

Transit of Epidermal Growth Factor through Coated Pits of the Golgi System

MARK C. WILLINGHAM and IRA H. PASTAN

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT Using a direct conjugate of epidermal growth factor (EGF) and horseradish peroxidase, we have followed the entry of EGF into KB (human carcinoma) cells. EGF initially was found bound diffusely to the entire cell surface at 4°C; on warming to 37°C, EGF was found clustered in clathrin-coated pits on the plasma membrane in 1 min or less. Within 1–2 min at 37°C, EGF began to accumulate in receptosomes within the cell and remained there for up to 10 min. At 10–13 min after warming to 37°C, EGF was found in thin reticular membranous elements of the Golgi system, as well as concentrated in the clathrin-coated pits present on these membranes. By 15 min after warming, EGF began to be delivered to lysosomes located near the Golgi system. These findings suggest that clathrin-coated pits in the Golgi reticular system accumulate EGF before delivery to lysosomes.

Many ligands that bind to specific cell surface receptors have been observed to undergo endocytosis through a specific morphologic pathway (reviewed in references 15 and 16). This pathway, called “concentrative adsorptive” or “receptor-mediated” endocytosis, utilizes a specialized region of the plasma membrane, the coated pit, which is covered on its cytoplasmic face by a latticework composed chiefly of the protein clathrin (17). A second population of smaller clathrin-coated structures are present in the Golgi system (5). Using α_2 -macroglobulin (α_2 M) as a ligand, it has been shown that α_2 M initially binds to plasma membrane receptors and these receptor-ligand complexes cluster into coated pits on the plasma membrane (24). At 37°C, this clustering process is followed within 1–2 min by endocytosis of the ligand into a specialized, uncoated vesicle, which we have termed a “receptosome” to emphasize its role in receptor-mediated endocytosis (25). Receptosomes migrate by saltatory motion in the cytoplasm and accumulate in the perinuclear Golgi region over a period of 10–30 min, depending on the cell type. After this considerable delay, α_2 M is found in small lysosomes in the Golgi region. A similar pathway has been shown for the internalization of a lysosomal enzyme, β -galactosidase (26), and low density lipoprotein (LDL) (20). Further, many viruses including Semliki Forest virus and vesicular stomatitis virus enter cells by coated pits and receptosomes (3, 9).

Because in the cell lines studied α_2 M is transferred rapidly through the Golgi system, it has been difficult to precisely track the route that α_2 M follows through the Golgi region to lysosomes. The part of the Golgi system responsible for this compartmentalization into lysosomes has been called by some workers “GERL” for Golgi-endoplasmic reticulum-lysosome

complex (13), and in many cell types it has a latticelike or reticular structure (12). The identification of this portion of the Golgi system has been made through the use of cytochemical markers—for lysosomal acid phosphatases. In many cultured cell lines, the amount of acid phosphatase is too low to use this criterion for its identification. In the reticular part of the Golgi system, a common morphologic element is the presence of small (600–800 Å) clathrin-coated pits (6). Because the coated pits at the cell surface function as points of clustering of receptor-ligand complexes, we suggested that the coated pits of the Golgi system might serve an analogous function (16). Recently, β -galactosidase was demonstrated to be concentrated in structures compatible in size and shape with the coated structures of the Golgi system (26). Other workers have suggested that the coated pits of the Golgi system might function as foci of concentration of materials destined for exocytosis (18). However, when the pathway of exocytosis of G protein of vesicular stomatitis virus was followed in cultured cells by electron microscopic immunocytochemistry, G protein was not detected in coated regions of the Golgi system (22). A review of the function and morphology of the Golgi system and its compartments has recently appeared (4).

To investigate the possible role of coated pits of the Golgi system in the delivery of extracellular ligands to lysosomes, we sought a ligand system with sufficiently high numbers of receptors and a sufficiently synchronous delivery of ligand to and through the Golgi system to allow us to analyze the intracellular organelles involved. This paper describes such a system, in which KB cells with $>10^6$ EGF receptors per cell (11) internalize a conjugate of epidermal growth factor (EGF) and horseradish peroxidase (EGF-HRP) in a highly synchro-

nous fashion, first through coated pits of the plasma membrane and receptosomes and then into the Golgi system. EGF accumulates in large amounts in the coated pits of the Golgi system 10–14 min after entry and before its delivery to lysosomes.

MATERIALS AND METHODS

Cells

KB (human carcinoma) cells were obtained from the American Type Culture Collection (Rockville, MD) and propagated in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, and 10% calf serum. Cells were subcultured at least 2 d before experiments.

EGF-HRP

It has been previously established that the α amino group of EGF can be used to couple it to ferritin (8) or α -lactalbumin (19) with retention of biological activity. We prepared a covalent conjugate of epidermal growth factor and horseradish peroxidase using an intermolecular disulfide interchange reaction (2).

Preparation of EGF-HRP

HRP (Sigma Chemical Co., St. Louis, MO) was derivatized with methyl-4-mercaptobutyrimidate (MMB) and dithionitrobenzoate (DTNB) as previously described to obtain one activated (SH) per molecule (2). 1 mg of EGF (Bethesda Research Laboratories, Bethesda, MD) in 0.5 ml of KPO, pH 8.5 mixed with 60,000 cpm 125 I-EGF was reacted for 6 h at 37°C with 6 mg of MMB and the derivatized EGF separated from low molecular weight reactants on a Sephadex G-25 (PD10) column. Under these conditions, one SH is introduced into each EGF molecule. Then EGF-SH (150 nmol) in 2 ml was mixed with 0.9 ml of HRP-TNB and incubated at 20°C for 18 h. By measuring release of nitrophenol at 412 nm, the reaction was found to go to 60% of completion. A 10% excess of cysteine (66 nmol) was added to react with free-SH groups, and the reaction mixture was concentrated to 1.0 ml on a PM10 Amicon filter (Amicon Corp., Lexington, MA) and applied to a 0.9 \times 50 cm G-75 column to separate EGF-HRP from unreacted EGF. The position of EGF was monitored by optical density and its content of 125 I. By this procedure, EGF-HRP is completely free of EGF but is mixed with a small amount of unreacted HRP.

Activity of EGF-HRP

EGF-HRP was compared with native EGF for its ability to displace 125 I-EGF from A431 cells. On a molar basis, EGF-HRP was 45% as active as native EGF in displacing 125 I-EGF (Willingham, Haigler, and Pastan, manuscript in preparation). When tested for its ability to induce surface ruffling in A431 cells (1), EGF-HRP induced ruffling to the same extent as unlabeled EGF when tested at equal concentrations (~100 nM, results to be presented elsewhere). KB cells showed no ruffling activity in response to either EGF or EGF-HRP (data not shown).

RESULTS

Steps of EGF Internalization

To determine the initial distribution of EGF on the cell surface, KB cells were exposed to EGF-HRP (50 nM) for 60 min at 4°C, washed, fixed, and preserved for electron microscopy. As shown in Fig. 1A and B, EGF was distributed diffusely over the cell surface; there was no significant concentration of the ligand in clathrin-coated pits. The specificity of the binding was established by the addition of a 10-fold excess (500 nM) of unlabeled EGF which substantially reduced the binding of EGF-HRP (Fig. 1C and D).

Clustering in Coated Pits

When cells were raised to 37°C for 1 min, EGF-HRP became concentrated in coated pits at the cell surface (Fig. 1E). At this time, significant amounts of EGF-HRP remained on the cell surface outside of pits in an unclustered state (Fig. 1E). Some clustering of EGF-HRP in coated pits could be detected after

warming cells to 37°C for as little as 10 s (data not shown). By 10 min at 37°C, almost all of the EGF-HRP had been cleared from the cell surface and could be detected within the cell.

Appearance of EGF-HRP in Uncoated Vesicles (Receptosomes)

Within 1–2 min at 37°C, receptosomes containing EGF-HRP were observed. At this time, a significant amount of EGF-HRP was still present on the cell surface. Between 2–9 min after warming to 37°C, the only intracellular site in which EGF-HRP was detected was the receptosomes.

Around the 10-min point, many of the receptosomes were found near the Golgi system (Fig. 1G), but others were found in more peripheral locations. At this time, vesicles in both locations were often found lying close to or in communication with narrow, elongated uncoated membranous elements (Fig. 1H).

At 10–13 min after warming to 37°C, EGF-HRP appeared in small (~700 Å in diameter), clathrin-coated structures of the Golgi system. Some of these structures were observed in the same section as Golgi stacks (Fig. 1I); others were not and probably were more peripheral in location (Fig. 1H). At 15 min, the first evidence that EGF-HRP had reached the lysosomal compartment was obtained (Fig. 1J).

In summary, the pathway of internalization of EGF-HRP is very similar to that observed for α_2 M (24, 25), β -galactosidase (26), and low density lipoprotein (LDL) (20): cell surface and coated pits of the cell surface; receptosomes; the Golgi region; and finally, lysosomes.

The Coated Structures of the Golgi System

In contrast to previous studies with other ligands in different cell types, many images of EGF-HRP in coated structures of the Golgi system and in uncoated tubular structures with which these coated structures communicated were obtained in KB cells (Fig. 2). Images of coated pits in the Golgi system containing EGF-HRP were easy to find 10–13 min after warming, averaging around 2–5 per cell profile in thin sections. Such images were never seen before 9 min after warming, and dropped off dramatically after 13–15 min. Since no localization of EGF-HRP in lysosomes was seen before 15 min, it is quite likely that this Golgi coated pit localization is a precursor to the delivery of EGF-HRP to lysosomes.

Golgi coated structures were seen in many different planes of section, often with the appearance seen in Fig. 2K–N in which their association with other membranous structures was not evident. However, since <50% of the images of coated profiles were not in obvious communication with reticular membrane structures, and since other images could easily be found showing clear continuity with reticular membranous structures (Fig. 2A–J, O–Q), we believe it is likely that the images in Fig. 2K–N represent tangential cross sections of coated pits, rather than isolated coated vesicles. The issue of whether coated pits pinch off to form vesicles or give rise directly to uncoated vesicles has been discussed elsewhere (21, 23, 27).

Concentration of EGF-HRP in Golgi Coated Pits

Fig. 2A, C, D, and O show examples in which EGF-HRP appeared to be concentrated in the clathrin-coated regions relative to the uncoated adjacent tubular elements. However, in other images (Fig. 2B, F, G, H, and P) it was not clear that

there was a discernible difference in concentration between coated and uncoated regions. It has previously been suggested that the coated regions of the Golgi system participate directly in the transfer of proteins from the Golgi system to lysosomes (16, 26). The finding that EGF-HRP was in some cases more highly concentrated in coated pits relative to adjacent uncoated tubular regions is consistent with this suggestion.

DISCUSSION

In this paper we have examined the pathway taken by EGF when it undergoes receptor-mediated endocytosis. EGF follows the same route previously shown for α_2 M, LDL, and β -galactosidase (reviewed in reference 16); after binding to cell surface receptors the ligand traverses coated pits, receptosomes, the Golgi region, and ultimately appears in lysosomes. In this study we have particularly focused on the events occurring in the Golgi region to determine which parts of the Golgi system participate in the transfer of ligands from receptosomes to lysosomes.

KB cells have a large number of EGF receptors (11). This enabled us to bind large amounts of the EGF conjugate to the cell surface at 4°C, raise the temperature to 37°C, and follow the entry of EGF into the cell and through various intracellular compartments as a synchronous wave. We previously used this approach with α_2 M on Swiss 3T3 cells, which have large numbers of α_2 M receptors, and were able to show that α_2 M traversed receptosomes and the Golgi region on the way to lysosomes (25). However, very few images of α_2 M in the various elements of the Golgi system were observed, probably because the ligand traversed the Golgi system very rapidly and asynchronously. In KB cells, many images of EGF-HRP were observed in the Golgi region 10–14 min after the first wave of EGF entry.

As shown in Fig. 1 *H*, receptosomes were seen in continuity with tubular elements of the reticular portion of the Golgi system (GERL), and EGF-HRP was found both in the receptosome and in the adjacent tubular element. At this same time, ligand also was detected in coated structures of the Golgi system (Fig. 1 *H*). Many of these coated pits were in continuity

with tubular elements of the Golgi system (Fig. 2), but we have not yet observed coated structures directly in continuity with receptosomes. Therefore, we believe that the most reasonable interpretation of these images is that receptosomes fuse with tubular elements of the Golgi system, allowing ligand to enter this portion of the Golgi system. Next, the ligand moves along the tubular elements and is concentrated in the coated pits of the Golgi system. From these coated pits, EGF is somehow transferred to lysosomes.

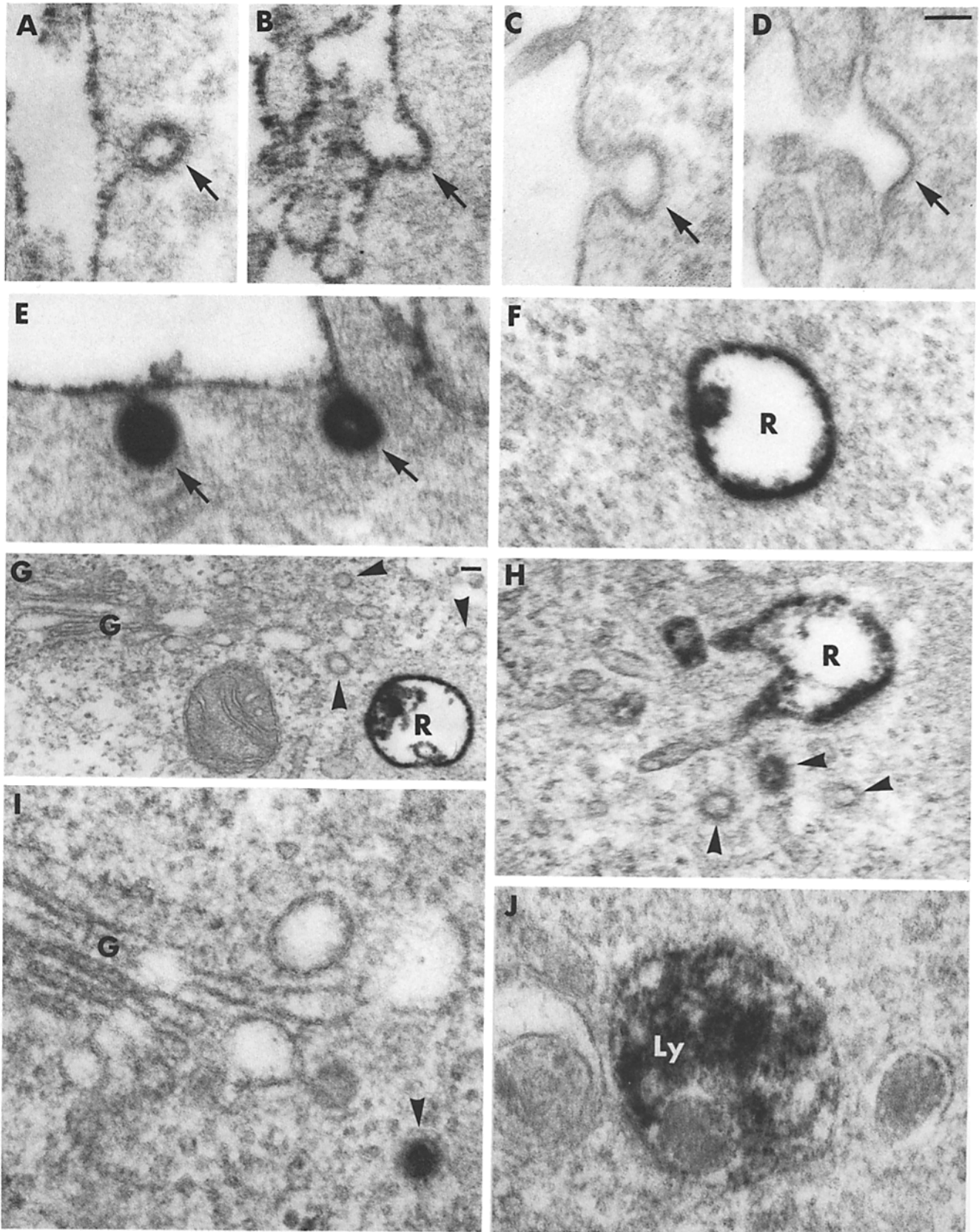
A number of images were observed in which EGF-HRP appeared to be more highly concentrated in coated pits of the Golgi system than in the contiguous tubular elements. This result suggests that coated pits of the Golgi system can concentrate ligands. If this is true, then coated pits of the Golgi system and those of the plasma membrane share this concentrative function. The entry of external ligands into Golgi elements has been reported previously for cell surface tracers such as cationic ferritin (10, 14) and for some lectins (6, 7). It is possible that a pathway similar to that shown in this paper for EGF may have been, at least in part, responsible for these observations. Cationic ferritin and lectins presumably bind to many types of receptors and other cell surface proteins, whereas EGF binds to only one receptor population.

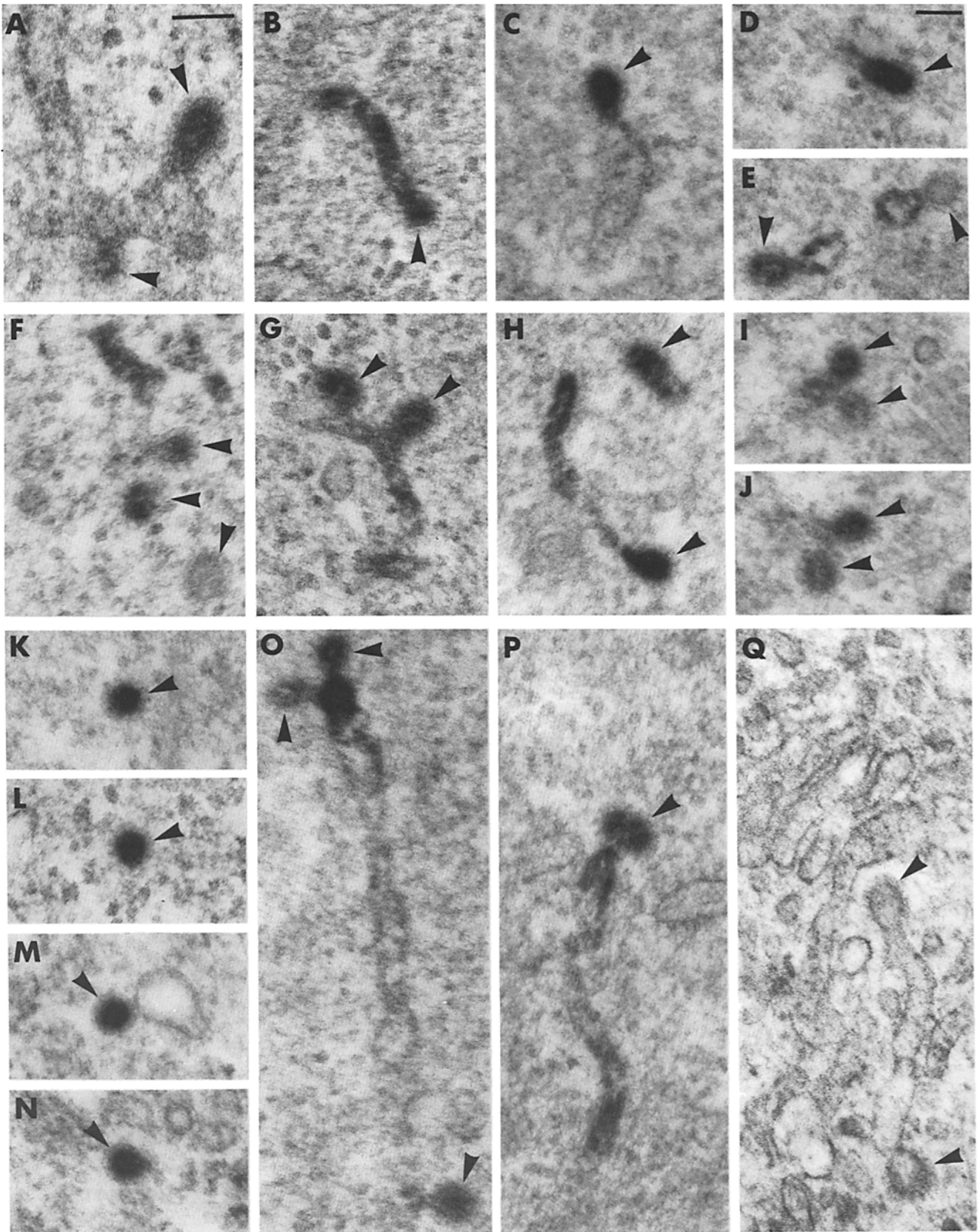
At the current time it is not known whether or not the EGF receptor accompanies the ligand into the cell and through the Golgi system. Immunocytochemistry with antibodies to the EGF receptor may contribute to the resolution of this question.

The entry of EGF into human epithelioid carcinoma cells (A431) has been studied by Haigler et al. (8). As in the present study, they found the initial distribution of EGF to be diffuse on the plasma membrane. With that conjugate, they observed EGF in clusters in coated pits as well as in uncoated regions of the membrane. They suggested that entry occurred through both coated and uncoated regions of the membrane. In the current study, all the EGF that we could detect was internalized through coated pits and not through uncoated regions of the membrane. The finding by Haigler et al. (8) that EGF enters by two routes may be due to the fact that A431 cells form extensive ruffles upon EGF addition, and ruffles give rise to pinocytotic vesicles (1). KB cells do not form ruffles upon EGF

FIGURE 1 The morphologic pathway of binding and internalization of EGF-HRP in KB cells. (A and B) After incubation and fixation at 4°C, EGF-HRP was diffusely distributed over the cell surface with no concentration whatsoever in coated pits (arrows). (C and D) Similar incubation in the presence of a 10-fold molar excess of unlabeled EGF showed that virtually all of the peroxidase reaction product seen in A and B was displaced. (E) Warming cells to 37°C for 1 min resulted in dramatic clustering of EGF-HRP in coated pits (arrows) on the cell surface. (F) After 5 min, much of the surface-bound EGF-HRP had been internalized into intracellular receptosomes (R). (G) 10 min after warming, many of these receptosomes were found in close proximity to peripheral elements of the Golgi system (G) in the region near Golgi coated structures (arrowheads) or (H) near more peripheral elements of the Golgi system containing coated structures (arrowheads). At 10 min or later, receptosomes (R) were often seen in an apparent continuity with these thin reticular Golgi elements. (I) Concentrated EGF-HRP was seen frequently between 10 and 13 min after warming to be present in coated structures at the edge of the Golgi system. (J) 15 min after warming, much of the internalized EGF-HRP was found in densely packed lysosomes forming at the margin of the Golgi system. At earlier times (5 or 10 min), no detectable EGF-HRP was found in such lysosomal structures. Lead citrate counterstain. Bars, 0.1 μ m. (A–F, H–J) \times 90,000; (G) \times 39,000.

FIGURE 2 Localization of EGF-HRP in Golgi coated pits between 10 and 13 min after warming. Cells treated as in Fig. 1 were examined carefully for localization of EGF-HRP in the clathrin coated pits of the Golgi system (arrowheads). (Q) In the absence of EGF-HRP, these structures appear often as pits in communication with thin reticular Golgi elements (arrowheads). (A–J, O, P) EGF-HRP was found in these structures between 10 and 13 min after warming cells to 37°C. Often the communication of these labeled pits with other membranous elements was visible. On occasion, these structures were seen in cross-section without obvious communication to other elements. (A, C, O) EGF-HRP is more concentrated in the coated pit regions than in adjacent uncoated elements. (E, P) Infrequently, some coated regions (arrowheads) did not show label in spite of the presence of label in adjacent membrane elements. (A, F, G, I, J, O, Q) Golgi coated regions often occur in groups adjacent to each other attached to the same uncoated cisternae. Lead citrate counterstain. Bars, 0.1 μ m. (A) \times 120,000 (B–Q) \times 90,000.





addition. A comparison of the internalization of EGF-HRP by various cell types will be reported elsewhere (Willingham, Haigler, and Pastan, manuscript in preparation).

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