EMBO reports

scientific report

An efficient genetic screen in mammalian cultured cells

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Received January 31, 2002; revised April 23, 2002; accepted May 17, 2002

Genetic approaches in mammalian cultured cells had limited success because the isolation of mutants and the identification of the mutated genes were often difficult. In the present report, we describe the establishment of a novel genetic screen in Cos-7 cells that allows rapid identification of polypeptides whose overexpression inhibits a certain cellular process. We demonstrate that this approach can be used successfully to isolate partial cDNAs whose overexpression specifically interfered with the clathrin-mediated endocytosis of transferrin.

INTRODUCTION

In higher eukaryotes, the endocytic uptake of specific substrates and plasma membrane receptors plays an essential role in many medically important processes (Marsh and McMahon, 1999). The classical endocytic pathway depends on the polymerization of the clathrin triskelions to drive vesicle formation (Schmid, 1997; Kirchhausen, 2000). The adaptor complex AP-2 (composed of α, β2, μ2 and σ2) links the cytoplasmic tails of receptors containing YxxØ or LL endocytosis motifs to clathrin cages (Kirchhausen *et al.*, 1997). A number of factors required for clathrin-dependent budding have been identified, yet the regulation of the process in specific cell types is not well understood. In addition, increasing evidence demonstrates the existence of poorly characterized clathrin-independent endocytic pathways, which participate in important physiological functions (Shin and Abraham, 2001).

To identify the machinery involved in the uptake of specific substrates in mammalian cells, we undertook a genetic approach that might be complementary to the biochemical studies performed by many laboratories. Genetics may facilitate the *in vivo* analysis of poorly characterized pathways and the identification of low abundant regulatory proteins that participate in clathrin-mediated endocytosis (CME). Most genetic screens performed in mammalian cells aimed to isolate recessive mutants in CHO cells (Krieger *et al.*, 1981; Hobbie *et al.*, 1994). Unfortunately, several drawbacks preclude the extended use of this approach. Only genes that encode proteins with no redundant counterparts can be identified, the isolation of clones bearing the mutations is time consuming and requires a stable chromosomal setting and, finally, the identification of the mutated genes might be difficult.

To overcome these problems, we have designed the first dominant interfering screen in mammalian cultured cells that allows rapid identification of partial cDNAs whose overexpression blocks a given process by competing with the endogenous functional proteins involved in that process. In the present report, we describe the methodology and demonstrate that it can be used to identify polypeptides whose overexpression inhibits CME of transferrin (Tf).

RESULTS

Identification of peptides whose overexpression inhibits CME using panning with antibody-coated plates

Cos-7 cells have been used to isolate cDNAs encoding proteins that are normally absent in this cell type (Aruffo and Seed, 1987a,b). For that purpose, a cDNA expression library in a vector bearing the SV40 origin of replication (SV40or) is transfected into Cos-7 cells under conditions that minimize the number of plasmids per cell. Cos-7 cells can replicate such plasmids because they express the viral large T antigen. Transfectants expressing the protein of interest are then isolated and plasmids encoding the polypeptide are recovered from the cell lysate (Hollenbaugh *et al.*, 1994). We reasoned that if the expression level of individual cDNAs sufficed, we could modify the method slightly and use it to identify polypeptides whose

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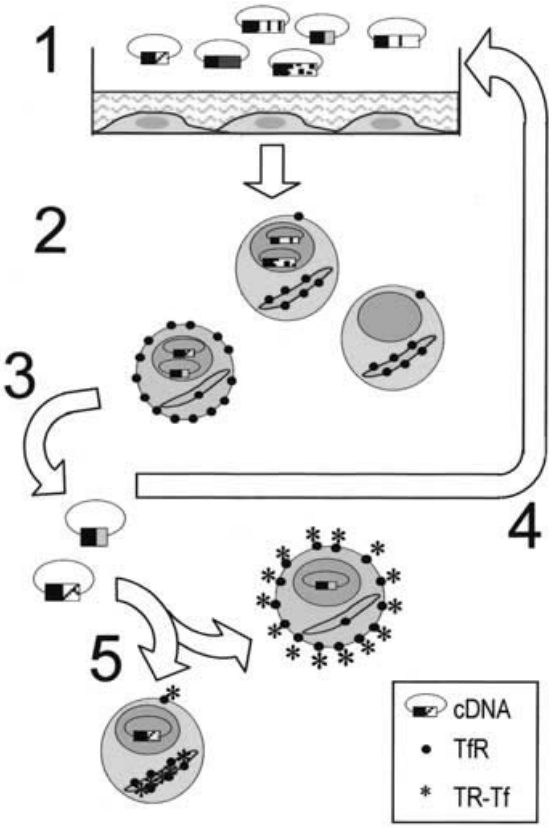

Fig. 1. A genetic screen in Cos-7 cells to isolate cDNAs whose overexpression interferes with CME. Cos-7 cells are transfected with a library of partial cDNAs fused to eGFP (1). One day after transfection, cells accumulating the TfR at the cell surface are selected using a monoclonal antibody against the TfR and anti-mouse IgG antibody-coated dishes or Cy5-labeled Tf and FACS (2). Selected cells are lysed and plasmids are recovered by electroporation into *E. coli* (3). Plasmids from individual colonies are either pooled and subjected to a new round of selection (4) or are separately purified to analyze the effect of overexpressing individual eGFP fusion proteins on the internalization of TR-Tf by fluorescence microscopy (5).

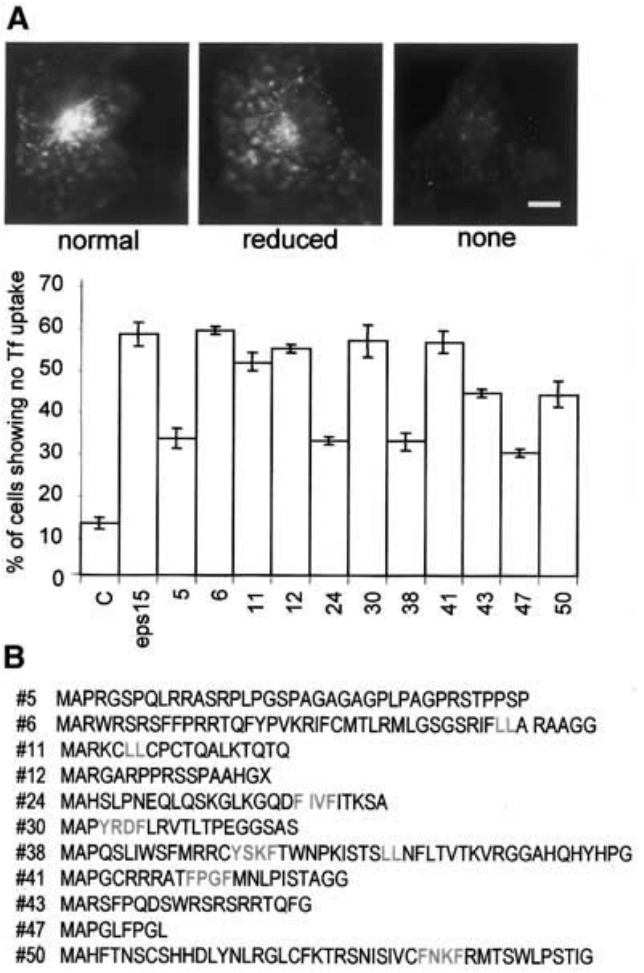

Fig. 2. Identification of short peptides whose overexpression inhibits CME by panning with antibody-coated plates. (**A**) Cos-7 cells transfected with pEGFP-N1 (C), pEGFP-C2-EΔ95/295 (eps15) or the plasmids isolated from the library (numbers) were incubated in the presence of TR-Tf. eGFP-expressing cells exhibiting normal, reduced or no uptake were scored. The graph represents the percentage of eGFP-expressing cells showing no Tf uptake. Bar = 10 μm. (**B**) Peptide sequences in frame with the eGFP initiation codon encoded by the inserts of the isolated plasmids. YxxØ and LL motifs are highlighted.

overexpression interfered with a given process, specifically with CME (Figure 1). To select cells with a block in CME, we used the observation that inhibition of this pathway alters the steady-state distribution of the recycling Tf receptor (TfR) and triggers its translocation from an endosomal compartment to the cell surface (Benmerah *et al.*, 1999) (Figure 1). Cells expressing the TfR at the cell surface could be isolated using a monoclonal antibody against the receptor and secondary antibody-coated plates. To establish the selection procedure, we used a cDNA encoding a mutant eps15 whose overexpression was previously shown to inhibit Tf uptake (eps15EΔ95/295; Benmerah *et al.*, 1999). Cos-7 cells were transfected with a mixture of plasmids encoding either eGFP (pEGFP-C2) or an eGFP fusion protein with the mutant eps15 (pEGFP-C2-EΔ95/295). Transfectants were then immunoselected using a monoclonal antibody against the TfR. Analysis of the molar ratio of the plasmids recovered from the isolated cells revealed a significant enrichment of pEGFP-C2-EΔ95/295 versus the empty vector with respect to the transfection mixture (from 10 to 35% of pEGFP-C2-EΔ95/295). No enrichment was observed when the antibody against the TfR was obliterated. This result suggested that the procedure preferentially selected cells expressing the mutant eps15 and, therefore, it could be used to screen for polypeptides whose overexpression blocked CME.

To validate our approach, we first screened an available library of short randomly primed partial cDNAs (maximum 200 bp) that were cloned after the initiation codon of eGFP in a modified pEGFP-N1 vector (MT-library; Bejarano and Gonzalez, 1999). We expected the screening procedure to enrich for plasmids encoding peptides bearing YxxØ motifs that would compete with that present in the TfR for binding to the AP-2 complex. Overexpression of peptides containing YxxØ motifs is known to inhibit Tf uptake (Marks *et al.*, 1996). One day after transfection, Cos-7 cells expressing the TfR at the cell surface were immunoisolated and plasmids were recovered from the cell lysate by electroporation into *Escherichia coli*. Bacteria were pooled and purified plasmids were subjected to a new round of selection. Plasmids were then isolated from 48 individual colonies and the

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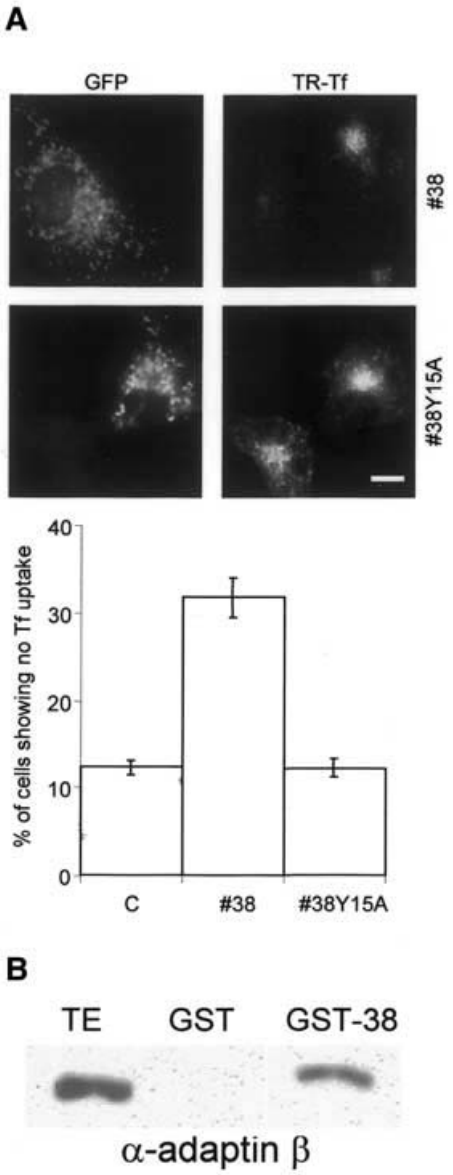

Fig. 3. Peptide 38 bears a bona fide Yxx∅ endocytosis motif. (**A**) Cos-7 cells transfected with pEGFP-N1 (C) or the indicated plasmids were assayed for their ability to internalize TR-Tf. The fluorescent signals from the eGFP and TR-Tf were analyzed by fluorescence microscopy using the appropriate filters. The graph represents the percentage of eGFP-expressing cells showing no uptake. Bar = 10 μm. (**B**) GST or GST fused to peptide 38 was bound to glutathione–Sepharose beads and incubated with a rat brain extract. Proteins bound to the beads were separated by SDS–PAGE and analyzed by immunoblotting using an antibody against adaptin β. Two micrograms of total rat brain protein extract were loaded as total (TE).

corresponding eGFP fusion proteins were overexpressed in Cos-7 cells to monitor the effect on the internalization of Texas Red Tf (TR-Tf). Overexpression of 11 of the 48 polypeptides significantly inhibited Tf uptake (Figure 2A). In contrast, overexpression of the eGFP fusion proteins of none of 17 randomly chosen plasmids from the original library inhibited CME (data not shown).

Analysis of the DNA sequences inserted after the eGFP initiation codon revealed that none encoded peptides that matched products from mouse ORFs. The inserts were cDNA fragments

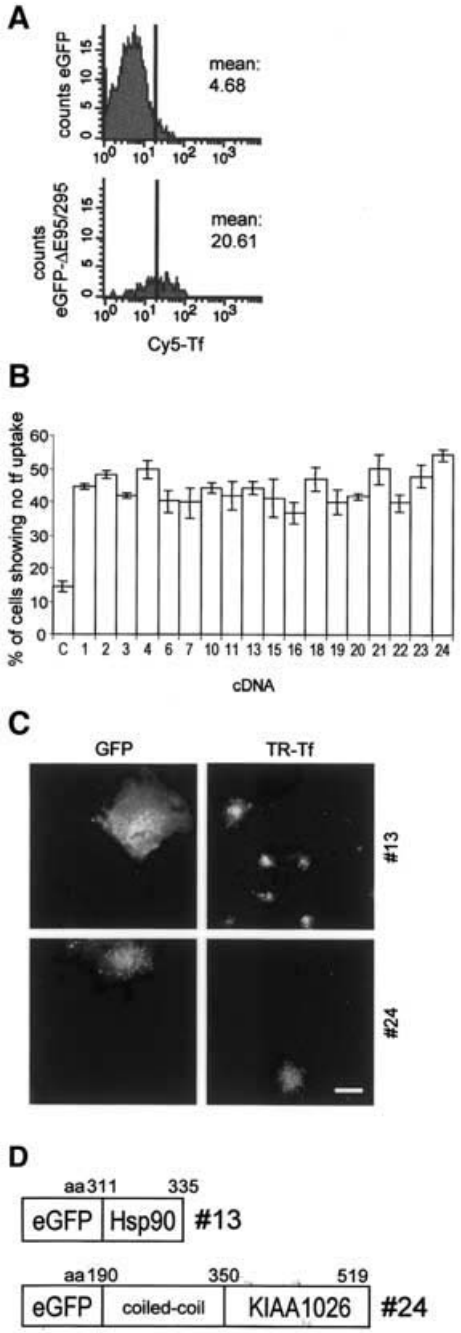

Fig. 4. Isolation of two cDNAs from human brain whose overexpression inhibits CME using FACS. (**A**) Cos-7 cells transfected with pEGFP-C2 (eGFP) or pEGFP-C2-EΔ95/295 (eGFP-EΔ95/295) were incubated in the presence of Cy5-Tf and analyzed by FACS. The *x*-axis represents the fluorescence intensity of the Cy5-Tf surface labeling per cell. The *y*-axis represents the number of transfected cells showing a particular fluorescence intensity. The bar indicates the intensity threshold used to screen the brain library (>2 × 10^1). (**B**) Cos-7 cells transfected with pEGFP-C2 (C) or the plasmids isolated from the human brain library (numbers) were incubated in the presence of TR-Tf. eGFP-expressing cells exhibiting normal, reduced or no uptake were scored. The graph represents the percentage of eGFP-expressing cells showing no uptake. (**C**) Cos-7 cells transfected with plasmids 13 and 24 were incubated with TR-Tf. The fluorescent signals from eGFP and TR-Tf were analyzed by fluorescence microscopy using the appropriate filters. Bar = 10 μm. (**D**) Scheme showing the structure of the eGFP fusion proteins encoded by plasmids 13 and 24.

Table I. Screening features

Library	MT-library (Bejarano and Gonzalez, 1999)	pEGFP-hB (this study)
Source	Mouse	Human brain
Vector	Modified pEGFP-N1	pEGFP-C2
Insert size	<200 bp (N-terminally fused to eGFP)	1200 bp (C-terminally fused to eGFP)
Original number of transfectants	3.4×10^6	3.4×10^6
Selection procedure	Panning with antibody-coated plates	FACS
Plasmids analyzed after selection	48	24
Plasmids inhibiting TR-Tf uptake	11	18

that either mapped in the non-translated regions or were out of frame. Nevertheless, analysis of the polypeptides in frame with the eGFP demonstrated that seven of them contained putative Yxx∅ or LL endocytosis motifs. Other inserts encoded for P-, G- and R-rich peptides, which did not include any obvious consensus sequences (Figure 2B). Mutation of the consensus Y in the putative endocytosis motif of peptide 38 completely abrogated its ability to prevent Tf uptake (Figure 3), thus demonstrating the contribution of this amino acid to the inhibition. Furthermore, a GST fusion protein with peptide 38 specifically pulled down the AP-2 complex from a rat brain extract (Figure 3B). These results suggested that overexpression of peptide 38 prevented CME of Tf by sequestering the adaptor complex via its Yxx∅ motif, and demonstrated the feasibility of a dominant interfering screen in mammalian cells.

Identification of protein fragments whose overexpression inhibits CME of Tf using FACS

Our final goal was to isolate partial cDNAs encoding protein fragments (rather than short peptides) whose overexpression blocked CME. We expected these cDNAs to encode distinct domains of proteins involved in the process. In essence, overexpression of such polypeptides would sequester their binding partners and prevent their interaction with the endogenous functional counterparts, thereby causing an endocytosis defect. To increase the probability of identifying cDNAs encoding domains of proteins involved in CME, we constructed a library of partial cDNAs from human brain with an average insert size of 1.2 kb. The inserts were C-terminally fused to eGFP (Table I). In addition, we improved the method to isolate cells exhibiting high surface TfR expression by using Cy5-labeled Tf and fluorescence-activated cell sorting (FACS) (Figure 4A). In contrast to the panning with antibody-coated plates, this method allowed fine-tuning of the selection stringency. One day after transfection with the human brain library, eGFP-expressing cells that exhibited high surface TfR expression were selected using FACS (Figure 4A) and plasmids were recovered from the isolated cells. The eGFP fusion proteins encoded by 24 individual plasmids were then expressed in Cos-7 cells to monitor the effect on the internalization of TR-Tf by fluorescence microscopy. Expression of 18 of the 24 polypeptides significantly inhibited Tf uptake (Figure 4B and C). Blast analysis of the peptide sequences in frame with eGFP revealed that two of them (13 and 24) showed 100% identity to products of human ORFs. cDNA 13 encoded amino acids 311–335 of the chaperone Hsp90α (NP_005339) and cDNA 24 encoded amino acids 193–520 of a thus far uncharacterized protein (KIAA1026) (Figure 4C and D).

DISCUSSION

The present report describes the establishment of a novel genetic approach in Cos-7 cells that allows fast identification of polypeptides whose overexpression specifically inhibits a certain process. When the polypeptides match protein fragments encoded within defined ORFs, such an approach could guide us to the cellular machinery involved in the process of interest. To our knowledge, this is the first dominant interfering screen successfully performed in mammalian cultured cells. The procedure permitted fast identification of polypeptides that specifically inhibited CME of Tf. Consistent with previous reports (Marks *et al.*, 1996), we found that five of the 11 inserts isolated from the MT-library encoded peptides bearing bona fide or degenerated Yxx∅ motifs that could compete with that present in the cytoplasmic tail of the TfR (Kirchhausen, 2000). Indeed, we demonstrated that one of these peptides bound the AP-2 complex and mutation of the consensus Y completely abolished its ability to inhibit Tf uptake. The LL motifs present in some of the peptides could also participate in the inhibition by titrating out the adaptor complex. However, the LL motif did not seem to contribute to the endocytic defect caused by overexpression of peptide 38, and mutation of the LL to AA in peptide 11 only partially reversed the inhibition (our unpublished data). These results are consistent with the observation that overexpression of peptides bearing LL motifs does not efficiently prevent internalization of Tf (Marks *et al.*, 1996). Finally, some of the peptides included P-rich stretches that could interact with SH3 domains of proteins involved in endocytosis (Kirchhausen, 2000). In fact, a GST fusion protein bearing peptide 5 specifically pulled down amphiphysin from a brain extract (our unpublished data).

Screening of a human brain expression library using the established procedure identified two protein fragments encoded within human ORFs. Considering the selection procedure used, overexpression of the corresponding eGFP fusion proteins did not affect synthesis or biosynthetic transport of the TfR to the plasma membrane. Thus, the endocytic defect installed upon overexpression of the polypeptides seemed to be quite specific. Interestingly, one of the isolated cDNA fragments encoded a peptide present in Hsp90. Hsp90 is an abundant cytosolic chaperone, which is quite restrictive with respect to the substrates it binds. In particular, Hsp90 forms complexes with different proteins involved in signal transduction. In all cases,

the interaction of Hsp90 with the client proteins proved to be important for the signal transducer (Richter and Buchner, 2001). Interestingly, one of these proteins is the Y kinase Src. Src has been involved in controlling clathrin assembly and endocytosis of the EGF receptor in A431 cells (Wilde *et al.*, 1999). Thus, an interesting possibility is that overexpression of peptide 13 disrupts the Src–Hsp90 complex and results in degradation of the kinase, thereby causing an endocytosis defect. The second polypeptide whose overexpression inhibited CME is encoded within an uncharacterized ORF, which was originally identified in a human brain cDNA (Kikuno *et al.*, 1999). This ORF encodes a polypeptide of 519 amino acids (KIAA1026) that shows high probability to form a coiled-coil structure between amino acids 173 and 350 (Lupas, 1996). Interestingly, the coiled-coil domain of the mouse Huntington interacting protein 1 (HipR) was recently implicated in recruitment of Hip1R to clathrin coated vesicles (Engqvist-Goldstein *et al.*, 2001). Detailed analysis of the cell biology and the interacting partners of the KIAA1026 protein would now be required to analyze its putative role in CME.

Other inserts isolated in the second screen did not encoded polypeptides in frame with eGFP that matched products of known ORFs. The cDNA fragments were part of the untranslated regions or were out of frame. Similar to the first screening though, most of the eGFP fusion proteins contained poly(P) stretches or YxxØ and LL motifs that could eventually explain the inhibition.

Our approach is extremely fast when compared with other genetic procedures in mammalian cultured cells. Mere isolation of a stable recessive mutant in CHO cells can last >3 months. However, the isolation and initial characterization of cDNAs using the established method were performed in 1 month. Furthermore, this approach might be applied to a wide variety of cell lines, independently of the stability of their chromosomal setting. For this purpose, the cell line of interest should be stably transfected with the SV40 large T antigen to allow replication of the plasmids. A number of these cell lines are already available at the American Type Culture Collection.

The use of this methodology to identify the machinery involved in a process of interest is only constrained by the establishment of a selection procedure to isolate cells exhibiting the adequate phenotypic defect. In the future, this approach might facilitate the study of specific cellular processes of medical relevance (i.e. apoptosis, development of the immune response in specific cell types, cell differentiation, pathogen invasion) that cannot be addressed in other, more genetically tractable, systems.

METHODS

Antibodies and chemicals. Monoclonal anti-TfR antibody (B3/25), affinity-purified sheep anti-mouse IgG (H+L) and Coated Vesicle Sampler Kit were purchased from Roche, Jackson ImmunoResearch and Becton Dickinson, respectively. Restriction enzymes and Vent DNA polymerase were from New England Biolabs. T4 DNA ligase and Klenow were from Roche, and *Pfu* Turbo from Stratagene. TR-Tf was from Molecular Probes. Cy5 labeling of Tf was performed with a monoreactive dye from Amersham-Pharmacia according to the manufacturer's instructions.

Plasmid and library construction and PCR mutagenesis. Recombinant DNA manipulations were carried out using standard techniques (Sambrook *et al.*, 1989). pGST-38 was generated by cloning the corresponding oligonucleotide in frame after the GST in pGEX-4T-2 (Pharmacia). Single amino acid exchange was carried out by site-directed mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene). Oligonucleotides were synthesized by Interactiva (Ulm, Germany). The eGFP-fused human library was constructed by subcloning 0.3–3 kb inserts of the pMyr human brain cDNA library (Stratagene) into pEGFP-C2 (Clontech).

Cell culture and transfections. Cos-7 cells were obtained from the DSMZ and cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were kept in a humidified 5% CO_2 atmosphere at 37°C. Transient transfection was performed using Lipofectamine 2000. Cells were analyzed 24 h after transfection.

Immunoselection of Cos-7 cells and plasmid recovery. Immunoselection of Cos-7 cells and recovery of plasmids were performed based on Hollenbaugh *et al.* (1994). Approximately 2×10^7 cells were transfected using 6 µg of library DNA and 10 µl of Lipofectamine 2000 per 10 cm plate. One day after transfection, cells were detached with 0.5 mM EDTA, 0.02% NaN_3 in PBS (PBS-EN), harvested, and resuspended in 0.8 ml of 5% calf serum (CS) in PBS-EN (PBS-ENCS) containing 1 µg/ml anti-Tf antibody. After 45 min incubation on ice, 0.8 ml of PBS-EN was added and cells were centrifuged for 4 min at 200 *g* through a 3 ml 2% ficoll gradient. The pellet was resuspended in 1 ml of PBS-EN and aliquots were pipetted through a nylon mesh into 8×6 cm plates coated with anti-mouse IgG antibody containing 3 ml of PBS-ENCS. After 3 h incubation at room temperature (RT), non-adhered cells were washed away with PBS-CS. Remaining cells were lysed with 0.4 ml of 0.6% SDS, 10 mM EDTA and incubated at RT for 20 min. The viscous mixture was pipetted into a microfuge tube and 0.1 ml of 5 M NaCl was added. After overnight incubation at 4°C, genomic DNA and debris were spin down at 14 000 r.p.m. for 4 min. Proteins were extracted with phenol–chloroform, and the DNA was ethanol precipitated in the presence of 20 µg/ml salmon sperm DNA. The pellets were resuspended in 100 µl of TE pH 7.5, pooled and reprecipitated. The final pellet was resuspended in 50 µl of TE pH 7.5 and transformed into *E. coli* by electroporation. Colonies were independently analyzed or harvested in LB to prepare a mini library for further rounds of selection.

Selection of Cos-7 cells expressing the TfR at the cell surface using FACS. Approximately 2×10^7 cells were transfected using 1.5 µg of library DNA and 15 µl of Lipofectamine 2000 per 10 cm plate. One day after transfection, cells were washed with PBS and incubated for 30 min at 37°C in DMEM containing 10 µM deferoxamine mesylate (DFO) to chelate Fe^{3+} from the endogenous Tf. Cells were then washed with PBS pH 7.6 containing 10 µM DFO to dissociate endogenous apo-transferrin and subsequently with PBS pH 7.6 to eliminate traces of DFO. Cells were then detached from the plate by trypsinization and resuspended in PBS pH 7.4 in the presence of 0.1% BSA and 13.2 µg/ml Cy5-Tf. After 15 min incubation on ice, cells were washed with PBS pH 7.4 and resuspended in DMEM containing 0.2% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 5% Cell Dissociation Buffer (Life Technologies). eGFP-expressing cells that exhibited high Cy5-Tf surface labeling were selected

using a FACS Vantage SE (Becton Dickinson) equipped with a Coherent Enterprise II argon laser (448 nm) and a filter with a band pass of 530/30 nm for the detection of eGFP, and a Coherent Unova 70C-SUV spectrum tuned at 648 nm and a filter with a band pass of 675/20 nm for the detection of Cy5.

Tf uptake in Cos-7 cells. Cos-7 cells were grown on R-collagen-coated glass coverslips. Cells were rinsed with PBS and pre-incubated in DMEM without FCS for 30 min. Cells were incubated at 37°C for 10 min in the presence of 20 µg/ml TR-Tf in DMEM containing 0.1% BSA. Cells were washed with cold PBS, fixed with 4% paraformaldehyde in PBS for 20 min at RT and mounted. The effect on CME was determined by scoring cells showing normal, reduced or no uptake of TR-Tf. At least 100 cells per experiment were scored. The averages and standard deviations of cells showing no uptake from three independent experiments were plotted.

GST pull-down. GST pull-downs from total rat brain protein extract were performed as described (Wigge *et al.*, 1997). Briefly, glutathione–Sepharose beads bound to 20 µg of GST or GST fused to the peptides were incubated with 0.5 ml of total rat brain extract (10 mg/ml) for 2.5 h at 4°C. Beads were washed with buffer A (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1% Triton X-100) containing protease inhibitors, and eluted with 6 bed volumes of buffer A containing 250, 350 or 450 mM NaCl. The eluates were pooled and subjected to a trichloroacetic acid precipitation. Precipitated proteins were resuspended in 20 µl of SDS–PAGE sample buffer. Ten microliters of each eluate and 2 µg of the total protein extract were separated by SDS–PAGE (Laemmli, 1970), transferred to nitrocellulose filters and analyzed by immunoblotting using the indicated antibody.

ACKNOWLEDGEMENTS

We thank A. Dautry-Varsat and C. Gonzalez for sending pEGFP-C2-EΔ95/295 and the MT-library, respectively. We thank A. Kehlenbach from the ZMBH cell sorting facility (granted by DFG, SFB52) for technical support, and B. Schwappach for critical reading of the manuscript and scientific advice. This work was supported by a grant from the DFG (SFB 352, C10).

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DOI: 10.1093/embo-reports/kvf131