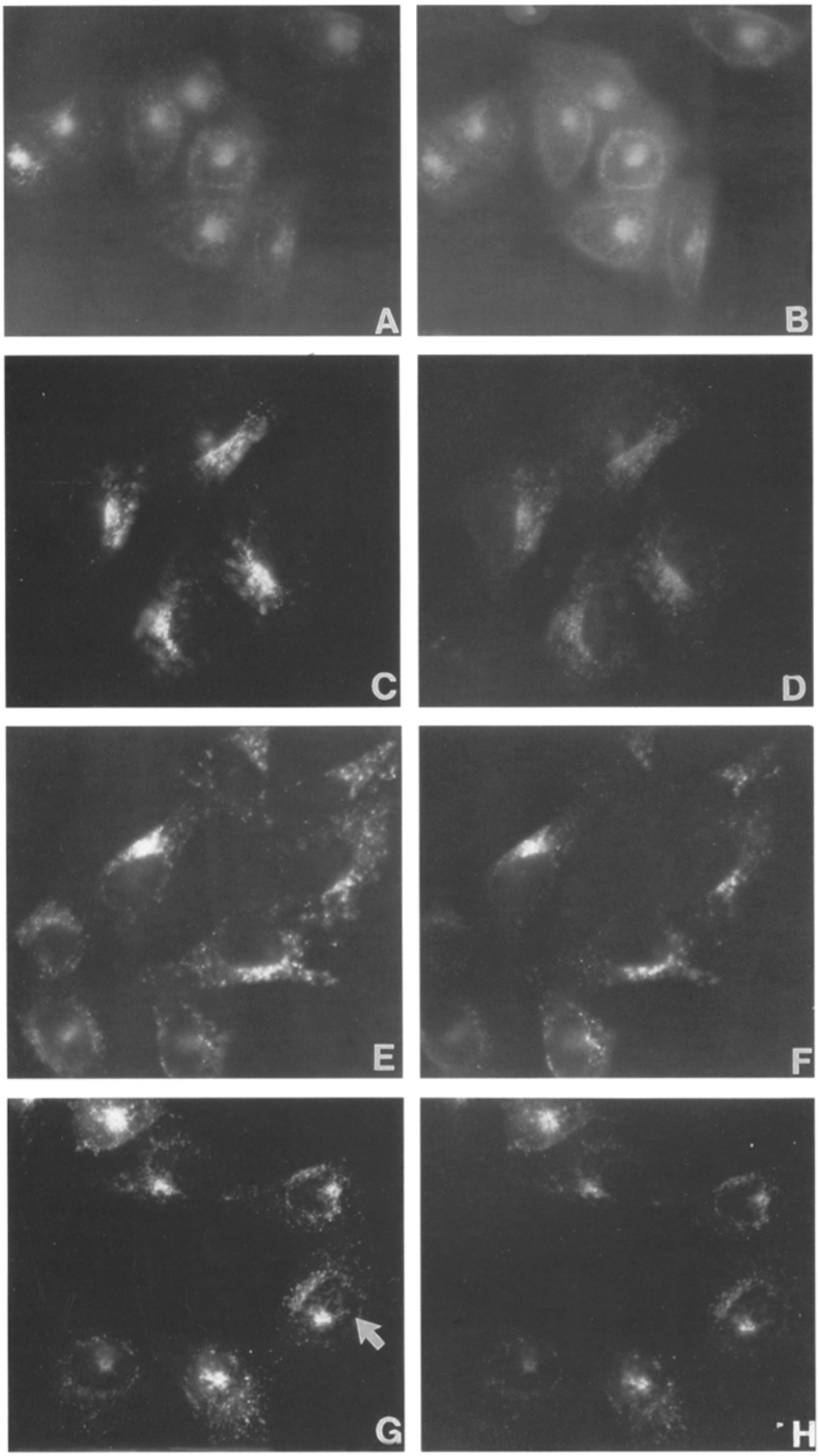

Figure 2. SDYQRL-TR and YQRL-TR constructs do not localize to the peri-centriolar recycling compartment. (*A* and *B*) Cells expressing the wild-type TR; (*C* and *D*) cells expressing the YQRL-TR construct; (*E* and *F*) cells expressing the SDYQRL-TR construct; (*G* and *H*) cells expressing the YQRL-TR. In *A*, *C*, *E*, and *G*, the distributions of Cy3-Tf after a 45-min incubation are shown. In *B*, *D*, *F*, and *H*, the distributions of the TR constructs detected with B3/25 mAb are shown for the same fields as in *A*, *C*, *E*, and *G*. A fluorescein-labeled rabbit anti-mouse antibody was used to visualize the distribution of B3/25. The cells were imaged by wide-field fluorescence microscopy using a 63× objective. In some cells expressing the YQDL-TR construct, Tf is found in both peri-centriolar and juxtanuclear structures (*G*, *arrowhead*).

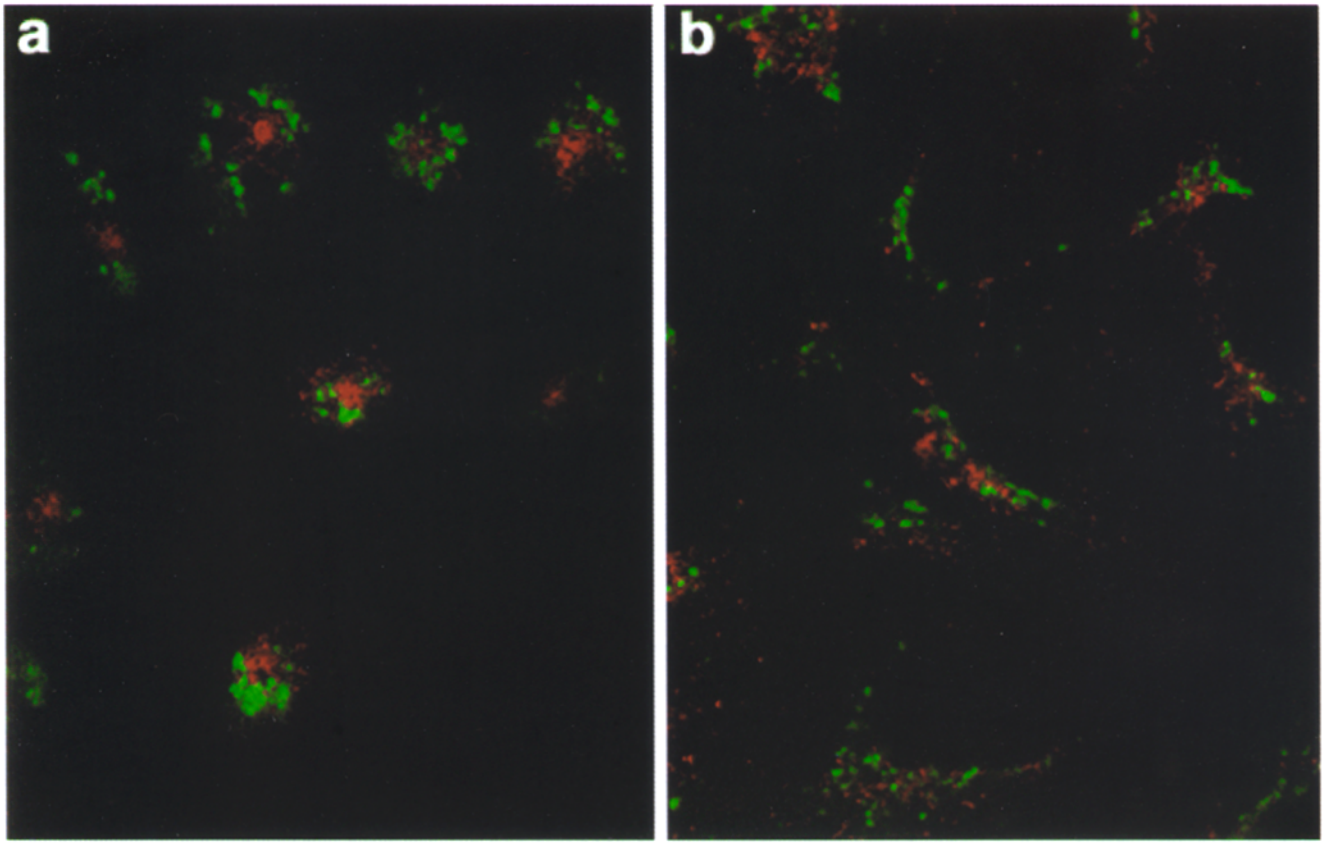

Figure 3. SDYQRL-TR does not colocalize with C_6-NBD-ceramide. The codistribution of wild-type TR and C_6-NBD-ceramide are shown in *a*, and the codistribution of SDYQRL-TR and C_6-NBD-ceramide are shown in *b*. Cells were incubated with Cy3-Tf for 45 min, washed, fixed, and stained with C_6-NBD-ceramide (Materials and Methods). In both panels, a single 400-nm focal plane imaged by a laser scanning confocal microscope is shown. In this pseudocolored image Tf is red, C_6-NBD-ceramide is green, and regions of overlap are yellow.

mera (TGN38-Tac), which contains the cytoplasmic and transmembrane domains of TGN38 fused to the extracellular domain of the T cell antigen (Humphrey et al., 1993). The TGN38-Tac chimera has been shown to localize to the TGN in a number of different cell types (Humphrey et al., 1993; Rajasekaran et al., 1994). The TGN-Tac chimera was labeled by incubation of cells with a fluorescent Fab of the anti-Tac mAb for 60 min followed by a 30-min chase in the absence of antibody. The cells were then fixed and stained with C_6-NBD-ceramide (Materials and Methods). It has previously been shown that the TGN-Tac chimera constitutively cycles between the plasma membrane and the TGN; thus, antibodies against the extracellular domain of Tac in the medium bind chimera on the surface and are internalized into the TGN (Humphrey et al., 1993). Single 400-nm optical sections were examined and TGN-Tac and C_6-NBD-ceramide were found to colocalize (Fig. 4). When the distribution of TGN-Tac was compared to SDYQRL-TR, no significant colocalization of the probes was observed (not shown). Thus, the juxtanuclear SDYQRL-TR compartment does not colocalize with the TGN labeled with either C_6-NBD-ceramide or TGN38-Tac. These results demonstrate that the failure of the SDYQRL-TR construct to localize to the TGN is not due to an inability of these cells to concentrate transfected TGN proteins in the TGN and thereby establishes that expression of SDYQRL-TR does not affect localization of proteins to the TGN.

SDYQRL-TR Juxtanuclear Endosomes Are Not Late Endosomes

To determine whether the SDYQRL-TR juxtanuclear endosomes are late endosomes, a compartment through which TGN38 might traffic on its way to the TGN, the distributions of SDYQRL-TR and mannose-6 phosphate receptor (M6P-R) were compared. Cells expressing SDYQRL-TR were incubated with cy3-Tf for 2 h, washed, fixed, and stained with an anti–M6P-R antibody (Materials and Methods). The cells were imaged with a scanning confocal microscope and a projection of a series of optical sections is shown in Fig. 5. The Tf label is in red and the M6P-R label is in green. There was little significant colocalization of Tf and M6P-R in cells expressing the SDYQRL-TR construct, demonstrating that the SDYQRL-TR juxtanuclear structures are not late endosomes.

SDYQRL-TR Juxtanuclear Endosomes Constitute a Functional Recycling Compartment

To further define the nature of the juxtanuclear compartment in cells expressing the SDYQRL-TR or YQRL-TR constructs, the kinetics of endocytic trafficking were exam-

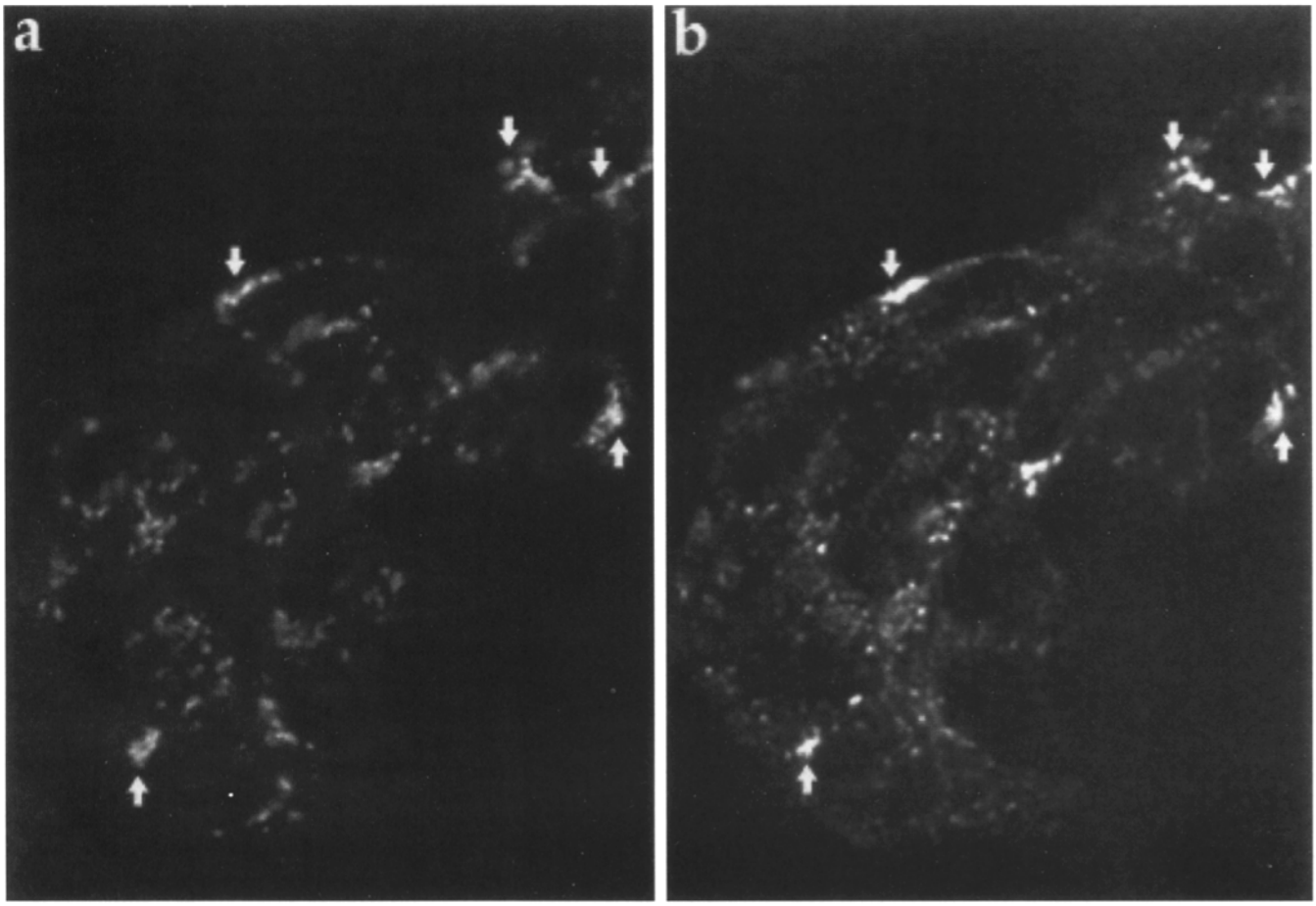

Figure 4. TGN38-Tac chimera codistributes with C_6-NBD-ceramide. The distributions of C_6-NBD-ceramide (*a*) and TGN38-Tac (*b*) in cells expressing SDYQRL-TR are shown. To label the TGN38-Tac construct, cells were incubated for 60 min with a Fab of mAb against the extracellular domain of Tac. The cells were washed, incubated for an additional 30 min, fixed, and stained with C_6-NBD-ceramide (Materials and Methods). The same single 400-nm focal plane imaged by a laser scanning confocal microscope is shown in both panels. The arrows note the colocalization of TGN-Tac, internalized from the surface, with C_6-NBD-ceramide label.

ined. To measure the rate at which SDYQRL-, YQRL-, and YQDL-TR constructs are returned to the cell surface, cells were incubated with iodinated Tf for 2 h, Tf bound to the surface was removed, and the Tf remaining cell associated and released into the medium as a function of time was measured (Materials and Methods). All the constructs were returned to the cell surface with kinetics indistinguishable from the recycling of the wild-type TR (Fig. 6 *A*). Thus, although the morphology of the compartment containing the SDYQRL-TR and YQRL-TR constructs is different from the peri-centriolar recycling compartment containing the wild-type TR, the rates of transport to the cell surface from these morphologically distinct compartments are the same.

The internalization rate constants for the various constructs were measured (Materials and Methods). SDYQRL-TR and YQRL-TR are internalized as rapidly as the wild-type TR, whereas the YQDL-TR is internalized at 50% the rate of the other constructs (Fig. 6 *B*). Thus, the SDYQRL and YQRL motifs are as potent internalization motifs as is the YTRF motif of the TR. The finding that substitution of aspartate for arginine reduces the internalization rate constant is in agreement with previous findings that YQDL sequence is not as potent an internalization motif as YQRL (Fuhrer et al., 1991; Humphrey et al., 1993).

The Juxtanuclear SDYQRL-TR Compartment Is More Acidic than the Peri-centriolar Recycling Compartment

To further characterize the juxtanuclear SDYQRL-TR compartment, its internal pH was measured. Cells were incubated with fluorescein-rhodamine–labeled Tf, and the rhodamine/fluorescein ratio was measured by confocal microscopy (Materials and Methods). The rhodamine/fluorescein fluorescence ratio (R/F) decreases as pH increases and is independent of the amount of probe in endosomes. In Fig. 7, frequency plots of R/F ratio for endocytic compartments from the central region of cells expressing either SDYQRL-TR or wild-type TR are shown. As shown previously, the peri-centriolar recycling compartment in cells expressing the wild-type TR has a pH of 6.5, which is more alkaline than both early endosomes (pH ~6.0) and late endosomes (pH 5.8) (Roederer et al., 1987; Yamashiro and Maxfield, 1987; Johnson et al., 1993; Presley et al., 1993). The R/F ratio of the juxtanuclear SDYQRL-

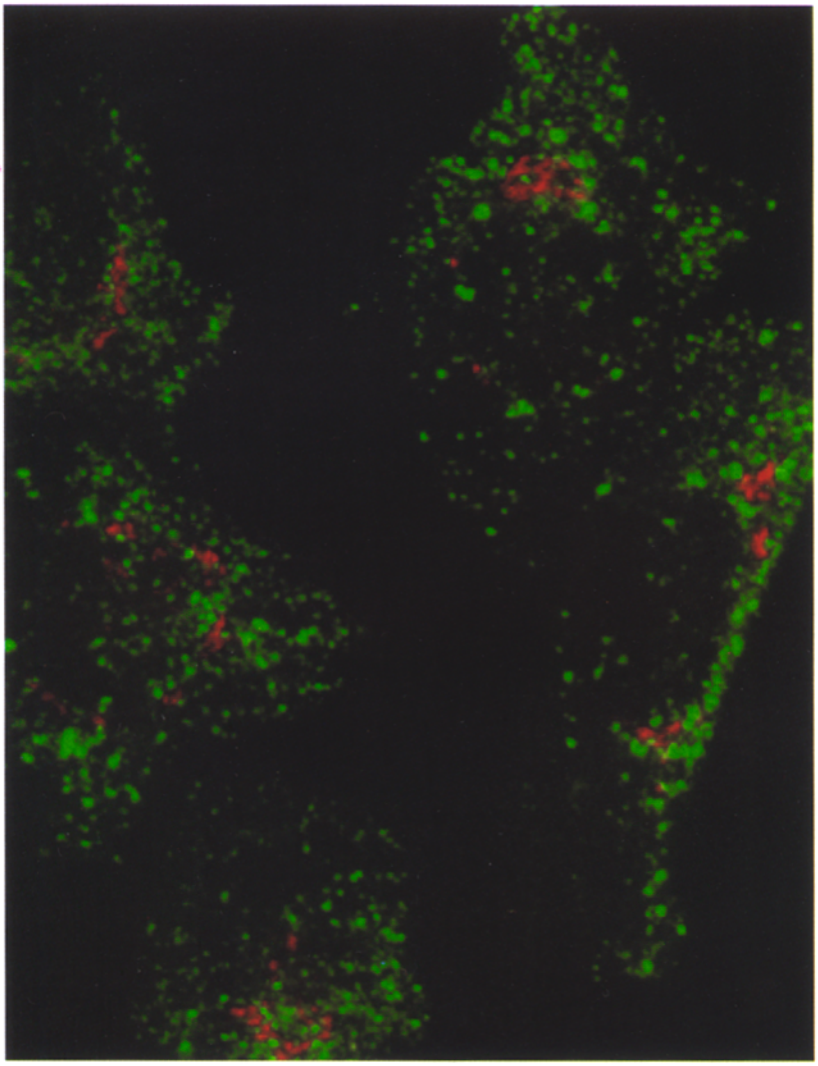

Figure 5. SDYQRL-TR is not in late endosomes. The codistributions of SDYQRL-TR and M6P-R are shown. SDYQRL-TR was labeled by a 45-min incubation with Cy3-Tf. The cells were fixed, permeabilized, and stained with an anti–M6P-R antibody. The anti–M6P-R antibody was detected using a fluorescein-labeled rabbit anti–mouse IgG. In this pseudocolored image, Cy3-Tf is in red and anti–M6P-R antibody is in green. A projection of successive focal planes imaged by a laser scanning confocal microscope is shown (Materials and Methods).

TR compartment is higher than the peri-centriolar recycling compartment in cells expressing the wild-type TR. The magnitude of this shift in R/F ratio is consistent with the juxtanuclear endosomes being 0.5 pH U more acidic than the peri-centriolar recycling compartment. Thus, both the morphology and the pH of the lumen of the SDYQRL-TR juxtanuclear compartment is different from the peri-centriolar recycling compartment in cells expressing the wild-type TR.

Cells Expressing SDYQRL-TR Accumulate Fe from Tf More Efficiently than Cells Expressing the Wild-Type TR

The biological role of the TR is to deliver iron to cells. The ability of cells to accumulate iron was examined (Materials and Methods). Cells expressing the SDYQRL-TR or YQRL-TR accumulate Fe faster than cells expressing the wild-type TR (Fig. 8). These results indicate that Fe is more efficiently accumulated per round of internalization of SDYQRL-TR or YQRL-TR. Although the reason for the increased efficiency of Fe accumulation in cells expressing SDYQRL- or YQRL-TR is not known, this difference provides additional evidence that the intracellular itinerary of these constructs is different from that of the wild-type TR.

The Morphology of the Peri-centriolar Recycling Compartment Is Altered by SDYQRL-TR

To examine the effect of SDYQRL-TR on the trafficking of other recycled molecules, the distribution of cellubrevin and fluorescent lipid NBD-sphingomyelin were examined. Cellubrevin, a v-SNARE involved in the fusion of recycling vesicles with the plasma membrane, colocalizes with Tf in the peri-centriolar recycling compartment of CHO cells (Galli et al., 1994). For these studies, the juxtanuclear SDYQRL-TR compartment was labeled with fluorescent Tf in a 2 h-incubation at 37°C. The cells were washed, fixed, and stained with an anti-cellubrevin antibody. In cells expressing SDYQRL-TR, cellubrevin is found in the juxtanuclear SDYQRL-TR compartment, whereas in cells expressing the wild-type TR, cellubrevin is in the peri-centriolar compartment (Fig. 9). Thus, expression of SDYQRL-TR changes the distribution of cellubrevin, a recycling membrane protein.

NBD-sphingomyelin internalized from the plasma membrane can be used as a marker of bulk membrane traffick-

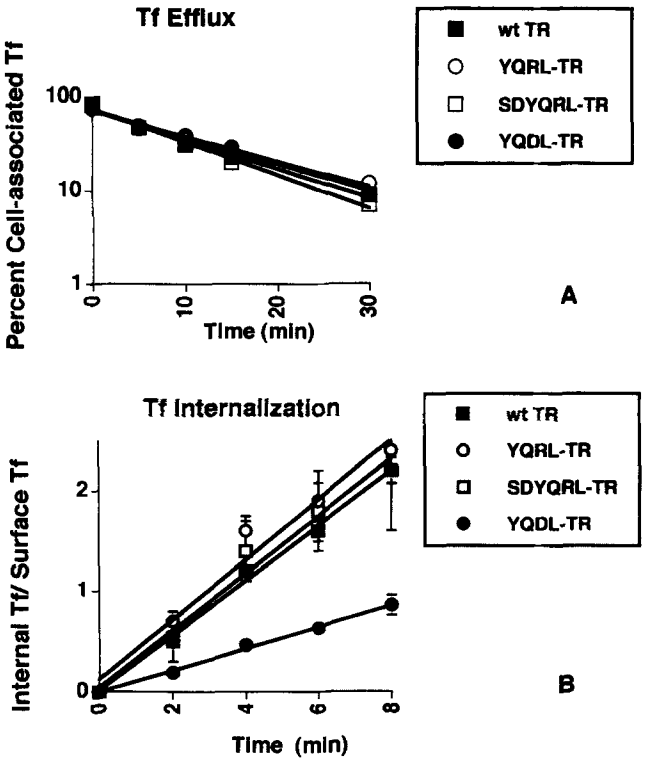

Figure 6. Endocytic trafficking kinetics. (*A*) The kinetics of Tf recycling are shown (Materials and Methods). The percent cell-associated Tf is the cell-associated Tf divided by the sum of the cell-associated Tf and Tf released into the medium. These data are the averages of at least three experiments ± SEM. (*B*) The kinetics of Tf internalization are shown (Materials and Methods). The data are presented as Tf internalized divided by the Tf bound to the surface at steady-state. These data are the averages of at least three experiments ± SEM.

ing (Pagano et al., 1989). In CHO cells, NBD-sphingomyelin follows the same intracellular itinerary as the wild-type TR (Mayor et al., 1993). To determine whether expression of SDYQRL-TR affects the distribution of this lipid marker of the recycling pathway, the distributions of endocytosed NBD-sphingomyelin and Tf in cells expressing SDYQRL-TR were determined (Materials and Methods). The two probes completely codistribute (Fig. 10). This result confirms that the juxtanuclear compartment in cells expressing SDYQRL-TR is the bona fide recycling compartment.

The Distribution of Clathrin Adaptin Complexes Are Not Affected by Expression of SDYQRL-TR

It has recently been shown that the SDYQRL motif binds the μ chains of both the Golgi- (AP-1) and plasma membrane- (AP-2) associated adaptin complexes (Ohno et al., 1995). AP-1 is localized to the *trans*-Golgi/TGN, where it plays a role in the formation of clathrin-coated vesicles that mediate transport between the TGN and the late endosomes. Therefore, it was of interest to determine whether AP-1 colocalizes with SDYQRL-TR. There is no significant overlap between the Tf-labeled juxtanuclear vesicles in cells expressing SDYQRL-TR and AP-1–labeled vesicles (Fig. 11). These results demonstrate that expression of

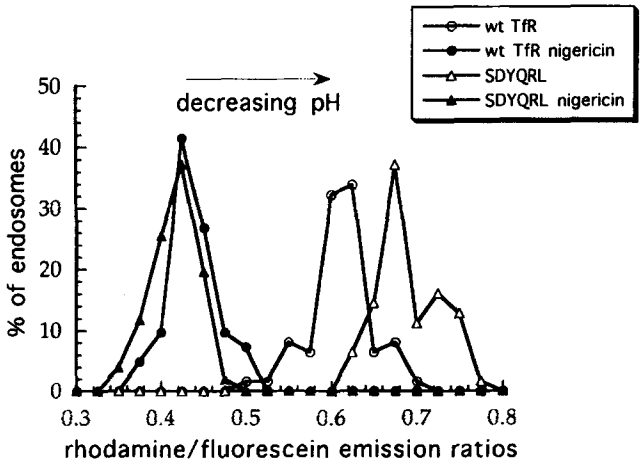

Figure 7. The juxtanuclear SDYQRL compartment is more acidic than the peri-centriolar recycling compartment. The pH in recycling compartments of cells expressing the SDYQRL and wild-type TR were measured as described (Materials and Methods). Shown is a histogram of TRITC/FITC emission ratios from the peri-centriolar recycling compartment in cells expressing the wild-type TR or the juxtanuclear vesicles in cells expressing SDYQRL-TR. In more acidic conditions, the FITC emission is less, and thus the ratio is higher. Cells expressing the SDYQRL construct have a distribution that is more acidic than for cells expressing the wild-type TR. When calibrated, this shift reflects a difference of ~0.5 pH U.

SDYQRL-TR does not alter the distribution of AP-1, and that AP-1 is not efficiently recruited to the juxtanuclear vesicles that contain SDYQRL-TR. In cells expressing SDYQRL-TR, AP-2 was concentrated in a punctate pattern on the plasma membrane, as it is in cells expressing the wild-type TR, demonstrating that expression of SDYQRL-TR does not alter the distribution of AP-2 (not shown).

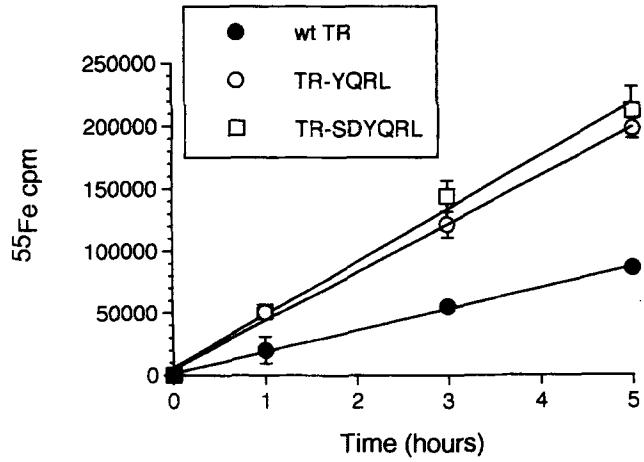

Figure 8. Cells expressing SDYQRL-TR accumulate Fe from Tf more rapidly than cells expressing wild-type TR. The results from a representative Fe accumulation experiment are shown. The Fe accumulation data are corrected for the number of Tf-binding sites on the surface of the cells at steady-state (Materials and Methods). Similar results were found in three other experiments.

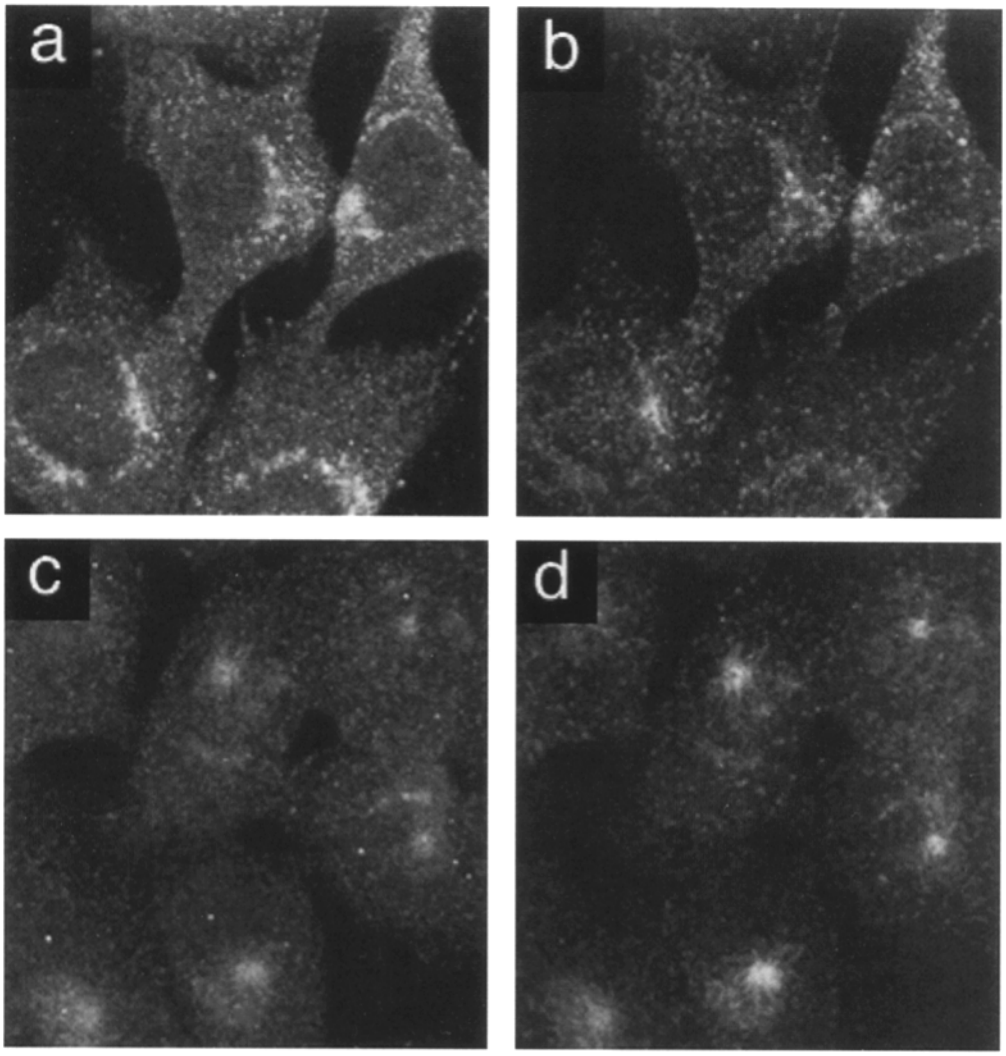

Figure 9. The distribution of cellubrevin is altered in cells expressing SDYQRL-TR. The SDYQRL-TR juxtanuclear compartment was labeled by a 2-h incubation with Cy3-Tf (*b*); cells were fixed, permeabilized, and stained with an anti-cellubrevin antibody (*a*). The anti-cellubrevin antibody was detected using a fluorescein-labeled rabbit anti–mouse IgG. In *c*, the wild-type TR peri-centriolar recycling compartment was labeled by a 2-h incubation with Cy3-Tf (*d*); cells were fixed, permeabilized, and stained with an anti-cellubrevin antibody (*c*).

Discussion

In this report, we show that expression of SDYQRL-TR or YQRL-TR constructs in CHO cells causes a reorganization of the endosomal recycling system. The CHO cell recycling compartment is compact, is usually located in focal planes toward the top of cells, is adjacent to the TGN, and has a pH of 6.5 (Yamashiro et al., 1984; Dunn et al., 1989; van der Sluis et al., 1992; McGraw et al., 1993). The juxtanuclear recycling compartment in cells expressing SDYQRL-TR or YQRL-TR constructs is punctate, is located in focal planes near the bottom of cells, is interspersed with the TGN, and has a more acidic pH. The differences in the rates of Fe accumulation are also consistent with the differences between the juxtanuclear recycling compartment in cells expressing SDYQRL-TR and the peri-centriolar endocytic recycling compartment in cells expressing the wild-type TR. Although the reason for the increased rate of Fe accumulation is not known, it may be linked to the more acidic pH of the juxtanuclear recycling compartment since pH regulates the release of Fe from Tf (Dautry-Varsat et al., 1983; Klausner et al., 1984; Sipe and Murphy, 1991). A possible explanation for the change in pH of the recycling compartment is that the composition of the proton pump, or regulators of the proton pump, may be different in the juxtanuclear compartment because of the altered membrane trafficking induced by expression of SDYQRL-TR. Two observations indicate that the juxtanuclear compartment in cells expressing the SDYQRL-TR or YQRL-TR constructs is the bona fide endocytic recycling compartment. First, two other recycling molecules, cellubrevin and sphingomyelin, colocalize with the SDYQRL-TR construct in the juxtanuclear endosomes. Second, the SDYQRL-TR construct is recycled back to the cell surface with the same kinetics as the wild-type TR.

We have not found any major differences in the behaviors of the YQRL-TR and the SDYQRL-TR constructs, thus the YQRL sequence is sufficient to cause a reorganization of the recycling compartment. Reorganization of the recycling compartment is a specific effect of the YQRL motif, since expression of the YQDL-TR does not affect the recycling compartment morphology to the degree that expression of YQRL-TR does. This finding indicates that reorganization of the recycling compartment by YQRL-TR is linked to the ability of the YQRL motif to target TGN38 to the TGN since mutation of arginine to aspartate within the context of the complete TGN38 cyto-

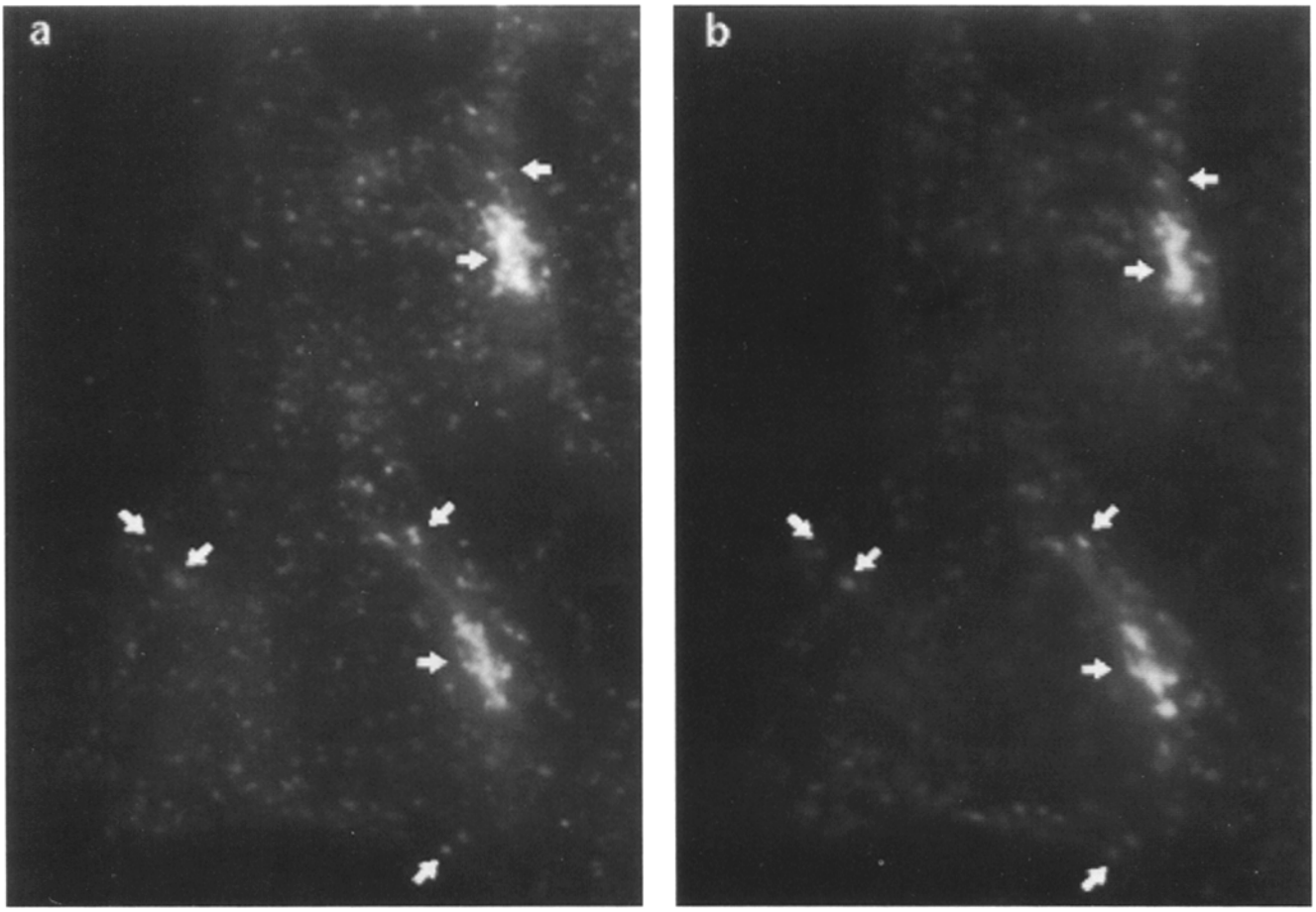

Figure 10. NBD-sphingomyelin is trafficked to the SDYQRL-TR juxtanuclear compartment. Cells expressing the SDYQRL construct were allowed to internalize Cy3-Tf for 40 min. For the final 10 min, the cells were labeled with NBD-sphingomyelin (Materials and Methods). The distribution of NBD-sphingomyelin is shown in *a* and the distribution of Cy3-Tf in *b*. The two labels were well colocalized (*arrows*).

plasmic domain abrogates TGN localization. (Humphrey et al., 1993).

Overexpression of chimeras containing the cytoplasmic domain of TGN38 saturate the TGN targeting/retention machinery (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). The failure of SDYQRL-TR to localize to the TGN is not due to the level of expression of SDYQRL-TR because TGN38-Tac chimera expressed in the same cell as SDYQRL-TR is concentrated in the TGN in these cells. Expression of SDYQRL in the context of the cytoplasmic domain of TGN38 does not cause a reorganization of the recycling compartment. A possible explanation for the different behaviors of these constructs is that the TGN38-Tac chimera is concentrated in the TGN, and therefore its concentration in endosomes at any given time will be low. This is not the case for the SDYQRL-TR. Because SDYQRL-TR is not sequestered in the TGN, it is rapidly trafficking between the plasma membrane and endosomal compartments and its steady-state concentration in endosomes will therefore be higher than that of TGN38.

Our findings indicate that the SDYQRL sequence is not sufficient for TGN localization. The localization of furin to the TGN requires two distinct motifs (Bosshart et al., 1994; Mollay et al., 1994; Schafer et al., 1995). Thus, it is possible that other TGN38 cytoplasmic sequences, in addition to the SDYQRL, are required for localization to the TGN. Furthermore, the transmembrane domain of the TGN38 can localize chimeric proteins to the TGN, indicating that in addition to cytoplasmic SDYQRL motif, the transmembrane domain of the TGN38 contains information that can localize proteins to the TGN (Ponnambalam et al., 1994).

Our finding that SDYQRL is insufficient to localize the TR to the TGN is not in agreement with previous studies. It has been reported that the SDYQRL is sufficient to localize reporter molecules to the TGN (Bos et al., 1993; Wong and Hong, 1993). In one study, YQRL was attached to the transmembrane domain of the LDL receptor by a linker of seven glycine residues (Bos et al., 1993). This construct, transiently transfected into cells, localized to a juxtanuclear region similar in morphology to the TGN. In another study, the YQRL sequence was attached to the transmembrane domain of glycophorin A by a linker of ten serine residues (Wong and Hong, 1993). In stably transfected CHO cells, this construct was found in the same region as the Golgi protein, mannosidase II. In both studies, morphology was assessed using conventional wide-field fluorescence microscopy. One possible explana-

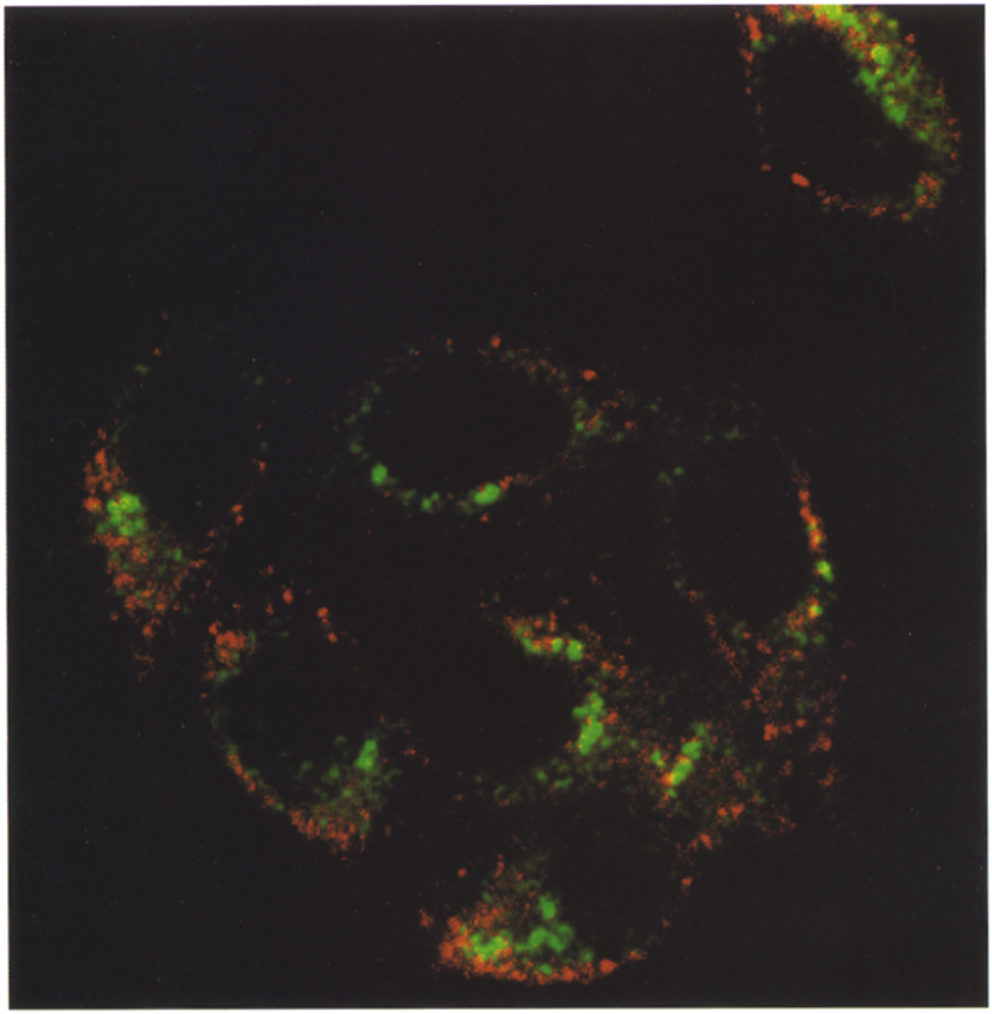

Figure 11. The Golgi-associated AP-1 adaptin complex does not colocalize with the juxtanuclear vesicles containing SDYQRL-TR. Cells expressing SDYQRL-TR were incubated for 2 h with cy3-Tf, washed, fixed, and stained with anti-AP-1 antibody (Materials and Methods). A single 400-nm-thick optical section is shown. The Cy3-Tf label is in red and the AP-1 label in green.

tion for the discrepancy between our findings and these other reports is that the juxtanuclear distribution observed for these YQRL-containing chimeras is not the TGN but, as we have found, a morphologically altered recycling compartment. We have used scanning laser confocal microscopy and two different markers of the TGN to establish that SDYQRL-TR is not in the TGN. Since the juxtanuclear compartment containing the SDYQRL-TR is interspersed with the TGN, it is not possible to distinguish the juxtanuclear compartment from the TGN based on morphology in single label experiments. In addition, two probes interspersed with one another may appear colocalized in wide-field fluorescence microscopy. An alternative explanation is that the SDYQRL sequence placed in the context of the TR cytoplasmic domain is somehow constrained so that it does not function as a TGN targeting/localization motif. The location of trafficking motifs within the cytoplasmic domains of proteins (e.g., distance from the membrane, local environment, and/or cytoplasmic domain structure) may influence the function of the motif. If this is the case, the location of SDYQRL in the cytoplasmic domain of the TR specifically affects TGN targeting without affecting SDYQRL-dependent internalization since the SDYQRL-TR is as rapidly internalized as the wild-type TR.

Tyrosine-based motifs and dileucine-like motifs provide the signals required for rapid internalization from the plasma membrane and for intracellular targeting to late endosomes, lysosomes, the basolateral membrane, and the TGN (for review see Roth, 1993; Trowbridge et al., 1993). These motifs are believed to function as molecular addresses that target proteins to their correct intracellular destinations. In support of this view, many of these motifs have been shown to direct motif-specific trafficking when transferred to heterologous proteins. It is generally interpreted that targeting signals cause proteins to be sorted into one pathway or another, whereas the properties of the pathways are believed to be determined by other molecules. For example, tyrosine-based internalization motifs are believed to target proteins to clathrin-coated pits on the cell surface, with the formation of coated pits being determined by clathrin, adaptins, and other proteins (Santini and Keen, 1996; for review see Schmid, 1993; Robinson, 1994). Our findings demonstrate that a TR containing the SDYQRL motif from TGN38 influences the physical characteristics (morphology and pH) of membrane compartments through which it traffics.

The mechanism by which SDYQRL-TR alters the recycling compartment is not clear. The molecular mechanisms that determine the morphology, location, and pH of intracellular compartments are not well understood. Membrane protein-targeting motifs are believed to regulate protein

targeting by interacting with adaptin protein complexes. These complexes recruit coat proteins to membranes, concentrate proteins in coated structures, and mediate the formation of coated vesicles (Schmid, 1993; Robinson, 1994; Seaman and Robinson, 1994). The possibility that the effect of the SDYQRL motif on the recycling compartment is mediated by the interactions of this motif with adaptin-like proteins is intriguing since it is known that membrane compartments are plastic and they can be dramatically altered by affecting coat protein binding. The effect of brefeldin A on the morphologies of membrane compartments is the most prominent example of the role of coat proteins in determining the morphologies of membrane compartments (Klausner et al., 1992). We have shown that expression of SDYQRL-TR does not affect the steady-state distributions of AP-1 and AP-2 adaptins, both of which are known to bind to the SDYQRL motif (Ohno et al., 1995), making it unlikely that interaction with these adaptins is responsible for the change in the recycling compartment. Recently, however, other adaptin-like proteins have been identified (Pevsner et al., 1994). It is possible that the SDYQRL motif also interacts with an as yet to be discovered adaptin. An additional possibility is that the SDYQRL motif of TGN38 is involved in the localization of TGN. Organelles require proteins to anchor and/or localize them to specific sites in the cell. In cells expressing the SDYQRL-TR construct, the SDYQRL motif may lead to an anchoring of recycling vesicles, which is appropriate for the TGN. Although these vesicles would be localized to the correct site they would not fuse with the TGN since they would not contain the appropriate molecules (e.g., SNAREs) required for fusion with the TGN.

The results presented in this report raise the possibility that other trafficking motifs may also play a role in regulating the physical characteristics of intracellular compartments, in which case the molecular mechanisms that determine intracellular trafficking of proteins may also determine the physical characteristics of intracellular membrane compartments. Future experiments will be directed towards understanding the underlying molecular mechanism responsible for the SDYQRL-TR reorganization of the recycling compartment.

The authors thank Drs. G. Baldini and W. Mallet for their critical reading of the manuscript. The authors thank Drs. P. De Camilli, F. Brodsky, and L. Traub for providing antibodies and Dr. J. Bonifacino for the TGN38-Tac construct. A.O. Johnson dedicates this work in the memory of her first mentor, Dr. Eric Holtzman.

This work was supported by grants from The Council for Tobacco Research (2919) and the National Institutes of Health (DK27083).

Received for publication 12 September 1996 and in revised form 27 October 1996.

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