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Detection of the Ligand Activity of the c-*erbB*-2 Protein in Calf Serum

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We established NIH3T3 derivatives in which wild-type c-*erbB*-2 or activated c-*erbB*-2 having a point mutation in the sequence coding for the transmembrane domain was expressed. These cell lines were termed RC and A4, respectively. A4 cells but not RC or NIH3T3 cells grew even in the presence of a low concentration (0.05%) of calf serum (CS), although the rate of their proliferation was low. In media containing 0.1% CS, both A4 cells and RC cells but not NIH3T3 cells could proliferate. Furthermore, RC cells induced foci formation when cultured in media containing 0.5% and 5% CS, while growth of the parental NIH3T3 cells was contact-inhibited under these conditions. These data suggest the presence of a factor(s) which activates protein-tyrosine kinase activity of the c-*erbB*-2 protein. In fact, the c-*erbB*-2 protein prepared from RC cells showed CS-dependent protein-tyrosine kinase activity when assayed in membrane fractions.

Key words: c-*erbB*-2 — Tyrosine kinase — Cell growth — Serum factor

The c-*erbB*-2 gene encodes a transmembrane glycoprotein which is similar to the epidermal growth factor (EGF) receptor in structure.[1] The c-*erbB*-2 gene is frequently amplified and overexpressed in many adenocarcinomas, including mammary carcinomas,[2–7] while the EGF receptor gene is often overexpressed in squamous carcinoma cells.[8] Overexpression of the c-*erbB*-2 protein has been shown to be correlated with lymph node metastasis and with a poor prognosis of breast cancer, suggesting that the c-*erbB*-2 protein plays an important role in the progression of this disease.[5–7]

Immunohistological study revealed that the c-*erbB*-2 protein is commonly located in fetal epithelial cells but hardly at all in postnatal tissues.[9] Therefore, this protein has been assumed to be a growth factor receptor that plays a role in mitogenic signalling in the fetal epithelium. Although there are several reports describing the presence of factors that stimulate the activity of the c-*erbB*-2 protein in the conditioned medium of transformed cells,[10–12] analysis of the molecular structure of its ligand is still required. In this study, we compared the effect of serum on cell growth between c-*erbB*-2 transfected cells and parental NIH3T3 cells. The results suggest that calf serum (CS) contains a factor(s) that stimulates the c-*erbB*-2-mediated signalling pathway.

We first examined serum dependency of growth of parental NIH3T3 cells and NIH3T3 cells transfected with expression plasmids containing either wild-type c-*erbB*-2 (RC cells) or c-*erbB*-2 mutated to encode Glu instead of Val-659 within the transmembrane domain (A4 cells). A4 cells but not RC cells showed transformed phenotype (Figs. 1 and 2). Both A4 and RC cells as well as NIH3T3 cells could grow in the presence of 0.25–1% CS (Figs. 1 and 2). Under these conditions, growth of NIH3T3 cells but not A4 or RC cells was contact-inhibited; namely both A4 and RC cells overgrew, showing narrow and broad rhomboid morphology, respectively (Fig. 2). In the presence of 0.05% CS, only A4 cells were able to proliferate (Fig. 3c). Interestingly, RC cells, though not transformed, proliferated a little in the presence of 0.1% CS (Fig. 3b), while parental NIH3T3 cells died under this condition. It is possible that this preferential growth stimulation of c-*erbB*-2-expressing cells by CS is mediated by up-regulation of kinase activity of the c-*erbB*-2 protein.

To test this possibility, we examined the level of expression and the kinase activity of the c-*erbB*-2 protein in these cells cultured at various concentrations of CS. Two sets of cells of each cell line were cultured in the presence of 5% CS. The medium in one set of cultures was replaced with medium containing 0.05% CS. After eight hours of incubation, the cell lysates of both sets were subjected to western blotting by using anti-c-*erbB*-2 antibodies (Fig. 4a). The level of expression did not change greatly when cells were cultured at the lower concentration of CS for the indicated period. Under the same experimental conditions except that the RC cells were also cultured in media containing 0.5% CS for eight hours, membrane fractions were prepared from the cell lysates and were subjected to *in vitro* kinase assay (Fig. 4b). The kinase activity of the normal c-*erbB*-2 protein was stimulated in a serum-dependent manner. In the presence of 5% CS, the kinase activity was similar to that of the mutant c-*erbB*-2 protein (Fig. 4b). Autophosphorylation activity of the mutant c-*erbB*-2 protein was high and independent of the CS concentration.

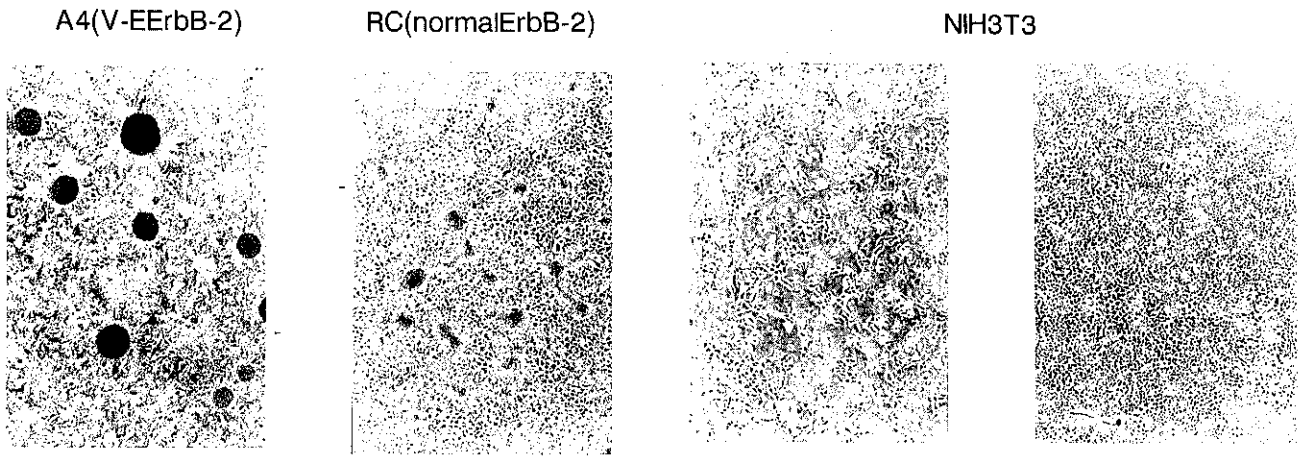

Fig. 1. Comparison of growth of A4, RC, and NIH3T3 cells. A4, RC, and NIH3T3 (left panel) were cultured in the ASF 104 medium with 0.25% CS. NIH3T3 cells (right panel) were also cultured in the same medium with 1% CS. Cells (2×10^4) of each cell line were grown in culture plates with 6 wells (3.5 cm diameter/well). Media were changed every two days. The photographs were taken on the tenth day (40-fold magnification).

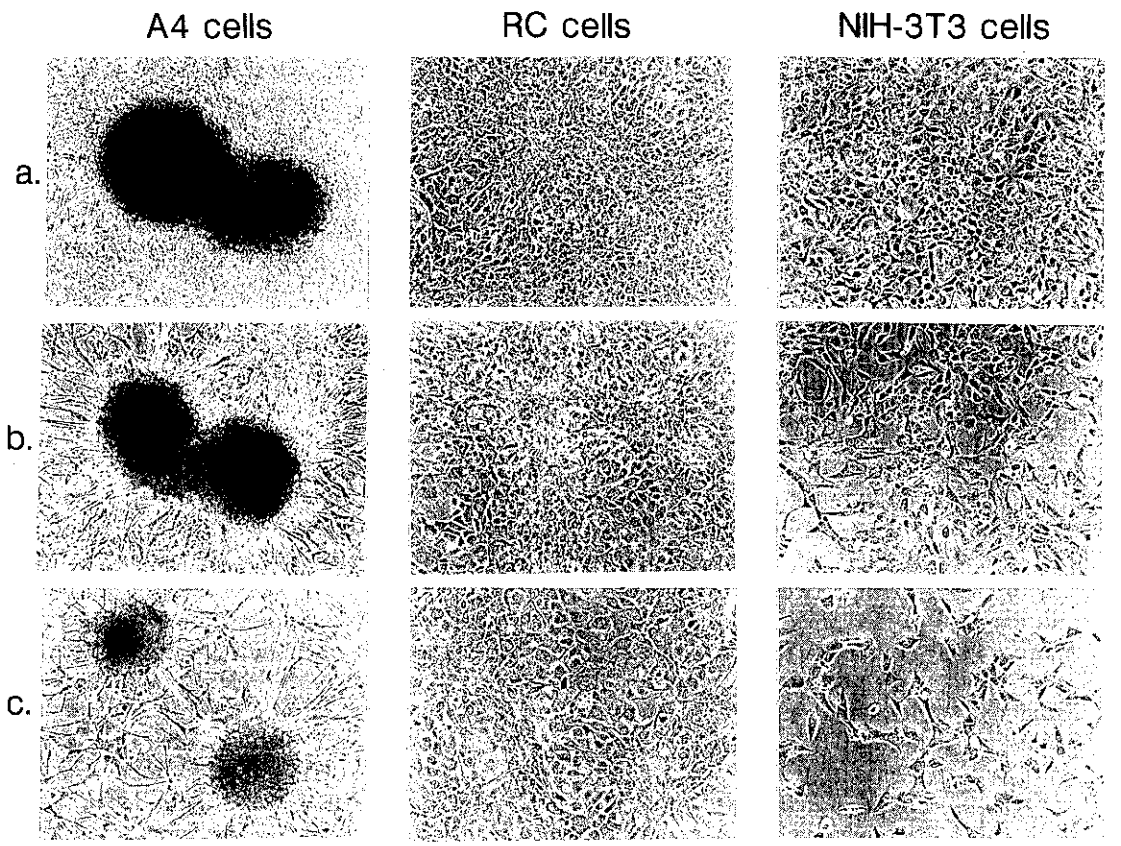

Fig. 2. Comparison of morphology of A4, RC and NIH3T3 cells. Cells (2×10^4) were incubated in the ASF-104 medium with 1% (a), 0.5% (b) and 0.25% (c) CS, respectively, in culture plates. The media were changed every two days. The photographs were taken on the tenth day (100-fold magnification).

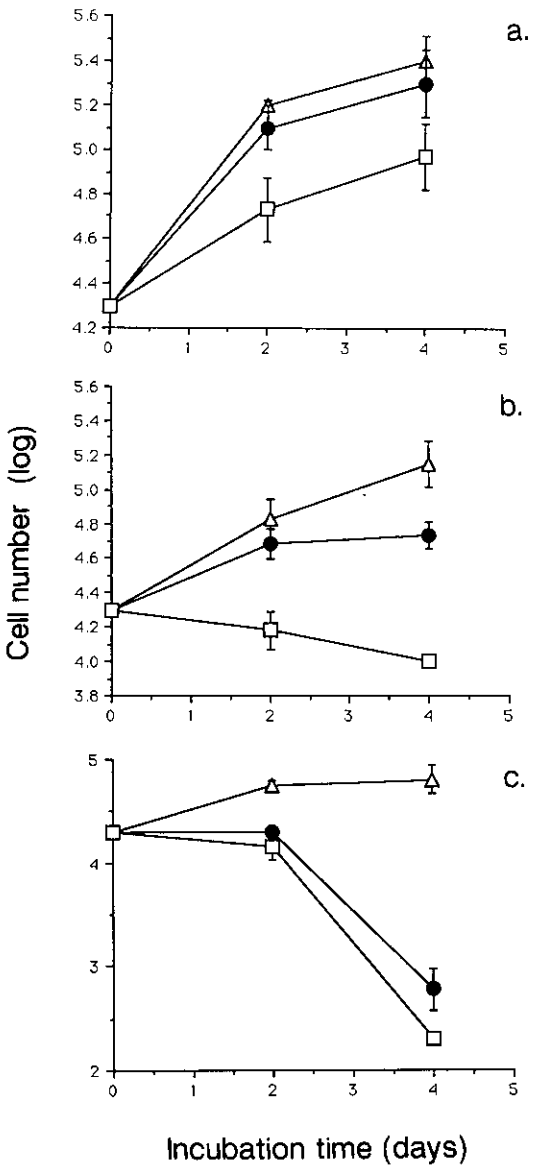

Fig. 3. Growth curves of A4, RC, and NIH3T3 cells. Cells (2×10^4) were inoculated in culture plates with 6 wells (3.5 cm diameter/well) in the ASF-104 media containing 1% (a), 0.1% (b), and 0.05% (c) CS, respectively. The cell number was counted on the second and fourth days of culture. The open triangles, closed circles, and open squares represent A4, RC, and NIH3T3 cells, respectively.

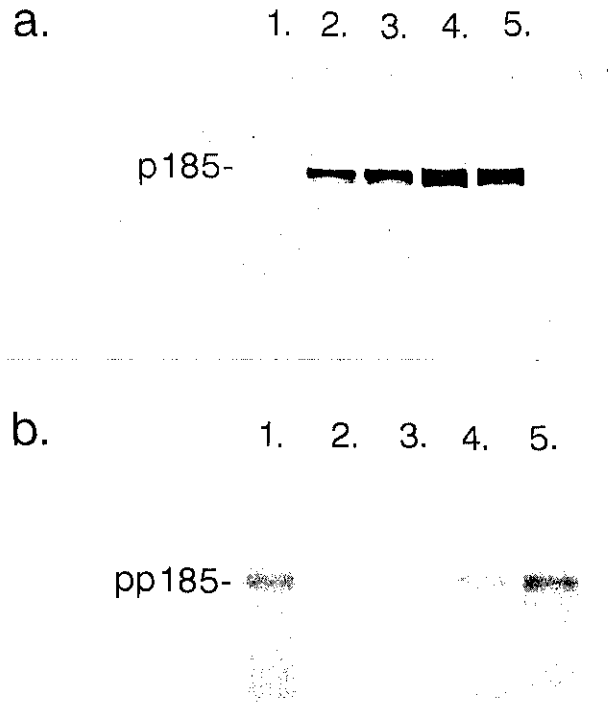

Fig. 4. Biochemical tests of the c-*erbB*-2 protein. a. Western blotting. Two sets of A4 (lanes 4 and 5), RC (lanes 2 and 3), and NIH3T3 (lane 1) cells (5% CS group: 5×10^5 and 0.05% CS group: 7.5×10^5 cells) were incubated in the ASF-104 medium with 5% CS. Then the media of one set were changed to that with 0.05% CS. After 8 h of culture with 5% CS (lanes 1, 3 and 5) and with 0.05% CS (lanes 2 and 4), cells were lysed with RIPA buffer (0.15 *M* NaCl/10 m*M* Tris-HCl, pH 7.4/10% Triton X-100/1% sodium deoxycholate/0.1% SDS/2 m*M* EDTA/14 m*M* 2-mercaptoethanol/aprotinin (20 μg/ml)/50 m*M* NaF/0.2 m*M* Na_3VO_4), the lysates from equal cell numbers were analyzed by western blotting with anti-c-*erbB*-2 polyclonal antibodies as described previously.[14] b. *In vitro* autophosphorylation. Lysates were prepared from A4 (lanes 1 and 5) and RC (lanes 2, 3 and 4) cells (5×10^4) cultured in ASF-104 media containing 0.05% (lanes 1 and 2), 0.5% (lane 3), and 5% CS (lanes 4 and 5). The c-*erbB*-2 protein was immunoprecipitated by using specific antibodies and the immune-complex was subjected to kinase assay as described previously.[14]

Therefore, CS was suggested to contain a factor(s) that stimulates the protein-tyrosine kinase activity of the c-*erbB*-2 protein. Up-regulation of the activity correlates well to the growth of the RC cells.

Mutations that substitute Glu for Val-659 within the transmembrane domain of the gene product are responsible for the transforming activity of the c-*erbB*-2 gene.[13, 14] Indeed, A4 cells expressing such mutant c-*erbB*-2 showed a typical transformed phenotype and could grow even with a limited amount of CS (0.05%). Studies with the mutant c-*erbB*-2 protein revealed that the transforming potential of the c-*erbB*-2 protein was correlated with activation of the protein-tyrosine kinase activity.[14, 15] However, since the ligand of the c-*erbB*-2 protein has not been identified, the precise mechanism of ligand-dependent activation of the c-*erbB*-2 kinase is yet to be

clarified. Previous reports showed that the protein-tyrosine kinase activity of a chimera of EGF receptor extracellular domain and the c-*erbB*-2 intracytoplasmic domain was stimulated by EGF,[16-18] suggesting that the c-*erbB*-2 gene product possesses mitogenic and transforming properties in the presence of the ligand. There are several reports showing that overexpression of the normal human c-*erbB*-2 gene product in NIH3T3 cells resulted in morphological transformation and conferred a tumorigenic phenotype upon these cells.[19,20] Analogous experiments with the EGF receptor also demonstrated a transforming potential of the overexpressed protein, only when activated by EGF or TGFα.[21,22] These data suggested that the medium contained the ligand for c-*erbB*-2 protein, though its level was not sufficient to transform NIH3T3 cells by activation of the mouse endogenous c-*erbB*-2 protein or exogenous human c-*erbB*-2 protein expressed at a lower level. However, there is no report that has examined this possibility. Here, we have clearly shown that CS contains a factor(s) that stimulates both protein-tyrosine kinase activity of the c-*erbB*-2 protein and growth of normal c-*erbB*-2-expressing cells. Overexpression of the c-*erbB*-2 protein is frequently observed in human mammary tumors and is reported to play a role in the spread of the tumor.[5,6] The overexpressed protein may be constitutively activated by its ligand, provided that the latter is present in human serum. Therefore, it is important to establish the molecular nature of the factor(s) in the serum.

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