Recruitment of Epidermal Growth Factor and Transferrin Receptors into Coated Pits In Vitro: Differing Biochemical Requirements

Christophe Lamaze, Takeshi Baba, Thomas E. Redelmeier, and Sandra L. Schmid

Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Submitted April 16, 1993; Accepted May 13, 1993

The biochemical requirements for epidermal growth factor (EGF) and transferrin receptor-mediated endocytosis were compared using perforated human A431 cells. Morphological studies showed that horseradish peroxidase (HRP)-conjugated EGF and gold-labeled anti-transferrin (Tfn) receptor antibodies were colocalized during endocytosis in vitro. The sequestration of both ligands into deeply invaginated coated pits required ATP hydrolysis and cytosolic factors and was inhibited by GTP γ S, indicating mechanistic similarities. Importantly, several differences in the biochemical requirements for sequestration of EGF and Tfn were also detected. These included differing requirements for soluble AP (clathrin assembly protein) complexes, differing cytosolic requirements, and differing sensitivities to the tyrosine kinase inhibitor, genistein. The biochemical differences detected between EGF and Tfn sequestration most likely reflect specific requirements for the recruitment of EGF-receptors (R) into coated pits. This assay provides a novel means to identify the molecular bases for these biochemical distinctions and to elucidate the mechanisms involved in ligand-induced recruitment of EGF-R into coated pits.

INTRODUCTION

Efficient internalization of nutrients, growth hormones, and other biologically important macromolecules into cells occurs via receptor-mediated endocytosis (Smythe and Warren, 1991). High affinity receptors on the cell surface bind ligands and are concentrated into specialized regions of the cell surface, termed coated pits. The coated pits invaginate to sequester the receptor-ligand complexes and then pinch off to form coated vesicles that carry the receptor-ligand complexes into the cell.

Receptors exhibit two types of behaviors with respect to their interaction with coated pits (Trowbridge, 1991). One class of receptors, for example, transferrin receptors (Tfn-R)¹ or low density lipoprotein receptors, are constitutively concentrated in coated pits and are efficiently internalized independent of ligand binding. The localization of this class of receptors to coated pits is deter-

mined by an "internalization" motif contained within the cytoplasmic domain of the receptor (Trowbridge, 1991). These internalization motifs consist of a tyrosine (or aromatic) residue presented in the context of a β -turn (Collawn *et al.*, 1990; Bansal and Gierasch, 1991; Eberle *et al.*, 1991). The second class of receptors, for example, epidermal growth factor receptors (EGF-R), platelet-derived growth factor receptors (PDGF-R), and insulin receptors, are efficiently internalized only upon ligand binding. The signals for ligand-induced recruitment of these receptors into coated pits are less well understood (Trowbridge, 1991). Data for both the EGF-R (Chang *et al.*, 1991) and the insulin receptor (Backer *et al.*, 1992) indicate that multiple β -turn containing motifs might function additively for efficient internalization. In addition, results for the insulin receptor (McClain *et al.*, 1987) and the PDGF-R (Sorkin *et al.*, 1991) indicate that the intrinsic tyrosine kinase activity of these receptors may also be required for their efficient internalization. Whether the EGF-R tyrosine kinase activity is also required for high efficiency endocytosis remains controversial (Glenney *et al.*, 1988; Felder *et al.*, 1990, 1992; Wiley *et al.*, 1991).

¹ Abbreviations used: AP, clathrin assembly or adaptor protein complex; MesNa, β -mercaptoethane sulfonate, sodium salt; Tfn, transferrin.

The concentration of both classes of receptors into coated pits presumably involves specific interactions between receptor tails and coat constituents (Robinson, 1992). However, many basic questions regarding the mechanisms responsible for recruitment of either class of receptors into coated pits remain. For example, do receptor tails recruit coat proteins to initiate coated pit assembly or do receptors migrate into assembled coated pits? Are other factors, not incorporated into coated pits or coated vesicles, required for receptor recruitment? What role do the clathrin-associated proteins (APs), also referred to as assembly or adaptor proteins, play in receptor recruitment? Do mechanistic differences, in addition to the requirement for receptor occupancy, exist between ligand-induced and constitutive receptor-mediated endocytosis?

To begin to address these important mechanistic issues, we have developed a cell-free assay system that faithfully reconstitutes early and late events in coated vesicle formation. This system uses perforated human A431 cells that are prepared by scraping them from their substratum so as to create large gaps in the cell surface rendering them fully accessible to exogenously added reagents. Assays based on the acquisition of inaccessibility to large and small probes have been developed that detect both the sequestration of receptor-bound ligand into deeply invaginated coated pits and its internalization into sealed coated vesicles. Extensive biochemical and morphological characterization of the constitutive receptor-mediated endocytosis of Tfn in perforated A431 cells have supported the physiological validity of this model system. Studies using this system have delineated four biochemically distinct events leading to coated vesicle formation (Smythe *et al.*, 1989, 1992a; Schmid and Smythe, 1991; Carter *et al.*, 1993; Schmid, 1993). These sequential events are: "priming," which involves AP binding to as yet unidentified membrane binding sites (Mahaffey *et al.*, 1990; Smythe *et al.*, 1992a; Peeler *et al.*, 1993); "coated pit assembly," which involves the recruitment of cytosolic clathrin to membrane-bound APs; "coated pit invagination," leading to the sequestration of bound ligand from bulky probes; and "coated vesicle budding," which requires a membrane fission event and leads to the internalization of bound ligand.

Here we use this assay system to measure EGF sequestration and compare the biochemical requirements for ligand-induced versus constitutive receptor-mediated endocytosis. Not unexpectedly, we show morphologically that EGF and Tfn are internalized via the same pathway in perforated A431 cells and biochemically that these processes share many requirements. Importantly, we have also identified several biochemical characteristics that distinguish endocytosis via these two classes of receptors. Differing cytosolic requirements for ligand-induced versus constitutive endocytosis provide the basis for identifying specific factors required for

EGF-R internalization. Characterization of these biochemical differences has begun to provide new insight into the mechanisms of ligand-induced recruitment of receptors into coated pits.

MATERIALS AND METHODS

Cell Lines and Reagents

A431 cells were cultured as previously described (Schmid and Smythe, 1991). Nucleotides and analogues were obtained either from Boehringer-Mannheim Biochemicals (Indianapolis, IN) or from Sigma Chemical (St. Louis, MO) and used as already described (Carter and Schmid, 1990). Tyrphostins, erbstatin, herbimycin A, and staurosporin were provided by Calbiochem (La Jolla, CA), and genistein and lavendustin A were from GIBCO (Gaithersburg, MD).

Bovine brain and K562 cytosols were prepared as described (Schmid and Smythe, 1991). Gel-filtered cytosol was obtained from freshly prepared bovine brain or K562 cytosol by chromatography on a Sephadex G25 column equilibrated with KSHM (100 mM potassium acetate, 85 mM sucrose, 20 mM Hepes [N-2 hydroxyethyl]piperazine-N'-2-ethane sulfonic acid), 1 mM MgOAc pH 7.4). Cytosols were stored as aliquots at -70°C after rapid freezing in liquid nitrogen. Bovine brain APs were prepared exactly as described by Smythe *et al.* (1992a).

Preparation of Enzyme-linked Immunoabsorbent Assay (ELISA) Plates

ELISA plates to detect transferrin were prepared as described elsewhere (Smythe *et al.*, 1992b; Carter *et al.*, 1993). ELISA plates to detect EGF were prepared following the same procedure except that rabbit anti-mouse EGF IgG (Genzyme, Boston, MA) was plated onto Maxisorb Immuno-module strips (Nunc Interlab, Thousand Oaks, CA) at a 1/500 dilution in 50 mM Na_2HCO_3 , pH 9.6. Plates were then treated as for transferrin plates.

Assays for Ligand Sequestration and Internalization

Perforated A431 cells were prepared as described (Smythe *et al.*, 1992b). Receptor grade mouse EGF (Upstate Biotechnology, Lake Placid, NY) was biotinylated via a cleavable disulphide bond using NHS-SS-biotin (Pierce Chemical, Rockford, IL). Two microliters of NHS-SS-biotin (164 mM in dimethyl sulfoxide [DMSO]), corresponding to a 20-fold molar excess, were added to 100 μl of 1 mg/ml EGF in phosphate-buffered saline (PBS) every 20 min during a 60-min incubation at room temperature. The reaction was quenched at 80 min by 2.5 μl of 1 M NH_4Cl . Unconjugated NH-SS-biotin was removed by gel filtration on a Sephadex G-10 spin-desalt column equilibrated in PBS. Conjugated EGF (BSS-EGF) was then resolved from free EGF using an immobilized monomeric avidin column (Pierce Chemical) following the manufacturer's instructions. The biotin used to elute BSS-EGF from the column was removed by a G-10 spin-desalt step. BSS-EGF (20 $\mu\text{g}/\text{ml}$) was aliquoted and stored at -70°C . Commercially available biotin-EGF (Boehringer Mannheim Biochemicals) was used when the cleavage of the disulphide bond (i.e., the internalization step) was not studied. EGF was used at a final concentration of 10 ng/ml (1.6 nM) in the assay.

The internalization and sequestration assays were performed as described in Carter *et al.* (1993) except that both BSST (Biotin-S-transferrin) and BSS-EGF were added to the same reaction. After processing for avidin inaccessibility or β -mercaptoethane sulfonic acid (MesNa) resistance as previously described (Smythe *et al.*, 1992b; Carter *et al.*, 1993), the cells were lysed in a final volume of $\sim 210 \mu\text{l}$. One hundred-microliter aliquots of the cell lysate were plated into microtiter wells containing 0.1 ml blocking buffer and coated with either anti-Tfn or anti-EGF antibodies. The plates were then processed as described (Carter *et al.*, 1993). All assays were performed in duplicate

the results from which differed by <5%. Backgrounds resulting from incubations performed in the absence of ATP and cytosol, perhaps due to intact cells in the preparation, accounted for <10% of the total signals and were subtracted from the results.

Morphological Characterization of Tfn-R and EGF-R Internalization in Perforated A431 Cells

As a morphological marker of Tfn-R, the monoclonal antibody (mAb) HTR-D65 (kindly provided by Suhaila White and Ian Trowbridge, The Salk Institute, La Jolla, CA) was conjugated with 10 nm colloidal gold (Amersham, Arlington Heights, IL) as previously described (Schmid and Smythe, 1991). Horseradish peroxidase (HRP)-conjugated mouse EGF (EGF-HRP) was kindly provided by Steven Wiley (University of Utah) and was prepared as previously described (Wiley *et al.*, 1991). Incubations for morphological studies were exactly as described for biochemical studies except that they were scaled-up fivefold (i.e., 200 μ l total volume, 1×10^6 cells/incubation), and the cells were incubated with both HTR-D65-gold and EGF-HRP. After incubation, the cells were returned to ice; pelleted to remove unbound HTR-D65-gold and EGF-HRP; and overlaid with 2.5% glutaraldehyde, 7% sucrose in 0.1 M cacodylate buffer, pH 7.2, for 30 min at 4°C. Pellets were washed twice with 0.1 M cacodylate buffer, 7% sucrose, and once with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.6. To detect HRP-containing structures, the pellets were incubated with 0.5 mg/ml 3,3-diaminobenzidine (Sigma) for 30 min at room temperature without hydrogen peroxide, followed by a 20-min incubation in this solution with 0.01% hydrogen peroxide. The incubated pellets were washed twice with Tris and fixed again with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The pellets were postfixed with 1% OsO₄ in 0.1 M cacodylate buffer, embedded in epon, and sectioned for electron microscopy by Dr. Cheng-Ming Chang of The Scripps Research Institute electron microscopy laboratory. The ultrathin sections were observed without stain under a Philips (The Netherlands) CM12 electron microscope at 100 kV. Quantification of gold and/or HRP-labeled structures was performed at the microscope by random examination of complete perforated cell profiles at a magnification of 8500.

RESULTS

Receptor-mediated Endocytosis of EGF in Intact A431 Cells

Assays previously developed for measuring the sequestration of Tfn into deeply invaginated coated pits and its internalization into sealed coated vesicles were adapted to measure events leading to receptor-mediated endocytosis of EGF. For these assays EGF was biotinylated via a cleavable disulfide bond (biotin-S-S-EGF or BSS-EGF). As has been established for BSST (Schmid and Smythe, 1991; Smythe *et al.*, 1992a; Carter *et al.*, 1993), when sequestered into deeply invaginated coated pits, BSS-EGF should become inaccessible to the large probe, avidin, while remaining accessible to the small membrane-impermeant reducing agent, MesNa. The internalization of BSS-EGF into sealed coated vesicles should render it both inaccessible to avidin and resistant to reduction by MesNa. BSS-EGF that remains unmasked by avidin or unreduced by MesNa can be quantitated using streptavidin-HRP after capture on ELISA plates coated with anti-EGF antibodies. Perforated A431 cells were incubated at 37°C in the presence of cytosol, an ATP-regenerating system, and 1.6 nM

BSS-EGF. Subsaturating concentrations of ligand were used to ensure efficient ligand-induced internalization (Wiley, 1988; Wiley *et al.*, 1991; Felder *et al.*, 1992). The data in Figure 1 show, as expected, that these two assays gave identical results when used to quantitate receptor-mediated endocytosis of BSS-EGF in intact A431 cells. Thus, in unperturbed intact cells, invaginated coated pits quantitatively bud to form coated vesicles carrying occupied EGF receptors into the cell.

Receptor-mediated Endocytosis of EGF in Perforated A431 Cells

A different result was obtained when perforated A431 cells were allowed to internalize BSS-EGF in vitro. Perforated cells were prepared by scraping adherent A431 cells from dishes so as to leave behind small areas of the plasma membrane. These otherwise intact cells were washed free of cytosol by low-speed centrifugation and

Figure 1. Receptor-mediated endocytosis of EGF in intact A431 cells. A431 cells grown to confluence on a 6-cm dish were dissociated for 5 min at 37°C in PBS containing 5 mM EDTA. Cells were harvested, washed in 10 ml PBS, 1 mM MgCl₂, and pelleted (1100 $\times$ g for 5 min). The cells were then resuspended in 1 ml of the assay mixture (PBS, 1 mM MgCl₂, 0.2% bovine serum albumin and 10 ng/ml BSS-EGF). Forty microliters of this assay mixture were incubated for the indicated times at 37°C before returning to ice, and analysis for either avidin inaccessibility (○) or MesNa resistance (●) was performed as described in MATERIALS AND METHODS. For this experiment, the A₄₉₀ obtained after a 2-min incubation to detect streptavidin-HRP was 0.484 and 0.455 for total cell associated BSS-EGF and BSST, respectively. The values obtained for avidin-inaccessible and MesNa-resistant ligands, respectively, were 0.173 and 0.049 for BSS-EGF and 0.230 and 0.122 for BSST. The backgrounds obtained for the MesNa resistance assay after a 30-min incubation on ice were 0.029 and 0.034 for BSS-EGF and BSST, respectively, representing signal:noise ratios for Tfn internalization of ~4. The backgrounds obtained for avidin inaccessibility were 0.023 and 0.040 for BSS-EGF and BSST, respectively, representing signal:noise ratios of >6 for both BSST and BSS-EGF. The data for this and all subsequent experiments are expressed as the percentage of total cell-associated ligand inaccessible to avidin or MesNa after subtraction of background as described in MATERIALS AND METHODS.

remained totally accessible to exogenously added reagents such as cytosolic proteins, peptides, or antibodies (Smythe *et al.*, 1992b). When perforated A431 cells were incubated at 37°C in the presence of cytosol, an ATP-regenerating system, and BSS-EGF, the ligand became efficiently sequestered into deeply invaginated coated pits (Figure 2A, ○). The acquisition of avidin inaccessibility occurred at >75% of the rate and extent of EGF sequestration seen in vivo (compare with Figure 1). However, despite very efficient sequestration, internalization of BSS-EGF into MesNa-resistant compartments was barely detected over ATP-independent backgrounds (Figure 2A, ●). This was not because of a lack of coated vesicle formation in this cell preparation, because significant internalization could be detected using biotinylated-transferrin (BSST) as ligand (Figure 2B, ●). These results are consistent with our previous findings that Tfn internalization in vitro results from the budding of previously formed coated pits (Smythe *et al.*, 1989, 1992a; Schmid and Smythe, 1991; Carter *et al.*, 1993). Because Tfn-R are constitutively internalized, Tfn can bind to unoccupied receptors that populate these late-stage intermediates of coated vesicle formation. Efficient EGF-receptor internalization occurs only in the presence of ligand (Wiley *et al.*, 1991) and so unoccupied EGF-receptors would, in general, not populate the preformed coated pits capable of budding. The low efficiency internalization of BSS-EGF observed was consistent with the low basal rate of internalization of unoccupied EGF-R (van't Hof *et al.*, 1989; Wiley *et al.*, 1991). These results suggest that the sequestration of EGF measured by avidin inaccessibility principally detects early events in coated vesicle formation.

Sequestration of EGF Requires ATP and Cytosol

To further characterize events involved in the efficient sequestration of EGF into perforated cells, ATP and cytosolic requirements were compared with those for Tfn sequestration. For these and most of the experiments that follow, perforated A431 cells were incubated with or without cytosol or ATP, as indicated, in the simultaneous presence of both BSST and B-EGF (biotin-EGF, a stable noncleavable biotinylated-EGF was used because the acquisition of MesNa resistance was insignificant). After incubation at 37°C and processing at 4°C for avidin inaccessibility as usual, one-half the cell lysate was applied to ELISA plates coated with anti-EGF antibodies, and one-half was applied to ELISA plates coated with anti-Tfn antibodies. In this way the ATP and cytosolic requirements, as well as other biochemical properties, could be directly compared in identical incubations in the same perforated cells. Control experiments showed that neither ligand affected the internalization of the other. The data in Figure 3 (■) shows, as previously observed (Schmid and Smythe, 1991), that sequestration of BSST was markedly stimulated by the

Figure 2. Receptor-mediated endocytosis of EGF in perforated A431 cells. Perforated A431 cells prepared as described in MATERIALS AND METHODS were incubated for the indicated times at 37°C in 40 μ l KSHM containing K562 cell cytosol (6 mg/ml), an ATP-regenerating system, 10 ng/ml BSS-EGF (A) and 2 μ g/ml BSST (B). Cells were then returned to ice and analyzed for either avidin inaccessibility (○) or MesNa resistance (●) as described in MATERIALS AND METHODS. The ATP and cytosol-independent backgrounds subtracted were obtained from cells incubated at 37°C for 30 min in presence of an ATP-depleting system and in absence of cytosol.

addition of ATP and cytosol. However, reproducible ATP and cytosol-independent signals were obtained, presumably reflecting invagination of a subpopulation of coated pits at later stages of assembly. In contrast, the sequestration of EGF in vitro was strongly dependent on addition of ATP and cytosol (Figure 3, ▨) consistent with the expectation that unoccupied EGF-receptors would not be present in coated pits at the later stages of assembly. These results indicated that the sequestration of EGF added in vitro was dependent on ATP and cytosol-dependent events early in coated vesicle formation.

Energy Requirements for EGF and Tfn Sequestration

We had previously demonstrated at least two ATP-requiring steps for receptor-mediated endocytosis of BSST

Figure 3. Comparison of ATP and cytosolic requirements for EGF and Tfn sequestration. Incubations of perforated A431 cells were for 30 min at 37°C in the presence or absence of K562 cytosol (6 mg/ml) and in the presence of either an ATP-regenerating or ATP-depleting system as indicated. Sequestration of ligand was assessed by avidin-inaccessibility for BSST (■) and B-EGF (▨). The backgrounds obtained after a 30-min incubation on ice have been subtracted and the results shown are the average ($\pm$ SD) of 15 independent experiments utilizing at least five different preparations of K562 cytosol.

in perforated cells (Schmid and Smythe, 1991). The data in Table 1 show that ATP hydrolysis is absolutely required for both early and late events in coated vesicle formation. The nonhydrolyzable analogue, ATP γ S, did not support either transferrin sequestration and internalization or EGF sequestration. When added in the presence of ATP, ATP γ S completely inhibited these events. The nonhydrolyzable analogue, AMPPNP, only weakly supported and partially inhibited each of these events. The data in Table 1 also show that none of the events leading to ligand sequestration or internalization was affected by the presence or absence of Ca²⁺. Control levels of ligand internalization and sequestration were obtained in the presence of 5 mM ethylene glycol-bis(aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and in the presence of 75 μ M CaCl₂. Higher concentrations of Ca²⁺ were slightly inhibitory. These results show that EGF and Tfn sequestration share several biochemical requirements, presumably reflecting common mechanisms for internalization.

GTP γ S Effectively Inhibits EGF Sequestration

We have previously shown by the differential effects of inhibitors that multiple GTP-binding proteins participate at distinct stages of coated vesicle formation in perforated A431 cells (Carter *et al.*, 1993). For example, the nonhydrolyzable analogue of GTP, GTP γ S, inhibited early during coat assembly and late during coated vesicle budding but not at intermediate stages involving coated pit invagination. As a result, the sequestration of BSST from avidin, which detects early, intermediate,

Table 1. ATP and calcium requirements for endocytosis of EGF and Tfn in perforated A431 cells

Additions to standard assay ^a	Transferrin		EGF
	Avidin signal % control	MesNa signal % control	Avidin signal % control
None	100	100	100
1 mM ATP γ S, no ATP	0	0	0
1 mM AMPPNP, no ATP	29	24	35
2 mM ATP γ S + ATP ^b	0	0	0
2 mM AMPPNP + ATP ^b	65	80	64
5 mM EGTA	100	102	103
75 μ M CaCl ₂	105	101	95
500 μ M CaCl ₂	76	88	67

^a Standard assay is performed with 800 μ M ATP and 5–6 mg/ml cytosol.

^b Standard assay conditions except ATP concentration is 250 μ M.

and late events in coated vesicle formation, could only be partially inhibited by GTP γ S. An example of this is shown in Figure 4 (○). When perforated A431 cells were incubated under standard assay conditions in the presence of increasing concentrations of GTP γ S, the extent of inhibition of BSST sequestration and internalization plateaued at $\sim$ 60%. In contrast, under identical conditions the sequestration of B-EGF from avidin was completely inhibited (>90% inhibition was obtained

Figure 4. GTP γ S effectively inhibits EGF sequestration. Perforated A431 cells were incubated for 30 min at 37°C as described in Figure 2 except that K562 cytosol (6 mg/ml) used was gel-filtered as described in MATERIALS AND METHODS. The data show the effect of increasing concentrations of GTP γ S on EGF sequestration (●) and Tfn sequestration (○). Also indicated is the level of protection when 1mM GTP was added to incubations containing 100 μ M GTP γ S.

with half-maximal inhibition occurring at $\sim 10 \mu\text{M}$ GTP γS). For each ligand, inhibition was prevented by inclusion of 1 mM GTP in the reaction mixture. These results parallel the effect of GTP γS on the early events in receptor-mediated endocytosis of Tfn (Carter *et al.*, 1993) and suggest that the recruitment of EGF-receptors into newly formed coated pits also requires GTP-binding proteins.

APs do not Stimulate EGF Sequestration

Results presented thus far are consistent with the interpretation that EGF sequestration *in vitro* is dependent on early events in coated vesicle formation. These events are ATP and cytosol dependent and GTP γS sensitive, as has been observed for the early events leading to Tfn sequestration (Schmid and Smythe, 1991; Smythe *et al.*, 1992a; Carter *et al.*, 1993). We have previously shown that coat assembly, the earliest event in coated vesicle formation, can be selectively measured by altering incubation conditions so as to take advantage of the stoichiometric requirement for coat proteins. Thus, when assays are performed at low cytosol concentrations, AP complexes become limiting. Supplementing these incubations with purified APs stimulates the sequestration of BSST into deeply invaginated coated pits. AP-stimulated sequestration of BSST provides an indirect measure of coated pit assembly and requires cytosolic clathrin and ATP (Smythe *et al.*, 1992a). We next examined the effect of APs on EGF sequestration. If EGF sequestration requires *de novo* coated pit assembly triggered by newly occupied receptors, then this reaction should be strongly dependent on APs. Surprisingly, the data in Figure 5 show that although BSST sequestration was markedly stimulated by APs, EGF sequestration was unaffected. This was true at all concentrations of either K562 or bovine brain cytosol (see below) and under all conditions tested. This result suggests either that EGF induces the rapid and efficient migration of occupied EGF-R into preformed coated pits before invagination or that APs are not required for EGF-R internalization. A trivial explanation for this latter possibility was that EGF sequestration occurs by a distinct pathway independent of clathrin-coated pits. It has, for example, been reported that EGF-R internalization into A431 cells can occur via smooth vesicles (Hopkins *et al.*, 1985; van't Hof *et al.*, 1989).

Internalization of EGF and Tfn Occurs via a Common Pathway in Perforated A431 Cells

Double-label ultrastructural studies were performed to determine whether EGF and Tfn were internalized via the same pathway in perforated A431 cells. Tfn-R internalization was followed using a gold-conjugated mAb, HTR-D65, directed against the extracellular domain of the Tfn-R. Previous studies had demonstrated that internalization of HTR-D65 was quantitatively and

Figure 5. Clathrin assembly protein complexes stimulate Tfn but not EGF sequestration. Perforated cells were incubated in a complete assay mixture except that K562 cell cytosol was at a limiting concentration (0.75 mg/ml). Increasing amounts of purified APs were added to the assay mixture that was incubated for 30 min at 37°C before returning to ice. The cells were then treated as indicated in Figure 2 to determine EGF sequestration (●) and Tfn sequestration (○).

qualitatively identical to Tfn (Schmid and Smythe, 1991). HRP-conjugated mouse EGF was used to follow internalization via the EGF-R. This ligand, generously provided by Steven Wiley (University of Utah), consisted of chromatographically purified monomeric mouse EGF conjugated to HRP (Wiley *et al.*, 1991). Its binding and internalization properties were tested and were indistinguishable from native EGF (see also Wiley *et al.*, 1991). Perforated A431 cells were incubated with gold-conjugated HTR-D65 and EGF-HRP (25 ng/ml) in the presence or absence of ATP and cytosol under standard reaction conditions except they were scaled up fivefold. Parallel biochemical analyses of BSST and B-EGF internalization were performed using the same cell preparation. The efficient ATP and cytosol-dependent sequestration achieved for both ligands is shown in Figure 7A.

The micrographs in Figure 6 show that HRP-EGF and gold-labeled anti-Tfn-R mAbs are predominantly located in the same endocytic structures under all incubation conditions. Because coat structures were difficult to detect on these unstained micrographs, the presence or absence of coats was not used as a criteria for identifying endocytic vesicles. The endocytic structures observed were placed in two categories for the quantitation shown in Figure 7: shallow pits (Figure 6e) and deeply invaginated pits (Figure 6, f and g and both labeled structure in h) or free vesicles (Figure 6, i and j). These latter two categories were grouped because it was impossible to unambiguously assign vesicles and deeply invaginated pits (see for example, Figure 6, h and i). The data in Figure 7 show that the percentage of double-labeled structures decreased slightly after incubation in

Figure 6. Double-label electron microscopic analysis of EGF and Tfn internalization into perforated A431 cells. Perforated cells were incubated with HRP-conjugated EGF (25 ng/ml) and gold-conjugated anti-Tfn-R antibody, HTR-D65, for 30 min at 37°C in the presence or absence of cytosol with or without ATP. After incubation the cells were fixed and processed for thin-section electron microscopy as described in MATERIALS AND METHODS. The sections were not stained so as to enhance detection of the HRP reaction product. (a-d) Low magnification views of perforated cells incubated under the indicated conditions. Bar, 1 μ m. (a) Incubation in the absence of ATP and cytosol. Arrows indicate clustered HTR-D65-gold particles at the cell surface that is uniformly labeled with the EGF-HRP reaction product. (b) Incubation with ATP in the absence of cytosol. Arrows indicate clustered HTR-D65-gold at the cell surface. Arrowheads indicate deeply invaginated coated pits containing both labels. (c) Complete incubation in the presence of ATP and cytosol. Arrowheads indicate that the EGF-HRP reaction product is lost from the cell surface. Arrows indicate endocytic structures labeled with HTR-D65-gold and detectable EGF-HRP reaction product. (d) Intact cell incubated in the absence of ATP and cytosol. Arrows indicate endocytic structures strongly labeled with HTR-D65-gold and HRP-EGF. (e-j) show high magnification images of double-labeled structures classified for purposes of quantitation as indicated. Bar, 0.1 μ m. (e) A shallow coated pit. (f-h) Deeply invaginated coated pits. (i and j) Endocytic vesicle profiles and/or deeply invaginated pits. The arrow in f indicates an invaginated pit that appears inaccessible to the clustered HTR-D65-gold. The arrowheads in i show the cell surface.

Figure 7. Quantitation of double-label electron microscopic analysis of endocytic structures involved in EGF and Tfn sequestration into perforated A431 cells. (A) The data shown represent the biochemical analysis of EGF and Tfn sequestration done in parallel to the morphological analysis shown in Figure 6. The extent of EGF sequestration (■) and Tfn sequestration (▨) under various assay conditions as

the presence of ATP (>60% of EGF-containing structures contained Tfn-R and vice versa) as compared with incubations in the absence of ATP and cytosol (~80% of EGF-containing structures contained Tfn-R and vice versa). The decrease in colabeling could, in part, reflect the more efficient ATP and cytosol-dependent internalization of HTR-D65-gold as compared with HRP-EGF. In addition, slower binding rates and reduced accessibility of the large gold-conjugated HTR-D65 as compared with the smaller HRP-conjugated EGF could result in exclusion of gold label from deeply invaginated pits that form rapidly in the presence of ATP and cytosol (see for example the accumulation of larger gold structures outside the invaginated pits in Figure 6, f and g).

Morphological examination confirmed that efficient sequestration of EGF and Tfn required ATP and cytosol. After incubation in the absence of ATP and cytosol, most of the double-labeled structures located at the cell surface were in shallow coated pits (>65%) (Figure 7C). Addition of ATP in the absence of cytosol resulted in a redistribution of ~50% of the double-labeled structures to deeply invaginated pits and/or vesicles, still principally located near the cell periphery (Figure 6b, arrows). Much of the HRP-EGF remained uniformly distributed on the cell surface, consistent with our results that recruitment of EGF-R to coated pits was strongly cytosol-dependent (see also below). Incubation in the presence of both ATP and cytosol resulted in the concomitant loss of disperse surface HRP-EGF labeling (Figure 6c, arrows) and redistribution of ~85% of double-labeled structures to deeply invaginated pits and/or vesicular profiles (Figure 7C).

The endocytic structures seen in perforated cells were identical to those observed in the occasional intact A431 cell distinguishable in our preparations by their cytosolic contents. However, internalization in these intact cells was independent of ATP and cytosol (Figure 6d). The use of HRP-activity to detect EGF in these double-label experiments precludes any quantitative conclusions regarding the redistribution of EGF compared with Tfn under the various incubation conditions. However, a

determined by inaccessibility of ligands to avidin is shown. (B) The data shown by the solid bars is the percentage of EGF-containing endocytic structures (detected by the diaminobenzidine reaction product) that also contained Tfn-R (detected by gold-labeled anti-Tfn-R antibody). The number of EGF-containing endocytic structures counted was 136, 163, and 114 for -/-, +/-, and ++ incubation conditions, respectively. The data shown by the striped bars are the percentage of Tfn-containing endocytic structures that also contained EGF. The number of Tfn-containing endocytic structures counted was 118, 182, and 118 for -/-, +/-, and ++ incubation conditions, respectively. Quantification of gold- and/or HRP-labeled endocytic structures was performed at the microscope by random examination of complete perforated cell profiles at a magnification of 8500. (C) The data shown are the percentage of total labeled endocytic structures under each incubation condition that were classified as described in Figure 6 and text as either shallow pits (■) or deeply invaginated pits and/or vesicles (▨).

qualitative comparison of the density of the HRP reaction in internalized structures observed in intact cells with those seen in perforated cells (Figure 6d versus Figure 6c, arrows) is consistent with our biochemical finding of inefficient internalization of EGF in vitro. The ATP and cytosol-dependent shift in labeled endocytic structures from originally shallow pits to deeply invaginated pits and/or vesicles mirrors results from the biochemical assays and supports the validity of these measurements for coated pit invagination and vesicle budding. Given these morphological results, the inability to stimulate EGF sequestration with AP complexes suggests that EGF binding induces the rapid recruitment of occupied receptors into preexisting coated pits.

Distinct Biochemical Requirements for EGF and Tfn Sequestration

Earlier studies in intact A431 cells had suggested that a component or components of the endocytic apparatus specifically required for ligand-induced EGF-R internalization might become limiting in cells expressing high levels of EGF receptors. At saturating ligand concentrations, EGF internalization into intact A431 cells was impaired, whereas Tfn internalization was unaffected (Wiley, 1988). Reduced EGF-R internalization rates at high EGF concentrations have also been observed in stably transformed cell lines expressing EGF-R (Wiley *et al.*, 1991; Felder *et al.*, 1992). We sought direct evidence for such a limiting cytosolic component by comparing the cytosolic requirements for Tfn and EGF sequestration in vitro. The data in Figure 8 show that Tfn sequestration was supported equally by cytosolic fractions derived from human K562 erythroleukemic cells or from bovine brain (□, ■, respectively). Half-maximal activity required ~1.5 mg/ml cytosol, whereas maximal activity was obtained in the presence of ~3 mg/ml cytosol from either source. In contrast, EGF sequestration in the same incubation required twofold more K562 cytosol for half-maximal and maximal sequestration (Figure 8, △). Bovine brain cytosol was four to fivefold less effective at supporting EGF sequestration as compared with Tfn sequestration and appeared to plateau at ~50% maximal activity. Cytosolic fractions obtained from HeLa cells and A431 cells were also tested but were less active than K562 cell cytosol. These results were consistent with the interpretation that a cytosolic component or components specifically required for EGF internalization was limiting and further showed that its concentration or activity may vary in cytosol obtained from different sources.

A Role for Tyrosine-Kinase Activity in EGF Internalization

Results from mutagenesis studies on a number of tyrosine kinase receptors including those for EGF (Wiley

Figure 8. Distinct cytosolic requirements for EGF and Tfn sequestration. Perforated cells are incubated as described in MATERIALS AND METHODS in the presence of increasing concentrations of either bovine brain cytosol (■, ▲) or K562 cytosol (□, △). The cells were treated as previously described to quantitate EGF sequestration (▲, △) or Tfn sequestration (■, □).

et al., 1991), PDGF (Sorkin *et al.*, 1991), and insulin (McLain *et al.*, 1987) have indicated that their intrinsic tyrosine kinase activity is required for efficient internalization of the receptor-ligand complex. Others have suggested that the tyrosine kinase activity of the EGF-receptor is not required for internalization but instead serves as a sorting signal for targeting receptors to the lysosomes (Felder *et al.*, 1990, 1992). We have examined the effect of tyrosine kinase inhibitors on EGF and Tfn internalization in intact cells and on sequestration in perforated A431 cells. The data in Figure 9 show that the internalization of EGF and Tfn into intact A431 cells was differentially affected by the presence of the tyrosine kinase inhibitor genistein. EGF internalization was completely inhibited in the presence of 100 μ M genistein (Figure 9A), whereas Tfn internalization was inhibited by <50% (Figure 9B).

The effect of genistein on EGF and Tfn sequestration was next examined in perforated cells. Because genistein is competitive with respect to ATP, in vitro assays were performed in the presence of 250 μ M added ATP (fourfold less than normal levels) using perforated cells prepared from untreated A431 cells. Reduction of ATP to this level had no effect on the efficiencies of EGF or Tfn sequestration. The extent of Tfn and EGF sequestration after a 30-min incubation in the presence of reduced ATP, cytosol, both ligands, and increasing concentrations of genistein is shown in Figure 10. As was observed in intact cells, genistein differentially inhibited EGF sequestration (●) over Tfn sequestration (○) in perforated cells. The differential effect was most dramatic at lower concentrations of genistein where it would be expected to be more selective for tyrosine kinases over other ki-

Figure 9. Effect of genistein on EGF and Tfn internalization in intact A431 cells. Intact A431 cells were prepared exactly as described in Figure 1 and incubated at 37°C for the indicated times in the presence (○, □) or absence (●, ■) of 100 μ M genistein. After return to ice, the cells were processed for avidin-inaccessibility to determine the extent of internalization of EGF (A) or Tfn (B). Preincubation of intact cells with genistein for 40 min had no effect on the extent of inhibition seen without preincubation.

nases. EGF internalization was inhibited by ~50% in the presence of 50 μ M genistein, whereas Tfn sequestration was reduced by only ~10%. At higher concentrations, where the genistein specificity might be lost, Tfn sequestration was inhibited by ~30%, whereas EGF sequestration was inhibited by >80%. These results suggest that a tyrosine kinase activity may be specifically required for EGF internalization both in vivo and in vitro.

DISCUSSION

EGF and Tfn Share a Common Pathway for Internalization In Vitro

Perforated A431 cells have been used to study coated vesicle formation and receptor-mediated endocytosis in vitro via constitutively internalized transferrin receptors

(Smythe *et al.*, 1989, 1992a; Schmid and Smythe, 1991; Carter *et al.*, 1993). Here we apply this system to study ligand-induced endocytosis via the EGF-R. Morphological examination of the structures involved in sequestration of HRP-conjugated EGF and gold-conjugated anti-Tfn-receptor antibodies into perforated A431 cells showed that these two ligands colocalized and are presumably internalized via the same endocytic pathway. These results are consistent with those of a number of investigators who have shown that EGF-R are internalized via clathrin-coated pits in a variety of cell types (Carpentier *et al.*, 1982; Hanover *et al.*, 1984; Schlesinger, 1986) including A431 cells (Willingham *et al.*, 1983). However, others have reported that EGF-R are not internalized via clathrin-coated pits in A431 cells (Hopkins *et al.*, 1985; van't Hof *et al.*, 1989). Protocols used in these latter studies may explain these differences. In one study (Hopkins *et al.*, 1985) cells were allowed to internalize prebound EGF for 5–8 min before fixation and labeling of surface EGF-R with gold-labeled antibodies, thus favoring detection of inefficiently internalized receptors. In the second study (van't Hof *et al.*, 1989) cells were incubated with gold-labeled EGF-R antibodies for 60 min at 37°C under conditions where substantial EGF-independent internalization occurred. Saturating concentrations of EGF were then added 5–10 min before fixation. It is possible, given the rapid internalization of occupied EGF-R ($t_{1/2}$ ~ 3–4 min) coupled to the inefficiency of EGF-R uptake (only ~30–40% of occupied receptors) in A431 cells, that productive early endocytic structures were underrepresented using these protocols.

Coated Vesicles Formed In Vitro Are Derived from "Fission Competent" Coated Pits

Only early events in endocytosis leading to the formation of deeply invaginated coated pits were efficiently

Figure 10. Effect of genistein on EGF and Tfn sequestration in semi-intact A431 cells. Perforated cells were incubated for 30 min at 37°C as described in MATERIALS AND METHODS in the presence of increasing concentrations of genistein. The cells were then returned to ice and treated as described for avidin-inaccessibility to determine EGF (●) and Tfn sequestration (○).

detected when EGF was used as a ligand, whereas both early and late events could be detected using Tfn (Schmid and Smythe, 1991). This result was consistent with our previously established working model for receptor-mediated endocytosis in vitro that suggested that Tfn is internalized only if it binds to Tfn-R that occupy more mature "fission competent" coated pits formed in vivo. Because Tfn-R are constitutively internalized, they will occupy all intermediates in the pathway of coated vesicle formation. Our working model further suggests that an event required to form a fission competent coated pit is not reconstituted in perforated A431 cells, resulting in the accumulation of ligand in deeply invaginated coated pits. Consistent with this model, we have reported that newly formed coated pits primed by AP binding become deeply invaginated but do not pinch off (Smythe *et al.*, 1992a). In the absence of ligand, most EGF-R are not associated with coated pits, thus our inability to detect internalization of EGF was also consistent with this working model. The nature of the fission competent coated pit or of the event required to render a coated pit competent for budding remains unknown.

What is the evidence that deeply invaginated coated pits are a bona fide intermediate along this pathway? We have previously shown that formation of deeply invaginated coated pits requires ATP and cytosolic factors including clathrin and APs (Schmid and Smythe, 1991; Smythe *et al.*, 1992a). Tfn sequestration is inhibited by GTP γ S (Carter *et al.*, 1993) and the recruitment of Tfn-R into these intermediates is inhibited by antibodies directed toward the Tfn-R cytoplasmic tail (Schmid and Smythe, 1991), suggesting a requirement for coat-tail interactions. We have also detected this intermediate in intact cells after ATP depletion (Schmid and Carter, 1990). Further morphological analysis is in progress to characterize the structure of the deeply invaginated coated pit that efficiently sequesters both receptor-bound EGF and Tfn.

Distinct Biochemical Requirements for the Recruitment of EGF- and Tfn-R into Coated Pits

Consistent with morphological studies demonstrating a common pathway for EGF and Tfn endocytosis, EGF sequestration shares several of the biochemical requirements previously demonstrated for early events leading to Tfn sequestration (Schmid and Smythe, 1991; Smythe *et al.*, 1992a, Carter *et al.*, 1993). We have shown, for example, that EGF sequestration in vitro also requires ATP hydrolysis and cytosolic factors and is inhibited by GTP γ S. More informative, however, are the biochemical properties that distinguish EGF and Tfn sequestration, namely soluble APs are limiting for Tfn but not EGF sequestration, higher cytosol concentrations are required to support EGF sequestration as compared with Tfn sequestration, Tfn sequestration is efficiently supported by bovine brain cytosol that only partially

and inefficiently supports EGF sequestration, and EGF sequestration is strongly inhibited by genistein that only partially inhibits Tfn sequestration. These differences were detected under identical incubation conditions with cells exposed simultaneously to both ligands and are likely to reflect important mechanistic distinctions between ligand-induced and constitutive receptor-mediated endocytosis.

The most striking and mechanistically informative distinction was that although APs markedly stimulate Tfn sequestration at low cytosol concentrations, they have no effect on EGF sequestration at any cytosol concentration. If EGF sequestration selectively measures early events in coated vesicle formation, why was this reaction independent of added APs? There are two possible mechanisms for ligand-induced internalization of receptors. Ligand binding could induce either the rapid migration of occupied receptors into preformed coated pits or the de novo assembly of coat proteins at the sites where occupied receptors have clustered. Our results provide strong evidence in favor of the former mechanism. The first detectable step in coated vesicle formation is the temperature-, cytosol-, and ATP-independent priming of membranes with APs (Mahaffey *et al.*, 1990; Smythe *et al.*, 1992a; Peeler *et al.*, 1993). This step is followed by the recruitment of clathrin from the cytosol that is unaffected by additional soluble APs (Smythe *et al.*, 1992a). The ligand-induced migration of EGF-R into AP-primed coated pits could occur either before, after, or along with the recruitment of clathrin.

How Are Receptors Recruited into Coated Pits?

Because EGF and Tfn colocalize to the same endocytic structures, the biochemical differences we detect are most likely due to differing requirements for the recruitment of receptors to these structures. AP complexes are thought to act as adaptors for recruiting constitutively internalized receptors into coated pits via interaction with a tyrosine-containing β -turn internalization motif in the cytoplasmic domains of receptors (Pearse and Robinson, 1990; Robinson, 1992). Deletion analysis has defined a more extended region (residues 993–1022) devoid of tyrosine-residues that is required for ligand-induced endocytosis of the EGF-R (Chang *et al.*, 1991). Whether the same "recruitor" molecule participates in both EGF and Tfn-R recruitment is not known. Our results suggest that APs are not required for the active recruitment of occupied EGF-R into coated pits. Direct evidence for the participation of APs in the recruitment of constitutively internalized receptors has also not been obtained. Thus, it remains possible that AP complexes interact with internalization motifs to retain receptors subsequent to their recruitment to coated pits (Schmid, 1992).

We have shown that AP complexes are a limiting component in cytosol derived from both bovine brain

and human K562 erythroleukemic cells required for Tfn sequestration (Smythe *et al.*, 1992a). Significantly higher cytosol concentrations were required for EGF sequestration than for Tfn sequestration, and cytosol from bovine brain was much less effective than cytosol from human K562 cells. Supplementing either of these cytosol sources with APs had no effect on EGF sequestration, suggesting that an additional component, more abundant or more active in K562 cytosol than in bovine brain cytosol, was specifically required for the ligand-induced recruitment of EGF-R into coated pits. Earlier studies in intact A431 cells, demonstrating that the efficiency of EGF internalization was reduced at saturating EGF concentrations whereas Tfn internalization was unaffected (Wiley, 1988), provided the first *in vivo* evidence for a limiting component of the endocytic apparatus specific for EGF uptake. The differences in cytosolic requirements observed for EGF sequestration *in vitro* are consistent with these *in vivo* findings and provide a unique assay for the detection and identification of these specific cytosolic factors.

Analysis of endocytic rates for mutant EGF-receptors lacking tyrosine kinase activity have indicated that the kinase activity is required for high affinity endocytosis of EGF (Glennay *et al.*, 1988; Wiley *et al.*, 1991). Furthermore microinjection of anti-phosphotyrosine antibodies inhibited EGF internalization in intact cells (Glennay *et al.*, 1988). However, other reports have suggested that kinase activity is instead required for down-regulation of receptors by preventing receptor recycling (Felder *et al.*, 1990, 1992). We have shown both in intact cells and *in vitro* that EGF internalization was strongly inhibited by genistein, a selective inhibitor of tyrosine kinases (Akiyama *et al.*, 1987), whereas Tfn sequestration was considerably less affected. These results provide support for the validity of our *in vitro* system, because they mirror the *in vivo* effects and indicate a fourth important biochemical distinction detected between ligand-induced and constitutive receptor-mediated endocytosis.

Is the EGF-R tyrosine kinase required for EGF sequestration *in vitro*? Inhibition by genistein occurred at concentrations comparable with those required for inhibition of EGF-stimulated phosphorylation *in vitro* and *in vivo* (half-maximal $\sim 50 \mu\text{M}$) (Akiyama and Ogawara, 1991). However, we have tested a number of other tyrosine kinase-specific inhibitors, including lavendustin A, herbimycin A, erbstatin, and tyrphostin, both *in vivo* and *in vitro* but found them either not to affect EGF or Tfn internalization or to inhibit both EGF and Tfn internalization (Lamaze and Schmid, unpublished). Most of these inhibitors, including genistein, are competitive with respect to ATP, and their specificities were determined *in vitro* using purified receptors or membrane fractions at μM ATP concentrations (Burke, 1992). Furthermore, many of these inhibitors require prolonged incubations to exert their effects on

intact cells (Faaland *et al.*, 1991; Burke, 1992). Under the conditions of our assay that includes the presence of crude cytosol, total cellular membranes, and $\sim 250 \mu\text{M}$ ATP, concentrations of inhibitors 10- to 1000-fold above the K_i reported from studies with isolated membranes were required. At these concentrations the specificity of these inhibitors is uncertain, thus inhibition of both Tfn and EGF internalization may reflect the direct or indirect involvement of other kinases. For example, staurosporine, a nonspecific kinase inhibitor, inhibited both EGF and Tfn internalization (Lamaze and Schmid, unpublished). Due to these inconsistencies, the differential effect of genistein observed does not allow for conclusions on the requirement of the EGF-R tyrosine kinase for internalization.

CONCLUSION

In summary, we have presented the first comparison of the biochemical requirements and properties of ligand-induced endocytosis via the EGF-receptor to constitutive endocytosis via the Tfn-receptor using perforated A431 cells. These studies have revealed important distinctions that highlight mechanistic differences between these two processes. Based on previous biochemical dissection of this assay system and our morphological studies, these differences most likely occur at an early stage involved in the recruitment of EGF receptors into newly formed coated pits. This assay therefore provides a means to identify factors specifically required for ligand-induced endocytosis of EGF-R and eventually to elucidate the mechanisms involved in receptor sorting into clathrin-coated pits.

ACKNOWLEDGMENTS

We are grateful to Gordon Gill for many helpful discussions and to L. Miya Fujimoto for preparation of cytosolic fractions and clathrin APs. This work was supported by National Institutes of Health grants GM42445 and CA27489 and by the Lucille P. Markey Charitable Trust to S.L.S. S.L.S. is a Lucille P. Markey Fellow. C.L. was supported by fellowship grants from La Ligue Nationale contre le Cancer and from the Philippe Foundation. This is The Scripps Research Institute manuscript number 7783-CB.

REFERENCES

- Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific kinases. *J. Biol. Chem.* 262, 5592-5595.
- Akiyama, T., and Ogawara, H. (1991). Use and specificity of genistein as inhibitor of protein-tyrosine kinases. *Methods Enzymol.* 201, 362-370.
- Backer, J.M., Shoelson, S.E., Weiss, M.A., Hua, Q.H., Cheatham, R.B., Haring, E., Cahill, D.C., and White, M.F. (1992). The insulin receptor juxtamembrane region contains two independent tyrosine/ β -turn internalization signals. *J. Cell Biol.* 118, 831-839.
- Bansal, A., and Gierasch, L.M. (1991). The NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation. *Cell* 67, 1195-1201.

- Burke, T.R. (1992). Protein-tyrosine kinase inhibitors. *Drugs Future* 17, 119–131.
- Carpentier, J.L., Gordon, P., Anderson, R.G.W., Goldstein, J.L., Brown, M.S., Cohen, S., and Orci, L. (1982). Co-localization of ^{125}I -epidermal growth factor and ferritin-low density lipoprotein in coated pits: a quantitative electron microscopic study in normal and mutant human fibroblasts. *J. Cell Biol.* 95, 73–77.
- Carter, L.L., Redelmeier, T.E., Woollenweber, L.A., and Schmid, S.L. (1993). Multiple GTP-binding proteins participate in clathrin coated vesicle-mediated endocytosis. *J. Cell Biol.* 120, 37–45.
- Chang, C.-P., Kao, J.P., Lazar, C.S., Walsh, B.J., Wells, A., Wiley, H.S., Gill, G.N., and Rosenfeld, M.G. (1991). Ligand-induced internalization and increased cell calcium are mediated via distinct structural elements in the carboxyl-terminus of the epidermal growth factor receptor. *J. Biol. Chem.* 266, 23467–23470.
- Collawn, J.F., Stangel, M., Kuhn, L.A., Esekogwu, V., Jing, S., Trowbridge, I.S., and Tainer, J.A. (1990). Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell* 63, 1061–1072.
- Eberle, W., Sander, C., Klaus, W., Schmidt, B., von Figura, K., and Peters, C. (1991). The essential tyrosine of the internalization signal in lysosomal acid phosphatase is part of a β turn. *Cell* 67, 1203–1209.
- Faaland, C.A., Mermelstein, F.H., Haashi, J., and Laskin, J.D. (1991). Rapid uptake of tyrophostin into A431 human epidermoid cells is followed by delayed inhibition of epidermal growth factor (EGF)-stimulated EGF receptor tyrosine kinase activity. *Mol. Cell. Biol.* 11, 2697–2703.
- Felder, S., LaVin, J., Ullrich, A., and Schlessinger, J. (1992). Kinetics of binding, endocytosis, and recycling of EGF receptor mutants. *J. Cell Biol.* 117, 203–212.
- Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J., and Hopkins, C.R. (1990). Kinase activity controls the sorting of the EGF receptor within the multivesicular body. *Cell* 61, 623–634.
- Glenney, J.R., Chen, W.S., Lazar, C.S., Walton, G.M., Zokas, L.M., Rosenfeld, M.G., and Gill, G.S. (1988). Ligand-induced endocytosis of the EGF-receptor is blocked by mutational inactivation and by microinjection of anti-phosphotyrosine antibodies. *Cell* 52, 675–684.
- Hanover, J.A., Beguinot, L., Willingham, M.C., and Pastan, I.H. (1984). Kinetics of transit of transferrin and epidermal growth factor through clathrin-coated membranes. *Cell* 39, 283–293.
- Hopkins, C.R., Miller, K., and Beardmore, J.M. (1985). Receptor-mediated endocytosis of transferrin and epidermal growth factor receptors: a comparison of constitutive and ligand-induced uptake. *J. Cell Sci. Suppl.* 3, 173–186.
- Mahaffey, D.T., Peeler, J.S., Brodsky, F.M., and Anderson, R.G.W. (1990). Clathrin-coated pits contain an integral membrane protein that binds the AP-2 subunit with high affinity. *J. Biol. Chem.* 265, 16514–16520.
- McClain, D.A., Maegawa, H., Lee, J., Dull, T.J., Ullrich, A., and Olefsky, J.M. (1987). A mutant insulin receptor with defective tyrosine kinase displays no biological activity and does not undergo endocytosis. *J. Biol. Chem.* 262, 14663–14671.
- Pearse, B.M.F., and Robinson, M.S. (1990). Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6, 151–171.
- Peeler, J.S., Donzell, W.C., and Anderson, R.G.W. (1993). The appendage domain of the AP-2 subunit is not required for assembly or invagination of clathrin-coated pits. *J. Cell Biol.* 120, 47–54.
- Robinson, M.S. (1992). Adaptins. *Trends Cell Biol.* 2, 293–297.
- Schlessinger, J. (1986). Allosteric regulation of the epidermal growth factor receptor kinase. *J. Cell Biol.* 103, 2067–2072.
- Schmid, S.L. (1992). The mechanism of receptor-mediated endocytosis: more questions than answers. *Bioessays* 14, 581–588.
- Schmid, S.L. (1993). Coated vesicle formation in vitro: conflicting results using different assays. *Trends Cell Biol.* 3, 145–148.
- Schmid, S.L., and Carter, L.L. (1990). ATP is required for receptor-mediated endocytosis in intact cells. *J. Cell Biol.* 111, 2307–2318.
- Schmid, S.L., and Smythe, E. (1991). Stage-specific assays for coated pit formation and coated vesicle budding in vitro. *J. Cell Biol.* 114, 869–880.
- Smythe, E., Carter, L.L., and Schmid, S.L. (1992a). Cytosol- and clathrin dependent stimulation of endocytosis by purified adaptors in vitro. *J. Cell Biol.* 119, 1163–1171.
- Smythe, E., Pypaert, M., Lucocq, J., and Warren, G. (1989). Formation of coated vesicles from coated pits in broken A431 cells. *J. Cell Biol.* 108, 843–853.
- Smythe, E., Redelmeier, T.E., and Schmid, S.L. (1992b). Receptor-mediated endocytosis in semi-intact cells. *Methods Enzymol.* 219, 223–234.
- Smythe, E., and Warren, G. (1991). The mechanism of receptor-mediated endocytosis. *Eur. J. Biochem.* 202, 689–699.
- Sorkin, A., Westermarck, B., Heldin, C.-H., and Claesson-Welsh, L. (1991). Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF β -receptor. *J. Cell Biol.* 112, 469–478.
- Trowbridge, I.S. (1991). Endocytosis and signals for internalization. *Curr. Opin. Cell Biol.* 3, 634–641.
- van't Hof, R.J., Defize, L.H.K., Nuijdens, R., de Brabander, M., Verkleij, A., and Boonstra, J. (1989). Dynamics of epidermal growth factor receptor internalization studied by Nanovid light microscopy and electron microscopy in combination with immunogold labeling. *Eur. J. Cell Biol.* 48, 5–13.
- Wiley, H.S. (1988). Anomalous binding of epidermal growth factor to A431 cells is due to the effect of high receptor densities and a saturable endocytic system. *J. Cell Biol.* 107, 801–810.
- Wiley, H.S., Herbst, J.J., Walsh, B.J., Lauffenburger, E., Rosenfeld, M.G., and Gill, G.N. (1991). Role of tyrosine kinase activity in endocytosis, compartmentation and down-regulation of the EGF-receptor. *J. Biol. Chem.* 266, 11083–11094.
- Willingham, M.C., Haigler, H.T., Fitzgerald, D.J.P., Gallo, M.G., Rutherford, A.V., and Pastan, I.H. (1983). The morphological pathway of binding and internalization of epidermal growth factor in cultured cells. Studies on A431, KB, and 3T3 cell, using multiple methods of labelling. *Exp. Cell Res.* 146, 163–175.