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Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation

(phosphotyrosine/Percoll gradient/epidermoid carcinoma cell)

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ABSTRACT **The down-regulation and internalization of the epidermal growth factor (EGF) receptors induced by two separate anti-EGF monoclonal antibodies (mAbs), IgG1 mAb-225 and -455, and by EGF was examined. mAb-225 competitively inhibits EGF binding and it is internalized to an extent comparable to EGF. The antibody down-regulates surface EGF receptors in a dose-dependent manner. In contrast, mAb-455 does not competitively inhibit the binding of EGF or mAb-225, but it specifically immunoprecipitates the EGF receptor. mAb-455 also down-regulates the EGF receptor. Unlike EGF, which elicits phosphorylation of the receptor at tyrosine, threonine, and serine residues, neither of these antibodies elicits phosphorylation of the EGF receptor in intact A431 cells or in KB cells. Our studies suggest that EGF-stimulated phosphorylation of the receptor is not required for the internalization of the ligand–receptor complex.**

Epidermal growth factor (EGF) is a potent mitogen for certain cultured cells and has been used extensively as a model for studying growth control (1, 2). Specific saturable receptors for EGF are present on a wide variety of tissues, including corneal cells, fibroblasts, lens glial cells, epidermoid carcinomas, granulosa cells, vascular endothelial cells, and choriocarcinomas (3, 4). EGF is mitogenic for a wide variety of tissue culture cells. After EGF receptor binding, the EGF receptor kinase autophosphorylates itself at at least three sites near the carboxyl terminus (5, 6). Phosphorylation of the receptor and other cellular substrates occurs at tyrosine residues (7, 8). The occupied receptors cluster in clathrin-coated pits and are internalized into endocytic vesicles; ultimately, EGF is degraded in lysosomes (1, 3). The relationship between phosphorylation of the receptor and internalization has not been established, although both occur within a few minutes of EGF binding.

Recent studies have shown that several human tumors express an increased number of EGF receptors (9–11). In some cases, as in A431 and human lung tumors, the increased receptor number is due to amplification of the EGF receptor gene (11–13).

A recent study from Pastan's laboratory (14) indicates that EGF induces internalization of the EGF receptor into receptosomes, followed by the loss of immunoreactive EGF receptor from the lysosome. Although the initial hormone–receptor interactions at the plasma membrane are required to obtain a biological response, the relevance of internalization of the hormone–receptor complex into the cell and the fate of the internalized receptor are not yet established.

The A431 epidermoid carcinoma cell line is unusual in that it displays an extremely high number of EGF receptors (15), and yet its growth is inhibited by concentrations of EGF that are mitogenic to other cell lines (16, 17). EGF receptor binding induces a series of morphological changes, such as rapid cell rounding in the absence of Ca^{2+} followed by the formation of ruffles and filopodia and, finally, retraction from the substratum (18, 19). EGF also stimulates the autophosphorylating membrane-associated protein kinase (20, 21) in these cells, although EGF inhibits, rather than stimulates, their growth.

In this report, we examine the relationship between receptor internalization and receptor autophosphorylation. We have previously shown that A431 cells internalize the anti-EGF receptor monoclonal antibody (mAb)-225 with similar kinetics to the internalization of EGF (22). In this report, we compare the internalization response of cells that exhibit different growth responses to EGF. A431 cells, which are growth-inhibited by EGF, were compared with KB cells, which are growth-stimulated by EGF (23). Furthermore, we examined the state of EGF receptor phosphorylation in both cell types after binding of EGF and antibody to the cell surface.

MATERIALS AND METHODS

Materials. mAb-225, -528, and -455 raised against human EGF receptors were purified from serum-free culture fluid conditioned with hybridomas as described (24, 25). Phosphate-free modified Dulbecco's modified Eagle's medium (DMEM) was purchased from Irvine Scientific. [^{32}P]Orthophosphate (28.5 Ci/nmol; 1 Ci = 37 GBq) was purchased from ICN. EGF was obtained from Collaborative Research (Waltham, MA).

Cell Lines and Cell Culture. The A431 human epidermoid carcinoma cell line was obtained from Gordon Sato Cell Sciences Center, Lake Placid, NY. KB cells were obtained from the American Type Culture Collection. Cells were grown at 37°C in a 1:1 (vol/vol) mixture of DMEM and Ham's F-12 nutrient medium (DMEM/F-12) containing 5% newborn calf serum in an atmosphere of 5% CO_2/95% air.

Down-Regulation of the EGF Receptor. Confluent 35-mm plates of A431 and KB cells were incubated in DMEM/F-12 containing minimal amounts of EGF (0.5% newborn calf serum) for 24 hr prior to experiments. EGF (20 nM), mAb-225 (20 nM), or mAb-455 (200 nM) was added for 0–4 hr at 37°C. Cells were placed on ice and washed three times with 0.5 ml of chilled DMEM/F-12 containing 0.2% bovine serum albumin and 0.02% NaN_3 (buffer A) and incubated with 0.5 ml of 50 nM ^{125}I-labeled EGF at 4°C for 1.5 hr. Cell-associated radioactivity was measured after washing cell layers with buffer A followed by solubilizing cells with 5 M NaOH.

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Abbreviations: EGF, epidermal growth factor; mAb, monoclonal antibody.

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Percoll Gradient Fractionation. Percoll gradient fractionation was performed as described (26), except that the Percoll concentration was 35%. Cells were disrupted by resuspending in 1.0 ml of acetate triethanolamine buffer (1 mM EDTA/10 mM acetic acid/10 mM triethanolamine, pH 7.4) and lysed by pipetting the suspension 20–40 times with a P1000 Pipetman (27).

Phosphorylation of the EGF Receptor and Immunoprecipitation. A431 (3.4×10^5) and KB (6.1×10^5) cells were preincubated at 37°C for 30 min in phosphate-free modified DMEM supplemented with L-glutamine (48.4 mg per 100 ml), sodium pyruvate (11.0 mg per 100 ml), and 4% newborn calf serum. Cells were further incubated in the same medium with [^{32}P]orthophosphate (0.4 mCi/ml) at 37°C for 15 hr. For the last 30 min, 20 nM EGF, 20 nM mAb-225, 1 μM mAb-455, or the equal volume of phosphate-free medium (for the control experiment) was added. After removing medium, the cell layer was rinsed with phosphate-buffered saline and lysed with 300 μl of RIPA buffer (0.05 M Tris·HCl, pH 7.7/0.15 M NaCl/1% Triton X-100/1.0% Na deoxycholate/0.1% NaDodSO$_4$/10 mM EDTA/100 μM Na_3VO_4/0.3 mM phenylmethylsulfonyl fluoride/5 mM iodoacetic acid) at 0°C for 30 min. Culture plates were rinsed with 200 μl of RIPA buffer and the rinse was combined with the above RIPA lysate. After removal of nuclei and insoluble material by centrifugation at 20,000 × *g* for 1 hr, the supernatant (250 μl) was subjected to immunoprecipitation with 2 μg of anti-EGF receptor mAb-528, 4.5 μg of rabbit anti-mouse antibody, and Pansorbin as described (28).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Immunoprecipitated ^{32}P-labeled EGF receptor was analyzed and further purified by NaDodSO$_4$/polyacrylamide gel electrophoresis as described (28).

Phosphoamino Acid Analysis. Analysis of phosphoamino acids of the ^{32}P-labeled EGF receptor purified by immunoprecipitation and NaDodSO$_4$/polyacrylamide gel electrophoresis was performed as described (29) with the modification that the gel band was digested directly with 6 M HCl as described (28).

RESULTS

Down-Regulation of the EGF Receptor. Two mAbs to the EGF receptor have been produced that have high affinity for and immunoprecipitate the EGF receptor from a variety of human cells. One of these, mAb-225 IgG1, blocks EGF binding, while the other, mAb-455 IgG does not block EGF binding. Both have antitumor effects on some human tumor xenografts in athymic mice (30). To investigate the effect of the antibodies on internalization of the EGF receptor, as compared to the effect of EGF, we determined whether the mAbs would elicit down-regulation of the EGF receptor. Two cell lines of epidermal origin were used in our investigations. A431 cells and KB cells were incubated with 20 nM EGF, 20 nM anti-receptor mAb-225, or 200 nM anti-receptor mAb-455 for 1–4 hr at 37°C. Subsequently, ^{125}I-labeled EGF binding capacity was measured to determine the number of EGF receptors available for binding. A 10-fold higher concentration of mAb-455 was used to obtain a similar level of receptor occupancy, because mAb-455 has 1/10th the binding affinity for the EGF receptor of either EGF or mAb-225 (25). Consistent with previous reports (14, 22, 23), EGF caused a rapid decrease in EGF binding (down-regulation) on both A431 and KB cell lines (Fig. 1). We have repeatedly observed ≈15% less EGF elicited down-regulation of the EGF receptors on A431 cells than on KB cells (Fig. 1). Anti-EGF receptor mAb-225 and -455 also elicited down-regulation of the EGF receptor in both cell lines (Fig. 1). When cells were incubated with EGF at 4°C (where no internalization of the receptor occurs), no decrease in EGF binding capacity was observed (22). Furthermore, if the cell layer was rinsed with acidic solution to remove any cell surface-associated ligands before incubation with ^{125}I-labeled EGF, a similar decrease in EGF binding was observed (data not shown). Therefore, the decrease of the EGF binding capacity shown in Fig. 1 is not simply due to a competition of ^{125}I-labeled EGF binding with unlabeled ligands. The fact that the anti-EGF receptor mAb-455, which does not block EGF binding (25), causes a dramatic decrease in EGF binding also supports the above conclusion. Furthermore, mAb-225 does not cause a measurable change in the amount of transferrin receptors present on A431 cells following massive EGF receptor internalization (C.L.M. and J. Castagnola, unpublished results).

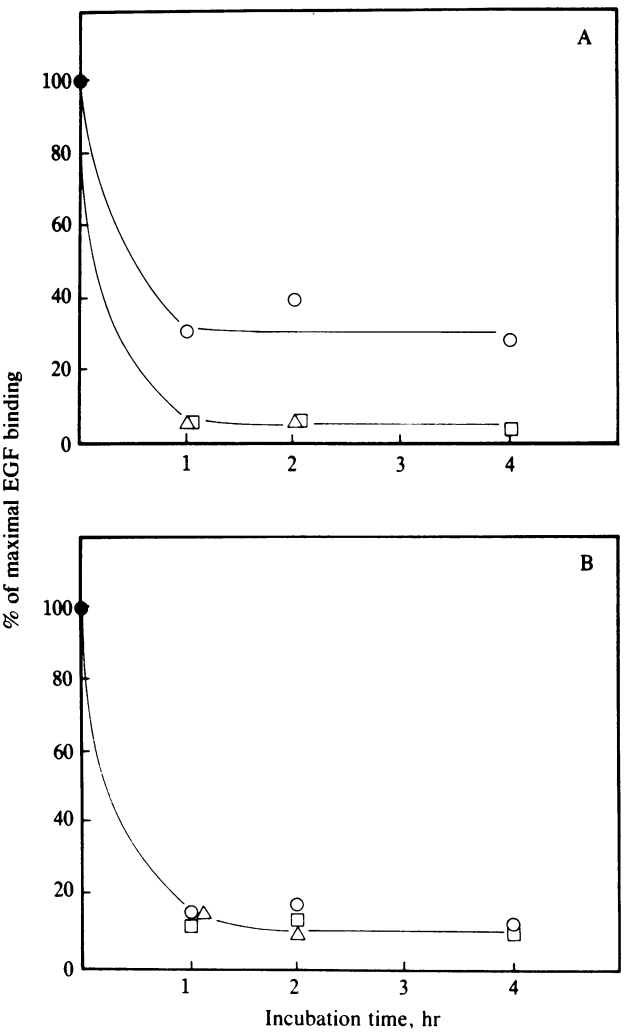

FIG. 1. Down-regulation of EGF receptor by EGF and anti-receptor mAbs. Confluent 3.5-cm plates of A431 (*A*) and KB (*B*) cells were cultured in DMEM/F-12 supplemented with 0.5% newborn calf serum. EGF (20 nM) (○), MaB-225 (20 nM) (△), and mAb-455 (200 nM) (□) were added for the indicated period of time at 37°C. The cell layer was rinsed with DMEM/F-12/0.5% newborn calf serum and incubated for another 1 hr at 37°C. Finally, the cells were incubated with ^{125}I-labeled EGF (50 nM) for 1.5 hr at 4°C. Cell-associated radioactivity was counted after solubilizing cells with 5 M NaOH.

Internalization of ^{125}I-Labeled EGF and ^{125}I-Labeled mAb. To investigate whether the ligands were localized to an intracellular compartment following down-regulation, KB cells were incubated with ^{125}I-labeled EGF or ^{125}I-labeled mAb for 10 min at 37°C and harvested for Percoll gradient centrifugation as described in *Materials and Methods*. After gradient centrifugation, 67% of ^{125}I-labeled EGF and 61% of ^{125}I-labeled mAb radioactivity was associated with an organelle peak that appeared at fractions 5–6 (Fig. 2). This

peak is known to contain receptosomes (26, 27). The density of this organelle-containing fraction was 1.04 g/ml. In previously reported experiments, we have demonstrated that this organelle fraction contains the early processed products of EGF (pI 4.2), but not the later-appearing products of pI 4.35 and 4.0, which are associated with a denser peak (1.07 g/ml) that contains lysosomal enzyme markers (27).

When cells were incubated with ^{125}I-labeled EGF or ^{125}I-labeled mAb for 1 hr at 4°C, under conditions where internalization of ligands was prevented, the majority of the isotope remained at the top of the gradient (fractions 1–3), not in association with cytoplasmic organelles sedimented into the gradient. The small peaks seen at fractions 5 and 6 in this experiment represent a portion of the iodinated ligands that migrate on the gradient at the same density as the organelles.

Control experiments that measure the adventitious binding of iodinated EGF or mAb to subcellular organelles in the homogenates are also shown in Fig. 2. KB cells were first harvested for Percoll gradient analysis, and then homogenates were incubated with ^{125}I-labeled EGF or ^{125}I-labeled mAb for 1 hr at 4°C. There was no detectable binding of either EGF or mAb to subcellular organelles during the incubation period and the experimental procedures; all of the radioactivity was found at the top of the gradients. Our results indicate that anti-EGF receptor antibody, as well as EGF, are quickly taken into the same subcellular organelle fraction.

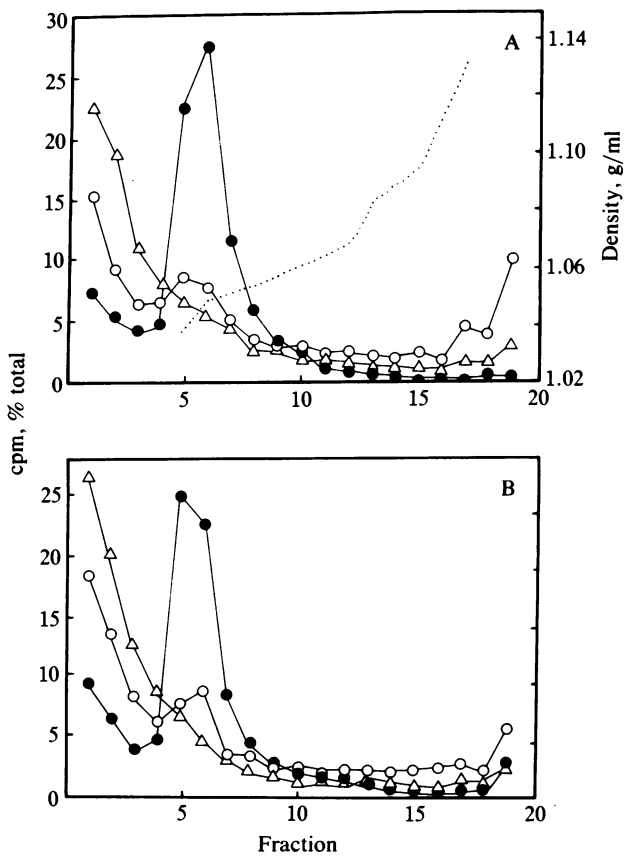

FIG. 2. Internalization of ^{125}I-labeled EGF and ^{125}I-labeled mAb-225. Confluent 10-cm plates of KB cells were incubated with ^{125}I-labeled EGF (5 ng/ml) (*A*) or ^{125}I-labeled mAb-225 (5 ng/ml) (*B*) either at 37°C for 10 min (●) or at 0°C for 1 hr. (○). Cells were then rinsed with ice-cold Hanks' balanced salt solution and harvested for Percoll gradient centrifugation as described in *Materials and Methods*. As a control (△) for the adventitious binding of both iodinated ligands to subcellular organelles in homogenates, the cells were first harvested for Percoll gradient analysis, and the homogenates were incubated with ^{125}I-labeled EGF or ^{125}I-labeled mAb-225 for 1 hr at 4°C. Radiolabeled ligands (8000 cpm) were added to each milliliter of homogenate to keep the ratio of radioactivity present in the homogenates similar to that observed after binding to cells. The density of the Percoll gradient is also shown (...).

Effect of EGF and mAb on Phosphorylation of the EGF Receptor. A431 cells and KB cells were incubated with [^{32}P]orthophosphate for 15 hr to equilibrate intracellular levels of ^{32}P-labeled nucleotide (29). Subsequently, the intact cells were incubated in the presence of either 20 nM EGF, 20 nM mAb-225, or 1 μM mAb-455 for 30 min. Total cellular protein was solubilized in the presence of inhibitors of proteases and phosphatases. After removing nuclei, EGF receptors were immunoprecipitated with anti-receptor antibody-528, rabbit anti-mouse antibody, and Pansorbin as described in *Materials and Methods*, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by autoradiography. Consistent with previous reports (8, 31), our results show that EGF stimulates phosphorylation of the EGF receptor 4-fold in intact A431 cells and 2-fold in KB cells (Fig. 3 and Table 1). However, neither mAb -225 nor -455 elicit phosphorylation of the EGF receptor.

Phosphoamino Acid Measurement of ^{32}P-Labeled EGF Receptors. Although none of our mAbs stimulated phosphorylation of the EGF receptor in intact cells, since the abundance of phosphotyrosine compared to phosphoserine and phosphothreonine is low, a possible increase in phosphorylation on tyrosine residue by mAb might not be detectable by a simple measurement of bulk ^{32}P radioactivity associated with the EGF receptor. Therefore, we determined the [^{32}P]phosphoamino acid content of the EGF receptor shown in Fig. 3 (see *Materials and Methods*).

The immunoprecipitated EGF receptor protein was excised from acrylamide gels and hydrolized with 6 M HCl at 110°C for 1 hr (28). Phosphoamino acids were purified by Dowex column chromatography as described in *Materials and Methods*, and analyzed by two-dimensional thin layer electrophoresis (Fig. 3*C*). EGF receptors from KB cells could not be analyzed for phosphoamino acid content because of the low level of ^{32}P incorporation into the receptor.

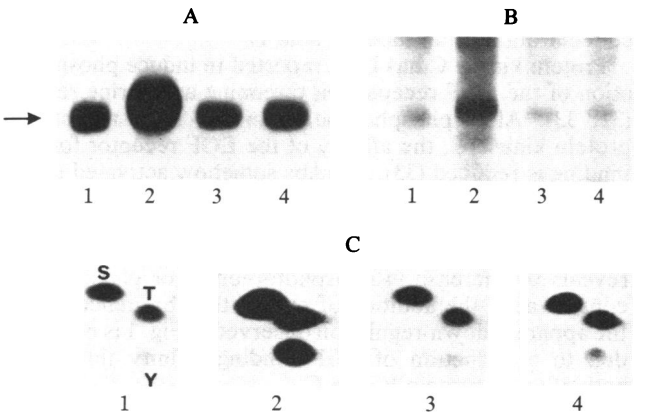

FIG. 3. Phosphorylation of EGF receptor *in vivo*. A431 (3.4×10^5 cells; *A*) and KB (6.1×10^5 cells; *B*) were labeled with [^{32}P]orthophosphate (0.4 mCi/ml) in phosphate-free DMEM supplemented with 4% newborn calf serum for 15 hr at 37°C. For the last 30 min, nothing (lanes 1), 20 nM EGF (lanes 2), 20 nM mAb-225 (lanes 3), or 1 μM mAb-455 (lanes 4) was added. Total cellular protein was solubilized with RIPA buffer (see *Materials and Methods*). The EGF receptor was immunoprecipitated with 50 nM mAb-528, 100 nM RAM and Pansorbin, and analyzed by 7.5% $NaDodSO_4$/polyacrylamide gel electrophoresis, followed by autoradiography. (*C*) Phosphoamino acid analysis of the EGF receptor. EGF receptor bands in *A* were excised and acid-hydrolyzed (see *Materials and Methods*). [^{32}P]Phosphoamino acids were separated by two-dimensional thin-layer electrophoresis and located by autoradiography. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

Table 1. Phosphorylation of the EGF receptor in intact cells in the absence or presence of EGF and mAbs

Treatment	A431 cells, total cpm × 10^{-3} in					KB cells, total cpm × 10^{-3} in
	P-Tyr	*P*-Thr	*P*-Ser	Phosphoamino acids recovered	EGF receptor before acid hydrolysis	EGF receptor before acid hydrolysis
Control	0.9 (1.0)	4.9 (1.0)	6.3 (1.0)	12.2 (1.0)	34.7 ± 1.0 (1.0 ± 0.03)	1.6 ± 0.02 (1.0 ± 0.02)
+ EGF	11.6 (12.4)	11.1 (2.2)	23.4 (3.7)	46.3 (3.8)	138.6 ± 4.6 (4.0 ± 0.13)	3.3 ± 0.12 (2.1 ± 0.08)
+ mAb-225	1.1 (1.3)	6.4 (1.3)	7.3 (1.2)	14.9 (1.2)	38.3 ± 4.5 (1.1 ± 0.13)	1.4 ± 0.16 (0.9 ± 9.19)
+ mAb-455	1.6 (1.8)	5.2 (1.0)	6.4 (1.0)	13.3 (1.1)	38.5 ± 0.8 (1.1 ± 0.02)	1.3 ± 0.20 (0.8 ± 0.13)

Experimental conditions are described in the legend of Fig. 3. Radioactivities in the EGF receptor and phosphoamino acids, which were separated on $NaDodSO_4$ polyacrylamide gels and thin-layer plates (Fig. 3), were measured. Numbers in parentheses are ratios of the radioactivity normalized to the control value.

As shown in Table 1, ≈35% of the radioactivity was recovered as phosphoamino acids, 30% was found as free phosphate (which resulted from acid hydrolysis), and the remaining 30% was associated with partially acid-digested oligophosphopeptides. The relative distribution of radioactivity described above was constant from sample to sample (Table 1). Furthermore, the relative ratios of radioactivity found in EGF receptors on $NaDodSO_4$ gel excised bands is 1 part (+ no addition)/4 parts (+ EGF)/1 part (+ mAb-225)/1 part (+ mAb-455). Similar relative ratios are found in phosphoamino acids determined by counting the radioactivity recovered from the two-dimensional thin-layer plate. Therefore, this analysis is likely to be representative of the abundance of phosphoamino acids associated with the EGF receptor.

EGF stimulated phosphorylation of the EGF receptor on tyrosine residues by ≈12-fold, on serine residues by ≈4-fold, and on threonine residues by ≈2-fold when compared to the untreated control. Phosphotyrosine accounts for 1/4 of the total phosphoamino acids found in the EGF receptor. However, mAb-225 did not cause any change in the amount or distribution of ^{32}P-labeled phosphoamino acids. mAb-455 failed to elicit an increase of phosphothreonine and phosphoserine; however, it appeared to stimulate the phosphorylation of tyrosine residues to a slight extent at 1 μM concentration of antibody (Table 1).

Protein kinase C has been reported to induce phosphorylation of the EGF receptor on threonine and serine residues (32, 33). After phosphorylation of the EGF receptor by protein kinase C, the affinity of the EGF receptor for EGF binding is reduced (33). If mAbs somehow activated protein kinase C, and did not actually induce internalization of EGF receptors, a reduction of EGF binding to the cell surface would still be observed. The phosphoamino acid analysis reveals no increase in phosphothreonine or phosphoserine content after the addition of mAbs, thereby indicating that the apparent down-regulation observed in Fig. 1 is not merely due to a reduction of EGF binding affinity through the activation of protein kinase C.

DISCUSSION

We have examined the internalization of EGF and two mAbs that specifically bind to human EGF receptors by using two human cell lines that exhibit different growth responses to EGF. The results indicate that anti-EGF receptor mAbs were internalized without measurable stimulation of receptor phosphorylation in both cell lines. In contrast, internalization of EGF, a natural ligand having the potential to induce cell proliferation, was accompanied by a stimulation of the receptor phosphorylation. However, both EGF and anti-EGF receptor mAbs were delivered to the same cytosolic fraction within 10 min, by Percoll gradient sedimentation (Fig. 2). Under similar experimental conditions, Beguinot *et al.* (14) have directly demonstrated, by immunoelectron microscopy using anti-EGF receptor antibody, the internalization of EGF receptors and their delivery to receptosomes within 5 min after the addition of EGF. These results strongly suggest that stimulation of EGF receptor phosphorylation is not coupled to the internalization of the ligand–receptor complex. Our data do not rule out the possibility that (*i*) EGF and EGF receptor antibodies enter the cell by different mechanisms; (*ii*) anti-receptor mAb induces a transitory labile phosphorylation of the receptor, which is lost by 30 min of incubation with the ligand. If phosphorylation of the receptor occurs in response to mAb binding, it is much less stable or less extensive than that elicited by EGF. Since EGF is known to induce receptor clustering prior to internalization, it is possible that the bivalent mAb may dimerize the receptor, facilitating internalization.

The biological role of EGF-stimulated receptor phosphorylation or internalization is still unclear. Recently, the tumor promotor, 4-phorbol 12-myristate acetate (PMA), which stimulates EGF receptor phosphorylation through the activation of protein kinase C, has been shown to induce internalization of the EGF receptor in KB cells (34). Upon PMA-induced internalization, EGF receptors were transferred to receptosomes and, subsequently, to elements of the transreticular Golgi. Furthermore, unlike the EGF-induced response, EGF receptors were not found in lysosomes. The different intracellular fate of EGF receptor molecules, after internalization and delivery to receptosomes, might be related to the different mode of phosphorylation of the EGF receptor. Detailed studies on lysosomal enzymes (35), which may support the hypothesis, show that phosphorylation of mannose endows lysosomal enzymes with a traffic signal for their intracellular transport. Our anti-EGF receptor mAbs may provide tools to explore the biological significance of EGF receptor phosphorylation.

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