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REDUCED BINDING OF EPIDERMAL GROWTH FACTOR BY AVIAN SARCOMA VIRUS-TRANSFORMED RAT CELLS

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Summary: Rat cells transformed by Rous sarcoma virus and Fujinami sarcoma virus bound 5-10% of the amount of epidermal growth factor (EGF) bound by normal cells. Scatchard plot analysis indicated that the reduction in binding by transformed cells was due to a decreased number of receptors rather than to altered binding affinity. In experiments with temperature sensitive mutants of Rous sarcoma virus and Fujinami sarcoma virus significant loss of EGF binding occurred within one hour of shift from non-permissive to permissive temperature. Conditioned media from various normal and transformed cell lines were examined for the ability to inhibit EGF binding to normal cells or to cause "down regulation" of EGF receptors. No activity of either type was found. EGF-dependent phosphorylation in isolated membrane preparations was also examined. Membranes from normal cells displayed EGF-dependent phosphorylation of a M_r 180,000 protein presumed to be the EGF receptor. This activity was absent in membranes from transformed cells. The data suggest a close correlation between activation of avian sarcoma virus transforming gene products and modulation of the EGF growth regulatory system.

Cells transformed by DNA-tumor viruses, RNA-tumor viruses, and chemical carcinogens proliferate under nutritional conditions which restrict the growth of their normal cell counterparts (1). Many of these transformed cells exhibit reduced *in vitro* requirements for particular growth factors such as epidermal growth factor (EGF) (1) and fibroblast growth factor (2), suggesting that the growth factor systems are activated or circumvented in the transformed cells. One possible explanation of this phenomenon has been presented by Todaro et al. (3), who propose

Abbreviations: EGF: epidermal growth factor; ASV: avian sarcoma virus; SRA: Schmidt-Ruppin strain subgroup A Rous sarcoma virus.

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that certain tumor cells produce their own growth factors which are secreted into the growth medium. In support of this hypothesis, EGF-like growth factors (termed sarcoma growth factors or transforming growth factors) have been isolated from culture fluids from a number of malignant cells (4, 5). Most of the cell lines which produce EGF-like factors bind reduced amounts of EGF (4, 5), perhaps due to interaction of the endogenously produced growth factors with EGF receptors.

However, loss of EGF requirement by all tumor cells is not readily explained in terms of production of endogenous factors. Cherington et al. (1) have shown that polyoma virus-transformed Syrian hamster cells no longer require EGF for growth in serum free medium, although polyoma virus transformed 3T3 cells bind normal amounts of EGF (6). Certain chemically transformed cells possess reduced numbers of EGF-receptors even though no EGF-like activity is present in culture supernatants (7) and SV40-transformed BHK cells produce factors which reduce EGF binding to normal cells without directly binding to the EGF-receptor (8).

Despite this general interest in growth factor interaction and neoplastic transformation, little is known about the role of growth factor systems in avian sarcoma virus (ASV)-transformed cells.

In this report EGF binding and EGF-dependent *in vitro* membrane protein phosphorylation are examined in ASV-transformed cells.

Materials and Methods

Cell Cultures. 3Y1 normal rat cells, Rous sarcoma virus Schmidt-Ruppin subgroup, strain A (SRA) transformed 3Y1 cells, and the ts225 3Y1 line infected by a temperature sensitive mutant of Fujinami sarcoma virus were generously provided by Dr. H. Hanafusa (The Rockefeller University). The LA23 NRK line infected with a temperature sensitive mutant of Prague A Rous sarcoma virus was a gift from P. Vogt (University of Southern California). RR1022 Schmidt-Ruppin strain subgroup D transformed rat cells were from A. Goldberg (The Rockefeller University). All cells were grown in DME containing 10% calf serum (Flow Laboratories). For the

temperature shift experiments the permissive temperature was 34.5°C and the non-permissive temperature was 40.5°C.

EGF Binding. Specific binding of [^{125}I]labeled EGF was performed as described by DeLarco and Todaro (9) except that 35mm dishes of subconfluent cells (approximately 5 x 10^5 cells/dish) were used. For "down regulation" experiments the normal culture medium was removed from 3Y1 cells and 2 ml of 24 hour conditioned medium from confluent cultures of various cell types were added. After 2 hours incubation at 37°C, [^{125}I]EGF binding was determined as above.

Membrane Preparation and in vitro EGF-stimulated Phosphorylation. Membrane fractions were prepared according to Thom et al. (10) except that cells were lysed in borate-EDTA buffer by pipetting and the lysate layered directly onto the 35% sucrose cushion. After centrifugation, the material at the 35% sucrose interface was used to assay EGF-stimulated phosphorylation of the M_r 180,000 protein presumed to be the EGF-receptor (11). EGF-stimulated phosphorylation in these membranes was carried out as (11).

Miscellaneous Methods. Binding of [^{125}I]insulin was done according to Raizada et al. (12). Protein was determined and SDS polyacrylamide gels were prepared as (13).

Materials. Receptor grade EGF was from Collaborative Research (Waltham, MA) and [^{125}I]labeled EGF was prepared according to Hollenberg and Cautrecasas (14) using carrier free Na ^{125}I from New England Nuclear (Boston, MA). [^{125}I]insulin and [γ-^{32}P]ATP were purchased from Amersham (Clearbrook, IL).

Results

Normal and ASV-transformed rat cells were examined for their ability to bind [^{125}I]EGF (Table 1). These data were collected using 0.4 ng/ml [^{125}I]EGF, although similar differences in binding were seen at EGF concentrations near the apparent dissociation constant. Transformed cells or cells infected with temperature sensitive mutants incubated at the permissive temperature bound 5-10% of the amount of EGF bound by control cells. Cells infected with several strains of Rous sarcoma virus and with a Fujinami sarcoma virus temperature sensitive mutant all exhibited reduced EGF binding. Loss of binding was not due to degradation of EGF since [^{125}I]EGF which had been incubated with transformed cells was bound at expected levels when subsequently added to normal cells. Binding of [^{125}I]insulin was not reduced in transformed cells.

Table 1. EGF binding to normal and avian sarcoma virus-transformed rat cells.

Cell Line	[^{125}I]EGF Bound (pg/10^6 Cells)
3Y1	18.2
SRA-3Y1	1.7
LA23 (40.5°C)	24.8
LA23 (34.5°C)	2.7
ts225 (40.5°C)	15.9
ts225 (34.5°C)	1.8
RAT-1	31.1
RR1022	1.6

Binding assays were performed as described in "Materials and Methods" using 0.4 ng/ml [^{125}I]EGF. LA23 and ts225 cells were maintained at 40.5°C or 34.5°C for at least 24 hours prior to assay.

Scatchard plot analysis (15) of EGF binding to 3Y1 cells and SRA - 3Y1 cells at 4°C revealed saturaturable binding with similar apparent dissociation constants (Kd) (1.2 to 1.4 nM) for both types of cells (Fig. 1). The total number of EGF binding sites was about 30,000 per cell for 3Y1 cells and about 2000 per cell for SRA-3Y1 cells.

The kinetics of EGF binding in temperature shift experiments using rat cells infected with temperature sensitive mutants of Rous sarcoma virus and Fujinami sarcoma virus are

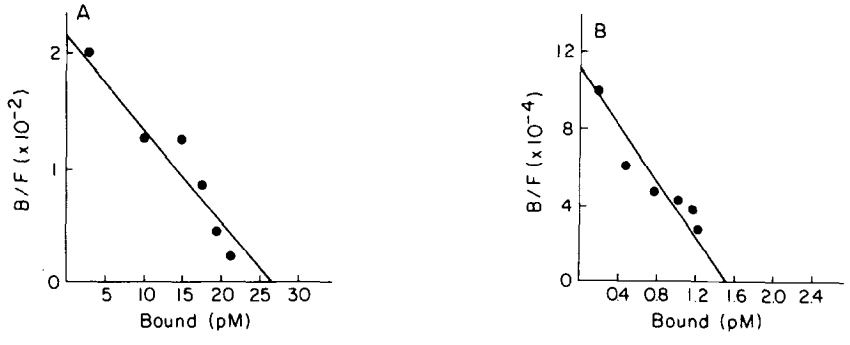

Fig. 1. Scatchard plot analysis of EGF binding to 3Y1 (A) and SRA-3Y1 cells (B). Binding assays were performed at 4°C for 2 hours at EGF concentrations from 1 ng/ml to 25 ng/ml essentially as described in "Materials and Methods".

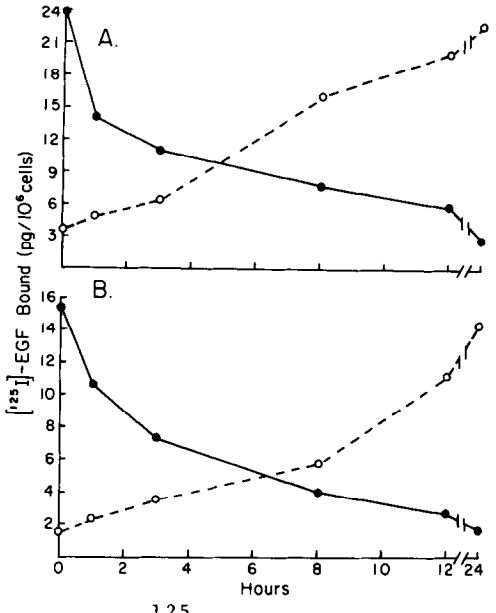

Fig. 2. Time course of [^{125}I]EGF binding following temperature shifts with LA 23 temperature sensitive Rous sarcoma virus infected cells (A) and with ts225 temperature sensitive Fujinami sarcoma virus infected cells (B). At T_0 cells were shifted from 40.5°C to 34.5°C (●-●) or from 34.5°C to 40.5°C (o-o). EGF binding was performed as described in "Materials and Methods".

presented in Fig. 2. For both cell lines shift from non-permissive (40.5°C) to permissive (34.5°C) temperature resulted in a rapid decrease in EGF binding. Significant loss is seen within one hour of downshift, with maximal loss reached at about 12 hours. When growth medium was replaced every hour with fresh media, similar results were found. Semi-logarithmic plots of the decrease in binding following shift from non-permissive to permissive temperature indicated that the reaction did not follow first order kinetics. Recovery of EGF binding following shift from permissive to non-permissive temperature was initially less rapid, but reached maximal levels by 12-16 hours. When 3Y1 cells grown at 40.5°C were shifted to 34.5°C, binding remained at 85-95% of the initial level. Temperature shift had no effect on EGF binding to SRA-3Y1 cells.

Since certain tumor cells secrete EGF-like factors (3, 4), culture media from ASV-transformed cells were examined for the

Table 2. Down regulation of 3Y1 cell EGF receptors by conditioned media and EGF.

Addition	[^{125}I]EGF Bound (pg/10^6 Cells)
None	18.1
3Y1 Medium	17.6
SRA-3Y1 Medium	16.4
LA23 Medium (34.5°C)	17.9
ts225 Medium (34.5°C)	18.3
5 ng/ml EGF	3.1

Assays were performed as described in "Materials and Methods" with 0.4 ng/ml [^{125}I]EGF.

ability to compete with EGF for receptor binding or to cause "down regulation" of EGF-receptors. No direct competing activity was found (not shown) and as shown in Table 2 none of the conditioned media from transformed cells induced loss of binding through "down regulation."

Fig. 3 demonstrates that membranes prepared from SRA-3Y1 cells or from 3Y1 cells grown in the presence of EGF no longer carry out *in vitro* EGF-stimulated phosphorylation of a M_r 180,000

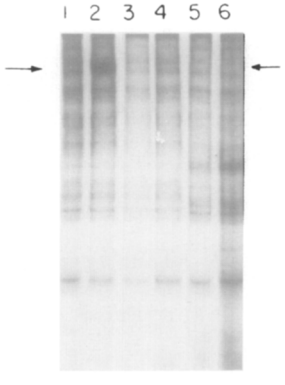

Fig. 3. EGF-stimulated phosphorylation of the EGF receptor in normal and ASV-transformed cells. Membranes were prepared from 3Y1 cells (1, 2), 3Y1 cells grown in the presence of 50 ng/ml EGF for 12 hours (3, 4) and from SRA-3Y1 cells (5, 6) as described in "Materials and Methods". For the samples in lanes 2, 4, and 6, membranes were incubated for 10' at 0°C with 100 ng/ml EGF prior to addition of 2.5 uM [γ-^{32}P]ATP. Arrows indicate the M_r 180,000 region of the gel.

protein presumed to be the EGF receptor (11). The absence of this activity in SRA-3Y1 membranes is probably not due to degradation of EGF receptors during membrane preparation since membranes from mixtures of 3Y1 and SRA-3Y1 cells display EGF-dependent phosphorylation.

Preincubation of normal 3Y1 membranes with crude conditioned media from avian sarcoma virus-transformed cells did not stimulate phosphorylation of the M_r 180,000 protein (not shown).

Discussion

The data indicate that functioning of the src or fps gene product leads to a rapid decrease in the EGF binding capacity of ASV-transformed rat cells. The mechanism by which this loss occurs is uncertain, although the Scatchard analysis indicates that ASV-transformation reduces the number of available EGF receptors without affecting their binding affinity. This is similar to the reduced EGF-binding exhibited by normal cells in which EGF receptors have been "down regulated" through prior exposure to EGF (16). The kinetics of the gain or loss of EGF binding by the temperature sensitive mutant infected cells in temperature shift experiments are also similar to those found in "down regulated" normal cells. Loss of EGF receptors through "down regulation" begins immediately upon EGF addition, while recovery of receptors after "down regulation" takes 10 to 20 hours (16). Consequently, the loss of EGF binding in ASV-transformed cells may be due to "activation" of the EGF system. This possibility is attractive since in the presence of EGF normal 3Y1 and RAT-1 cells acquire many of the altered growth characteristics associated with ASV-transformation. These include altered morphology, reduced serum requirements, increased solute transport, and density independent growth (not shown).

Production of endogenous growth factors by the tumor cells could account for these findings. However, several lines of evidence argue against the accumulation of diffusable EGF-like factors in ASV-transformed cell media: 1) The loss of EGF binding by LA 23 cells or ts225 cells when shifted from 40.5°C to 34.5°C is very rapid, precluding the build-up of endogenous factors in the medium, 2) Conditioned media from transformed cells do not compete with EGF for receptor binding or induce "down regulation" of EGF-receptors, 3) In in vitro studies with isolated membranes, transformed cell media does not stimulate phosphorylation of the Mr 180,000 EGF-receptor.

The present study suggests a more direct relationship between activation of ASV-transforming gene product protein kinase (17, 18) and activation of the EGF growth regulatory system. The availability of antiserum against the EGF-receptor should be useful in more directly examining this problem, especially in regard to the role of phosphorylation of the EGF receptor in receptor function.

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