Still, ANT did not affect the effect of oestradiol. We therefore conclude that in contrast to GnRH and AG, ANT did not prevent the augmenting effect of oestradiol. Although both GnRH, AG and ANT firmly bind to the GnRH receptors of the gonadotrophs [13], receptor binding is not sufficient to exhibit intrinsic GnRH activity: for this the receptor-ligand complex must be internalized [14, 15]. GnRH antagonists are not internalized after binding to the GnRH receptor [16]. The present results therefore suggest that, like induction of LH release, prevention of the augmenting effect of oestradiol requires internalization of the GnRH/Ag-receptor complex. Interaction between GnRH/AG and oestradiol, therefore, probably does not take place at the level of the binding of ligands to the GnRH receptor.

Probably it is not a question of binding of ligands to the oestrogen receptor, either, as we demonstrated recently that the positive effect of the non-steroidal oestrogen analog clomiphene on the GnRH-induced LH response is *not* prevented by GnRH [17], in spite of the fact that this drug firmly binds to the oestrogen receptor [18].

We conclude that superpotent GnRH agonists are also superpotent with regard to prevention of the positive effect of oestradiol, and the GnRH antagonists are ineffective in this respect. We suggest that GnRH/AG-oestradiol interaction does not take place at the level of the receptors of GnRH or oestradiol.

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0014-4754/91/070716-03$1.50 + 0.20/0

EGF receptor induction and insulin-EGF overlap in *Tetrahymena*

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Received 22 October 1990; accepted 28 January 1991

Summary. Offspring generations of *Tetrahymena* pretreated (imprinted) with insulin showed a greater binding capacity for the hormone than offspring of untreated ones. The epidermal growth factor (EGF) imprinted for insulin to a greater degree than insulin itself, and vice versa: insulin imprinted for EGF more efficiently than EGF itself. These phenomena can be explained by the overlap of insulin and EGF on one another's receptors in *Tetrahymena*.
Key words. Tetrahymena; EGF; insulin; hormonal imprinting; receptors.

Although the hormone receptors of higher organisms are genetically encoded, they begin to function only after adaptation to the appropriate hormone [1]. Receptor adaptation (amplification) takes place as a rule in the early postnatal period, when primary interaction with the appropriate hormone gives rise to hormonal imprinting that accounts for stabilization of the binding capacity characteristic of adulthood [2]. Unicellular organisms do not have encoded receptors, but possess certain membrane structures which are able to recognize environmental signal molecules. These structures can be imprinted by hormones of higher vertebrates, responding to these by modification of the binding capacity and of certain functional parameters [3, 4]. It follows that hormonal imprinting can also take place at the unicellular level and account for formation of genuine receptors which persist over many offspring generations. For example, primary interaction of the unicellular organism *Tetrahymena* with insulin resulted in the formation of specific insulin receptors, as substantiated by displacement and saturation experiments [5]. The insulin molecule displayed a full imprinting potential also after deprivation of its 5-C termi-

nal amino acid or N-terminal phenylalanine group, whereas insulin dimers failed to induce imprinting[6]. In view of this various questions arose: (1) whether molecules which have receptors to a certain extent similar to the insulin receptor could also induce a receptor for insulin, and vice versa, (2) whether insulin could induce a receptor for such molecules and, last but not least, (3) to what extent insulin and the molecules in question would overlap on one another's receptors. We therefore examined insulin and EGF, whose receptors belong to the same (tyrosine kinase) receptor family for receptor forming potential and overlapping in a unicellular model system.

Tetrahymena pyriformis GL cells were used as the model organism. They were cultured in 0.1% yeast extract containing 1.0% Bacto tryptone medium (Difco, Michigan, USA), and used in the logarithmic phase of growth.

A part of the mass culture was not treated, to serve as control, and parts were treated (imprinted) with 10^{-6} M insulin (Insulin Semilente MC, Novo, Copenhagen, Denmark) for 1 h, or 10^{-6} M EGF (mouse epidermal growth factor, Sigma, St. Louis, USA), also for 1 h. Primary exposure to insulin or EGF is referred to in the figures as '1st treatment'.

After the above treatment the cultures were thoroughly washed in plain medium and were returned to plain medium for 24 h. Subsequently they were fixed in 4% formalin solution (in PBS, pH 7.2), washed thoroughly in PBS, and treated again for binding experiments.

The intensity of fluorescence was assayed with a Zeiss Fluoval cytofluorimeter in all experimental series. The analogous signals emitted by the cytofluorimeter were transformed to digital signals for analysis with a Hewlett Packard HP 41 CX calculator, that was programmed for determination of mean values (group averages), standard deviation and significance (by Student's t-test) of intergroup variation. Three replica experiments were performed in each series and the figures show the mean values of three replica assays.

Examination of the control, insulin-imprinted and EGF-imprinted groups for FITC-insulin binding

The control and the EGF-imprinted cultures were assigned to three groups, of which one was not treated (the original control cells served as absolute control in this series), the second was exposed to 10^{-6} M insulin, and the third to 10^{-6} M EGF for 20 min at room temperature (22 °C). After treatment the cells were washed in PBS and were incubated in the presence of FITC-labeled insulin (FITC = fluorescein isothiocyanate, isomer I; BDH, England) for 45 min.

The insulin-imprinted cultures were assigned to two groups, of which one was not treated and the other was re-exposed to 10^{-6} M insulin for 20 min. Both were subsequently incubated in the presence of FITC-insulin. The protein concentration of the conjugate was 0.1 mg/ml and the FITC/protein ratio was 0.34.

After incubation the cells were thoroughly washed in PBS, spread on slides, dried and assayed for intensity of fluorescence. The results of this experimental series are shown in figure 1.

Examination of insulin binding by anti-insulin-antibody tracing (monoclonal mouse anti-insulin, Amersham, England)

In this experimental series FITC-labeled anti-mouse goat IgG (HUMAN, Budapest) was used as second antibody. The protein content of the conjugate was 0.1 mg/ml, its FITC/protein ratio was 1.45.

The control and the insulin-imprinted and EGF-imprinted cultures were assigned to four groups for treatment according to the following scheme:

First treatment (imprinting)	Second treatment	First antibody (anti-insulin AB)
1. Control, insulin or EGF	–	–
2. Control, insulin or EGF	–	+
3. Control, insulin or EGF	10^{-6} M insulin for 20 min	–
4. Control, insulin or EGF	10^{-6} M insulin for 20 min	+

After the second (insulin) treatment all cultures (including the untreated ones) were washed in PBS. The cultures of the second and fourth groups were thereafter incubated in the presence of anti-insulin antibody for 20 min at room temperature. Subsequently all cultures were washed in PBS, incubated in the presence of FITC-labeled anti-mouse antibody for 45 min, washed again in PBS, spread on slides, dried, and assayed for intensity of fluorescence (fig. 2).

Examination of binding by anti-EGF-receptor-antibody tracing (monoclonal anti-epidermal growth factor receptor, mouse ascites fluid; Sigma, USA)

In this experiment, too, FITC-labeled anti-mouse goat IgF was used as second antibody.

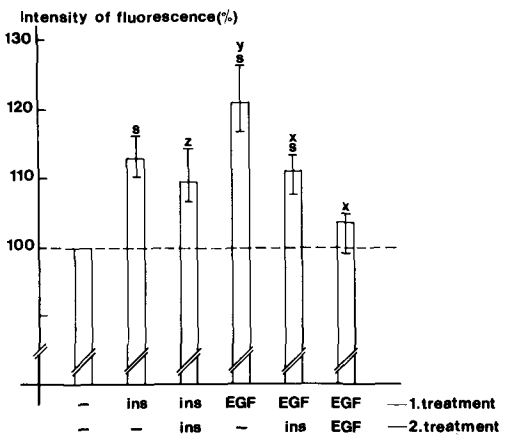

Figure 1. FITC-insulin binding to insulin, EGF or double-treated *Tetrahymena* (1. treatment = imprinting, 2. treatment = treatment after fixation) s = $p < 0.01$ to control; z = $p < 0.05$ to control, x = $p < 0.01$ to EGF imprinted, y = $p < 0.01$ to insulin imprinted.

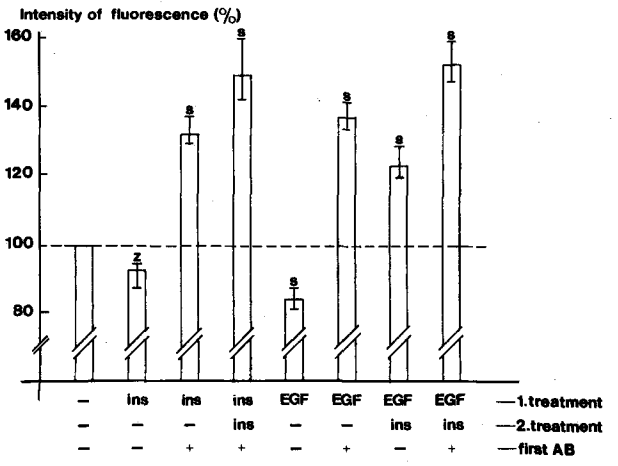

Figure 2. Study of insulin binding to insulin or EGF treated *Tetrahymena* by anti-insulin antibody (first AB = mouse monoclonal antibody). Determination by FITC-labeled anti-mouse goat IgG. s = $p < 0.01$ control; z = $p < 0.05$ to control.

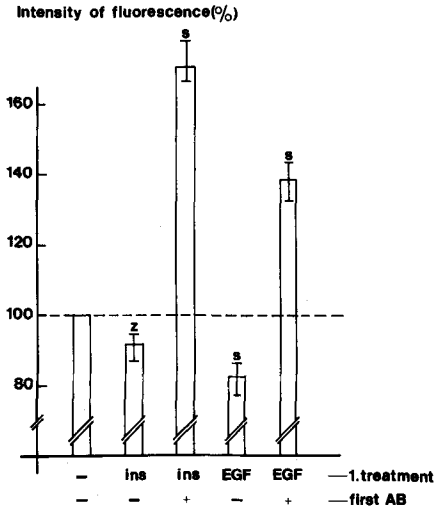

Figure 3. Binding of EGF receptor antibody (from mouse) to insulin, EGF or double-treated *Tetrahymena*. Determination by FITC-labeled anti-mouse goat IgG. s = $p < 0.01$ to control, z = < 0.05 to control; s = $p < 0.01$ to imprinted.

The control, insulin-imprinted and EGF-imprinted cultures were assigned to two groups of which one was incubated only in the presence of the second antibody, whereas the other was also incubated in the presence of the first antibody (anti-EGF-receptor antibody) for 20 min. Further treatment was as described in paragraph 2. The results are shown in figure 3.

Examination of binding of anti-EGF (mouse epidermal growth factor antiserum developed in rabbit; Sigma, USA)

The scheme of this experiment corresponded in every respect with that described in paragraph 2, except that 10^{-6} M EGF was used instead of 10^{-6} M insulin. The anti-rabbit goat IgG-FITC conjugate used as second antibody contained 0.1 mg/ml protein and its FITC/protein ratio was 1.65. The results of the fourth experimental series are shown in figure 4.

As demonstrated in earlier studies, primary exposure of *Tetrahymena pyriformis* to insulin also increased the binding capacity of the cell membrane for insulin in the present experiments (fig. 1), in which insulin binding increased markedly over the control 24 h after primary interaction, i.e. in the sixth to eight offspring generation. Since earlier studies [5] had demonstrated the specificity of induced insulin receptors, in the present experiments we did not employ binding kinetic analysis. However, the fact that addition of non-labeled insulin to fixed cells decreased the binding capacity for labeled insulin indicated that primary exposure had induced formation of a specific insulin receptor. The insulin (and EGF) dose for provocation of imprinting was higher (10^{-6} M) than the K_d of mammalian receptors (10^{-9} M); however, in previous experiments this dose had brought about the most powerful imprinting in *Tetrahymena*. For the inhibition of the binding of labeled hormone the same dose was also used for saturation of receptors. However, these doses

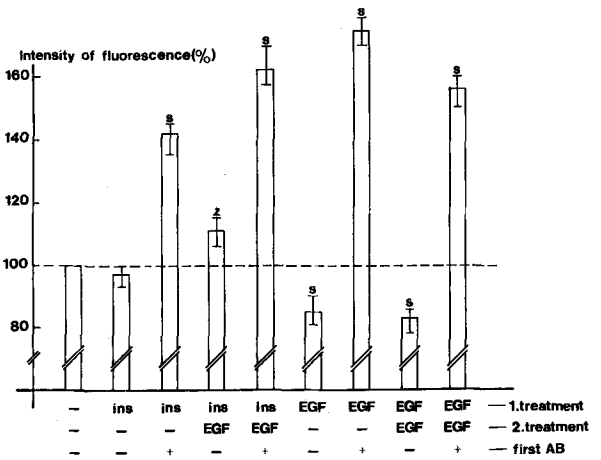

Figure 4. Study of the binding of rabbit EGF antibody by FITC-labeled anti-rabbit goat IgG. s = $p < 0.01$ to control, z = $p < 0.05$ to control.

did not lead to the binding data becoming unspecific, as these were measured using labeled hormones.

According to the literature there are certain overlaps in the effects of insulin and epidermal growth factor, and in the structure of their receptors [7–11] in mammals. This stimulated us to study the overlapping in the case of hormonal imprinting in organisms at a phylogenetically lower level. The experiments demonstrated that pretreatment (imprinting) with EGF increased the binding capacity of *Tetrahymena* for insulin to a significantly greater degree than insulin imprinting itself, and addition of non-labeled insulin to the fixed cells significantly reduced the degree of binding. Use of EGF instead of insulin for the second exposure had a still greater (statistically significant) depressive effect on the binding of labeled insulin.

The experimental results therefore indicated that EGF induced (amplified) a binding site for insulin in *Tetrahymena*, from which it was concluded that the related receptor structures differ to a smaller extent at the unicellular than at the vertebrate level. At the same time it seemed surprising that EGF increased the binding capacity for insulin to a greater degree than insulin itself, to judge from the greater inhibition of insulin binding by EGF than by insulin in the fixed cells. The same was suggested by the results of the experiments with anti-insulin antibody, although the latter also detected the insulin added for primary exposure. On the other hand, increase in the antibody binding by the fixed cells after the second exposure to insulin was obvious (fig. 2), and it also occurred after the second exposure to EGF, since the latter, too, gave rise to an increase in the binding capacity for insulin. Thus, although the use of insulin for primary exposure in both series prevents conclusions on qualitative changes, the fact remains that insulin and EGF gave rise to the same tendency of receptor activity on the second exposure.

Pretreatment (imprinting) with EGF induced the formation of receptors for EGF, as shown by binding experiments with the anti-EGF-receptor antibody (fig. 3). Surprisingly, imprinting with insulin induced the formation of significantly more receptors for EGF and, vice versa, EGF imprinted more efficiently for insulin reception (binding) than insulin itself (fig. 1).

Experiments with the anti-EGF antibody (fig. 4) strongly suggested that the cells internalized and stored the EGF used for treatment, to judge from the significant (70%) increase of intracellular EGF over the control.

Since the active group of both the insulin receptor and EGF receptor is tyrosine kinase, it is not surprising that the two receptors resemble one another in structure and even in antigenicity. This can explain why imprinting by the two hormones induced receptors of overlapping binding affinity. However, it remains to be explained why both insulin and EGF induced receptors more effectively for the other substance than for themselves. The experiments demonstrate that insulin and EGF receptors are related in structure, and that there is more overlap hormone-binding to them at lower levels of phylogenesis than in higher organisms.

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0014-4754/91/070718-04$1.50 + 0.20/0

L-tyrosine induces tyrosinase expression via a posttranscriptional mechanism

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Received 25 September 1990; accepted 23 October 1990

Summary. Exposure of hamster amelanotic melanoma cells to L-tyrosine caused a time-dependent increase of tyrosinase protein concentrations, tyrosinase activity and level of cell pigmentation. In contrast, Northern blot analysis using mouse tyrosinase cDNA showed a steady level of tyrosinase mRNA. Thus in hamster melanoma cells the stimulation of intracellular tyrosinase concentration by L-tyrosine is mediated mainly via a posttranscriptional mechanism.
Key words. L-tyrosine; melanogenesis; tyrosinase; melanoma.

Melanogenesis is a multistep process of L-tyrosine transformation into melanin, which in vivo is under the strict control of multiple gene products[1,2]. It starts with the hydroxylation of L-tyrosine to L-dopa and oxidation of L-dopa to dopaquinone, both catalyzed by tyrosinase (monophenol: oxygen oxidoreductase, E.C. 1.14.18.1)[1]. Two main components of the melanogenic apparatus, tyrosinase and melanosomes, are synthesized separately[1–3]. Melanin synthesis requires the translocation of tyrosinase from the trans-Golgi-reticulum (TGR) to