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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L-tyrosine induces tyrosinase expression via a posttranscriptional mechanism

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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

melanosomes. In addition to its role as a precursor to melanin, L-tyrosine can also act as an inducer and positive regulator of melanin synthesis, melanosome formation and tyrosinase activity[4,5]. To study the molecular mechanism of this regulation, we followed the sequence of tyrosinase gene expression after exposure of cultured hamster amelanotic melanoma cells to L-tyrosine.

Materials and methods

Cell culture. The Bomirski AbC1 hamster amelanotic melanoma cells were cultured in low tyrosine Ham's F-10 medium plus 10% horse serum[4]. To study melanogenesis the semiconfluent cultures were exposed to an increased L-tyrosine concentration (200 µM) for 0, 9, 24 and 48 h, the cells were harvested and pelleted, and the melanin content was judged macroscopically.

SDS-PAGE and Western blotting. Cell pellets were lysed in 0.1 M phosphate buffer, pH 6.8, plus 1% Triton X-100, 1 mM PMSF and 0.01% aprotinin. Cell extracts were centrifuged at 15,000 × g for 30 min at 4 °C and supernatants were used for further analysis. Equal amounts of proteins were separated by electrophoresis on a 0.1% SDS/8% polyacrylamide gel under nonreducing conditions according to a nondenaturating modification of the method of Laemmli[6,7]. Separated proteins were blotted to Zeta Probe blotting membranes as described previously and processed further for dopa oxidase activity of tyrosinase or immunostained as described previously[6,7].

Dopa oxidase activity of tyrosinase. The membranes were washed briefly in phosphate buffer and stained with 5 mM L-dopa in 0.1 M sodium phosphate buffer, pH 6.8, at 37 °C[6,7].

Immunostains. After blocking with 10% milk in 20 mM Tris, 0.5 M NaCl, pH 7.5 (TBS), the membranes were washed in TTBS (TBS plus 0.05% Tween 20 plus 1% BSA), and afterwards probed with polyclonal antibodies against purified tyrosinase[8] (1:200 dilution; gift of Dr J. Laskin), antibodies PEP_7[9] (1:50 dilution; gift of Dr V. Hearing), and rabbit nonimmune serum. Polyclonal antibody PEP_7 was raised against synthetic peptide corresponding to aa 519–533 from the C terminus of the mouse tyrosinase protein[9]. After washing with TTBS, the membranes were incubated with goat anti-rabbit IgG (1:500 dilution) conjugated to horseradish peroxidase. The immune complexes were visualized by staining with diaminobenzidine and H_2O_2[7].

RNA blotting. Total RNA was isolated by the guanidinium hydrochloride method[10]. Poly $(A)^+$ mRNA was extracted using 'Fast track' mRNA isolation kit[7]. Five µg of poly $(A)^+$ mRNA or 40 µg of total RNA were electrophoretically separated on a formaldehyde/1% agarose gel[7]. RNA was transferred to Zeta Probe blotting membranes by capillary transfer. The membranes were photographed under UV light and dried for 2 h at 80 °C under vacuum. The membranes were prehybridized in hybridization buffer (50% deionized formamide, 7% SDS, 5 X Denhardt's solution, 5 × SSC, 20 mM NaH_2PO_4, pH 7.0, and 100 µg/ml denaturated salmon sperm DNA) at 42 °C for 12 h. The heat denaturated, random prime-labelled tyrosinase cDNA clone Mty811C[11] (gift of Dr B. Kwon) (sp. act. 6×10^8 cpm/µg) was added to the hybridization buffer and hybridization was performed for 12–24 h at 42 °C. The membranes were washed for 5 min with 2 × SSC plus 0.5% SDS and 15 min with 2 × SSC plus 0.1% SDS at room temperature, then with 0.2 × SSC plus 0.1% SDS at 68 °C for 2 h. A final 5-min rinse in 0.2 × SSC plus 0.5% SDS was performed before exposing the hybridized blots to Kodak XAR film at − 70 °C.

Results and discussion

As a basic model, we used Bomirski's Ab melanosome and melanin deficient hamster amelanotic melanoma cells[12], in which L-tyrosine induced melanogenesis and tyrosinase activity[4,13]. This induction was specific for L-tyrosine since D-tyrosine, N-acetyl-tyrosine, L-phenylalanine, L-tryptophan and L-valine were ineffective in the melanogenic effect, and was time and dose dependent with submaximal stimulation of tyrosinase activity using 200 µM L-tyrosine in the culture medium[4].

Exposure of hamster amelanotic melanoma cells to 200 µM L-tyrosine in the culture medium induced melanin synthesis after 24 h of culture with optimal melanization after 48 h, as evidenced by a jet black cell pellet (fig. 1). The induction of melanin synthesis was preceded and further accompanied by an increase in dopa oxidase activities which appeared as proteins of MW 68 kD, 85–90 kD and 135 kD, an increase of intracellular concentration of proteins of MW 68 kD and 135 kD detected by polyclonal anti-tyrosinase antibodies[8], and of proteins of MW 68 kD and 80 kD detected by anti-tyrosinase antibodies PEP_7[9] (fig. 2a–c). The dopa oxidase positive protein with a MW of 85–90 kD was not recognized by anti-tyrosinase antibodies and therefore it may not be tyrosinase. The proteins of 68 kD and 135 kD correspond to hamster tyrosinase characterized previously[6,7]. The protein of 80 kD MW was detected previously by Pomerantz's anti-tyrosinase antibody in hamster malignant[6] and mouse normal and malignant[14] melanocytes. Therefore, the induction of melanogenesis by L-tyrosine

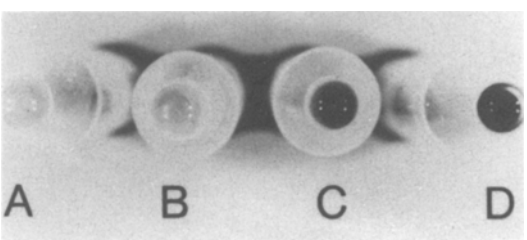
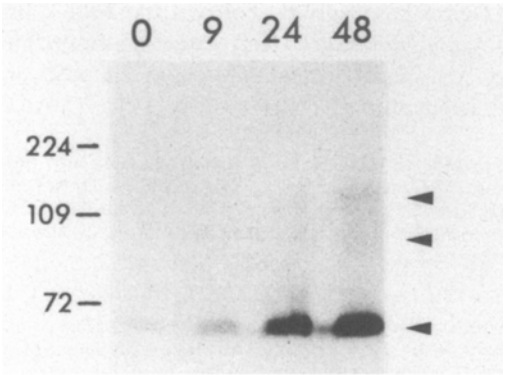
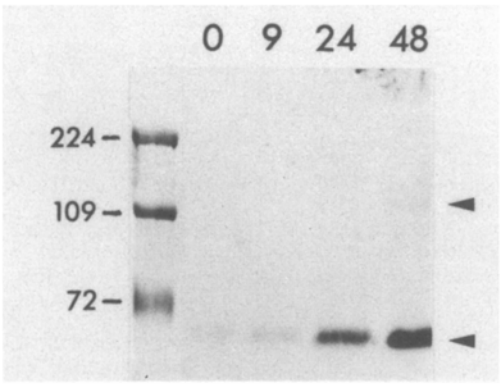


Figure 1. The effect of L-tyrosine on melanin synthesis. The Bomirski AbC1 hamster amelanotic melanoma cells were cultured in low tyrosine Ham's F-10 medium plus 10% horse serum. The semiconfluent cultures were exposed to 200 µM L-tyrosine for 0, 9, 24 and 48 h.





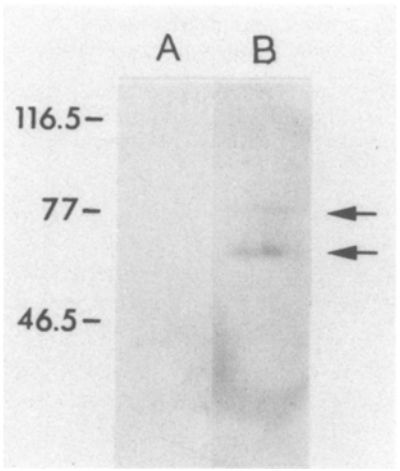
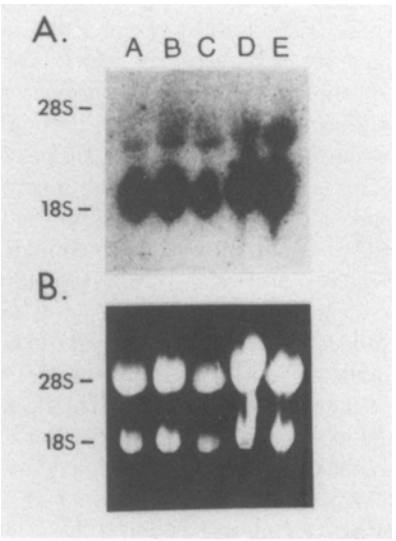


Figure 2. The effect of L-tyrosine on tyrosinase. The AbC1 cells were exposed to 200 μM L-tyrosine for 0 (0), 9 (9), 24 (24) and 48 (48) h in *A* and *B*, and for 0 (A) and 48 (B) h in *C*. Cell pellets were lysed, extracts centrifuged and supernatants separated under nonreducing conditions on 0.1 % SDS/8 % polyacrylamide. The proteins were blotted to nylon membranes and processed for dopa oxidase activity of tyrosinase (*A*) or immunostained (*B* and *C*) as described previously[6,7]. The immune complexes were visualized by staining with diaminobenzidine and H_2O_2. Arrow heads: dopa positive proteins. Arrows and arrow heads: specific proteins recognized by anti-tyrosinase antibody. Left panels: MW standard (kD). *A*, Dopa oxidase activity. *B*, Immunostain with polyclonal antibodies against purified tyrosinase[8]. *C*, Immunostain with antibodies PEP_7 against synthetic peptide corresponding to aa 519–533 from the C terminus of the mouse tyrosinase protein[9].

is accompanied by an increased abundance of tyrosinase proteins of an approximate MW of 68 and 135 kD and of a protein of an apparent MW of 80 kD recognized by anti-tyrosinase antibodies that does not express a functional tyrosinase activity.

To study changes in tyrosinase mRNA levels we used tyrosinase cDNA clone Mty811C[11]. Northern blot analysis of total RNA shows a steady level of tyrosinase mRNA after exposure of cultured melanoma cells to L-tyrosine for 0, 3, 9, 24 and 48 h (fig. 3a). The above results using time points 0 and 48 h after exposure to L-tyrosine were reproduced in three separate experiments (not shown). In addition, Northern blot analysis



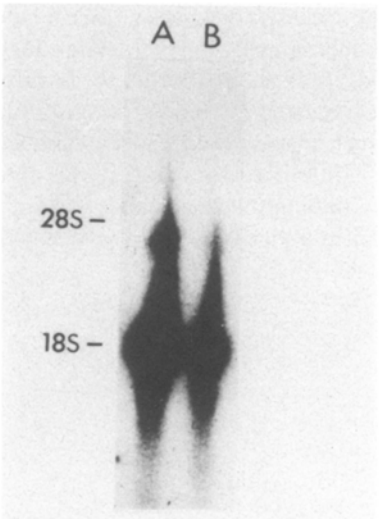


Figure 3. The effect of L-tyrosine on tyrosinase mRNA. The AbC1 cells were exposed to 200 μM L-tyrosine for 0 (A), 3 (B), 9 (C), 24 (D) and 48 (E) h in *A*, and for 0 (A) and 48 (B) h in *B*. Five μg of poly $(A)^+$ mRNA or 40 μg of total RNA were electrophoretically separated through formaldehyde/1 % agarose gels, transferred to nylon membranes, hybridized with ^{32}P-labelled tyrosinase cDNA (Mty811C), and exposed on Kodak XAR film[7,11]. *A*, Northern blot of total RNA. (A), hybridization to Mty811C tyrosinase cDNA probe. (B), UV photographs of transferred RNA. *B*, Northern blot of poly $(A)^+$ mRNA.

of poly A^+ mRNA further confirms the above results showing a steady level of tyrosinase mRNA after exposure of melanoma cells to L-tyrosine (fig. 3b). Thus the inducing effect of L-tyrosine on tyrosinase protein concentration is mediated mainly via a post-transcriptional mechanism.

Previously we have shown that the induction of melanogenesis by L-tyrosine is accompanied by de novo formation of melanosomes and translocation of tyrosinase from trans-Golgi reticulum (TGR) to these organelles[4,13]. This may suggest that L-tyrosine, via induction of melanosome formation and facilitation of tyrosinase transport to the organelle, protects the enzyme from entering a degradative pathway and allows its accumulation in this organelle. On the other hand cycloheximide (protein synthesis inhibitor) prevented the induction of tyrosinase activity by L-tyrosine[4], which is in agreement with the hypothesis suggesting the existence of a control point for tyrosinase activity at the level of tyrosinase mRNA translation[15,16] and suggests a positive regulation of this process by L-tyrosine. In other systems the positive regulation of ferritin translation by iron is well documented[17]. At present we cannot conlude definitively which of the two proposed pathways is operating in the described system.

Induction of melanogenesis by L-tyrosine in undifferentiated malignant melanocytes is an example of substrate induction of the metabolic pathway that can change dramatically the metabolic status and behavior of the target cell[5]. Tyrosinase is a key regulatory element in this system. It regulates tyrosine activity via its hydroxylation to biologically active L-dopa[1,3], and it inactivates L-dopa via its oxidation and accelerates the rate of melanogenesis[1,3]. Melanogenesis consumes intracellular oxygen and the end-product is melanin[1,3]. Melanin inhibits tyrosinase activity[18], can act as a buffer for intracellular calcium[19], and reversibly binds several bioregulatory molecules such as serotonin, catecholamines and prostaglandins[20]. In order to understand this autoregulatory circuit it is of paramount importance to clarify the molecular mechanism underlying the in vivo substrate regulation of tyrosinase activity. The first step in this direction has already been taken: L-tyrosine has been shown to induce tyrosinase expression mainly via a posttranscriptional mechanism(s).

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