# Association of human transferrin receptor with GABARAP

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**Abstract A yeast two-hybrid screen identified a specific interaction between the cytoplasmic domain of transferrin receptor (TfR) and GABARAP, a 14 kDa protein that binds to the γ2 subunit of neuronal $GABA_A$ receptors. The specificity of the TfR–GABARAP interaction was demonstrated by in vitro binding assays with purified proteins and by co-immunoprecipitation of GABARAP with endogenous TfR from HeLa cell lysates. Replacement of the YTRF internalization motif with ATRA within the cytoplasmic domain of TfR reduced interaction with GABARAP in the yeast two-hybrid screen and in vitro binding assays. The intracellular location of GABARAP using chimeric GABARAP–GFP showed that the majority of GABARAP was located in perinuclear vesicles. Our results show that GABARAP plays a more general role outside the confines of neuronal cells and $GABA_A$ receptors. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Transferrin receptor; γ-Aminobutyric acid type A receptor-associated protein; Protein trafficking; Endocytic motif

## 1. Introduction

The classical transferrin receptor (TfR) is a type II integral membrane protein responsible for delivery of iron-laden transferrin to the endosomal compartment. TfR-mediated internalization of transferrin is regarded as a standard example of endocytosis. The cytoplasmic domain of transferrin receptor contains a YXXΦ internalization motif, where X is any residue and Φ is a residue with a bulky hydrophobic side chain, involved in the rapid endocytosis of the receptor [1]. The internalization of TfR and other transmembrane proteins containing tyrosine-based motifs depends on direct or indirect association of the receptor cytoplasmic domain with heterotetrameric AP-2 (reviewed in [2]). However, some [3] but not all receptors [4,5] with tyrosine-based motifs compete with each other for endocytosis, a process that is potentially mediated by a set of adapter proteins or different domains of the same protein during the early stages of endocytosis. In this study, a yeast two-hybrid strategy was employed to search for proteins that interact with the endocytic motif of TfR. Collawn and co-workers [6] found that specific placement of a second YTRF in the TfR cytoplasmic domain increased the rate of endocytosis two-fold. We decided to exploit this finding by using a version of the TfR cytoplasmic domain containing the wildtype (residues 20–23) and a second (residues 31–34) YTRF motif as the bait in a yeast two-hybrid screen.

A specific interaction with γ-aminobutyric acid type A receptor-associated protein (GABARAP) dominated the results from the screen. To test whether the interaction observed in the yeast two-hybrid screen was also detectable in mammalian cell culture, we performed immunoprecipitation experiments and found that GABARAP co-precipitated with TfR. GABARAP is a putative microtubule-associated protein that was originally identified through its interaction with a cytoplasmic loop of the γ2 subunit of $GABA_A$ receptor [7]. Although the biological functions of GABARAP in neurons are not fully established, this small 14 kDa protein is speculated to be involved in the clustering of the $GABA_A$ receptor at nerve synapses and/or in $GABA_A$ receptor trafficking in the nerve body [8–10]. GABARAP belongs to a family of proteins that is conserved from plants to man. In humans, three closely related homologues have been identified in a variety of tissues by Northern analysis and RT-PCR [11].

We demonstrate that GABARAP engages in a specific interaction with the intact cytoplasmic domain of TfR and that binding is attenuated upon introduction of two mutations within the endocytic signal motif. Surprisingly, GABARAP was not located at the plasma membrane as judged by immunofluorescence and its overexpression had no effect on the endocytosis of TfR. Instead, the association of GABARAP with TfR cytoplasmic domain is most likely important for proper trafficking and sorting of a class of plasma membrane proteins along either a biosynthetic or degradative pathway. Our results indicate that GABARAP is playing a more generalized function in cells than binding exclusively to neuronal $GABA_A$ receptors.

## 2. Materials and methods

### 2.1. Generation of the cytoplasmic domains of the TfR

Plasmid pCDTR-1 encoding human TfR (a gift of Dr. A. McClelland [12]) was the original PCR template for all TfR constructs. All constructs listed were confirmed by restriction mapping and sequencing. A 5′*Nco*I restriction site and a stop codon at C62 followed by a *Bam*HI restriction site were engineered for insertion of TfR(2–62) into pET-11d vector (Novagen). For the TfR cytoplasmic domain lacking an internalization signal, primers were designed to create alanine substitutions at Y20 and F23. A primer containing a *Nde*I restriction site

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*Abbreviations:* Tf, transferrin; TfR, transferrin receptor; GST, glutathione *S*-transferase; $GABA_A$, γ-aminobutyric acid type A; GABARAP, $GABA_A$ receptor-associated protein; MBP, maltose binding protein; hrGFP, humanized green fluorescent protein; NETT, 150 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.4, 1% Triton X-100

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and a primer containing a stop site at the DNA encoding S63 with a *Bam*HI restriction site were used to amplify the TfR(1–63;A20A23) to insert into pET-11c vector (Novagen). The constructs were transformed into *Escherichia coli* strain BL21(DE3). Protein expression was induced at $OD_{600} \approx 0.8$ with 1.0 mM IPTG for 3–4 h at 32°C. Cells were collected by centrifugation ($5000 \times g$, 30 min, 4°C), washed, and resuspended in lysis buffer (25 mM piperazine, 1.0 mM EDTA, pH 6.5) and stored at −80°C. Thawed cell pellets were disrupted in a French pressure cell and centrifuged ($100\,000 \times g$, 30 min at 4°C). Both the TfR(2–62) and TfR(1–63;A20A23) cytoplasmic domains were purified by column chromatography on Q Sepharose Fast Flow resin (0.15–0.5 M NaCl) followed by a Superdex 75 gel filtration column (Amersham Pharmacia Biotech). The identities of the TfR cytoplasmic domains were confirmed by automated Edman sequencing as described [13].

### 2.2. Yeast two-hybrid screen

A yeast two-hybrid screen was carried out as previously described [14]. The yeast strains were from Dr. Stan Hollenberg, Vollum Institute, OHSU. The cDNA library was constructed from HeLa cell mRNA using random hexamers and the TimeSaver cDNA synthesis kit (Amersham Pharmacia Biotech). The cDNAs were amplified by polymerase chain reaction (PCR) and inserted into the plasmid, pVP16, carrying the viral VP16 activation domain to create a VP16/cDNA library [14]. The TfR cytoplasmic domain consisting of a second YTRF internalization sequence in place of residues 31–34 was inserted into a pLex-A fusion vector pBTM116-ADE2 and transformed into *Saccharomyces cerevisiae* L40(MATa). The library was transformed into the MATa strain L40 carrying pBTM116-ADE2 TfR(1–63;31–34YTRF). Screening was done in the presence of 3-aminotriazole (5 mM). Surviving colonies were further selected by assaying for β-galactosidase reporter gene activity to confirm presence and interaction of bait and prey. Specificity of interaction was further tested as described previously [14] by curing colonies of pBTM116-ADE2 TfR(1–63;31–34YTRF) followed by mating them with MATα strain AMR70 carrying control bait pBTM116-ADE2 TfR(1–63; A20A23), which lacks an internalization sequence. Plasmid DNA was isolated from colonies that grew with the bait plasmid but not with the control plasmid, transformed into electrocompetent *E. coli* strain HB101, sequenced, and subjected to BLAST analysis.

### 2.3. Generation of GST/GABARAP and MBP/GABARAP chimeric proteins

The full length GABARAP was generated with primers to include the known sequence of GABARAP and ligated into pGEM-T (Promega). Primers were used to create *Bam*HI sites to subclone the entire coding region of GABARAP cDNA into pMAL-c2 to obtain the maltose binding protein (MBP)/GABARAP chimera. pGEX-3X/GABARAP(36–117) was generated from *Bam*HI and a partial *Eco*RI digest of GABARAP from a positive plasmid in the yeast two-hybrid screen and inserted into pGEX-3X (Amersham) via *Bam*HI and *Eco*RI sites to create the glutathione *S*-transferase (GST)/GABARAP chimera. MBP/GABARAP was purified on amylose resin (New England Biolabs) and the GST/GABARAP chimeric proteins were purified on glutathione-coupled columns (Sigma) as per manufacturer's instructions.

### 2.4. Antibody production and purification

Two antibodies against GABARAP were raised in rabbits. Anti-GABARAP-1 (rabbit #14588) was generated against a chimeric GST/GABARAP (residues 36–117) isolated from an SDS–PAGE gel. Anti-GABARAP-2 (rabbit #16234) was generated from full length GABARAP cleaved and purified from a MBP/GABARAP fusion protein cleaved with factor Xa (New England Biolabs). Both antibodies were affinity-purified against MBP/GABARAP covalently bound to Affigel-10 (Bio-Rad) as per manufacturer's directions.

### 2.5. Cell culture and establishment of stable cell lines

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Primers encoding *Bam*HI sites were used to amplify GABARAP for insertion into pUHD-10-3. The correct orientation of the insert was verified. Tetracycline-repressible plasmid pUHD10-3(GABARAP) was co-transfected with a hygromycin-resistant plasmid (pcDNA3) into HeLa cells expressing the tTA fusion protein (gift from Dr. Sandy Schmid, Scripps Institute, La Jolla, CA, USA) as described [4,15]. Transfected cells were selected in DMEM containing 10% fetal bovine serum, G418 (400 μg/ml), hygromycin (250 μg/ml) and doxycycline (20 ng/ml). Following a double selection using the GABARAP-1 antibody on Western blots of cell lysates positive colonies were identified. The humanized green fluorescent protein (hrGFP)/GABARAP chimera plasmid was created by amplifying the GABARAP gene from the pGEM-T(GABARAP) plasmid using primers that inserted a *Sac*I site before the start of the GABARAP gene. An *Xba*I site was added 3′ of the GABARAP coding region and ligated into the *Sac*I-*Xba*I digested phrGFP-N1 vector (Stratagene) containing the hygromycin-resistance gene. The plasmid encodes hrGFP followed by a five amino acid linker (Glu, Leu, Pro, Gly and Arg) fused to the N-terminus of GABARAP. HeLa cells expressing hrGFP/GABARAP were selected with hygromycin as described above. Positive subclones were confirmed by fluorescence microscopy.

### 2.6. In vitro binding, immunoprecipitation, and gel electrophoresis

Affigel-10 bound GST or GST/GABARAP(36–117) was incubated for 90 min at 4°C in NETT (150 mM NaCl, 5 mM EDTA, 10 mM Tris base pH 7.4, 1% Triton X-100) plus COmplete™ Mini EDTA-free protease inhibitor cocktail (Roche) with either 3 μg of purified TfR(2–62) or TfR(1–63; A20A23). Samples were centrifuged ($1300 \times g$, 5 min, 4°C), aspirated and the pellet was resuspended in NETT. The suspension was layered on top of 1.0 ml of NETT containing 15% sucrose and centrifuged ($14\,000 \times g$ for 2 min at ambient temperature).

Immunoprecipitation of TfR, SDS–PAGE, and Western blots were carried out as previously described [16]. Blots were probed with either anti-TfR (h68.4, Zymed) or anti-GABARAP-1 at 1:10 000 for 1 h followed by a 1 h incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:10 000 dilution; Boehringer Mannheim) and detection with an enhanced chemiluminescence substrate (Pierce).

### 2.7. Iodination and uptake protocol

The iodination of holo-Tf and the measurement of internalized [$^{125}$I]Tf were previously described [4,17]. Briefly, cells in 35 mm dishes were incubated with [$^{125}$I]Tf (50 nM) at 37°C, 5% $CO_2$. At specified times, cells were cooled on ice, washed and surface Tf removed by 0.5 M NaCl/0.5 M acetic acid. The number of surface TfRs for each uptake experiment was determined after incubating cells with [$^{125}$I]Tf (50 nM) on ice for 90 min. Cells were washed four times, and solubilized as described [4].

### 2.8. Immunocytochemistry

HeLa cells grown on glass coverslips were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and blocked with bovine serum albumin (2.5 mg/ml) or 10% fetal bovine serum. Coverslips were then incubated for 1 h with the following: anti-TfR monoclonal antibody 4093 (1:200; a gift from Vonnie Landt, Washington University, St. Louis, MO, USA) and/or GABARAP-2 antibody (1:250). Coverslips were washed with PBS, incubated with Alexa Fluor 594 goat anti-mouse and/or Alexa Fluor 488 goat anti-rabbit (1:750 dilution; Molecular Probes) for 1 h, washed in PBS and mounted on ProLong Antifade (Molecular Probes). Cells were visualized with a Nikon fluorescent microscope or with the Applied Precision Deltavision® image deconvolution system.

## 3. Results

### 3.1. Yeast two-hybrid screen

A yeast two-hybrid screen was designed to identify proteins that interact with TfR cytoplasmic domain. The bait plasmid was constructed by fusing the LexA DNA binding domain to the TfR cytoplasmic domain containing a second YTRF internalization signal at residues 31–34. This substitution enhances the TfR internalization rate two-fold [6,17–19]. A HeLa cDNA library encoding small protein binding motifs fused to the VP16 activation domain was transformed into yeast strain L40 (MATa) expressing the bait. A screen of $6.8 \times 10^7$ poten-

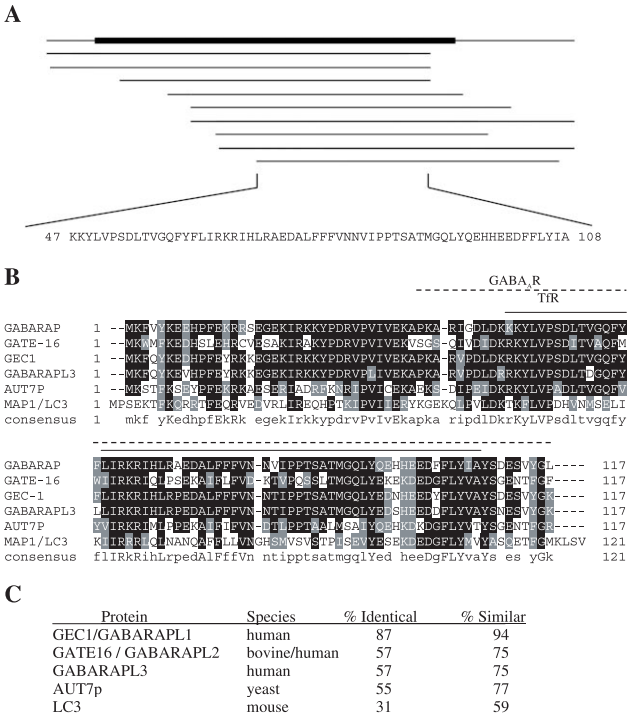


Fig. 1. Mapping the TfR binding domain of GABARAP. A: The top line represents GABARAP mRNA with the translated region in bold. Nine overlapping cDNA fragments are shown, followed by the translated consensus transferrin receptor interacting region, residues 47–108. B: Comparison of the sequences between GABARAP-related proteins, guinea pig GEC1 (human GABARAPL1), bovine GATE-16 (human GABARAPL2), human GABARAPL3, yeast AUT7p, and human Map1A/1B/LC3. Black boxes denote identical residues and gray boxes denote residues of similar charge or hydrophobicity. Alignments were done using ClustalW (Baylor College of Medicine Molecular Biology Computation Resource). C: The percent sequence identity and similarity between GABARAP and its family members [7,11,28–30].

tial colonies resulted in the selection of 231 colonies that interacted with the bait and did not interact with the control bait lacking an internalization signal. Examination of a randomly selected set of 35 library inserts yielded 29 sequences (82%) encoding portions of the same GABARAP open reading frame. The common region of overlap shared by all of the GABARAP sequences spanned residues 47–108 (Fig. 1A). The intracellular loop of the γ2 subunit of the $GABA_A$ receptor interacts with residues 36–117 of GABARAP, indicating that these two receptors are docking to either the same site or overlapping sites on GABARAP. GABARAP belongs to a family of closely related proteins. No other member of the human GABARAP family was identified in these or subsequent yeast two-hybrid clone sequences (Fig. 1B,C). These results identified a possible GABARAP-TfR interaction specific for this family member.

### 3.2. GABARAP interacts directly with purified TfR

The yeast two-hybrid screen identified GABARAP as a protein that interacts with TfR cytoplasmic domain containing a second YTRF motif. A GST/GABARAP fusion protein consisting of GABARAP residues 36–117 (GST/GABARAP (36–117)) was created to test the ability of GABARAP to interact with purified TfR cytoplasmic domain in vitro. Western blot analysis demonstrated that purified TfR(2–62) interacted with immobilized GST/GABARAP(36–117) whereas purified TfR(2–62) did not interact with immobilized GST alone. In control experiments, only low levels of TfR(1–63; A20A23) were associated with GST/GABARAP(36–117) (Fig. 2A). GABARAP fused to MBP was also capable of capturing TfR(2–62) under similar in vitro conditions and no TfR(2–62) was isolated using MBP alone (data not shown), confirming

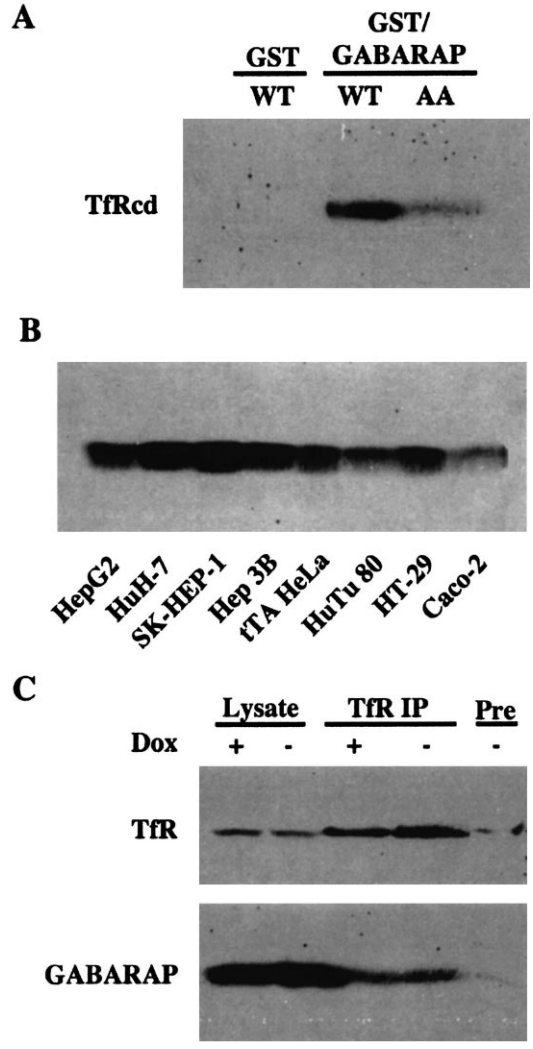


Fig. 2. Association of TfR and GABARAP. A: GST or GST/GABARAP(36–117) bound to Affigel-10 was incubated with either 3 μg of TfR(2–62) (WT) or TfR(1–63;A20A23) (AA). The samples were run on SDS–PAGE gels, transferred to nitrocellulose, probed with anti-TfR antibody, and observed with enhanced chemiluminescence. B: Equal protein concentrations of lysates from a variety of cell lines were run on a 13% polyacrylamide gel, transferred and probed with anti-GABARAP-1. The antibody used to detect the cytoplasmic domain of the TfR (h68.4) reacts equally well on western blots with the native and the mutated A20A23 cytoplasmic domains (data not shown). C: Lysate (50% of amount immunoprecipitated) from tTA HeLa cells that overexpress GABARAP upon the removal of doxycycline (Dox−) were preabsorbed with Pansorbin (Pre) followed by incubation of the supernatant with sheep anti-TfR and Pansorbin (TfR IP). Pellets from the incubations were extracted with 2×Laemmli buffer, loaded onto 13% polyacrylamide gels, transferred and immunodetected with anti-TfR (h68.4) and anti-GABARAP-1.

that GABARAP binds to the native TfR cytoplasmic domain in a specific fashion.

### 3.3. GABARAP is expressed in a variety of cell lines

GABARAP was first identified through its association with the γ2 subunit of the $GABA_A$ receptor [7,9]. However, $GABA_A$ receptor expression is restricted to neurons. In contrast, Northern blot analysis of multiple human tissues has shown GABARAP mRNA is ubiquitous [20]. All human cell lines that we have tested show expression of GABARAP immunoreactive proteins (Fig. 2B). These results indicate that GABARAP has a more generalized function than its association with the $GABA_A$ receptor.

### 3.4. GABARAP co-immunoprecipitates with TfR in cultured cells

The wide expression patterns of GABARAP and TfR and the direct association of TfR cytoplasmic domain with GABARAP led us to examine the interaction between these two proteins in human cell lines. Full length GABARAP was stably transfected into tTA HeLa cells under control of the tetracycline-repressible promoter [15]. Anti-TfR antibodies were used to immunoprecipitate TfR from tTA HeLa cell extracts either endogenously expressing or overexpressing GABARAP (Fig. 2C). GABARAP was detected in all lanes where cell extract was immunoprecipitated with antibodies against TfR. Only a small amount of endogenous GABARAP was associated with TfR, consistent with the possibility that GABARAP binds to multiple receptors.

In summary, these results demonstrate that GABARAP specifically interacts with TfR in a yeast two-hybrid screen, in an in vitro GST pulldown system, and in vivo, forming endogenous GABARAP–TfR complexes that can be immunoprecipitated from solubilized cell extracts.

### 3.5. GABARAP expression level does not influence Tf internalization

If GABARAP functions by interacting with the endocytic motif of TfR, overexpression of GABARAP could affect the rate of TfR endocytosis. However, overexpression of GABARAP (Fig. 3A) resulted in no significant change in transferrin uptake (Fig. 3B) and no substantial change in the distribution of TfR between the plasma membrane and internal compartments (results not shown). Lack of an effect on endocytosis opens several possibilities. GABARAP might be sequestered in an intracellular compartment and interact with TfR in a process that is distinct from endocytosis. GABARAP may already be present in excess over TfR, precluding any overexpression phenotype. Efforts to reduce GABARAP expression with antisense technology were unsuccessful (results not shown). Functional redundancy within the GABARAP family may protect the cell against loss of a specific member. With regard to this last possibility, we stress that GABARAP itself was the only family member that binds TfR with sufficient affinity to be detected in our yeast two-hybrid screen.

### 3.6. Immunolocalization of GABARAP in cells

Two approaches were taken to determine the intracellular localization of GABARAP. Anti-GABARAP-2, a polyclonal antibody to recombinant GABARAP, was generated and affinity-purified. In addition, HeLa cells were stably transfected with an hrGFP/GABARAP chimera in order to distinguish

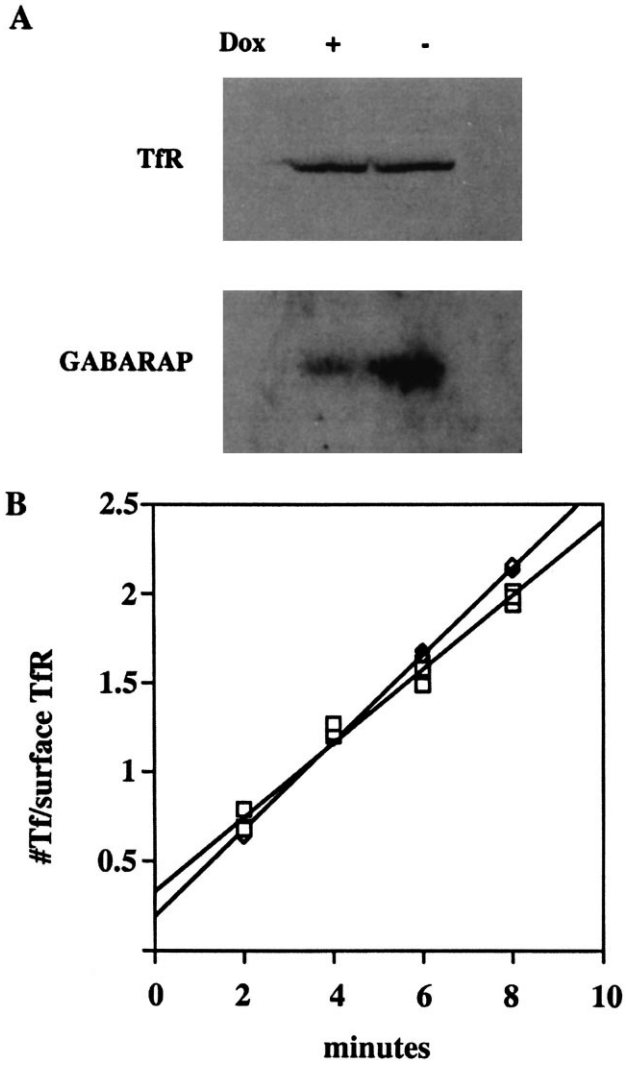


Fig. 3. GABARAP overexpression effects on TfR. A: tTA HeLa cells transfected with GABARAP under the tetracycline-repressible promoter were induced to express GABARAP by the withdrawal of doxycycline for 3 days; solubilized lysates from $10^6$ cells were loaded onto a 13% polyacrylamide gel, transferred to nitrocellulose, and detected with antibodies to TfR and anti-GABARAP-1. B: The effect of GABARAP overexpression on TfR endocytosis was examined by measuring the rate of [$^{125}$I]transferrin uptake per surface TfR into cells. The rates of Tf uptake in uninduced cells (plus doxycycline (square)) and in cells that had been induced to express GABARAP (diamond) were 0.208 Tf/TfR/min, $R^2$ 0.988 and 0.245 Tf/TfR/min, $R^2$ 0.996, respectively. The experiment was repeated three times with similar results.

GABARAP from family members that could potentially cross-react with the polyclonal antibody (i.e. GABARAPL1, L2, and L3). Endogenous GABARAP was detected in a perinuclear and scattered pattern with anti-GABARAP-2 (Fig. 4A). Preincubation of affinity-purified anti-GABARAP-2 with purified recombinant GABARAP abolished fluorescence, confirming signal specificity (Fig. 4B). Similar findings were obtained when HeLa cells stably expressing chimeric hrGFP/GABARAP were examined, but the immunofluorescence pattern was more scattered and occasional large vesicles were noted (Fig. 4D). Since large vesicles were never seen in cells expressing endogenous GABARAP and since anti-GABAR-

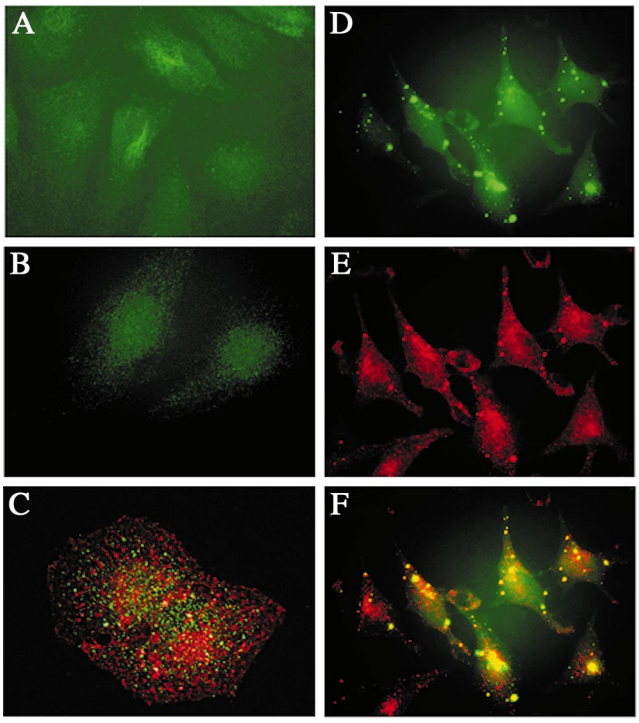


Fig. 4. Distribution of hrGFP/GABARAP, GABARAP/hrGFP and GABARAP. A: HeLa cells were permeabilized and stained for endogenous GABARAP with affinity-purified anti-GABARAP-2 antibody. B: As a control the affinity-purified anti-GABARAP-2 antibody was preincubated with purified GABARAP and then incubated with fixed and permeabilized HeLa cells as in A. C: HeLa cells were permeabilized and stained for endogenous GABARAP (green) and TfR (red); images were acquired and analyzed with Image Restoration Microscopy (deconvolution). Pictured is a single 0.5 μm *z*-section one third up from the bottom of the cell. D: HeLa cells expressing hrGFP/GABARAP. E: The same field incubated with an affinity-purified antibody, GABARAP-2. F: The images were superimposed to show colocalization of the hrGFP/GABARAP and the anti-GABARAP-2 immunofluorescence.

AP-2 recognized all hrGFP/GABARAP positive vesicles, the discrepancy may be due to overexpression of the chimera in some cells. Another difference is that anti-GABARAP-2 recognized additional vesicles (Fig. 4E,F), which we attribute to cross-reactivity with other family members. The cellular locations of TfR and endogenous GABARAP were examined in thin optical sections by confocal microscopy, and minimal overlap of GABARAP with TfR (Fig. 4C) was observed. These results suggest that GABARAP is not primarily associated with endocytic vesicles and that the association between GABARAP and TfR is most likely transient. As in the $GABA_A$ receptor γ2 subunit/GABARAP interaction in hippocampal neurons [10,21], essentially no colocalization of GABARAP with its binding partner, TfR, could be discerned in HeLa cells. GABARAP is a small protein, and we cannot formally exclude the possibility that the GABARAP epitope is masked in certain complexes, for example at the plasma membrane. The similar patterns seen with two different detection methods, namely visualization of the hrGFP/GABARAP chimera and immunodetection of endogenous GABARAP with an affinity-purified polyclonal antibody, argue against this explanation. Overall, our findings are consistent with GABARAP involvement in trafficking of TfR along another pathway such as a biosynthetic or degradative pathway. TfR is a relatively stable protein with a half-life of approximately 16–24 h [22,23], and therefore only a small fraction of the total TfR pool would be expected to be trafficking through these pathways at any given time.

## 4. Discussion

The TfR/Tf delivery of iron to cells is an extensively studied example of receptor-mediated endocytosis, yet little is known about the intracellular interactions of the TfR cytoplasmic domain. We have identified a specific interaction between TfR and GABARAP through a yeast two-hybrid system. The specificity of the positive two-hybrid interaction was corroborated by pulldown experiments in which purified TfR cytoplasmic domain was captured with immobilized GST/GABARAP(36–117)-coupled beads. Finally, the affinity of the interaction was high enough to allow co-immunoprecipitation of a small fraction of endogenous GABARAP with TfR from HeLa cell extracts. Taken together, our results point to a direct interaction between these two proteins and establish that GABARAP has a more universal trafficking function and is important in cells other than neurons. The latter finding is consistent with GABARAP expression in tissues that do not express the $GABA_A$ receptor.

The yeast two-hybrid bait included a second tyrosine-based internalization motif (YTRF) to tailor the search for proteins interacting with the endocytic motif of TfR and a control bait lacking both internalization signals to eliminate false positives. Despite implementation of this approach, we did not find any evidence that GABARAP is directly involved in TfR endocytosis. In particular, the lack of effect of GABARAP overexpression and its almost complete absence from endocytic vesicles do not support the involvement of GABARAP in endocytosis. Rather, GABARAP most likely plays a role in the trafficking of TfR along a biosynthetic or degradative pathway. Alterations in the cytoplasmic domain of the TfR can slow the progression of the TfR through the biosynthetic pathway [13]. In addition, GABARAP could also participate either in the cycling of TfR back to the Golgi [24] or in polarized sorting to the basolateral membrane. Efforts are under way to characterize the GABARAP-associated vesicles seen by us and others [20] to distinguish between these possibilities.

The function of GABARAP is not known. It was originally identified in a yeast two-hybrid screen as interacting with the γ2 subunit of the $GABA_A$ receptor. At first, GABARAP was thought to cluster $GABA_A$ receptors at the nerve synapse but was later proposed to function in the trafficking of this receptor. The paralogues and homologues of GABARAP may offer clues as to the function of this small but versatile linker protein. GABARAP and a family member, GATE-16, interact with NSF [21,25]. GATE-16 also interacts with the v-SNARE GOS-28 in a NSF-dependent manner in a proposed intra-Golgi transport mechanism [25]. NSF is released prior to the fusion of the v-SNARE/t-SNARE complex, however the origin and effect of the GABARAP–NSF interaction is unknown. In light of these examples and our results, it is more likely that GABARAP is an adapter protein that undergoes dynamic association with NSF as part of a receptor trafficking network as opposed to a scaffolding protein. GABARAP family member APG8/Aut7p of *S. cerevisiae* is involved in starvation-induced autophagocytosis and vegetative cyto-

plasm to vacuole targeting, and Sec18p, the yeast homologue of NSF, functions in vacuole trafficking [26]. These observations suggest a possible role for GABARAP in lysosomal degradation targeting.

Recently, the high resolution crystal structures of monomeric GABARAP (1.6 Å) and an unanticipated second GABARAP crystal form (1.9 Å) were determined [27]. The latter structure represents an extended, oligomeric GABARAP network and places the biological relevance of observed in vitro tubulin–GABARAP interactions on firmer ground. Thus, the structure of GABARAP is in accord with its putative role as a microtubule-associated protein that sequesters target proteins through a specific docking site(s) on the face opposite the microtubule binding domain. Current studies in this and other laboratories aim to elucidate the precise role of GABARAP in the control of receptor trafficking and receptor density.

At present, the few known binding partners of GABARAP have all been identified in neuronal cells. We extend the range of possible functions of GABARAP by reporting interaction with TfR in a biological pathway that is entirely distinct from neurotransmitter receptor trafficking. Except in the case of the $GABA_A$ receptor $\gamma 2$ subunit [7,9], little is known about specific residues that interact with the GABARAP docking site. To date, it is not established whether binding partners utilize the same or an overlapping docking site on GABARAP or whether more than one partner binds simultaneously. We are now investigating the TfR–GABARAP interface through a combination of biochemical and structural techniques.

The current state of knowledge is most consistent with GABARAP belonging to a family of proteins that serve to concentrate proteins for packaging into vesicles. In addition, with its microtubule binding domain that is distinct from both the TfR and the $GABA_A$ receptor binding domain, GABARAP could serve to tether vesicles to microtubules during transport from one organelle to another. The precise function of the GABARAP protein family remains to be determined.

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