Down-Modulation of erbB2 Activity is Necessary but not Enough in the Differentiation of 3T3-L1 Preadipocytes

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Abstract   The high incidence of obesity-related pathologies, led to the study of the mechanisms involved in preadipose cell proliferation and differentiation. Here, we demonstrate that modulation of erbB2, plays a fundamental role during proliferation and adipogenic induction of preadipocytes. Using 3T3-L1 cells as model, we demonstrate that EGF (10 nM, 5 min) in addition to stimulate receptor tyrosine phosphorylation of both erbB2 and EGFR, is able to induce the heterodimer erbB2-EGFR. We treated proliferating 3T3-L1 cells with two inhibitors, AG 825 (IC_{50} 0.35 μM, 54 times more selective for erbB2 than for EGFR, IC_{50} 19 μM), and AG 879 (IC_{50} of 1 μM for erbB2 versus 500 μM for EGFR). We found that both inhibited the proliferation on a dose-dependent basis, reaching a 30% maximal inhibition at 100 μM ($P < 0.001$) for AG825, and a 20% maximal inhibition at 10 μM ($P < 0.001$) for AG 879. These results involve erbB2 in 3T3-L1 proliferation. When studying the differentiation process, we found that the action of MIX–Dexa immediately activates MEK, JNK and p38 kinases. We observed that PD98059 and SP600125 (MEK–ERK and JNK inhibitors, respectively) added 1 h prior to the MIX–Dexa induction produced a decrease in erbB2 expression after 6 h, which is even greater than the one produced by the inducers, MIX–Dexa. This work supports erbB2 as a key factor in 3T3-L1 adipogenesis, acting mostly and not only during the proliferative phase but also during the differentiation through modulation of both its expression and activity. J. Cell. Biochem. 104: 274–285, 2008.   © 2007 Wiley-Liss, Inc.

Key words: erbB2; EGFR/erbB1; adipogenesis; 3T3-L1; tyrphostin

The predetermined fibroblasts from adipose tissue undergo, in the presence of glucose and other hormonal factors, a differentiation process so that they become adipose cells or adipocytes [for review, see Ailhaud, 1997; Mandrup and Lane, 1997; Morrison and Farmer, 2000]. The adipocyte is not only a reservoir for energy but, also and mainly, a center of metabolic regulation. When the adipose tissue cannot longer accommodate the excess of lipids, fat accumulates in other tissues and the individual becomes obese. The high incidence of obesity and associated pathologies, such as diabetes and cardiovascular diseases, led to the need of understanding the process of adipogenesis [Must et al., 1999]. The study of the mechanisms involved in cell proliferation and differentiation as well as its adipogenic control could contribute to the treatment of the abovementioned pathologies.

The murine 3T3-L1 fibroblastic cell line [Green and Kehinde, 1974] has been used as a model system to study the mechanisms involved in the adipogenic process. The spontaneous

but slow process of differentiation can be accelerated, once confluence is reached, with isobutyl-methylxanthine (MIX) or any other cAMP-increasing agent, dexamethasone (Dexa), and insulin like growth factor-1 (IGF-1) or supraphysiological concentrations of insulin (INS) [Smith et al., 1988]. After hormonal induction confluent preadipocytes undergo mitotic clonal expansion, growth arrest and then a second round of cell division to allow transcription factors to act so that cells finally express adipocyte-specific gene products [Cornelius et al., 1994]. Several transcription factors, including members of the peroxisome proliferator activated receptor (PPAR) and CAAT enhancer binding protein (C/EBP) families, act cooperatively and sequentially to trigger the differentiation program [Lowell, 1999]. Some growth factors and their receptors play pivotal roles in this process, such as IGF-1R and EGFR. 3T3-L1 cells, although capable of proliferating, are unable to differentiate in medium with serum, deprived from peptide and steroid hormones, in general, and INS and IGF-1 in particular [Smith et al., 1988]. It has been shown that IGF-1 and EGF are important mitogenic factors for 3T3-L1 cells [Boney et al., 1998, 2000, 2001]. In a serum-free culture system, a differentiation rate similar to that obtained with insulin is achieved with IGF-1 only in combination with EGF [Schmidt et al., 1990], making EGF the most potent serum component in supporting the exponential growth necessary to reach confluency. EGF has also been described to have sometimes controversial effects on adipose differentiation because it blocks maturation of adipocyte precursor cells into mature adipocytes both in vivo and in vitro [Serrero, 1987; Serrero and Mills, 1991] but enhances adipogenesis in the already differentiating adipocytes in vitro [Adachi et al., 1994]. The high levels of EGF found in ovariectomized mice (10 times higher than in young females) produce adipocyte hiperdystrofy [Kurachi et al., 1993]. One possible explanation could be differences in postreceptor signaling since EGFR levels have been previously described either to remain constant [Reed et al., 1977; Adachi et al., 1994] or, as we previously found, to decrease throughout differentiation [Hardy et al., 1995; Boney et al., 1998; Pagano and Calvo, 2003]. Another explanation could come from a possible interaction with erbB2, an EGFR family related member. Both of them are transmembrane receptors with intrinsic tyrosine kinase activity (type I TKR), as IGF-1R, and dimerize in order to transduce their signal. 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]. Frequently, signaling from heterodimers presents distinct features compared to the corresponding homodimers as a way of diversifying the signal [Olayioye et al., 1998]. Since it has been demonstrated that erbBs role in cell proliferation, differentiation and survival is of extreme importance [for a review see Olayioye et al., 2000; Olayioye, 2002] we dedicated ourselves to investigate their participation, particularly that of erbB2, in the progress of the adipogenic program.

In a previous work, we studied erbB1/EGFR, erbB2, erbB3 y erbB4 as well as ligands, heregulin and EGF, in terms of expression, activation and modulation during the adipogenic process [Pagano and Calvo, 2003]. On a protein level, we found out that the expression of erbB2 and EGFR increased during the proliferative phase, significantly decreasing thereafter in response to adipogenic induction (MIX, Dexa, and FBS), with a similar pattern for both receptors. The progress towards a differentiated state was accompanied by an increase in the expression of mature heregulin or neuregulin 1, type I, an erbB specific ligand, which could be responsible for preventing the activation of erbB2 in response to exogenous heregulin. Although we could not find typical erbB3 and erbB4 isoforms in that same study, we detected different specific minor bands through Western blot possibly corresponding to isoforms already described. If this were the case, these isoforms could function as receptors for neuregulin and hence, as correceptors for erbB2 and/or EGFR as well. Preliminary results allow us to say that 3T3-L1 cells in fact do express erbB3 (RT-PCR experiments, unpublished results, manuscript in preparation).

In the present study, our aim was to elucidate the specific role of erbB2 in 3T3-L1 adipogenesis. We show that both erbB2 and EGFR are tyrosine-phosphorylated in response to EGF, forming a heterodimer and, thus, we present evidence that an interaction between these two receptors is possible in these cells. Pharmacological inhibition of the receptor with the use of tyrphostins AG 825 and AG 879 during the

proliferation of 3T3-L1 cells results in a partial inhibition of this process, demonstrating the participation of erbB2 at this stage. Reduced receptor activity by tyrphostin treatment during adipogenic induction potentiates the effect of the inducers, but cannot replace them, thus indicating that the decrease in the expression/activation of erbB2 could favor the adipogenic process. We confirmed the activation of three different signaling pathways upon adipogenic induction: MAPK, JNK, and p38. ErbB2 expression after adipogenic induction was further downmodulated by the MAPK and JNK inhibitors (PD98059 and SP600125, respectively), possibly suggesting that the activation of these kinases, in response to induction, could retard the down-regulation of erbB2. These results constitute, to our knowledge, the first evidence of the role of erbB2 in 3T3-L1 adipose 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) were routinely cultured in Dulbecco's modified Eagle's medium (Hyclone) with 4 mM L-glutamine, 4.5 g/L glucose, 10^{-6} M biotin, 0.11 g/L sodium piruvate and supplemented with 10% FBS (Gen SA, Bs. As.) plus antibiotics (Gibco, Life Technologies, Rockville, MD) 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) in DMEM-10% FBS. After 48 h, the medium was removed and replaced by DMEM-10% FBS 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 Blot

Cell monolayers were washed and collected in ice-cold PBS and centrifuged at 4,000 rpm (1,500g) 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, 1 μg/ml of leupeptin, pepstatin, aprotinin,

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 (13,500g) 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) 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.

ErbB2/EGFR Heterodimerization by EGF

We basically followed the protocol described by Wada, Qian and Greene [Kokai et al., 1988; Wada et al., 1990]. Briefly, proliferating cells (2×10^6/dish) 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, or mock treated (0.1% BSA) for 5 min at 37°C. Stimulation was stopped by placing the cells on ice and washing them twice with ice-cold PBS plus 400 μM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 400 μM Na_3VO_4 and extracted directly in lysis buffer: 150 mM NaCl, 10 mM sodium phosphate [pH 7.4], 1% digitonin [according to Qian et al., 1992], 2 mM EDTA, 10 mM sodium pyrophosphate, 400 μM Na_3VO_4, and protease inhibitors (1 μg/ml aprotinin, benzamidin, leupeptin, and 1 mM AEBSF). The lysates were clarified by centrifugation at 12,000 rpm (13,500g) at 4°C for 20 min. Protein concentration was determined when supernatant was transferred to a fresh tube. To immunoprecipitate the erbB2-EGFR complexes equal amounts of protein from each treatment (2,000 μg) were precleared with protein LA-agarose beads (Sigma) and rabbit normal IgG for 30 min on ice with continuous mixing. After a

quick centrifugation at low speed the precleared samples were first incubated with the specific antibody (erbB2 or EGFR) for at least 2 h on ice and then with protein LA-agarose beads for another 2 h or ON. Immune complexes were collected by centrifugation at 1,500*g*, washed three times with PBS plus protease and phosphatase inhibitors and boiled for 5 min in sample buffer and subjected to SDS–PAGE. Tyrosine phosphorylation and receptor dimerization were determined by WB, using a specific anti-P-Tyr antibody (SC PY99) and anti-erbB2 or anti-EGFR, respectively, to detect the coprecipitation of the opposite receptor.

Antibodies

Rabbit policlonal anti-EGFR (Santa Cruz 1005 sc-03, 1:1,000 dilution); rabbit policlonal anti-erbB2 (Santa Cruz Neu C-18, 1:1,000); mouse monoclonal anti-phosphotyrosine (Santa Cruz PY99, 1:500); rabbit policlonal anti-ERK (Santa Cruz, sc-154, 1:500); mouse monoclonal anti-phosphoERK (Santa Cruz sc-7383, 1:500); goat policlonal anti-JNK (Santa Cruz 474G, 1:500); mouse monoclonal anti-phosphoJNK (Santa Cruz sc-8254, 1:500); goat policlonal anti-p38 (Santa Cruz 535G, 1:500); mouse monoclonal anti-phospho p38 (Santa Cruz sc-7973, 1:250); rabbit anti-goat (Santa Cruz sc-2768, 1:1,000); goat anti-rabbit (Santa Cruz sc-2054, 1:2,000); rabbit anti-mouse (Amersham Life Sciences RPN 2108, 1:2,000).

Proliferation Assay by MTT

Briefly, cells were plated in 96-well culture dishes (3,000 cells/well), allowed to adhere and to proliferate for 1 day, and then treated for 48 h with the inhibitor (tyrphostin), with or without the mitogen (EGF). Near the end of the proliferation assay half of the medium (100 μl) was removed and 10 μl from a 5 mg/ml MTT solution (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) were added to each well. The cells were incubated at 37°C for 5 h for allowing MTT cleavage to occur. Acid/alcohol (0.04 N HCl in 2-propanol) was added to the cells (100 μl) to convert phenol red into a yellow color that does not interfere with MTT formazan measurement, and to dissolve the precipitates to provide a homogeneous solution for measurement. Within the next hour the absorbance was measured on an ELISA reader at 570 nm. All readings were performed in duplicate and values of proliferation were calculated as a percentage of the absorbance measured on control cells (no inhibitor, no EGF treatment).

### Triglyceride Determination

Triglyceride accumulation was determined by using a commercial kit (Wiener lab., Rosario, Argentina) based on the Trinder enzymatic method. In this work cells plated on 24-well dishes were washed with PBS and scraped with a rubber policeman in deionized water (100 μl/well). Following three rounds of sonication, 50 μl of each sample were incubated with the enzimatic mix at room temperature and absorbance was determined at 490/505 nm 20 min later. All readings were performed in duplicate and values expressed as triglyceride (μg/well).

RESULTS

EGF Induces erbB2-EGFR Heterodimerization in 3T3-L1 Cells

In a previous work we demonstrated that 3T3-L1 cells express EGFR as well as the related receptor erbB2 [Pagano and Calvo, 2003]. Both of them are strongly up-modulated during the proliferation phase of the cell culture and down-modulated once the cells are induced to differentiate with MIX–Dexa, in the presence of 10% FBS. At this point, differentiated cells express much lower receptor levels than their counterpart undifferentiated ones. As EGF is known to exert mitogenic activity [Schmidt et al., 1990] and to induce differentiation on these cells [Adachi et al., 1994], we studied its capacity to activate erbB2 and EGFR and to promote their heterodimerization.

3T3-L1 cells were allowed to proliferate and, before reaching confluence, they were serum-starved overnight to rule out a possible basal or serum effect that could affect the activation status of these receptors. Then, cells were stimulated with EGF, serum, or none (BSA), followed by erbB2 or EGFR immunoprecipitation from digitonin (a mild detergent) protein extracts. By Western blot analysis we demonstrated that EGF (10 nM, 5 min, 37°C) was able to induce the heterodimer (Fig. 1A, right and left panels) in addition to stimulate receptor tyrosine phosphorylation (Fig. 1B). The immunoprecipitated EGFR (IP: EGFR) from EGF-treated cells and then, immunoblotted against
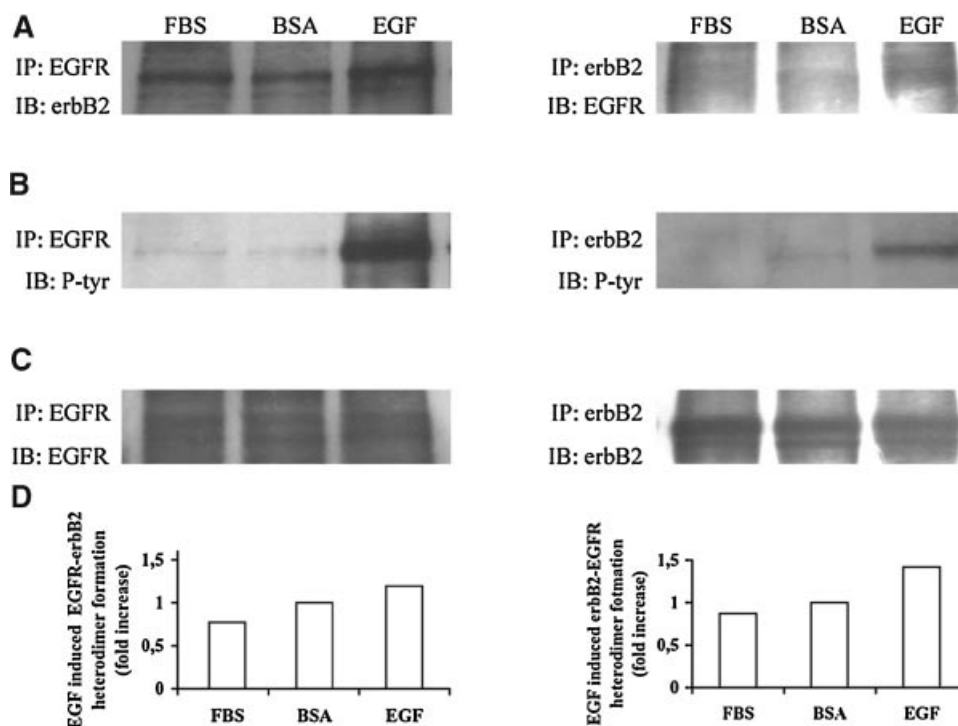
Fig. 1. EGF induces erbB2-EGFR heterodimerization in 3T3-L1 cells. Proliferating cells cultured in DMEM-10% FBS were serum-starved overnight and then treated (three 100×20-mm dish per condition) for 5 min at $37°C$ with: 10% FBS, 1% BSA, 10 nM EGF. Digitonin extracts (2 mg protein) were immunoprecipitated (IP) with anti-EGFR and anti-erbB2 (Santa Cruz, sc-03 and Neu C-18, 1:1,000) antibodies and eluates were resolved by 7% SDS–PAGE and immunoblotted (IB) using antibodies against (**A**), the opposite receptors to demonstrate heterodimerization; (**B**) phosphotyrosine, thereby confirming receptor activation;

(**C**) the same receptors used for immunoprecipitation in order to control for equal sample loading and IP effectivity. **D**: Densitometric analysis of heterodimer formation (gels in panel A). Values are expressed as fold increase in erbB2-EGFR formation (arbitrary units) over the control (BSA), previously referred to the total specific protein value after densitometrying bands in panel (C). Band intensities were quantified using the Scion Image software (NIH Image for Windows). Shown is an experiment out of four.

erbB2 (IB: erbB2), showed that EGF stimulated heterodimerization (Fig. 1A, left panel). Likewise, immunoprecipitated erbB2 samples immunoblotted against EGFR confirmed this result (Fig. 1A, right panel). EGF induced receptor tyrosine phosphorylation as well as serum attenuation are shown as an internal control that the system is responding adequately as demonstrated in our previous study [Pagano and Calvo, 2003, see the Discussion section for further explanation on this item]. Immunoprecipitation and immunoblotting were also performed sequentially with the same antibody (anti-EGFR or anti-erbB2, according to the case), to control equal loading as well as expression levels for each receptor (Fig. 1C, left and right, respectively). The densitometric analysis (Fig. 1D) showed that EGF differentially induced the formation of heterodimer erbB2-EGFR.

Tyrphostins, by erbB2 Blockage, Inhibit 3T3-L1 Cells Proliferation

To evaluate erbB2 importance on these cells proliferation, we decided to reduce receptor activity by using a class of compounds known as "tyrphostins" which, by competing with the ATP binding site in the kinase domain, interfere with receptor activity [Levitzki and Gazit, 1995]. Since erbB receptors share a great homology of sequence, both in nucleotides and in aminoacids, with a certain degree of overlapping in their functions, we chose two different tyrphostins for their high selectivity for erbB2 over EGFR. The compound AG 825 is 54 times more selective for erbB2 (IC_{50} 0.35 μM) than for EGFR (IC_{50} 19 μM). By treating proliferating 3T3-L1 cells with AG 825 we found that it inhibited the proliferation on a dose-dependent manner (Fig. 2A), reaching a
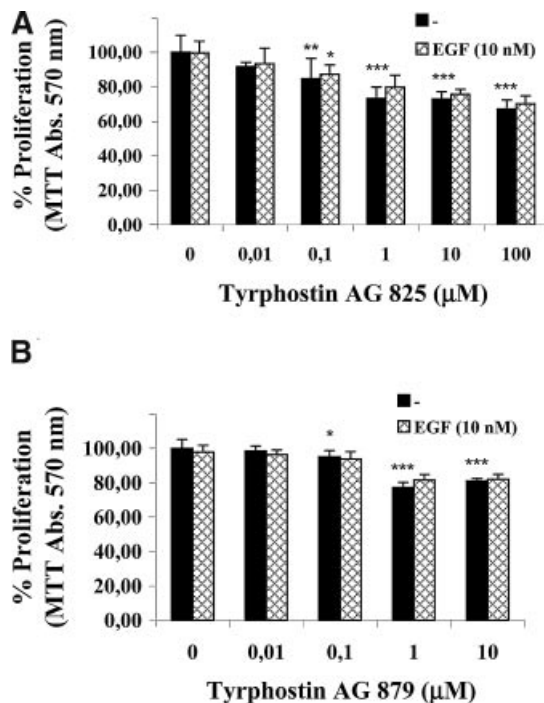
Fig. 2. Blockade of erbB2 by tyrphostins inhibits 3T3-L1 proliferation. Proliferating cells were treated with different tyrphostin concentrations (**A** AG 825, **B** AG 879) with (patterned bars) or without EGF 10 nM (black bars) for 2 days. Cellular proliferation was assessed by the MTT method. Values are expressed referred to as percentages of the control condition (no treatment or tyrphostin 0 μM without EGF, first column, 100%) and represent the mean ± SD (n = 8). Statistic analysis: one-way ANOVA followed by Student–Newman–Keuls post-test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Shown is the statistical significance for every tyrphostin concentration regarding the control condition and when not other way marked, significance is the same for every pair of tyrphostin concentration (EGF treated or not). Shown is a representative experiment out of three. All experiments involving tyrphostins included carrier controls (DMSO) (not shown).

maximum of 30% inhibition at 100 μM ($P < 0.001$, compared to control: tyrphostin 0 μM without EGF or first column). This effect started at a concentration of 0.1 μM ($P < 0.01$).

We also used AG 879, an even more selective inhibitor for erbB2 (IC_{50} 1 μM) than for EGFR (IC_{50} 500 μM), to confirm the previous result as well as the possible role of this receptor on this cell line. AG 879 also significantly reduced 3T3-L1 proliferation (Fig. 2B) starting at 0.1 μM ($P < 0.05$) and reaching its maximal inhibition (20 %) at 10 μM ($P < 0.001$) since 100 μM was toxic for the cells (not shown). EGF was unable to prevent receptor inactivation and proliferation inhibition by the inhibitors (Fig. 2 patterned bars).

Tyrphostins, by erbB2 Blockage, Stimulate 3T3-L1 Differentiation, Induced by MIX–Dexa

Once we established erbB2 involvement in 3T3-L1 preadipocytes proliferation, we then studied its participation in their differentiation. The aim was to evaluate whether erbB2 inhibition (by tyrphostin treatment) could mimic the effect of MIX–Dexa induction. Since we used both tyrphostin AG 825 and AG 879 in proliferation experiments, with similar results, we decided to perform differentiation assays only with AG 879.

Tyrphostin AG 879 was added for 1 h prior to the addition of induction mixture or media alone, always in the presence of 10% FBS. Then, AG 879 treated cells, with MIX–DEXA plus serum or serum alone, were allowed to differentiate. The experiment was ended when fully differentiation in control cells (without AG 879, with MIX–Dexa) was observed by Oil Red O staining of lipid droplets (not shown). Triglyceride determination demonstrated that AG 879 potentiated the differentiation induced by MIX–Dexa ($1 \, \mu M \, P < 0.001$ and $0.1 \, \mu M \, P < 0.01$) but was unable to induce it by itself (Fig. 3, compare full bars against striped ones).
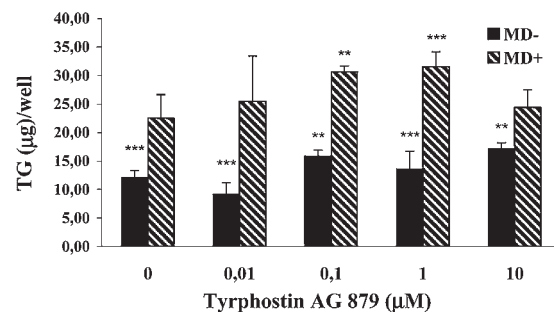
Fig. 3. Blockade of erbB2 by tyrphostin AG 879 potentiates 3T3-L1 differentiation induced by MIX–Dexa but it is not able to induce differentiation by itself. Post-confluent cells were treated with AG 879, a highly selective inhibitor of erbB2, 1 h before the MIX–Dexa treatment. Insulin was added 2 days after. Triglyceride production was determined by the Trinder method 5 days after insulin addition. Values are expressed as the media ± SD (n = 4). Statistic analysis: one-way ANOVA followed by Student–Newman–Keuls post-test ($**P < 0.01$, $***P < 0.001$). Shown is the statistical significance for every condition regarding the control condition (routinely differentiated cells, with MIX–Dexa without AG 879, first striped bar). Note that all but two conditions are significantly different from the control. Differences between MIX–Dexa treated or not, are always statistically different from each other but not shown due to space limitations. Shown is a representative experiment out of three.

Tyrphostin Effects on erbB2 Tyrosine Phosphorylation in 3T3-L1 Cells

As a control of erbB2 inactivation by the inhibitors, we studied its tyrosine phosphorylation after different doses of AG 825 with or without EGF. Tyrphostin AG 825 inhibited EGF stimulated erbB2 tyrosine phosphorylation starting at a concentration of 0.1 μM (Fig. 4A), thus suggesting that AG 825 inhibition on proliferation would be caused by erbB2 inactivation.

Although the differences between the IC$_{50}$ for tyrphostin AG 825 ensure selectivity for erbB2 over EGFR, we also studied EGFR tyrosine phosphorylation in the presence of the inhibitor (data not shown). In this case, AG 825 inhibited EGF stimulated phosphorylation only at higher concentration (1 μM) and with evident lower potency.

MIX–Dexa Induced 3T3-L1 Cells Display Activated MEK, JNK, and p38 Kinases

It has been described that the stimulation with MIX–Dexa triggers several signaling pathways, including that of the mitogen activated protein kinases (MAPKs). Because of the controversy indicating that p42/p44 or ERK1/2 activation block adipogenesis [Font de Mora et al., 1997; Boney et al., 2000] while the opposite is also suggested [Prusty et al., 2002], we decided to analyze the role of MAPKs, JNK (c-jun N-terminal Kinase) and p38 in 3T3-L1 differentiation, by first analyzing their activation after MIX–Dexa treatment. We found that ERK1/2, JNK and p38 are immediately activated after the induction of differentiation with MIX–Dexa (Fig. 5A–C, see 5, 15 and 30 min), with an apparent second peak of activation for ERK1 at day 1 (24 h). Different kinases showed different activation/deactivation patterns: P-ERK1 began to deactivate after 30 min, but ERK2 showed a sustained level of phosphorylation, while P-JNK reached a maximum at 30 min and P-p38 began to deactivate from 5 h on.

MEK and JNK Inhibition Effects on erbB2 Expression in 3T3-L1 Cells

We previously observed that erbB2 expression in 3T3-L1 cells significantly decreased after a few hours of MIX–Dexa treatment (Pagano and Calvo, 2003). Since we also found ERK1/2, JNK and p38 activation after this induction, we
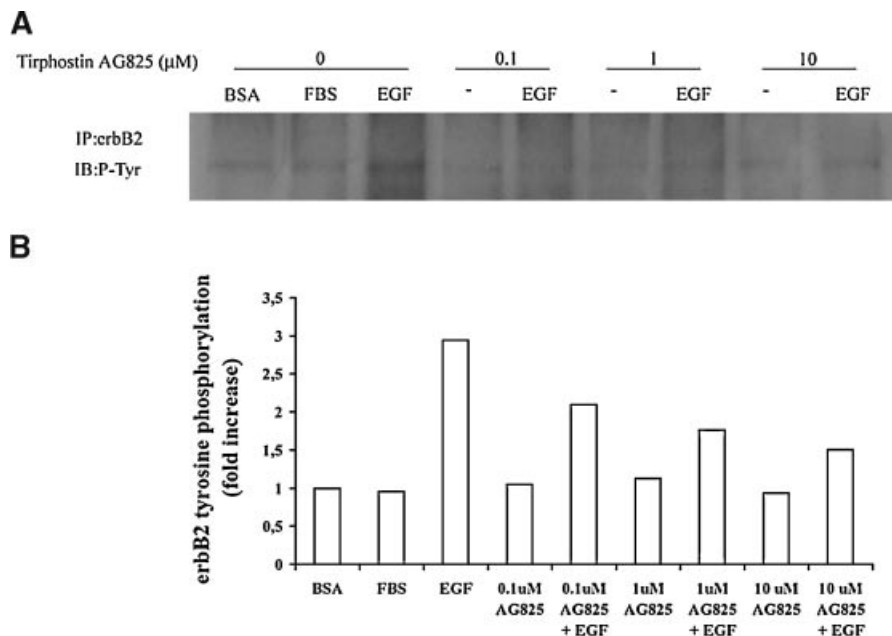
Fig. 4. Effects of tyrphostin AG 825 on erbB2 tyrosine phosphorylation. Proliferating 3T3-L1 cells were serum deprived for 18 h and then treated with or without EGF in the presence of different concentrations of tyrphostin AG 825. **A**: erbB2 was immunoprecipitated (500 μg/condition) with a specific antibody and its tyrosine phosphorylation determined by Western Blot, and (**B**), densitometric analysis was performed. Band intensities were quantified using the Scion Image software (NIH Image for Windows). Values are expressed as fold increase in tyrosine phosphorylation (arbitrary units) over the control (BSA), previously referred to the total protein (erbB2 immunoblot) value. Shown is an experiment out of two.
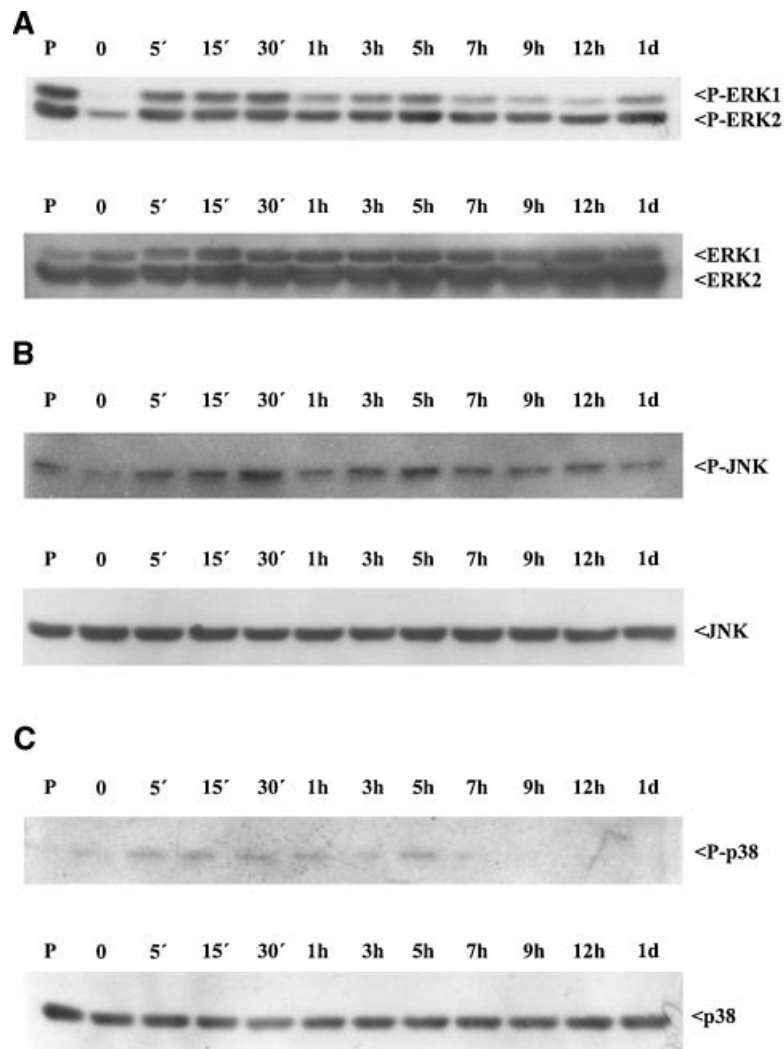
Fig. 5. ERK1/2, JNK, and p38 activation after MIX-dexamethasone induction of differentiation. Post-confluent (growth arrested) 3T3-L1 cells were routinely stimulated with MIX−Dexa and samples were taken at different times after induction (**P** stands for ''cells in the proliferation state''). Kinase activation was determined in protein extracts by WB (60 μg/well): (**A**) phospho-ERK1/2 (P-ERK1/2); (**B**) phospho-JNK (P-JNK); (**C**) phospho-p38 (P-p38). Shown are the WB for total ERK1/2, JNK, and p38 as a control of kinases expression level and/or equal loading (**lower panels**). The experiment shown here is representative of three such experiments.

decided to evaluate the effects of MEK and JNK inhibition on erbB2 expression after MIX−Dexa induction. We observed that treatment with PD98059 or SP600125, both inhibitors of MEK and JNK respectively, for 1 h prior to the MIX−Dexa induction produced an even greater decrease in erbB2 expression after 6 h (Fig. 6), when compared to MIX−Dexa alone.

DISCUSSION

To further understand potential functions of the erbB family of tirosine kinase receptors, in particular those of erbB2, during adipocytic differentiation, we used the 3T3-L1 murine preadipocyte cell line. Heterodimer formation has demonstrated to be important in many cellular processes in different cell types and tissues, such as mammary gland epithelial cells [Schroeder and Lee, 1998; Darcy et al., 1999, 2000], neural progenitors migration in neural crest and trabeculae formation in the heart [Lee et al., 1995; Britsch et al., 1998], showing unique emerging properties that differ from those of homodimers [Olayioye et al., 2000]. Since erbB2 can heterodimerize with other erbB family members upon binding of their respective ligands [Graus-Porta et al., 1997] and we have
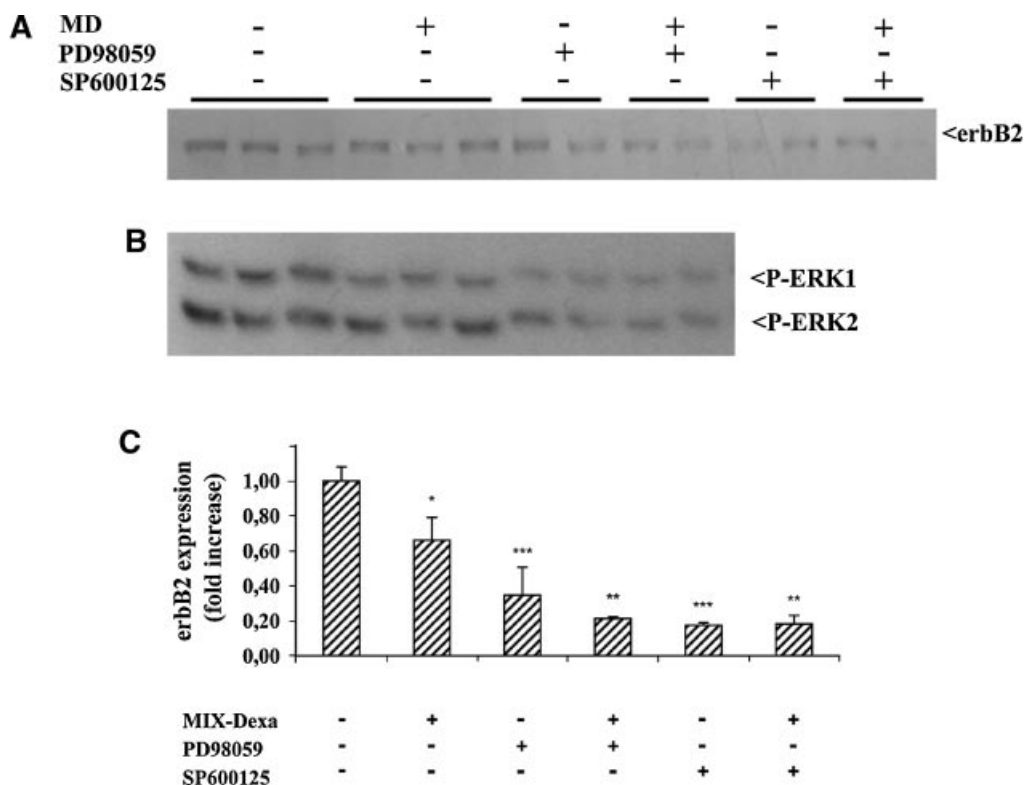
Fig. 6. MEK and JNK inhibition effects on erbB2 expression in 3T3-L1 cells. Post-confluent (growth arrested) 3T3-L1 cells grown in 6-well dishes were treated with the MEK inhibitor (PD98059) or the JNK inhibitor (SP600125). One hour later cells were routinely induced to differentiate with MIX–Dexa and after 6 more hours the experiment was ended by protein extraction. WB determination (40 µg/well): (**A**) erbB2 expression; (**B**) kinase inhibition (P-ERK1/2); (**C**) densitometry. Conducted experiments included carrier controls (DMSO, not shown). Band intensities were quantified using the Scion Image software (NIH Image for Windows) and data were subjected to statistical analysis by one-way ANOVA, followed by the Tukey post-test. Values are expressed as fold increase in erbB2 expression (arbitrary units) over the control at 6 h without MD and without inhibitors, and represent the mean $\pm$ SEM (n = 2). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. For statistical analysis, each condition was referred to the respective control, MD− or MD+ (inhibitors treated samples without MD are referred to the MD− control—first bar—while inhibitors treated samples with MD are referred to MD+ control—second bar). Shown is an experiment out of two.

previously demonstrated erbB2 and EGFR expression and modulation during 3T3-L1 adipose differentiation [Pagano and Calvo, 2003] it was mandatory to find out whether EGF was able to promote erbB2-EGFR heterodimerization. Here, we demonstrate erbB2-EGFR dimer formation in 3T3-L1 cells in response to EGF.

We also studied other heterodimers possibilities, such as heregulin mediated erbB2-erbB3 or erbB2-erbB4 complexes (data not shown). In a previous study, we showed that 3T3-L1 cells express HRG/NRG1 endogenously, and that erbB2 does not phosphorylate in tyrosine upon HRG addition [Pagano and Calvo, 2003]. Although we also established that we could not find typical erbB3 and erbB4 isoforms, the true NRG1 receptors, we detected different specific bands through Western blot possibly corresponding to already described smaller isoforms

that could function as co-receptors for erbB2 and/or EGFR, avoiding further tyrosine phosphorylation after exogenously added NRG1, if the cells were already auto-stimulated. In order to demonstrate this hypothesis, we stimulated 3T3-L1 cells with NRG1. Nor the cells increased their proliferation neither erbB2 and/or EGFR showed any increase in P-tyr. Supporting the idea of a possible erbB2/erbB3 heterodimer, we are able to demonstrate expression of mRNA for erbB3 in 3T3-L1 cells by RT-PCR (E Pagano & JC Calvo, unpublished work, manuscript in preparation).

Although one may wonder why FBS has not a stronger effect on the phosphorylation status of EGFR and/or erbB2, as it is normally used to propagate the cells and also should contain EGF, we have previously established that the EGF- induced increase in erbB2 tyrosine

phosphorylation is attenuated in the presence of serum and that serum itself produces a lower degree of tyrosine phosphorylated receptor than the control condition (BSA) [Pagano and Calvo, 2003], possibly due to the presence of other factors. Growth hormone, for example, has been described to decrease tirosine phosphorylation of erbB2 in 3T3-F422A fibroblasts [Kim et al., 1999].

By reducing erbB2 activity with specific pharmacological inhibitors, the tyrphostins AG 825 and AG 879, we demonstrated that erbB2 is partially responsible for 3T3-L1 proliferation and that the lack of its activity during adipogenic induction favored differentiation (Figs. 2 and 3). Since both tyrphostins are selective for erbB2 over EGFR, being AG 825 more potent, we evaluated receptor tyrosine phosphorylation as a consequence of EGF stimulation in the presence or absence of the inhibitor (Fig. 4).

Given that the IC_{50} for EGFR is 19 μM, it would be possible to think that EGFR phosphorylation inhibition accounts for erbB2 inactivation, since this effect has already been described for another tyrphostin, AG 1478, which by specifically inhibiting EGFR also inhibits erbB2 activation and heterodimer formation [Lenferink et al., 2001]. For that reason, we chose to also use AG 879, a more selective erbB2 inhibitor than AG 825, which caused inhibition of 3T3-L1 cells proliferation but it reached a maximal inhibition of 20 % at a 10 times lower dose (Fig. 2B). Considering that by using AG 825 it is conceivable a mixed inhibition of erbB2 plus EGFR, especially at the higher doses, the high specifity of AG 879 leaves little doubt about the tyrphostins target. It is possible, then, that 3T3-L1 proliferation is mediated by both receptors, erbB2 and EGFR, in addition to the very well documented IGF-1R mitogenic activity in this system [Smith et al., 1988; Boney et al., 1994, 1998, 2000, 2001].

Since we found a physiological role for erbB2 in 3T3-L1 cells proliferation it was tempting to think that it could be involved in their differentiation too. AG 879 treatment prior to MIX−Dexa induction demonstrated that a partial erbB2 inactivation increases the differentiation rate but it is not enough to induce it by itself (Fig. 3). This result was to be expected if we consider that different signals are required to promote the expression of essential transcription factors for adipogenesis to proceed [revised by Mandrup and Lane, 1997; Lowell, 1999]. Simple blockade of mitogenic signals could not be enough since the cells would be, in the first place, devoided of PPARγ activation.

Supporting the idea that an excessive erbB2 inhibition could result deleterious for the differentiation process, data from the MAPK and JNK inhibitors experiment (Fig. 6) suggest that inhibition of the start differentiation signal (at the stage of Mix−Dexa treatment) also produces a decrease in erbB2, at the expression level, and eventually, at its activity. It seems that erbB2 and differentiation are connected, in a very delicate balance so that if erbB2 signaling is too dampened so it is differentiation.

MEK/ERK signaling pathway participation in adipogenesis regulation has been a matter of discussion, with some authors showing that mitogen-stimulated ERK attenuates adipogenic gene expression [Font de Mora et al., 1997], while others demonstrated that MEK1 activation, restricted to the first minutes after adipogenic induction, promotes differentiation facilitating the expression of main transcription factors [Prusty et al., 2002]. This indicates that the effect could be either positive or negative depending upon the exact moment of MEK activation during the whole process. In this work, we show that ERK1 activation after 5 min of adipogenic induction in 3T3-L1 cells is clearly stronger than that of ERK2, but the latter seems to remain active during the first 24 h post induction, while ERK1 begins to diminish its activity an hour after stimulation, descending even more after 7 h of treatment. Apparent second peak of ERK1 activation was observed at 24 h, which is generally associated with initiation of differentiation [Nguyen et al., 1993]. Other authors described this peak as early as 12 h of treatment, but they included insulin in its differentiation cocktail, arguing that ERK1 activation is caused by MIX and insulin action [Prusty et al., 2002]. It is obvious that there must be other factors involved in ERK1 activation since we do not use insulin until 3 days after MIX−DEXA treatment and ERK1 is equally activated.

We previously demonstrated that erbB2 expression diminished after MIX−DEXA treatment in 3T3-L1 cells [Pagano and Calvo, 2003]. We looked for ERK and JNK signaling in this process and we found that PD98059 or SP600125 treatments (starting 1 h prior to MIX−DEXA induction), specific inhibitors of

ERK and JNK respectively, produced an even larger decrease in erbB2 expression, as compared to control (Fig. 6). This result indicates that ERK and/or JNK signaling inhibition or dampening would favor erbB2-decreased expression, and that these signaling pathways may be delaying it.

In this work we demonstrated that JNK is activated immediately after adipogenic induction (Fig. 5B) with a maximal activation 30 min later, after which, it decreases. There is no other previous work in this regard since JNK activation is frequently related to the insulin resistance in adipocytes rather than to the normal cellular outputs of proliferation and differentiation. Here we present that the specific inhibition of JNK produced a greater decrease in erbB2 expression after 6 h of MIX–Dexa treatment (Fig. 6). The reasons for this phenomenon are not clear and so it remains to be elucidated the potential role of JNK after hormonal induction and which signals it triggers that affect erbB2 expression in 3T3-L1 adipocytes.

In this study we demonstrate that p38, as well as ERK and JNK, activate after 5 min of adipogenic induction, continuing for the next 5 h (Fig. 5C). Similar results are reported by Engelman et al. [1998], where they verify that p38 activity is high in the initial steps of differentiation but it dramatically decreases as soon as fibroblasts differentiate into adipocytes.

All the data presented in this manuscript support EGFR, erbB2, ERK, JNK, and p38 signaling as key factors during the adipogenic differentiation that are worth pursuing. Of outstanding interest are the relationships between the results presented herein and modulation of PPARγ activity through natural or pharmacological ligands currently used to treat diabetes and fatty acid synthetase regulation by erbB2 and vice versa [Menendez et al., 2004]. A deep knowledge of the mechanisms that underlie adipogenesis initiation and conclusion is required in order to, eventually, defeat obesity and its related pathologies.

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