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ErbB2 and EGFR Are Downmodulated During the Differentiation of 3T3-L1 Preadipocytes

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Abstract The expression of receptors belonging to the epidermal growth factor receptor subfamily has been largely studied these last years in epithelial cells mainly as involved in cell proliferation and malignant progression. Although much work has focused on the role of these growth factor receptors in the differentiation of a variety of tissues, there is little information in regards to normal stromal cells. We investigated erbB2 expression in the murine fibroblast cell line Swiss 3T3L1, which naturally or hormonally induced undergoes adipocyte differentiation. We found that the Swiss 3T3-L1 fibroblasts express erbB2, in addition to EGFR, and in a quantity comparable to or even greater than the breast cancer cell line T47D. Proliferating cells increased erbB2 and EGFR levels when reaching confluence up to 4- and 10-fold, respectively. This expression showed a significant decrease when growth-arrested cells were stimulated to differentiate with dexamethasone and isobutyl-methylxanthine. Differentiated cells presented a decreased expression of both erbB2 and EGFR regardless of whether the cells were hormonally or spontaneously differentiated. EGF stimulation of serum-starved cells increased erbB2 tyrosine phosphorylation and retarded erbB2 migration in SDS–PAGE, suggesting receptor association and activation. Heregulin-α1 and -β1, two EGF related factors, had no effect on erbB2 or EGFR phosphorylation. Although 3T3-L1 cells expressed heregulin, its specific receptors, erbB3 and erbB4, were not found. This is the first time in which erbB2 is reported to be expressed in an adipocytic cell line which does not depend on non EGF family growth factors (thyroid hormone, growth hormone, etc.) to accomplish adipose differentiation. Since erbB2 and EGFR expression were downmodulated as differentiation progressed it is conceivable that a mechanism of switching from a mitogenic to a differentiating signaling pathway may be involved, through regulation of the expression of these growth factor receptors. J. Cell. Biochem. 90: 561–572, 2003.

Key words: erbB2; EGFR; adipocyte differentiation; fibroblasts; 3T3-L1 cells; tyrosine phosphorylation; heregulin; cAMP; dexamethasone; insulin; EGF

The epidermal growth factor receptor (EGFR, erbB1) and erbB2 belong to a subfamily of transmembrane receptors with intrinsic tyrosine kinase activity (type I TKR), which also includes the related proteins erbB3 and erbB4. They all share a similar structure: a glycosylated extracellular binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain. Binding of a specific ligand to one of the erbB receptors triggers the formation of specific receptor homo- and heterodimers, being erbB2 the preferred signaling partner [Graus-Porta et al., 1997]. Their role in cell proliferation, differentiation, and survival has been demonstrated in a large number of normal and tumorigenic cell lines, but usually in epithelial cells [Hynes and Stern, 1994; Olayioye et al., 2000]. To our knowledge, there are very few in vivo reports of erbB2 expression in stromal cells: the gestational decidualized

Abbreviations used: EGF, epidermal growth factor; EGFR or erbB1, EGF receptor; DEXA, dexamethasone; MIX, isobutyl-methylxanthine; INS, insulin, HRG, heregulin; IGF-1R, insulin like growth factor-1 receptor; FBS, fetal bovine serum.

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stromal cells and placenta in humans [Press et al., 1990]; the postimplantation period in mice [Lim et al., 1997]; and, to a limited extent, the stromal mammary cells during pregnancy [Schroeder and Lee, 1998; Darcy et al., 2000]. Regarding erbB2 expression in fibroblastic cell lines, there are reports in Rat-1 and 3T3-F422A fibroblasts [Stern and Kamps, 1988; Kim et al., 1999].

The murine 3T3-L1 fibroblastic cell line [Green and Kehinde, 1974] has been a useful model system for studying mechanisms that direct progenitor cells to terminally differentiate into functional adipocytes. It has been described that the spontaneous but long process of differentiation can be accelerated once confluence is reached by treating the cells with isobutyl-methylxanthine (MIX) or any other cAMP increasing agent, dexamethasone (DEXA) and pharmacological concentrations of insulin (INS) or physiological doses of insulin like growth factor-1 (IGF-1) [Smith et al., 1988]. In a completely serum-free culture system, a rate of adipose conversion similar to that obtained with insulin is achieved with IGF-1 only in combination with EGF [Schmidt et al., 1990]. EGF is by far the most potent serum component in supporting the exponential growth phase necessary to obtain confluency and in preserving the ability of 3T3-L1 cells to undergo adipose conversion [Schmidt et al., 1990]. EGF has been described to have distinct and sometimes controversial effects on adipose differentiation. EGF blocks maturation of adipocyte precursor cells into mature adipocytes both in vivo and in vitro [Serrero, 1987; Serrero and Mills, 1991] but it enhances adipogenesis in the already differentiating adipocytes [Adachi et al., 1994]. This has been thought to be achieved by differences in the postreceptor signaling since, curiously, EGFR levels have been previously described either to remain constant [Reed et al., 1977; Adachi et al., 1994] or to decrease throughout differentiation [Hardy et al., 1995; Boney et al., 1998].

Since no ligand has been identified yet for erbB2, its activation depends on binding of transforming growth factor-α (TGF-α), amphiregulin or EGF to the EGFR, or upon binding of neuregulins/heregulins to erbB3 and/or erbB4 [Sliwkowski et al., 1994; reviewed in Olayioye et al., 2000 and in Carraway and Burden, 1995 and references therein] and subsequent heterodimerization. Given that EGF presents some controversial effects on the differentiation process of 3T3-L1 cells, some could be ascribed to the formation of EGFR/erbB2 heterodimers. There is growing evidence pointing towards biologically relevant interactions between the ErbB system and the IGF-1R [Roudabush et al., 2000; Balaña et al., 2001], another type of tyrosine kinase receptor, which is activated by non EGF family related factors, such as IGF-1 and insulin and has an important role in adipose differentiation [Smith et al., 1988; Boney et al., 1998]. This and the fact that IGF-1 mediates transactivation of the EGF receptor in the mitogenic signaling pathway [Roudabush et al., 2000], led us to think of a possible role for erbB2 in this preadipocyte cell line.

In this study, our aim was to investigate erbB2 along with EGFR/erbB-1 expression and modulation in the Swiss 3T3L1 cells during proliferation and naturally or hormonally induced differentiation.

MATERIALS AND METHODS

Cell Culture

Swiss 3T3-L1 preadipocytes (fibroblasts, embryo, mouse, ATCC CCL 92.1) purchased from the ABAC (Asociación Banco Argentino de Células, Pergamino, Buenos Aires) were routinely cultured in Dulbecco's modified Eagle's medium (Hyclone) with 4 mM L-glutamine, 4.5g/L glucose, 10^{-6} M biotin, 0.11g/L sodium pyruvate, and supplemented with 10% FBS (Gen SA, Bs. As.) plus antibiotics (Gibco, Invitrogen Corporation, Carlsbad, CA) and 2 days after confluence, differentiation was induced by the addition of dexamethasone (DEXA, 0.1 μM) and 3-isobutyl-1-methylxanthine (MIX, 500 μM). After 48 h, the medium was removed and replaced by DMEM containing insulin (INS, 2 μM). Experiments were usually finished after 5 days of insulin treatment, when 80–90% of the culture was differentiated into adipocytes (refractive droplets at conventional microscope).

Preparation of Cell Lysates for Western Blotting

Cell monolayers were washed and collected in ice-cold PBS and centrifuged at 4,000 rpm (1,500*g*) at 4°C for 10 min. Pellets were solubilized in Triple-detergent lysis buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM NaF, 10 μg/ml of leupeptin, pepstatin, aproti-

nin, spermin, spermidin, sodium metavanadate and 1 mM AEBSF) for 20 min on ice, followed by sonication. The lysates were clarified by centrifugation at 12,000 rpm (13,500*g*) at 4°C for 15 min. Proteins boiled in sample buffer (50 mM Tris-Cl [pH = 6.8], 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol) were subjected to a 7% SDS–PAGE at 100 V for 1.30 h. Protein transfer was done onto nitrocellulose membranes (Protran™, Schleicher & Schuell) and checked by Ponçeau Red staining. After blocking with 5% milk in TTBS (10 mM Tris-HCl [pH = 8], 150 mM NaCl, 0.05% Tween-20), filters were probed with specific antibodies. Proteins were visualized with peroxidase coupled antibodies, using the ECL (Amersham Pharmacia Biotech, Buckinghamshire, England) detection system and stripping and reprobing of blots were accomplished according to the manufacturer's suggestions. Densitometric analysis was performed with the NIH Scion Image program. One way ANOVA, followed by a post-test (Student-Newman-Keuls or Tukey) was used with statistical purposes where mentioned.

For Figures 1 and 3, erbB2 and EGFR time expression curves were determined as follows: we prepared three gels for each experiment, loading duplicate samples from the proliferative period in one gel, MIX-DEXA-treated samples in another and non-treated samples in the last one. The same amount of T47D was loaded in each one of the gels to allow comparison between them, since T47D erbB2 and/or EGFR expression do not vary. We then performed densitometric analysis on each time-point and referred it to the T47D value from its own gel and after that, to the day 1 value (control). We finally obtained the average between samples from the same time-point and plotted all of them in a time-curve.

ErbB2/ EGFR Phosphorylation by EGF

Proliferating cells were serum starved by incubation for 24 h with 0.1% [w/v] BSA. Cells were treated with 10 nM EGF (Gibco, Life Technologies) in the same medium, 10% (v/v) FBS, EGF + FBS, or mock treated (0.1% BSA) for 5 min at 37°C (the same protocol was followed, when indicated, for heregulin α1 and β1, 1 nM, Neo Markers, Fremont, CA). Stimulation was stopped by placing the cells on ice and washing them twice with ice-cold PBS. Cells lysates were obtained by scraping cells in PBS plus protease and phosphatase inhibitors, cen-

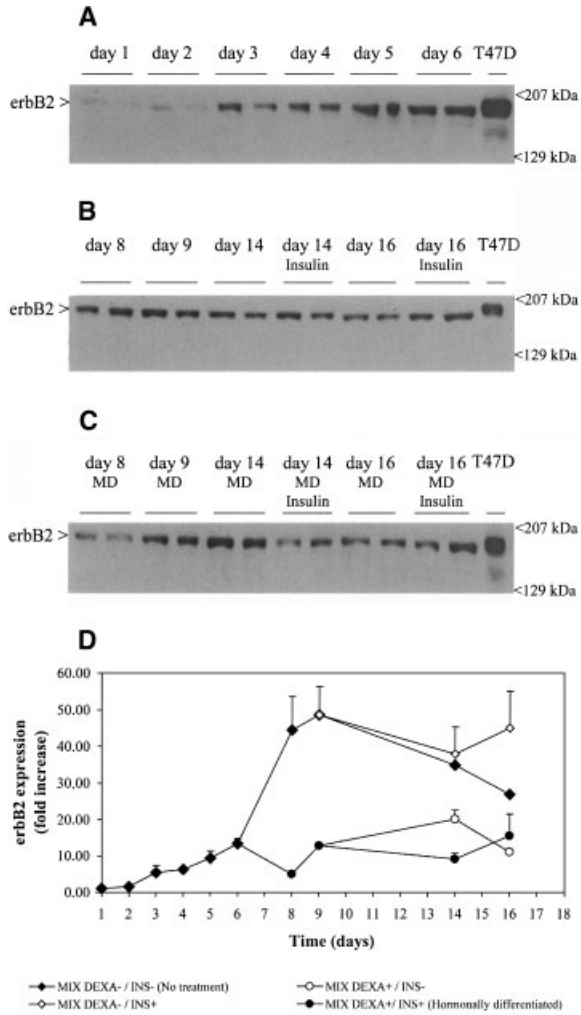

Fig. 1. ErbB2 p185 expression and modulation in 3T3-L1 cells. Cells were cultured in DMEM–10% FBS and proteins from whole detergent extracts (8 μg protein per well) were resolved by a 7% SDS–PAGE and immunoblotted with an anti-erbB2 antibody (Santa Cruz, Neu C-18, 1:1000). A reactive band (185 kDa) was observed in extracts from: (**A**) Proliferating cells between day 1 and 6 after seeding; (**B**) Post-confluent undifferentiated 3T3-L1 cells, with or without INS on days 14 and 16 after seeding; and (**C**) Post-confluent 3T3-L1 cells, induced to differentiate 2 days after confluence was reached, with MIX+DEXA (MD) on days 8 and 9 after seeding, with or without INS on days 14 and 16. Differentiation was followed by measuring the triglyceride accumulation in replicate cultures (data not shown). T47D breast cancer cell line was used as an internal control to allow comparison between gels. The experiment shown here is representative of three such experiments. **D**: The data in 1 (A), (B), and (C) were subjected to densitometric analysis as described under ''Materials and Methods.'' Briefly, band intensities were quantified using the Scion Image software (NIH Image for Windows) and, then, subjected to statistical analysis by one-way ANOVA, followed by the Student-Newman-Keuls post test. Values are expressed as fold increase over day 1 and represent the mean ± SEM (n = 4). Statistical significance is not shown due to its complexity but it is explained in the text.

trifuging at low speed and resuspending with 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM FNa, 5 mM EDTA, 5 mM EGTA, 10% glycerol, 1 μg/ml of leupeptin, pepstatin, aprotinin, 2 mM orthovanadate, 1 mM AEBSF, and 1 mM benzamidin. Extracts were kept at −70°C until use. For immunoprecipitations, equal amount of protein (200 μg) were precleared with protein LA-agarose beads (Sigma) and rabbit normal IgG for 30 min on ice with continuous mixing. The precleared samples were first incubated with the specific antibody (erbB2 or EGFR) for 2 h and then with protein LA-agarose beads for 1 h. Immune complexes were collected by centrifugation at 1,500*g*, washed three times with extraction buffer, boiled for 5 min in sample buffer and subjected to SDS–PAGE. Tyrosine phosphorylation was determined by WB, using a specific anti-P-Tyr antibody (SC PY99).

Antibodies

Policlonal anti-EGFR (Santa Cruz 1005 sc-03, 1:1,000 dilution); policlonal anti-erbB2 (Santa Cruz Neu C-18, 1:1,000); monoclonal anti-erbB3 (Neo Markers, Ab-7 2C3, 1:200); policlonal anti-erbB4 (Neo Markers, Ab-2, 1:200); monoclonal anti-phosphotyrosine (Santa Cruz PY99, 1:500); policlonal anti-HRG (Santa Cruz C-20 SC-348, 1:200); goat anti-rabbit (Santa Cruz sc-2054, 1:2000); anti-mouse (Amersham Life Sciences RPN 2108, 1:2000).

RESULTS

ErbB2 Expression in 3T3-L1 Cells

To further understand potential functions of the EGFR subfamily during adipocytic differentiation, we used the EGF and IGF-1 responsive 3T3-L1 murine preadipocyte cell line. It has been published that EGF, through its receptor, produces a 2–3-fold stimulation in 3T3-L1 preadipocytes proliferation but is unable to initiate adipocyte differentiation [Smith et al., 1988], being somehow inhibitory, even though it can promote adipogenesis of already differentiating adipocytes [Adachi et al., 1994]. Since erbB2 can heterodimerize with other ErbB family members upon binding of their respective ligands [Graus-Porta et al., 1997] and the IGF-1R can transactivate the EGFR [Roudabush et al., 2000], it was interesting to find out whether these preadipocytes express erbB2. Figure 1A, B, and C depicts the pattern of erbB2 expression (185 kDa) in proliferating and post-confluent 3T3L1 cells, hormone-treated or not, as detected by Western blotting. The densitometric analysis (Fig. 1D) showed that, in untreated cells (see closed diamonds), this expression significantly increased throughout proliferation (from day 1 to day 6, $P < 0.001$) and post confluence periods, peaking at day 8–9 to a maximum of fourfold above confluence (day 6 vs. 8 and 9, $P < 0.001$), and slightly decreasing after 14–16 days of culture. On the other hand, the treatment of cells with the inducers dexamethasone (DEXA) and 3-isobutyl-1-methylxanthine (MIX), for 48 h (see Fig. 1D, closed circles) significantly reduced erbB2 expression as compared with untreated cells (circles against diamonds at days 8 and 9, $P < 0.001$). By the end of the differentiation period, erbB2 levels in differentiated cells were much lower than in untreated cells (compare closed diamonds with closed circles, $P < 0.001$ at day 14 and $P < 0.01$ at day 16) but similar to the ones reached at confluence. It seems that inducers prevented erbB2 levels to rise in the growth-arrested period. In untreated cells (without the MIX/DEXA addition) insulin usually enhanced erbB2 expression (Fig. 1D, open diamonds at day 16) but, in differentiated cells, it could not counteract the inhibitory action of the inducers (Fig. 1D, compare open with closed circles).

Since erbB2 modulation by hormones is complex, we wanted to elucidate whether the decrease in erbB2 expression, observed in differentiated cells as compared to undifferentiated ones, occurred to the same extent in spontaneously differentiated cells. To analyze this, we took samples from control cells at confluence, hormonally differentiated cells, undifferentiated control cells at the time when treated cells accomplished their differentiation and spontaneously differentiated cells (almost a month in culture). Figure 2 shows that erbB2 expression is significantly decreased in differentiated cells ($P < 0.05$), regardless of how they were differentiated (see Fig. 2A, hormonal and spontaneous differentiation vs. control).

EGFR Expression in 3T3-L1 Cells

It has been described that EGFR levels either remain constant [Adachi et al., 1994; Reed et al., 1977] or decrease through the differentiation of 3T3-L1 cells [Hardy et al., 1995; Boney et al., 1998]. To confirm the EGFR modulation in

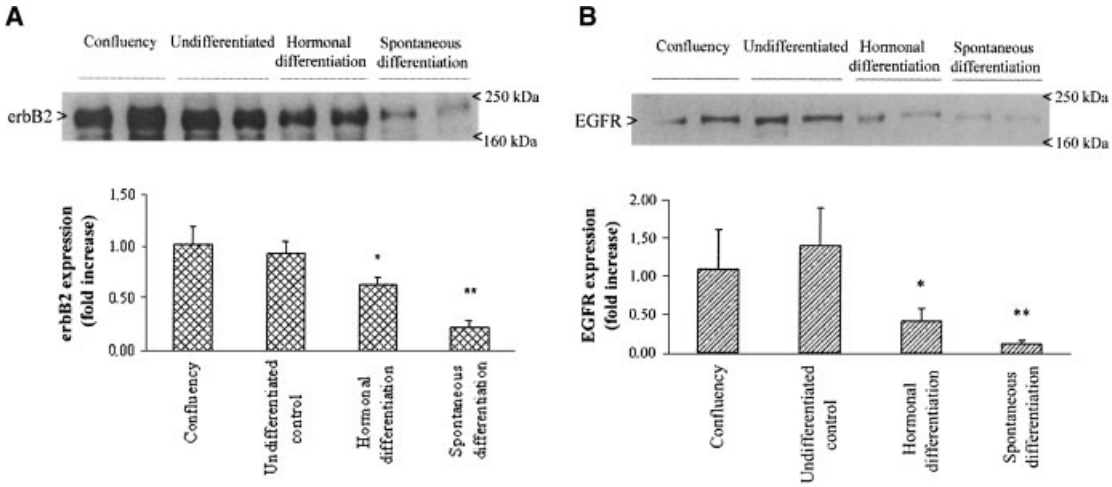

Fig. 2. Decrease in receptor expression in differentiated cells. Whole extracts (10 μg protein per well) from cells reaching confluency, hormonally differentiated cells, undifferentiated control cells (cells that were not subjected to hormonal treatment but sample was taken at the same time when treated cells accomplished their differentiation) and spontaneously differentiated cells (almost a month in culture) were subjected to SDS–PAGE and blotted against (**A**) erbB2 and (**B**) EGFR. Densitometric analysis is shown for each receptor. Band intensities were quantified using Scion Image software and, then, subjected to statistical analysis by one-way ANOVA, followed by the Tukey post-test. Values are expressed as fold increase over the 6 h control and represent the mean ± SEM (n = 2). $^{*}P < 0.05$ and $^{**}P < 0.01$.

these cells, throughout the complete cycle of differentiation from fibroblast (preadipocytes) to adipocytes, we analyzed it by Western blot, as we did for erbB2. We observed the expression of two reactive bands for erbB-1 (Fig. 3A–C), one with the expected molecular weight for the typical EGFR (170 kDa), and the other possibly corresponding to the type III variant (vIII) of 145 kDa. As observed for erbB2, there was an increase in EGFR expression of approximately 10-fold over control (day 1 vs. day 6, $P < 0.001$) during the proliferation period, reaching a maximum at day 8 (i.e., 2 days after confluence, day 6 vs. day 8, $P < 0.01$). As culture progressed, by day 16 EGFR expression diminished reaching pre-confluence levels (see Fig. 3D, closed diamonds at day 16). In differentiated cells, it was extremely downregulated by day 8–9 of the cycle (in Fig. 3D, compare closed diamonds with closed circles at day 8, $P < 0.01$) and, after that, it remained quite constant even when insulin was added to the medium. By day 14, there was a significant drop in EGFR expression in treated cells as compared with untreated ones (see circles against diamonds, $P < 0.01$). However, by day 16, there seemed to be no difference between differentiated or undifferentiated cells. In order to clarify this, we performed an experiment that allowed us to determine that EGFR expression was significantly diminished in differentiated cells compared to undifferentiated or even confluency controls (see Fig. 2B). The same result was observed in hormonal ($P < 0.05$) and spontaneously ($P < 0.01$) differentiated cells thus justifying the apparently contradictory result seen in Figure 2 by day 16. As for erbB2, this can be explained through the fact that spontaneous differentiation (untreated cells) goes slowly to a loss of receptors, which hormonal induced cells achieve first, so as soon as we move further in time (from day 14 to day 16, for example) differences in receptor expression get smaller.

Rapid Effects of MIX-Dexamethasone on Receptor Expression

To better understand the cause for this drop in the receptor expression, we performed a series of experiments to determine whether MIX/dexamethasone had any effect on this decrease or this was a consequence of differentiation itself. By treating proliferating cells with MIX/DEXA for 6, 12, 24, and 48 h (see Fig. 4), we determined that after 24 h of treatment, MIX/DEXA ($P < 0.001$) had an inhibitory action on erbB2 expression.

We, then, decided to study the effect on growth-arrested cells, which are the ones to be subjected to the differentiation program. We cultured 3T3-L1 cells 2 days beyond confluence and started MIX/DEXA treatment for 6, 12, 24, and 48 h (see Fig. 5A). We observed that erbB2

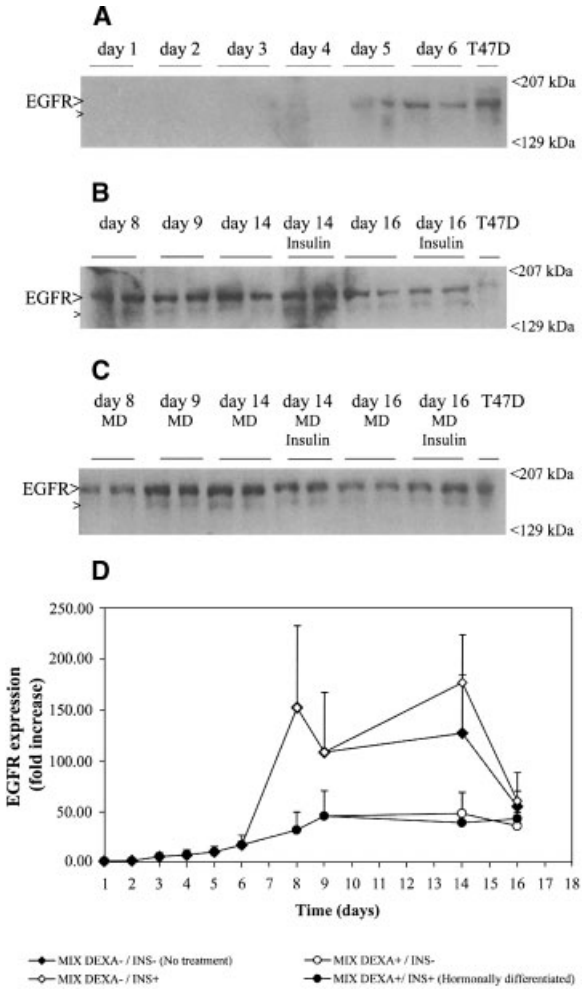

Fig. 3. EGFR expression and modulation in 3T3-L1 cells. Aliquots (8 μg protein per well) from the same extracts as shown in Figure 1 were resolved by 7% SDS–PAGE and immunoblotted with anti-EGFR antibody (Santa Cruz 1005 SC-03, 1:500). Two bands were recognized in these extracts: the 170 kDa band, indicates the expected molecular weight for the EGFR, and the 145 kDa band, presumably belonging to the vIII variant of EGFR. **A**: Proliferating cells. **B**: Post confluent undifferentiated cells. **C**: Post-confluent differentiated cells. **D**: Densitometric analysis performed as described before under ''Materials and Methods'' and in Figure 1.

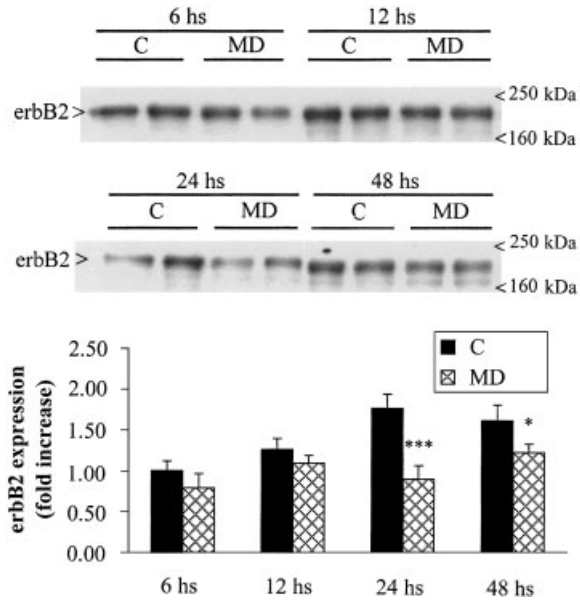

Fig. 4. Rapid effect of MIX-dexamethasone on erbB2 expression in proliferating cells. Proliferating cells were cultured on 6-well plates and treated as indicated, and protein extracts were obtained at 6, 12, 24, and 48 h. Samples were loaded onto a 7% SDS–PAGE (10 μg protein per well) and blotted against erbB2. Densitometric analysis shows in black, proliferating control 3T3-L1 cells (C) and in pattern, MIX-dexamethasone treated cells (MD). Statistical analysis was performed by one-way ANOVA, followed by the Tukey post-test. Values are expressed as fold increase over the 6 h control and represent the mean ± SEM (n = 3). $*P < 0.05$ and $***P < 0.001$.

expression was diminished by this treatment by 12 h ($P < 0.01$) and on ($P < 0.001$). Also, EGFR levels were lower at 24 h ($P < 0.001$, Fig. 5B). Furthermore, cells exposed to inducers for up to 7 days showed an even stronger inhibition in their receptor levels (data not shown).

EGF Induces Tyrosine Phosphorylation of ErbB2 in 3T3-L1 Cells

Since it is known that tyrosine phosphorylation accounts for receptor activation, we tried to evaluate whether there was any signal of it on erbB2 in 3T3-L1 cells. In a preliminary experiment, we found that one of the major phosphotyrosine reactive bands corresponded to a molecular weight of 185 kDa (data not shown), the same MW as for erbB2. However, no significant differences were found in the tyrosine phosphorylation status of total cell extracts neither when comparing spontaneous against hormonally differentiated cells nor between any stage of differentiation. To rule out a possible basal or serum-induced stimulation that could prevent a further increase, proliferating cells were serum-starved and stimulated with EGF, serum, both or none. ErbB2 was immunoprecipitated and its tyrosine phosphorylation determined through Western blotting. Figure 6 shows that EGF (10 nM, 5 and 10 min) increased erbB2 tyrosine phosphorylation by 2.5-fold in comparison with the non-stimulated cells (BSA 0.1%), by sixfold over the serum-stimulated cells and by fivefold over the undepleted cells (no serum starvation).

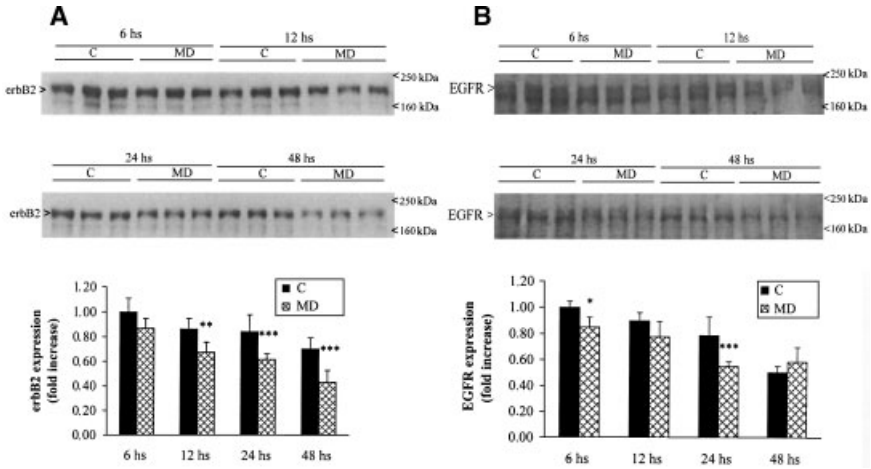

Fig. 5. Rapid effect of MIX-dexamethasone on erbB2 and EGFR expression in growth arrested cells. Growth arrested cells (2 days after reaching confluence) were cultured on 6-well plates and treated as indicated, and protein extracts were obtained at 6, 12, 24, and 48 h. Samples were loaded onto a 7% SDS–PAGE (10 μg protein per well) and blotted against erbB2 (**A**) and EGFR (**B**). Densitometric analysis shows in black, proliferating control 3T3-L1 cells (C) and in pattern, MIX-dexamethasone treated cells (MD). As for Figure 4, statistical analysis was performed by one-way ANOVA, followed by the Tukey post-test. Values are expressed as fold increase over the 6 h control and represent the mean ± SEM [n = 3]. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

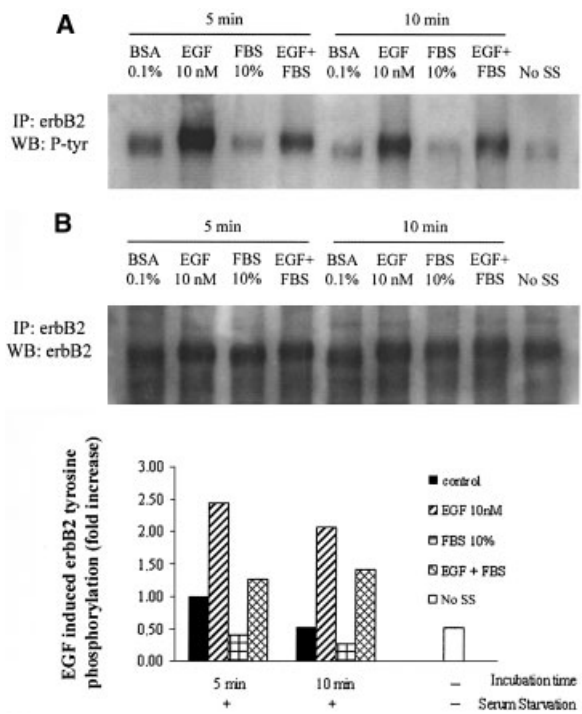

Fig. 6. EGF induces tyrosine phosphorylation of erbB2 in 3T3-L1 cells. 3T3-L1 cells were serum-starved for 24 h in DMEM–0.1% BSA (one 100 × 20-mm dish per condition) and then, they were treated as indicated in the figure: DMEM–0.1% BSA, 10 nM EGF, 10% FBS, 10 nM EGF + 10% FBS for 5 and 10 min (as a control, a sample that was not serum deprived, named ''no SS,'' was loaded in the last lane). Detergent extracts (200 μg protein) were immunoprecipitated with anti-erbB-2 and eluates were resolved by 7% SDS–PAGE and immunoblotted sequentially with (**A**) anti-phosphotyrosine (Santa Cruz., PY99, 1:500) and (**B**) anti-erbB2 antibodies. **C**: Densitometric analysis performed as indicated under ''Materials and Methods.''

EGF but not Heregulin Promotes Tyrosine Phosphorylation of ErbB2 and EGFR in 3T3-L1 Cells

We, then, determined the effect of other type I TKR ligands on the tyrosine phosphorylation status of the receptors. In Figure 7, we demonstrated that only EGF (10 nM) was capable of inducing an increase in receptor tyrosine phosphorylation when compared with heregulin-α1 and -β1 (10 nM). It should also be pointed out that the degree of erbB1 tyrosine phosphorylation induced by EGF was higher than the one of erbB2 (see Fig. 7A,B).

When performing the immunoblots to verify an equal loading of the samples, we observed that EGF caused a shift on the erbB2 migration in SDS–PAGE (see Fig. 7C).

Heregulin Expression in 3T3-L1 Cells

Since we could not find any effect of heregulin on receptor phosphorylation and as a way to elucidate whether this was a self-stimulated system, we looked for the expression of heregulin, which is a direct ligand for erbB3 and erbB4 but not for erbB2 or EGFR, although it may allow a heterodimer formation with them. As determined by Western blotting we found that 3T3-L1 cells showed at least two positive bands for heregulin, of 105 and 44 kDa each, which corresponded to the precursor and cleaved forms, respectively. We also observed that, in 3T3-L1 cells, the precursor

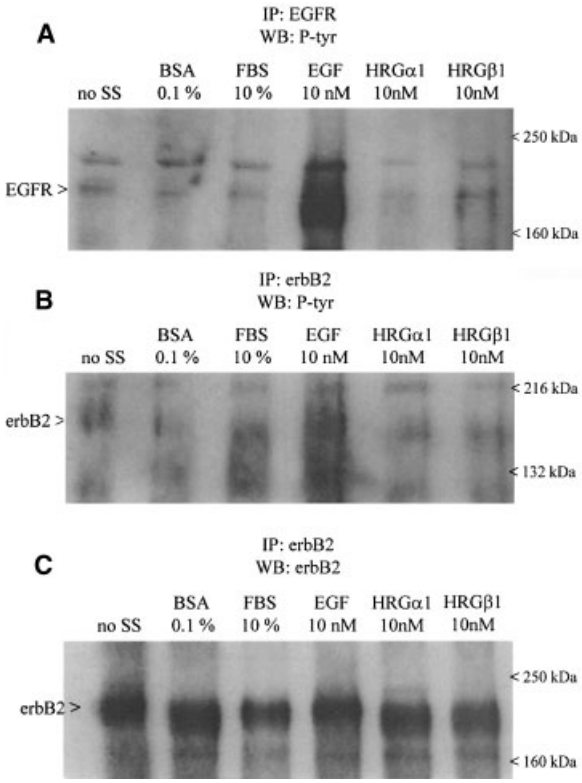

Fig. 7. EGF but not heregulin promotes tyrosine phosphorylation and migration of erbB2 in 3T3-L1 cells. As for Figure 6, cells were serum-starved (SS) and then treated for 5 min with the following factors: none (DMEM–0.1% BSA), 10% FBS, 10 nM EGF, 10 nM heregulin α1 and β1. First lane shows no starvation (no SS). Detergent extracts (200 μg protein) were immunoprecipitated with anti-erbB2 (**A**) and anti-EGFR (**B**) antibodies and eluates were resolved by 7% SDS–PAGE and immunoblotted against an anti-phosphotyrosine antibody. **C**: Control of an equal loading for the samples immunoprecipitated in (A). ErbB-2 blotting shows that EGF but not heregulin neither serum can induce a shift in the migration of erbB2.

form seemed to be downmodulated in adipocytes (Fig. 8).

ErbB3 and ErbB4 Expression in 3T3-L1 Cells

Given that exogenously administered heregulin had no effect on receptor tyrosine phosphorylation (neither on cell proliferation, data not shown), we needed to verify the presence of the other members of the EGFR family, erbB3 and erbB4, since they are the true receptors for it. We did not find the classic receptor forms (of the correct molecular mass), by performing Western blotting of whole extracts and immunoprecipitated protein samples. Nevertheless, in Figure 9, it can be noticed the presence of several specific erbB3 and erbB4 reactive bands in 3T3-L1 cells, that were also present in the control cell line T47D.

DISCUSSION

In this work, we demonstrated for the first time that the normal murine fibroblast preadipocytic cell line, Swiss 3T3-L1, expresses erbB2, a member of the type I tyrosine kinase receptor subfamily, largely described to be involved in many different cellular outputs ranging from proliferation, differentiation and embryogenesis to apoptosis, transformation, and carcinogenesis. 3T3-L1 cells expressed erbB2 in a quantity comparable to or even greater than the breast cancer cell line T47D, suggesting some relevance in this system. We found that the expression of erbB2 increased 10-fold during the proliferative stage. The addition of the

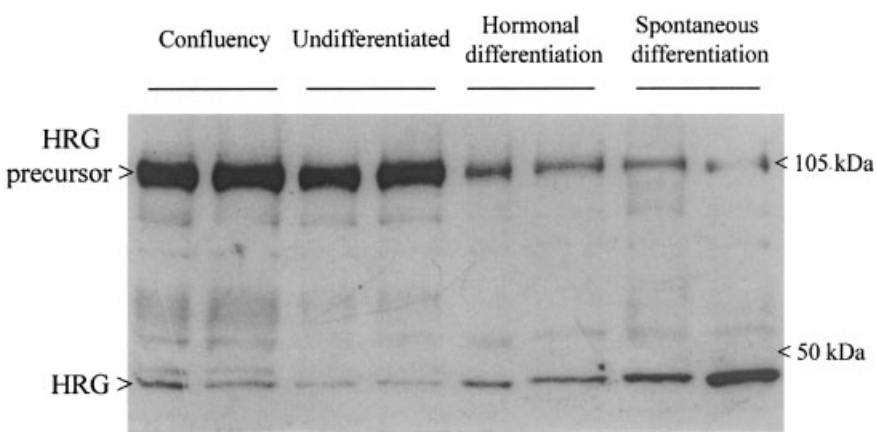

Fig. 8. Heregulin expression in the 3T3-L1 cell line. Whole extracts from 3T3-L1 cells reaching confluency, hormonally differentiated, undifferentiated control (cells that were not subjected to hormonal treatment but processed at the same time as the differentiated cells) and spontaneously differentiated (almost a month in culture) were subjected to SDS–PAGE and blotted against a polyclonal anti-heregulin antibody (Santa Cruz C-20 SC-348, 1:200). At least two positive bands were observed for heregulin, of 105 and 44 kDa each, corresponding to the precursor and cleaved forms, respectively.

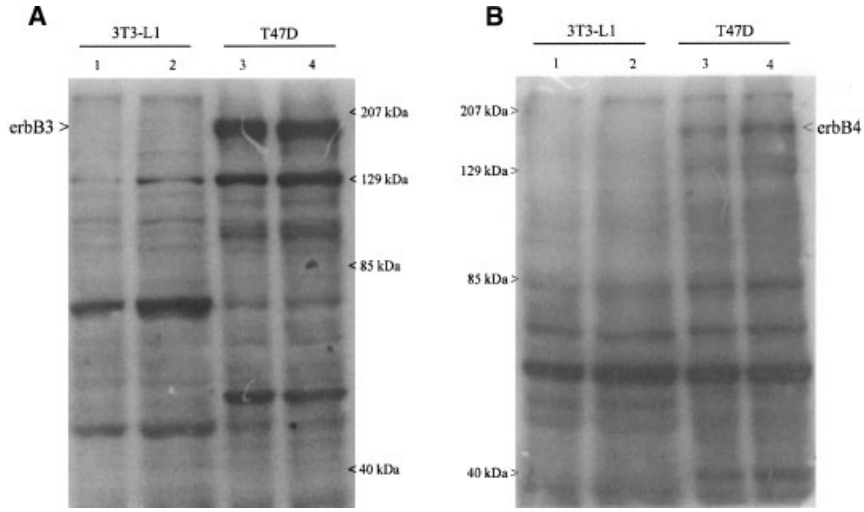

Fig. 9. ErbB3 and erbB4 reactive bands in 3T3-L1 cells. Whole extracts from 3T3-L1 cells were subjected to SDS–PAGE and blotted against (**A**) anti-erbB3 (Neo Markers, monoclonal, Ab-7 2C3, 1:200) and (**B**) anti-erbB4 (Neo Markers, policlonal, Ab-2, 1:200). **Lanes 1** and **3**: 50 μg protein per well; **lanes 2** and **4**: 100 μg protein per well. It can be noticed the presence of several specific reactive bands in 3T3-L1 cells for erbB3 and erbB4, as compared with the control cell line T47D, although none being the classic forms (180 kDa).

differentiation inducers (MIX and dexamethasone) kept erbB2 in that level during the growth arrest period. Otherwise it would have increased even higher, up to fourfold over the confluency level. The hormonal induction of the adipocytic differentiation led to a faster loss of receptors than in spontaneously differentiated cells. Nevertheless, regardless of how they were differentiated, erbB2 was downmodulated as differentiation progressed. We found it difficult to establish a clear modulation by insulin. It is possible that its non consistent effects depended on the differentiation status of the cells at the very moment when the sample was taken, as well as on a balance between its effects as a growth factor, mainly acting through the IGF-1 receptor, and as a lipogenic hormone, promoting the glucose and amino acid uptake that leads to lipid droplets accumulation.

We found that the treatment with MIX and dexamethasone had rapid effects on erbB2 expression in 3T3-L1 cells, since it was significantly diminished after 24 h in proliferating cells and after 12 h in growth-arrested cells. So, a genomic effect on the erbB2 promoter cannot be discarded, although it should be oppositely different from previous reports in the literature. It was described that cAMP, as well as confluence, as different ways to arrest cell growth, increased promoter activity, mRNA and protein levels in breast cancer cell lines [revised by De Bortoli and Dati, 1997]. Dexamethasone also increased both erbB2 mRNA and protein levels in endometrial and ovarian carcinoma cells [Karlan et al., 1994; Markogiannakis et al., 1997]. As differentiating agents, dexamethasone is frequently used because it stimulates the production of prostacyclin (a cAMP elevating and Ca^{2+} mobilizing agent) [revised by Ailhaud, 1997], and MIX because upon inhibiting the phosphodiesterase, it increases even further the cAMP levels, which initiates a cascade of trancriptional events leading to the expression of many proteins mediating adipocyte function. Some authors have demonstrated that undifferentiated preadipocytes express many inhibitory proteins that must be repressed to allow differentiation to proceed. This could be the case for erbB2 since its promoter has binding sites for the transcription factors AP-2α and SP-1, which transcriptional activity repression is necessary for PPARγ and C/EBPα gene expression, transcription factors that, both, play key roles in the differentiation process [reviewed by Morrison and Farmer, 2000]. In other systems, such as the well-known neuronal cell line PC12, cAMP or nerve growth factor can induce differentiation through the sustained activation of the extracellular signal-regulated kinase (ERK) while EGF alone can not, because it induces only a transient activation, thus resulting in proliferation [Yao et al., 1998]. Being erbB2 the preferred heterodimerization partner for EGFR, and given that it is a potent mitogenic

signaling receptor, it would be reasonable to think that the proliferation signal mediated by growth factors receptors should be "turned off" in order to "turn on" the differentiation process.

We detected two specific bands for EGFR in 3T3-L1 cells, the classical receptor (170 kDa) and probably the variant type III (EGFRvIII, 145kDa). We found that, as for erbB2, also EGFR diminished in adipocytes as compared with preadipocytes. This was in agreement with Hardy et al. [1995] who observed a decrease in 3T3-L1 EGFR levels from 10,000 binding sites/cell in preadipocytes to less than 5,000 in mature adipocytes. Some other authors, have found, however, through Scatchard analysis [Reed et al., 1977] and through RT-PCR [Adachi et al., 1994] two classes of EGF receptors (between 25,000 and 40,000 sites per cell) that are present in both undifferentiated and differentiated 3T3-L1 cells but at similar abundance. It is not clear what the function of this secondary receptor could be, since it is ligand independent.

We found that the treatment with MIX and dexamethasone had downmodulating effects on EGFR expression, the same feature as for erbB2. Even though these factors are agents that usually upmodulate EGFR transcription (2–3-fold stimulation) [reviewed by Bates and Hurst, 1997], other factors also present in the serum, such as retinoic acid or thyroid hormone, could influence negatively on it. In some tissues, cAMP can induce phosphorylation on serine residues of the EGFR, thus decreasing its signal transduction and promoting receptor down-regulation [Barbier et al., 1999]. Dexamethasone, by glucocorticoid receptor occupation, has been described to cause a rapid inhibition of signaling proteins recruitment to EGFR, thus leading to decrease its mitogenic signal [Croxtall et al., 2000].

Although a decrease in erbB2 and EGFR expression was observed after the MIX-DEXA treatment, we cannot conclude that this decrease results in the induction of differentiation or it is a consequence of it.

Since the auto or heterologous phosphorylation of ErbBs members is a main event in their signaling pathways, it was important to study this event on these cells. We stimulated previously serum-starved cells with EGF and immunoprecipitated erbB2 and found a significant increase in erbB2 tyrosine phosphorylation in response to EGF that was attenuated in the presence of serum. This was an important finding since it indicated that EGF was able to activate erbB2 in 3T3-L1 cells and that, at least part of its effects, could be explained through an EGFR–erbB2 heterodimer. We chose EGF and not TGF-α for example, because EGF plays an important role as a mitogenic agent when administered to preadipocytes, where it stimulates cell division with a 2–3-fold increase in cell number [Smith et al., 1988] although it can not initiate adipocyte differentiation. In a serum-free culture system, it is by far the most potent serum component in supporting the exponential growth phase necessary to reach confluency which, in time, triggers differentiation [Schmidt et al., 1990]. It is noteworthy that in the absence of serum it preserves the ability of 3T3-L1 cells to undergo adipose conversion and, in combination with IGF-1, brings the cells to a maximal differentiation rate comparable to that obtained with high insulin concentrations [Schmidt et al., 1990]. Other investigators have found that EGF exerted a sustained MAPK activity stimulation in proliferating 3T3-L1 cells that did not decrease substantially in differentiating cells, somehow contributing to its description as a potent mitogenic and antiadipogenic agent [Boney et al., 1998]. It seems likely that the mitogenic effect fades away as differentiation program begins. A similar situation, with a cAMP-activated MAPK pathway, can be observed in PC12 neuronal cells, where EGF alone cannot induce differentiation while EGF plus cAMP produce the same effect as NDF [Yao et al., 1995]. The possibility of different signaling mediated by EGF through EGFR/erbB2 dimers remains to be clarified.

It could be hypothesized that there could be a cross-talk between erbB2–EGFR and the IGF-1R in the 3T3-L1 cells, as it was observed for the EGF-dependent mitogenic signaling through erbB2 phosphorylation modulated by GH in 3T3-F442A fibroblasts, which also leads to adipocyte differentiation in these cells [Kim et al., 1999], since it is well known that a functional IGF-1R is required for the mitogenic activity of the EGFR [Coppola et al., 1994]. IGF-1 is able to transactivate EGFR through a metalloproteinase-mediated cleavage of heparin binding-EGF (HB-EGF) in COS-7 cells [Roudabush et al., 2000] and lately, it was described to increase HB-EGF mRNA in 3T3-L1 cells as well [Mulligan et al., 2002]. In addition, we have previously described that 3T3-L1 cells IGF-1 production increases whereas IGF-1R

decreases with differentiation [Zizola et al., 2002]. It is encouraging that the differentiation signal transduced by IGF-1R shares some features with the one by erbB2 (PI3-K, Akt, etc.).

We could see that EGF induced a shift in erbB2 migration on SDS–PAGE that neither HRG α nor β were able to do. This could be due to changes in phosphorylation, as seen by others [Kim et al., 1999], who described that GH induces retardation on SDS–PAGE migration of erbB2 in 3T3-F442A fibroblasts due to increased serine/threonine phosphorylation (via PKA), while decreases tyrosine phosphorylation, a common feature to downregulate receptor activity.

Heregulin, the human homologue of NDF (neu differentiation factor), can stimulate erbB2 tyrosine phosphorylation through heterodimer formation with erbB3 and erbB4 and cause differentiation of many human breast cancer cells [Peles et al., 1992]. As we did not find any increase in erbB2 or EGFR tyrosine phosphorylation when heregulin was added to the medium, we conclude that the direct receptors were present, if so, at very low levels in these cells to provide a significant number of binding sites to transduce a differential signal through them, or the system provided a ligand that acted by an autocrine way.

To further test this issue, we checked for the presence of heregulin in 3T3-L1 cells and we found both the precursor as well as the mature forms. This could be the reason why no further increase in tyrosine phosphorylation was observed when adding HRG to the medium, being the system already activated. Typical erbB3 and erbB4 isoforms were not observed in 3T3-L1 cells as compared with the control cell line T47D, although several isoforms for each of them have been described [Lee and Maihle, 1998; Junttila et al., 2000; Srivinisan et al., 2001].

All these results, taken together, point towards an involvement of EGF and its family of receptors, in the changes that take place during adipose differentiation. Although no cause–effect relationship could be drawn from the results presented herein, nevertheless the changes in receptor abundance and the possibility of a heterodimeric signaling through EGF or another erbB ligand, observed along proliferation and/or differentiation are worth pursuing. Further experiments will deal with the intracellular pathways put into motion when those changes take place.

To our knowledge, this is one of the very few reports on erbB2 expression in non-malignant, non-transformed, non-epithelial, differentiating cells.

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REFERENCES

Adachi H, Kurachi H, Homma H, Adachi K, Takashi I, Morishige K, Matsuzawa Y, Miyake A. 1994. Epidermal growth factor promotes adipogenesis of 3T3-L1 cell in vitro. Endocrinology 135:1824–1830.

Ailhaud G. 1997. Obesity and the adipocyte. Molecular mechanisms of adipocyte differentiation. J Endocr 155: 201–202.

Balaña ME, Labriola L, Salatino M, Movsichoff F, Peters G, Charreau EH, Elizalde P. 2001. Activation of erbB-2 via a hierarchical interaction between erbB-2 and type I insulin-like growth factor receptor in mammary tumor cells. Oncogene 20:34–47.

Barbier A, Poppleton H, Yigzaw Y, Mullenix J, Wiepz G, Bertics P, Patel T. 1999. Transmodulation of epidermal growth factor receptor function by cyclic AMP-dependent protein kinase. J Biol Chem 274:14067–14073.

Bates NP, Hurst HC. 1997. Transcriptional regulation of type I receptor tyrosine kinases in the mammary gland. J Mammary Gland Biol Neoplasia 2:153–163.

Boney CM, Smith RM, Gruppuso PA. 1998. Modulation of insulin-like growth factor 1 mitogenic signaling in 3T3-L1 preadipocyte differentiation. Endocrinology 139: 1638–1644.

Carraway KL III, Burden SJ. 1995. Neuregulins and their receptors. Curr Opin Neurobiol 5:606–612.

Coppola D, Ferber A, Miura M, Sell C, D'Ambrosio C, Rubin R, Baserga R. 1994. A functional insulin-like growth factor 1 receptor is required for the mitogenic and transforming activities of the epidermal growth factor receptor. Mol Cell Biol 14(7):4588–4595.

Croxtall JD, Choudhury Q, Flower RJ. 2000. Glucocorticoids act within minutes to inhibit recruitment of signaling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. Br J Pharmacol 130:289–298.

Darcy KM, Zangani D, Wohlhueter AL, Huang RY, Vaughan MM, Russell JA, Ip MM. 2000. Changes in erbB2 [her-2/neu], erbB3, and erbB4 during growth, differentiation, and apoptosis of normal rat mammary epithelial cells. J Histochem Cytochem 48:63–80.

De Bortoli M, Dati C. 1997. Hormonal regulation of type I receptor tyrosine kinases expression in the mammary gland. J Mammary Gland Biol Neoplasia 2: 175–185.

Graus-Porta D, Beerli RR, Daly JM, Hynes NE. 1997. ErbB-2, the preferred heterodimerization partner of all erbB receptors, is a mediator of lateral signaling. EMBO J 16:1647–1655.

Green H, Kehinde O. 1974. Sublines of mouse 3T3 cells that accumulate lipid. Cell 1:113–116.

Hardy RW, Gupta KB, McDonals JM, Williford J, Wells A. 1995. Epidermal growth factor (EGF) receptor carboxiterminal domains are required for EGF-induced glucose transport in transgenic 3T3-L1 adipocytes. Endocrinology 136:431–439.

Hynes NE, Stern DF. 1994. The biology of erbB-2/HER-2 and its role in cancer. Biochim Biophys Acta 1198:165–184.

Junttila TT, Sundvall M, Maatta JA, Elenius K. 2000. ErbB4 and its isoforms: Selective regulation of growth factor responses by naturally occurring receptor variants. Trends Cardiovasc Med 10:304–310.

Karlan B, Jones J, Slamon D, Lagasse L. 1994. Glucocorticoids stabilize Her-2/neu messenger RNA in human epithelial ovarian carcinoma cells. Gynecol Oncol 53: 421–426.

Kim S, Houtman JCD, Jiang J, Ruppert M, Bertics PJ, Frank SJ. 1999. Growth hormone-induced alteration in erbB-2 phosphorylation status in 3T3-F442A fibroblasts. J Biol Chem 274:36015–36024.

Lee H, Maihle N. 1998. Isolation and characterization of four alternate c-erbB3 transcripts expressed in ovarian carcinoma-derived cell lines and normal human tissues. Oncogene 16:3243–3252.

Lim H, Dey SK, Das SK. 1997. Differential expression of the *erbB2* gene in the periimplantation mouse uterus: Potential mediator of signaling by epidermal growth factor-like growth factors. Endocrinology 138:1328–1337.

Markogiannakis E, Georgoulias V, Margioris A, Zoumakis E, Stournaras C, Gravanis A. 1997. Estrogens and glucocorticoids induce the expression of c-erbB2/NEU receptor in Ishikawa human endometrial cells. Life Sci 61:1083–1095.

Morrison RF, Farmer SR. 2000. Symposium: Adipocyte function, differentiation and metabolism. Hormonal signaling and transcriptional control of adipocyte differentiation. J Nutr 130:3116S–3121S.

Mulligan C, Rochford J, Denyer G, Stephens R, Yeo G, Freeman T, Siddle K, O'Rahilly S. 2002. Microarray analysis of insulin and insulin-growth factor-1 (IGF-1) receptor signaling reveals the selective up-regulation of the mitogen heparin-binding EGF-like growth factor by IGF-1. J Biol Chem 277(45):42480–42487.

Olayioye MA, Neve R, Lane HA, Hynes NE. 2000. The ErbB signaling network: Receptor heterodimerization in development and cancer. EMBO J 19:3159–3167.

Peles E, Bacus SS, Koski RA, Lu HS, Wen D, Ogden SG, Levy RB, Yarden Y. 1992. Isolation of the neu/HER-2 stimulatory ligand: A 44 kDa glycoprotein that induces differentiation of mammary tumor cells. Cell 69:205–216.

Press MF, Cordon-Cardo C, Slamon DJ. 1990. Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene 5:953–962.

Reed BC, Kaufmann SH, Mackall JC, Student AK, Lane MD. 1977. Alterations in insulin binding accompaying differentiation of 3T3-L1 preadipocytes. Proc Natl Acad Sci USA 74:4876–4880.

Roudabush FL, Pierce KL, Maudsley S, Khan KD, Lutrell LM. 2000. Tansactivation of the EGF receptor mediates IGF-1-stimulated Shc phosphorylation and ERK1/2 activation in COS-7 cells. J Biol Chem 275:22583–22589.

Schmidt W, Pöll-Jordan G, Löffler G. 1990. Adipose convertion of 3T3-L1 cells in a serum-free culture system depends on epidermal growth factor, insulin-like growth factor-1, corticosterone, and cyclic AMP. J Biol Chem 265:15489–15495.

Schroeder JA, Lee DC. 1998. Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. Cell Growth Differ 9:451–464.

Serrero G. 1987. EGF inhibits the differentiation of adipocyte precursors in primary cultures. Biochem Biophys Res Commun 146:194–202.

Serrero G, Mills D. 1991. Physiologic role of EGF on adipose tissue development in vivo. Proc Natl Acad Sci USA 88: 3912–3916.

Sliwkowski MX, Schaefer G, Akita RW, Lofgren JA, Fitzpatrick VD, Nuijens A, Fendly BM, Cerione RA, Vandlen RL, Carraway KL III. 1994. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J Biol Chem 269:14661–14665.

Smith PJ, Wise LS, Berkowitz R, Wan C, Rubin CS. 1988. Insulin-like growth factor-1 is an essential regulator of the differentiation of 3T3-L1 adipocytes. J Biol Chem 263:9402–9408.

Srivinisan R, Leverton K, Sheldon H, Hurst H, Sarraf C, Gullick W. 2001. Intracellular expression of the truncated extracellular domain of c-erbB3/HER3. Cell Signal 13:321–330.

Stern DF, Kamps MP. 1988. EGF-stimulated tyrosine phosphorylation of p185neu: A potential model for receptor interactions. EMBO J 7:995–1001.

Yao H, Labudda K, Rim C, Loda M, Stork PJ. 1995. Cyclic adenosine monophosphate can convert epidermal growth factor into a differentiating factor in neuronal cells. J Biol Chem 270:20748–20753.

Yao H, York RD, Misra-Press A, Carr DW, Stork PJ. 1998. The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated kinases and gene expression by nerve growth factor. J Biol Chem 273:8240–8247.

Zizola C, Balaña ME, Sandoval M, Calvo JC. 2002. Changes in IGF-1 receptor and IGF-1 mRNA during differentiation of 3T3-L1 preadipocytes. Biochimie 84:975–980.