Analysis of Clathrin-mediated Endocytosis of Epidermal Growth Factor Receptor by RNA Interference*

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To identify proteins that participate in clathrin-mediated endocytosis of the epidermal growth factor receptor (EGFR), 13 endocytic proteins were depleted in HeLa cells using highly efficient small interfering RNAs that were designed using a novel selection algorithm. The effects of small interfering RNAs on the ligand-induced endocytosis of EGFR were compared with those effects on the constitutive internalization of the transferrin receptor. The knock-downs of clathrin heavy chain and dynamin produced maximal inhibitory effects on the internalization of both receptors. Depletion of αa, β2, or μ2 subunits of AP-2 reduced EGF and transferrin internalization rates by 40–60%. Down-regulation of several accessory proteins individually had no effect on endocytosis but caused significant inhibition of EGF and transferrin internalization when the homologous proteins were depleted simultaneously. Surprisingly, knock-down of clathrin-assembly lymphoid myeloid leukemia protein, CALM, did not influence transferrin endocytosis but caused significant inhibition of EGF and AP180 are monomeric proteins. Clathrin lattice serves as an organizing scaffold for the proteins that carry out cargo sorting, membrane invagination, vesicle scission, and uncoating. These accessory molecules include adaptor/scaffold proteins and enzymes, such as dynamin GTPase, kinases, and phosphatases (5). The sequence of events during coated vesicle formation is understood at the morphological level. However, the molecular mechanisms of the individual steps of clathrin-mediated endocytosis (CME) remain to be elucidated.

The endocytosis of the EGF receptor (EGFR) has been one of the most popular experimental systems to study the mechanisms of CME (6). Over the years a number of models have been proposed and numerous proteins have been implicated. However, these models are difficult to reconcile with each other, and the views on the mechanisms of EGFR endocytosis remain controversial. One source of the discrepancies is that the important feature of EGFR endocytosis, the saturability of the specific internalization pathway, is often overlooked (7, 8). In many types of cells, occupancy of the large number of EGFRs leads to their internalization through the slow clathrin-independent pathway because the rapid clathrin-dependent pathway becomes saturated (8, 9). Therefore, to comparatively analyze CME of the EGFR under different experimental conditions, low EGF concentrations and cell lines expressing moderate receptor levels must be used.

Recent progress in application of RNA interference (RNAi) methods in mammalian cells led to important advances in elucidation of the mechanisms of EGFR endocytosis. We first used RNAi to demonstrate an essential role of Grb2 in the CME of EGFR (10). More recently, two groups performed analysis of EGFR endocytosis in cells depleted of clathrin and AP-2 by RNAi (11, 12). Both studies reported no effect of AP-2 depletion on EGFR internalization despite the dramatic reduction in the number of coated pits, while they observed contrasting effects of the clathrin knock-down on EGFR endocytosis. These studies raised the question whether EGFR utilize coated pits for internalization and proposed that, even if they do utilize coated pits, these receptors are internalized through coated pits not containing AP-2.

To assess the importance of clathrin, AP-2, and other endocytic proteins for EGFR endocytosis, we used novel algorithm to generate functional siRNAs to efficiently suppress the ex-
pression of these proteins. This large scale RNAi analysis revealed that clathrin, dynamin, AP-2, Eps15/15R, and Rab5 have general roles in the CME, whereas CALM, similarly to Grb2, is required specifically for EGFR internalization.

EXPERIMENTAL PROCEDURES

Antibodies—The monoclonal antibodies to clathrin heavy chain (TD.1) were obtained from American Type Culture Collection (Manassas, VA); EEA.1, Rab5a, and PP1 were from BD Transduction Laboratories (Los Angeles, CA); Tsg101 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); α-actinin was from Chemicon, Inc.; and GFP was from Zymed Laboratories Inc. (South San Francisco, CA). Polyclonal antibodies to clathrin heavy chain II was from Affinity Bioreagents, Inc. (Golden, CO); rabbit polyclonal antibodies Ab262 specific to β-adaptins and Ab20 to CALM were described previously (13, 14). Polyclonal antibodies to clathrin light chains A and B were kindly provided by Dr. F. Brodsky (University of California at San Francisco), epsin 1 by Dr. L. Traub (University of Pittsburgh), and Eps15 Ab577 and Eps15R Ab860 by Dr. P. P. Di Fiore (European Cancer Institute, Milan, Italy).

siRNA Design and Synthesis—siRNAs were selected using an advanced version of the rational design algorithm that ensures siRNA had less than 16 bp of consecutive homology to untargeted molecules. For each gene, four siRNA duplexes with the highest scores (SMARTscores) were selected, and a BLAST search was conducted (Human EST data bank) to eliminate potential for off-target effects. Only those sequences with more than three mismatches against unrelated genes were selected (15). All duplexes were synthesized in-house as 21-mer with UU overhangs using a modified method of 2′-acetyl labile orthoester chemistry (16), and the anti-sense strand was chemically phosphorylated to ensure maximized activity (17). Grb2 siRNA was described in Ref. 10. Sequences were presented in the “Supplemental Materials.” μ2 siRNA was provided by Dr. M. Marsh (University College, London). siRNA to α-adaptin was synthesized as described previously (11).

Cells and Transfections—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, antibiotics, and glutamine. siRNA duplexes were resuspended in 1× siRNA Universal buffer (Dharmacon, Inc.) to 20 μM prior to transfection. HeLa cells in 12-well plates (50–60% confluent; 1 ml of DMEM/fetal bovine serum per well) were transfected twice with 4 μl of 20 μM siRNA duplex and 3 μl of LipofectAMINE 2000 reagent (Invitrogen) in 100 μl Opti-MEM medium according to manufacturer’s recommendations at 24-h intervals. For transfections, in which two or three siRNA duplexes were included, the amount of each duplex was decreased so that the final siRNA concentration remained constant between experiments. Cells were placed into normal culture medium 12 h prior to experiments, which were performed 3 or 4 days after the initial transfection. Transfections of α-adaptin siRNA were performed using Oligofectamine reagent (Invitrogen) as described previously (11).

Western Blot Analysis—The cells were lysed by scraping from the dish by rubber policeman into Triton X-100/glycerol solubilization buffer, and the lysates were electrophoresed as described previously (14). Western blot analysis was performed with several antibodies followed by detection using enhanced chemiluminescence system. Several x-ray films were analyzed to determine the linear range of the chemiluminescence signals, and the quantifications were performed using densitometry. In experiments with Eps15R-targeted siRNAs, cell lysates were subjected to immunoprecipitation with Ab860, and Eps15R was detected in immunoprecipitates by Western blot as described above.

FEP Fusions—To generate the fusion of YFP with Rab5b or Rab5c (YFP-Rab5b or YFP-Rab5c), a DNA fragment encoding the full-length human Rab5b or Rab5c was obtained by PCR using 5′ flanking sequences (Stratagene) with a SacI restriction site introduced into the 5′ end and a KpnI site into the 3′ end and cloned into pEYFP-C1 vector (Clontech, Palo Alto, CA). GFP-CALM and YFP-Rab5a constructs were described previously (11, 14, 18).

Internalization of 125I-EGF and 125I-Transferrin—Mouse receptor-grade EGF (Collaborative Research Inc.) and iron-saturated human Tf (Sigma) were iodinated as described previously (10). Cells grown in 12-well dishes were incubated with 125I-EGF (1 ng/ml) or 125I-Tf (1 μg/ml) in binding medium (DMEM, 0.1% bovine serum albumin) at 37 °C, and the ratio of internalized and surface radioactivity was determined during linear 5-min time course to calculate specific internalization rate constant k, as described previously (10).

Fluorescence Microscopy—Mock- or siRNA-transfected cells grown on glass coverslips were incubated with 2 ng/ml EGF-rhodamine conjugate (EGF-Rh) (Molecular Probes, Inc.) for 5 min at 37 °C, fixed with paraformaldehyde, and processed for imaging as described previously (10). A Z-stack of 12 two-dimensional images was acquired for each experimental variant under identical settings through Cy3 filter channel and deconvoluted using SlideBook 4.0 software.

RESULTS AND DISCUSSION

Thirteen coated pit and endosomal proteins were chosen to study using siRNA method based on their possible involvement in the CME of EGFR and availability of antibodies. Individual and pools of siRNAs were designed and synthesized using an improved version of the rational design algorithm (19). Briefly, the algorithm was developed by performing a statistical analysis of siRNA (and microRNA) and led to the identification of critical sequence and thermodynamic attributes that are common among functional silencing reagents. siRNA duplexes were transfected (individually or in pools) into HeLa cells, and the level of silencing was quantitated by Western blotting at 2, 3, and 4 days after transfection.

Fig. 1 shows that the majority of siRNAs and siRNA pools...
effectively depleted coated pit proteins by 90–95% of levels observed in mock-transfected cells. In most cases, the extent of knock-down was maximal at 3 days after siRNA transfection. Interestingly, CALM was completely eliminated 2 days after transfection. The effectiveness of siRNAs targeted to CALM was confirmed in experiments, in which these siRNAs completely down-regulated GFP-tagged CALM (data not shown). In three instances (β2-adaptin, Eps15, and Eps15R) maximal silencing effects were observed 4 days following transfection. It was difficult to determine the precise extent of Eps15R depletion, because Eps15R is expressed as several splice variants which can be ubiquitylated, thus running as multiple immunoreactive bands (20, 21).

Fig. 1 also illustrates the effective depletion of four endosomal proteins, Rab5a, Rab5b, Rab5c, and EEA1. For Rab5a, greater than 99% of the protein (and a YFP-tagged version) were eliminated by all four siRNA duplexes. Because antibodies that recognize endogenous Rab5b and Rab5c were not available, YFP-tagged Rab5b and Rab5c were generated and expressed in HeLa cells. The experiments with CALM and Rab5a validated the use of heterologously expressed fusion proteins for testing the efficiency of siRNAs. As was the case with Rab5a, levels of both YFP-Rab5b and YFP-Rab5c were reduced by greater than 99%, as determined using an antibody directed against YFP.

Thus in all cases, with the exception of Eps15R, either a single siRNA or an siRNA pool was available that reduced silencing by >90%, and about 50% of all duplexes reduced protein levels by >95%. These results represent a significant improvement over those obtained using conventional design methodologies (22). More importantly, the high efficiency of gene knock-down achieved with multiple siRNAs enabled us to utilize biochemical assays to examine the functional consequences of protein down-regulation.

One or several siRNAs for each gene were selected to test the functional effects of protein silencing on the internalization rates of two receptors, for Tfn and EGF. Tfn receptor (TfnR) is constitutively endocytosed through clathrin-coated pits. Internalization of EGFR through coated pits is induced by the ligand. Both receptors are endogenously expressed in HeLa cells at moderate levels. The measurements of the uptake rates of 125I-Tfn and 125I-EGF were performed using short time course experiments at 37 °C to avoid contribution of the recycling in the uptake kinetics and using low ligand concentration to avoid saturation of the CME pathway (for EGF).

Fig. 2A shows effects of the depletion of each of 13 proteins individually on Tfn and EGF internalization. The largest reduction of internalization rates of both TfnR and EGFR was caused by knock-down of clathrin heavy chain. The residual uptake (20–25%) mainly consists of a clathrin-independent component and a coated vesicle-mediated endocytosis, presumably, due to a small pool of untransfected cells (<5% based on immunofluorescence analysis; data not shown). During preparation of this manuscript, two groups reported contradictory results of siRNA knock-down of clathrin heavy chain on EGFR internalization (11, 12). Our 125I-EGF internalization data strongly support the essential role of clathrin in EGFR internalization (11). The lack of such role reported by Hinrichsen et al. (12) could be explained by an inefficient depletion of clathrin (80%) and/or their internalization assay that favors measurements of the clathrin-independent endocytosis due to the high concentrations of labeled EGF used. To substantiate this view using an independent endocytosis assay, the effect of clathrin heavy chain siRNA on the uptake of EGF-Rh was analyzed using fluorescence microscopy. In mock-transfected cells EGF-Rh was efficiently accumulated in endosomes after 5 min of continuous endocytosis (Fig. 2B). In contrast, in cells depleted of clathrin, almost no punctate rhodamine fluorescence was observed, indicative of the effective block of endocytosis (Fig. 2B).

The strong inhibitory effect of dynamin II depletion by siRNA on the uptake of 125I-EGF (Fig. 2A) and EGF-Rh (Fig. 2B) supports the data obtained with dominant-negative mutants of dynamin (23) and is consistent with the view that EGFR is internalized through the pathway of CME. Interestingly, for dynamin II and a number of other genes there appears to be a threshold in the extent of protein down-regulation that must be reached to cause observable functional effects. For instance, incomplete depletion of dynamin II by duplex 1 (85% depletion) resulted in a significantly lesser functional consequence (only 30% reduction of internalization rates) than that produced by duplex 2 or a pool of duplexes (94% depletion; >70% rate reduction) (data not shown). These data emphasize that high potency of siRNA is essential to reveal the function of targeted molecules and indicates that complete
protein elimination is a requirement for the correct accession of the gene function.

In contrast to the essential role of clathrin heavy chain, individual (Fig. 2A) or simultaneous down-regulation of clathrin light chains A and B (Fig. 3) did not affect internalization rates. Thus, clathrin light chains do not appear to play an essential role in CME under the conditions of our internalization assays. This observation is consistent with studies using endonuclease interfering RNA that revealed no effect of clathrin light chain A depletion on Tfn endocytosis (24).

Unexpectedly, CALM-targeted siRNA had partial but specific inhibitory effect on EGFR endocytosis (Fig. 2A). The endocytic rates were reduced on average by 45% in six independent experiments with two siRNA duplexes. Because CALM depletion did not affect Tfn endocytosis, this protein is apparently not an essential component of the clathrin-associated endocytic apparatus, a finding that is surprising in light of previous observations that overexpression of the CALM clathrin binding domain blocked endocytosis (14). It is likely that the effect generated by the CALM mutant was the result of interference with other clathrin binding proteins rather than disruption of specific CALM functions. Likewise, the lattice assembly function of CALM/AP180 demonstrated in vitro (4) may be accomplished by other proteins in vivo.

Our previous siRNA studies (10) demonstrated Grb2-mediated mechanism of EGFR endocytosis. As shown in Fig. 2C, Grb2 knock-down specifically reduced the rates of EGFR internalization to the extent comparable with that caused by clathrin depletion, suggesting that Grb2 is responsible for most of the EGFR uptake through the CME pathway. Since the simultaneous depletion of Grb2 and CALM by siRNAs did not produce an additive effect (data not shown), it is unlikely that CALM is involved in Grb2-independent endocytosis. It is possible, however, that CALM participates in one of the mechanisms linking EGFR-Grb2 complex to coated pits.

The depletion of β2-adaptin reduced the endocytosis rates of EGF and Tfn by 50–60% (Fig. 2A). In addition, partial inhibition of EGFR endocytosis in cells transfected with siRNA targeted to the μ2 subunit of AP-2 was observed (25) (Fig. 3B). Thus, our RNAi data are consistent with the general role of AP-2 in CME. This view was, however, challenged by Motley et al. (11) who reported that siRNA knock-down of AP-2 does not affect EGFR internalization and by Conner and Schmid (26) who reported normal EGFR internalization rates in cells where AP-2 was sequestered by overexpressed adaptor-associated kinase 1. The most conceivable explanation of the discrepancies between these studies and our results is that different methods to measure internalization rates were used. The latter studies used a technique of cell preincubation with 125I-EGF at 4°C followed by the chase incubation at 37°C. Long incubation of cells with EGF at 4°C allows effective recruitment of ligand-receptor complexes into coated pits (10) and could be, therefore, sufficient to fully recruit activated EGFR into coated pits remaining in AP-2 depleted cells. In contrast, we measured internalization rates by incubating cells with the labeled ligand directly at 37°C without a 4°C preincubation, a condition that is more physiological and that exposes differences in the rates of the initial steps of internalization.

To provide additional evidence for the role of AP-2 in EGFR endocytosis, α-adaptin siRNA used by Motley et al. (11) was tested in our internalization assays. Fig. 2D shows that under these conditions the depletion of α-adaptin reduced EGFR endocytosis to the extent comparable with that observed in β2-depleted cells (Fig. 2A). In contrast, there was no difference in the internalization rates of EGFR in mock-transfected and siRNA-transfected cells when these rates were measured after 4°C incubation (Fig. 2D). In summary, our data with siRNAs targeted to AP-2 indicate that there is no significant difference in the AP-2 requirement for CME of EGFR and TfnR. The incomplete inhibition of TfnR endocytosis by AP-2 siRNAs in our experiments may be due to a lower threshold of AP-2 depletion that was necessary for the functional effects as compared with this threshold of clathrin or dynamin depletion sufficient for such effects. Similarly to the experiments with clathrin heavy chain and dynamin II siRNAs, the inhibition of EGFR endocytosis by AP-2 depletion tended to be slightly less pronounced than that of TfnR endocytosis. This can be attributed to the ability of EGFR to be internalized through clathrin-independent pathways.

As in the case of clathrin, AP-2, and dynamin, none of siRNAs targeted to several accessory proteins produced EGFR-specific effects. 15–30% reduction of internalization rates of both receptors was observed in cells transfected with Eps15 or Eps15R siRNAs (Fig. 2A). Eps15 and Eps15R have significant sequence homology, identical domain architecture, and similar protein interaction motifs (27). When both Eps15 and Eps15R were depleted by a combination of two siRNAs, the rates of internalization of 125I-Tfn and 125I-EGF were decreased by 40% (Fig. 3), suggesting that these proteins have important but redundant roles in CME. Overexpression of Eps15 fragments containing binding sites for α appendage domain of AP-2 effectively inhibited endocytosis of TfnR and EGFR (28, 29). However, a number of other coated pit proteins interact with the same domain of AP-2 (30). Therefore, the dominant-negative effects of Eps15 mutants did not provide a direct evidence for a specific function of Eps15 in endocytosis, because these effects could be due to interference of the Eps15 mutants with other α appendage interactions.

Depletion of all three highly homologous Rab5 isoforms re-
sulted in a 50% reduction of internalization rates of TfnR and EGFR (Fig. 3), whereas siRNAs targeted to Rab5a, Rab5b, and Rab5c did not affect internalization when transfected individually (Fig. 2A). Based on overexpression of mutants of Rab5 mutants, Rab5a isoform was implicated into the specific regulation of EGFR endocytosis (31). Our RNAi experiments showed that Rab5a is redundant, and the elimination of a combination of three Rab5 isoforms is necessary to reveal the role of Rab5a in CME. On the other hand, knock-down of EEA.1 resulted in a 50% reduction of internalization rates of TfnR and pit. 

function of Rab5 in CME may be mediated by the pools of Rab5 localized and associated with EEA.1 in early endosomes, the function of Rab5s in CME. On the other hand, knock-down of EEA.1 complexes with other effectors.

To summarize, this RNAi analysis has provided new insights in the function of the individual components of clathrin-coated pits in EGFR internalization. Further detailed investigation of the consequences of the depletion of endocytic proteins is necessary to elucidate the precise function of these proteins. This study demonstrates that the specific and efficient gene silencing by siRNA can be used as an approach to functionally analyze the entire cellular machineries, such as the clathrin-coated pit.

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