Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5521–5525, August 1986
Cell Biology

Epidermal growth factor receptor is increased in multidrug-resistant Chinese hamster and mouse tumor cells

(reverse transformation/vincristine resistance/actinomycin D resistance/plasma membrane glycoproteins)

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Communicated by Lloyd J. Old, April 7, 1986

ABSTRACT **Multidrug-resistant sublines of Chinese hamster lung and mouse tumor cells selected for high levels of resistance to vincristine or actinomycin D have increased numbers of epidermal growth factor (EGF) receptors compared to control cells. Evidence for this increase was found in six of six resistant cell lines with the use of receptor binding or immunoprecipitation techniques. Levels of ^{125}I-labeled EGF binding to intact actinomycin D-resistant cells derived from DC-3F or CLM-7 Chinese hamster lines are increased 3- to 10-fold compared to controls. Scatchard analysis of these data suggests that increased binding is a result of increased receptor number rather than altered affinity of receptor for ligand. Affinity-labeling and immunoprecipitation studies confirmed the finding of increased receptor amount in resistant hamster and mouse cells. Multidrug-resistant variants of DC-3F cells overproduce a plasma membrane glycoprotein (gp150–180) with several physicochemical properties that resemble those of EGF receptor. However, electrophoretic transfer blots with a polyclonal antibody to gp150–180 show that EGF receptor and gp150–180 are probably different molecules. Resistant cells described in this report manifest a normalized phenotype compared to transformed, tumorigenic, drug-sensitive parental cells. EGF receptor increase in resistant variants may be associated with this reverse transformation.**

Chinese hamster lung and mouse tumor cells selected for resistance to a diverse group of widely used cancer chemotherapeutic agents including antibiotics, such as actinomycin D or daunorubicin, or plant alkaloids, such as vincristine, are cross-resistant to other members of this group and are termed multidrug-resistant (1–4). An additional phenotypic characteristic of these cells with acquired multidrug resistance in our laboratory is a marked normalization of cell morphology and *in vitro* growth patterns and diminution of oncogenic potential *in vivo* (3, 5, 6). Resistance is quantitatively associated with decreased rate of drug accumulation (1, 2, 5). A specific plasma membrane glycoprotein species ($M_r \approx$ 150,000–180,000) is overproduced in multidrug-resistant Chinese hamster (DC-3F) cells (3, 4, 7, 8). A number of investigators have reported a similar species in other resistant hamster and human cells (9–12). No certain role for this membrane protein (gp150 in our previous literature and now designated gp150–180) in the development of resistance has been described.

Observation of normalized or reverse transformed phenotype of the resistant variants described in this report suggested the possibility of growth factor receptor changes in these cells. Polypeptide growth factors and the receptors to which they bind are intrinsically linked to growth control (13, 14) and possibly to tumorigenesis (15, 16). Initially, receptor for epidermal growth factor (EGF) was investigated because of the similarity between its molecular weight (M_r 170,000) (17) and pI (7.0) (18) and the molecular weight and pI of gp150–180, M_r 150,000–180,000 and 7.1, respectively (8). EGF binding to its receptor induces proliferation of target cells and, *in vitro*, can generate a phenotypic response associated with cellular growth (14, 19). The concomitance of increased receptor with development of resistance as a result of chronic exposure to xenobiotic agents is reported here. A brief report of these findings has been published (20).

MATERIALS AND METHODS

Materials. EGF was purchased from Collaborative Research (Waltham, MA); Na^{125}I and radioactive protein standards were from Amersham; [γ-^{32}P]ATP (2000–3000 Ci/mmol; 1 Ci = 37 GBq) and other radioisotopes were from New England Nuclear; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and other chemicals were from Sigma. Gel electrophoresis materials and nitrocellulose were purchased from Bio-Rad, and mouse monoclonal antibody to EGF receptor was from Oncor (Gaithersburg, MD). Polyclonal antibody to purified mouse liver EGF receptor was a gift from Stanley Cohen. Vincristine was a gift from Eli Lilly.

Cell Culture. Origins and phenotypic characteristics of the cell lines have been described (1–8, 21). Human epidermoid, A431, and human fibroblast, WI-38, cells were obtained from the American Type Culture Collection. All cultures are grown in Eagle's minimum essential medium (MEM)/Ham's F12 medium (1:1) supplemented with 5% fetal bovine serum. Experiments were conducted with cells in middle-to-late exponential growth phase. For some experiments cells were metabolically labeled with [^{35}S]methionine (10 μCi/ml, 800 μCi/mmol) in methionine-free medium for 18 hr or [6-^{3}H(N)]glucosamine (10 μCi/ml, 10–30 μCi/mmol) in glucose-reduced medium for 24 hr.

EGF Binding Assays. EGF was iodinated (300 Ci/μg) by the chloramine-T method (22, 23) and separated from ^{125}I by elution with 0.05 M potassium phosphate (pH 7.4) from a column of Sephadex G-25 (0.9 × 27 cm). Binding assays were carried out according to procedures described by Das *et al.* (24).

Cross-Linking of EGF to Its Receptor. Cells were incubated with 1 nM ^{125}I-labeled EGF in 1.0 ml of binding buffer for 60 min at 22°C as for binding assays. Procedures for cross-linking EGF to its receptor with 2 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide have been published (25). Electrophoresis was carried out on 7.5% acrylamide gels (0.075 × 14 × 11 cm) (26). This gel system was used throughout the study except where noted.

***In Vitro* Phosphorylation of Isolated Plasma Membranes.** Procedures for the isolation of plasma membranes have been

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Abbreviation: EGF, epidermal growth factor.

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published (7). Conditions used for phosphorylation of isolated plasma membranes were those described by King *et al.* (27) for phosphorylation of EGF receptor. Two-dimensional gel electrophoresis was carried out as described by O'Farrell *et al.* (28) and as previously used in this laboratory (8, 29).

Immunoprecipitation. [^{35}S]Methionine-labeled cells were lysed in a buffer containing 0.01 M Tris·HCl (pH 7.4), 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% $NaDodSO_4$, and 1 mM phenylmethylsulfonyl fluoride. Procedures have been reported for the precipitation of EGF receptor by mouse monoclonal or rabbit polyclonal (30) antibody by using protein A-Sepharose (30). Radioactivity in gel bands was measured with a liquid scintillation procedure.

Generation of Rabbit Polyclonal Antibodies and Electrophoretic Transfer Blotting. Rabbits received three intravenous injections at 10- to 18-day intervals of 5×10^7 DC-3F/VCRd-5L cells, which contain a high amount of gp150–180 (8), and were bled 6 days after the last injection. Serum was absorbed on 10^9 DC-3F cells per ml of serum for 2 hr (twice) and 16 hr (once) at 4°C.

Electrophoretic transfer blots were prepared according to procedures described by Towbin *et al.* (31). Cells were lysed in 20 mM Hepes buffer (pH 7.2) containing 1% Triton X-100. Aliquots of 100,000 × *g* supernatants containing 100 μg of protein were subjected to electrophoresis on 7.5% gels and the proteins were transferred to nitrocellulose. After blocking in 5% powdered milk in 0.1 M Tris buffer (pH 7.4) the sheets were incubated in absorbed polyclonal antibody (1:50 dilution in the milk solution). Detection of antigens was completed with the use of peroxidase-conjugated goat anti-rabbit antiserum and 4-chloro-2-naphthol and hydrogen peroxide as substrate.

RESULTS

Cell Lines and EGF Binding. The cell lines used in this study are listed in Table 1. CLM-7T is a spontaneously transformed tumorigenic Chinese hamster bone marrow line, whereas CLM-7N cells are nontumorigenic (6). CHNF 12, 18, 20, and 22 are independently derived, early passage normal or near-normal Chinese hamster bone marrow cell strains. Binding studies were carried out for all cells listed in Table 1 under a variety of conditions, including temperatures of 4°C, 22°C, and 37°C, various stages of cell growth, various times of incubation, and presence or absence of substances known to inhibit lysosomal degradation of EGF (32). The cells listed in Table 1 fell into two categories with respect to measurement of EGF binding: those for which B_{max} values could readily be determined under any conditions used and those for which specific binding could not be obtained in most experiments. The distinction between these two categories is not known. Schaudies *et al.* have reported that different cell lines can possess receptors with similar binding properties but process EGF in a dissimilar manner (33). Receptor amount in those cells that fell into the second category was measured by immunoprecipitation.

Scatchard plot analyses of binding data from representative experiments for DC-3F, DC-3F/AD X, CLM-7T, and CLM-7/AD XV are shown in Fig. 1. The mean B_{max} values derived from three to seven independent experiments are given in Table 1. The levels of EGF binding are in line with published values for cells such as Chinese hamster ovary (34). Affinity of the receptor for its ligand in resistant and sensitive cells appears to be similar (DC-3F and DC-3F/AD X, K_d = 1.0 nM; CLM-7T and CLM-7/AD XV, K_d = 0.4 nM). The increase in receptor is not related to differences in cell volume or size because affinity-labeling and immunoprecipitation studies, in which comparisons are based on equal amounts of cell protein rather than cell number, reveal approximately the same level of increase (Figs. 2–4, Table 1).

Affinity Labeling of EGF Receptor and Immunoprecipitation. Fig. 2 demonstrates that ^{125}I-labeled EGF binds to a M_r 170,000 protein (EGF receptor) in DC-3F and DC-3F/AD X cells. Densitometric analysis of the data reveals a 3-fold increase in level of binding to the resistant cells. Representative results of immunoprecipitation of EGF receptor from mouse tumor MAZ, MAZ/VCR, QUA, and QUA/ADj cells are shown in Fig. 3. Densitometric analysis and measurement of amounts of radioactivity in the EGF receptor bands from three experiments showed increases of 1.7-fold in MAZ/VCR compared to MAZ and 4.2-fold in QUA/ADj compared to QUA (Table 1). Immunoprecipitation of EGF receptor from DC-3F, DC-3F/VCRd-5L, and DC-3F/AD X revealed increases of 1.8- and 2.5-fold in DC-3F/VCRd-5L and DC-3F/AD X cells, respectively (Fig. 4*C* and Table 1).

Comparison of gp150–180 and EGF Receptor. A characteristic plasma membrane glycoprotein (M_r 150,000–180,000), designated gp150–180, is overproduced in resistant DC-3F/VCRd-5L and DC-3F/AD X cells compared to controls (Fig. 4*A*). The major *in vitro* phosphorylated protein in isolated plasma membranes of these cells has a molecular weight corresponding to that of gp150–180 or EGF receptor

Table 1. EGF receptor in multidrug-resistant and control cells

Cell line	Fold increase in resistance	Transformation phenotype*	B_{max} for EGF binding, fmol per 10^6 cells†	Immunoprecipitation of EGF receptor†‡
DC-3F	1	T	0.9 ± 0.08	0.013 ± 0.005
DC-3F/VCRd-5L	2750	N	1.4, 4.1§ (1.5, 4.6)	0.023 ± 0.001 (1.8)
DC-3F/AD X	2450	N	2.6 ± 0.47 (3.0)	0.032 ± 0.005 (2.5)
CLM-7N	1	N	2.5 ± 0.8	
CLM-7T	1	T	0.4 ± 0.04	
CLM-7/AD XV	415	N	4.0 ± 1.9 (10)	
CHNF 12		N	19.4 ± 8.0	
CHNF 18		N	8.3 ± 1.1	
CHNF 20		N	18.1 ± 7.7	
CHNF 22		N	18.7 ± 5.2	
MAZ	1	T		0.022 ± 0.01
MAZ/VCR	3939	N		0.042 ± 0.01 (1.7)
QUA	1	T		0.027 ± 0.01
QUA/ADj	9355	N		0.11 ± 0.05 (4.2)

*T, transformed as determined by tumorigenicity testing and/or morphology; N, near normal or normalized.
†Numbers in parentheses are ratios of resistant to appropriate control cell values.
‡Percent of radioactivity incorporated into and immunoprecipitated as EGF receptor from lysates of cells metabolically labeled with [^{35}S]methionine. Bands containing EGF receptor were excised for measurement of radioactivity.
§Results of two different experiments. Measurement of EGF binding to these cells was difficult (see text).

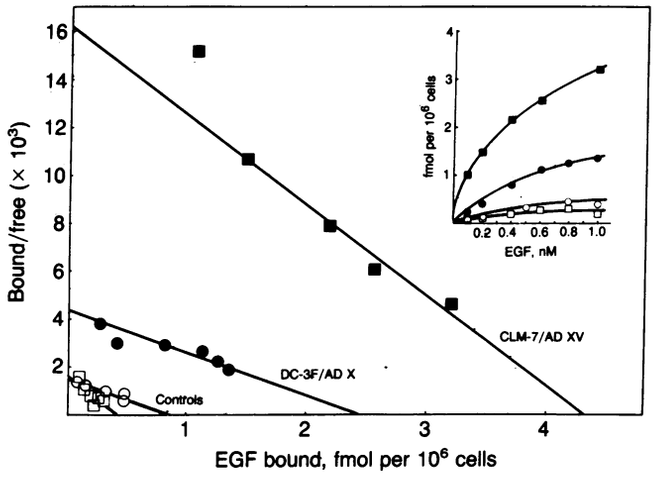

FIG. 1. Scatchard analyses of ^{125}I-labeled EGF binding to DC-3F (○), DC-3F/AD X (●), CLM-7T (□), and CLM-7/AD XV (■) cells. Cells in monolayer were incubated with ^{125}I-labeled EGF (0.05–1 nM) in the presence or absence of 100 nM unlabeled EGF for 60 min at 22°C. (*Inset*) Representative binding curves. The value at 1.05 fmol of EGF bound per 10^6 cells for CLM-7/AD XV, specifically excluded in the Scatchard plot, was reproducible and may indicate the presence of a high-affinity class of EGF receptor in those cells.

(Fig. 4*B*). EGF increased the level of phosphorylation of this protein by 1.5- to 2-fold (Fig. 4*B*, lanes 4 and 6). EGF receptor in A431 cells is phosphorylated *in vitro*, and the level of phosphorylation is enhanced 3-fold in the presence of EGF (27). Gp150–180, labeled *in vitro* with [γ-^{32}P]ATP (Fig. 4*D*) or labeled metabolically with [^{35}S]methionine (Fig. 4*E*), [^{3}H]glucosamine (ref. 8), or $^{32}P_i$ (data not shown), has a pI of 7.1. Gp150–180 in DC-3F is visualized only after prolonged film exposure; however, EGF receptor is readily shown to be present in DC-3F by EGF binding (Fig. 1) and by immunoprecipitation (Fig. 4*C*).

Analysis of gp150–180 with Polyclonal Antibody Raised Against DC-3F/VCRd-5L Cells. A representative electrophoretic transfer blot of DC-3F, DC-3F/VCRd-5L, and DC-3F/AD X proteins analyzed with the polyclonal antibody to gp150–180 is shown in Fig. 5, lanes 1–3. Densitometric analysis of these lanes indicates a >50-fold increase in

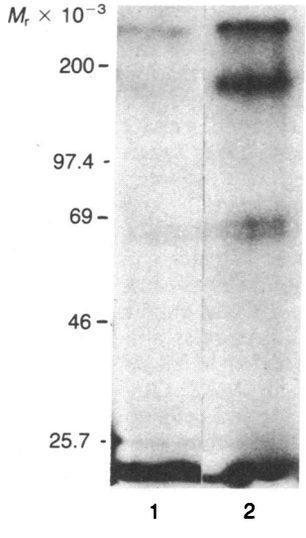

FIG. 2. Radioautogram of affinity-labeled EGF receptor in DC-3F (lane 1) and DC-3F/AD X (lane 2) cells. Ligand–receptor cross-linking procedures are given in the text. Two hundred micrograms of protein, measured by the Lowry procedure (35), was analyzed for each sample.

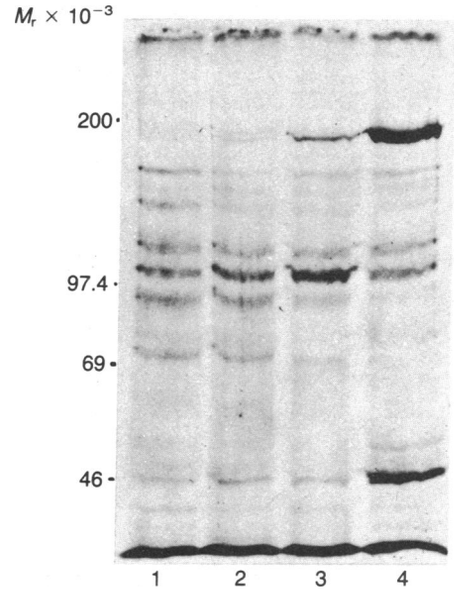

FIG. 3. Radioautogram of [^{35}S]methionine-labeled proteins immunoprecipitated with rabbit polyclonal antibody to EGF receptor. Aliquots containing 1 × 10^6 cpm of MAZ (lane 1), MAZ/VCR (lane 2), QUA (lane 3), and QUA/ADj (lane 4) cell proteins were analyzed.

gp150–180 in resistant cells. MAZ/VCR and QUA/ADj, but not MAZ or QUA, cells also contain proteins that cross-react with this antibody (data not shown). There is no cross-reacting material in A431 cells (data not shown). A431 cells have high levels of EGF receptor (in our hands, the B_{max} value is about 600 fmol per 10^6 cells). The discrepancy between the large increase in amount of gp150–180 and the low-level increase in EGF receptor in resistant cells and the lack of material in A431 cross-reactive with gp150–180 antibody suggest that the polyclonal antibody recognizes a species distinct from EGF receptor.

In initial attempts to ascertain whether EGF receptor increase is associated with the normalization aspect of the resistant cell phenotype we examined EGF receptor content of four early-passage Chinese hamster bone marrow populations. These cells have near-normal morphologies and *in vitro* growth patterns and are drug-sensitive. Plating efficiencies in a soft agar assay (36) are 0.001% or less for CHNF 12 and CHNF 20, 1.0% for the more rapidly growing CHNF 22 strain, and 16.4% for CHNF 18, the only strain showing early signs of spontaneous morphological transformation. The latter shows the lowest level of EGF binding of the four CHNF strains (Table 1). However, all four strains have higher levels of binding, ranging from 9- to 49-fold, than spontaneously transformed Chinese hamster DC-3F and CLM-7T cells (Table 1). CHNF 12 and CHNF 20, the strains with the lowest soft agar plating efficiency, also synthesize a protein (M_r 150,000) that is recognized by a component of the gp150–180 polyclonal antibody (Fig. 5, lanes 4 and 6).

Further indication of a relationship between state of transformation and EGF receptor content was demonstrated by binding studies of CLM-7 cells prior to and after transformation. The B_{max} for EGF binding of CLM-7N (nontumorigenic) cells is 6.3-fold higher than that of CLM-7T (tumorigenic) (Table 1).

Study of Drug Sensitivity of Cells with High Levels of EGF Receptor. To investigate the possibility that high levels of EGF receptor *per se* would render a cell intrinsically resistant to vincristine or actinomycin D, A431 and WI-38 cells, which contain high levels of receptor, were challenged with drug. The cells were found to be 5–10 times more sensitive to

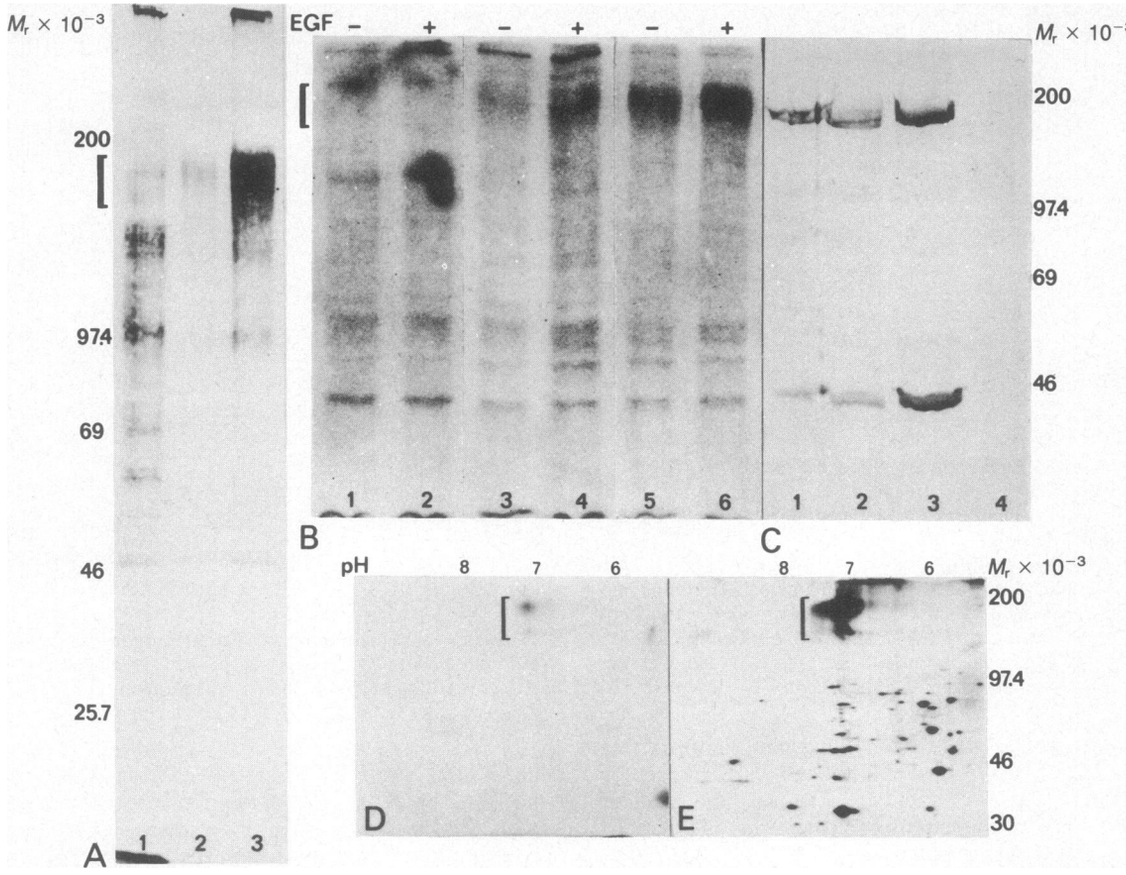

FIG. 4. Physicochemical characteristics of gp150–180 and of immunoprecipitated EGF receptor in DC-3F cells and sublines. (*A*) Fluorogram of isolated plasma membrane proteins metabolically labeled with [^{3}H]glucosamine (1×10^5 cpm per sample) from DC-3F (lane 1), DC-3F/VCRd-5L (lane 2), and DC-3F/AD X (lane 3) cells. Proteins were separated by electrophoresis on NaDodSO$_4$/acrylamide (5–13% gradient) gels ($0.075 \times 14 \times 30$ cm). Bracketed gp150–180 is the major membrane glycoprotein in both resistant sublines. (*B*) Radioautogram of isolated plasma membranes (30 μg per reaction mixture) from DC-3F (lanes 1 and 2), DC-3F/VCRd-5L (lanes 3 and 4), and DC-3F/AD X (lanes 5 and 6) phosphorylated *in vitro* with [γ-^{32}P]ATP (15 μM, 1×10^6 cpm) in the absence (−) or presence (+) of 100 nM EGF. Entire reaction mixtures were examined on gels (26). (*C*) Fluorogram of [^{35}S]methionine-labeled EGF receptor from DC-3F (lane 1), DC-3F/VCRd-5L (lane 2), and DC-3F/AD X (lane 3) cells (5×10^6 cpm per sample) immunoprecipitated with monoclonal antibody to mouse EGF receptor. Lane 4 is a preimmune serum control. (*D*) Radioautogram of two-dimensional gel analysis of DC-3F/AD X cell plasma membranes phosphorylated *in vitro* in the presence of EGF. Products of the reaction (a duplicate of that shown in *B*, lane 6) were examined by isoelectric focusing (pH 3–10) followed by second-dimension electrophoresis on 7.5% acrylamide gels. (*E*) Radioautogram of two-dimensional gel analysis of 2×10^5 cpm of isolated plasma membrane from DC-3F/VCRd-5L cells metabolically labeled with [^{35}S]methionine. Gp150–180 is designated by brackets.

actinomycin D and 2–7 times more sensitive to vincristine than hamster or mouse control cells.

DISCUSSION

Increase in EGF receptor coincident with development of multidrug resistance in cells selected with vincristine or actinomycin D was observed in six independently derived sublines of Chinese hamster and mouse tumor cells. Cells that have not been exposed to the xenobiotic agents and have high EGF receptor levels as a phenotypic characteristic appear not to be intrinsically resistant to these agents.

EGF receptor was the initial receptor studied in this context because some of its characteristics are similar to those of gp150–180, as described in this report. However, whereas EGF receptor and gp150–180 may have some properties in common, they are probably distinct species. DC-3F/AD X cells, for example, show a 3-fold increase in EGF receptor by EGF binding, EGF affinity labeling, and immunoprecipitation compared to DC-3F but have a >50-fold increase in gp150–180. Whether EGF receptor is part of the gp150–180 family of proteins or is in some way related to gp150–180 remains to be investigated. As yet not fully explained is the effect of EGF on level of phosphorylation of gp150–180 (Fig. 4*B*).

Considerable information about regulation of EGF receptor synthesis and receptor modulation associated with changes in state of transformation has been reported (37–40). In the overall view, EGF receptor levels may be decreased, increased, or not changed in cancer cells as compared to their normal counterparts (39). Jetten has shown that 3T6 and NRK cells treated with retinoic acid manifest more normal characteristics and have 2- to 3-fold higher EGF binding levels than untreated cells (37). Other investigators have shown that Chinese hamster embryo cells lose cell-surface EGF receptors gradually during the course of neoplastic progression (38). In our experience phenotypically more normal cells (multidrug-resistant cells, CLM-7N, CHNF 12, 18, 20, 22) have higher levels of EGF receptor than transformed cells (DC-3F, MAZ, QUA, CLM-7T). EGF receptor decrease in transformed cells compared to normal controls can be a result of production of transforming growth factor-α (TGF-α) which can down-regulate the receptor (39). However, in several types of tumors EGF receptor levels are higher than in the normal counterparts because of increased numbers of receptor gene copies (39). Whether modulation of receptor number in the cells studied for this report is a result of gene amplification, altered rate of transcription, TGF-α production, or another mechanism will be a subject for future study.

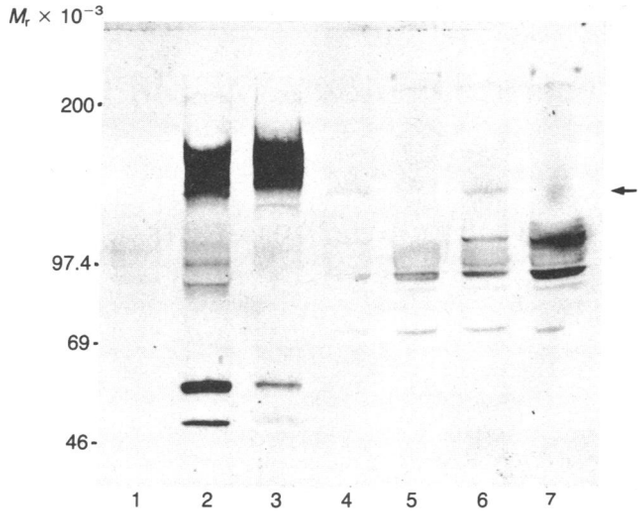

FIG. 5. Electrophoretic transfer blot analysis of DC-3F (lane 1), DC-3F/VCRd-5L (lane 2), DC-3F/AD X (lane 3), CHNF 12 (lane 4), CHNF 18 (lane 5), CHNF 20 (lane 6), and CHNF 22 (lane 7) cell proteins with absorbed polyclonal antibody to gp150–180. The M_r 150,000 cross-reacting material in CHNF 12 and 20 is shown by an arrow. Additional cross-reacting material is also of interest. The M_r 150,000 band is indicated because of its correspondence with gp150–180.

One testable hypothesis of why multidrug-resistant cells might have increased EGF receptor levels is that altered or decreased transport of required nutrients is involved in the drug exclusion mechanism of multidrug-resistant cells. Increased receptor amount may assist in increasing uptake of certain nutrients to maintain cell viability.

An intriguing corollary to the possibility that normalization is part of the resistance phenotype is that gp150–180 (or a member of that family of proteins) may be associated with state of transformation. This was suggested by the finding of cross-reacting material in CHNF 12 and 20 (strains with low plating efficiency in soft agar) to the polyclonal antibody that recognizes gp150–180 (Fig. 5). The cross-reacting material (M_r 150,000) is not seen in DC-3F cell samples.

EGF receptor level has been shown to be elevated in cells selected for high levels of resistance to vincristine or actinomycin D by several different criteria. This modulation may be an inherited, epigenetically controlled trait of multidrug-resistant cells associated with the observed cell normalization.

We thank Dr. David Donner for participation and Katherine Schneider for technical assistance in this study. This investigation was supported in part by Grants CA-28595, CA-08748, and CA-315525 from the National Institutes of Health.

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