Expression of erythroblastic leukemia viral oncogene homolog (erbB) mRNAs and possible splice variants in 3T3-L1 preadipocytes

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Received February 3, 2011; Accepted June 3, 2011

DOI: 10.3892/mmr.2011.517

Abstract. Previously, we studied the erythroblastic leukemia viral oncogene homolog (erbB) family of tyrosine kinase growth factor receptors in terms of protein expression, modulation and activation in the 3T3-L1 cell line. In the present study, the presence of full-length erbB mRNAs, and splice or proteolytic erbB variants, was evaluated using RT-PCR. Epidermal growth factor receptor (EGFR)/erbB1 expression was confirmed. Wild-type (wt) ErbB2, human erbB2 (HER2)-extracellular domain (ECD) and herstatin mRNA expression were analyzed. Restriction analysis confirmed wt expression. 3T3-L1 cells exhibited HER2-ECD and herstatin mRNA expression, although at extremely low levels (compared to the control cell lines). ErbB3 cDNA was amplified using mouse mammary tumors and rat acines as positive controls. ErbB4 was not positively identified in these cells. In conclusion, this study demonstrates that 3T3-L1 cells express EGFR/erbB1, erbB2 and erbB3, and possibly, certain erbB2 splice or proteolytic variants, which are worth pursuing.

Introduction

The erythroblastic leukemia viral oncogene homolog (erbB) family of tyrosine kinase growth factor receptors consists of transmembrane proteins that dimerize in order to transduce their signal. This homolog includes the epidermal growth factor receptors (EGFRs) erbB1, erbB2, erbB3 and erbB4. These receptors share a similar structure and a high degree of homology (1), i.e. 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, with erbB2 as the preferred signaling partner (2). Signaling from heterodimers usually presents distinct features compared to the corresponding homodimers as a way of diversifying the signal (3). It was demonstrated that the role of erbB in cell proliferation, differentiation and survival is of extreme significance (4,5). ErbB2 overexpression and amplification were observed in a variety of human epithelial tumors and were correlated with an unfavourable prognosis in patients with breast and ovarian cancer (6). Heterodimeric receptor combinations containing erbB2, particularly erbB2-erbB3, exhibit superior signal-transducing and cell-growth stimulating capabilities (7-9). The evidence indicates that erbB2 confers an autocrine growth advantage to tumor cells.

Various growth factor receptors (VEGFR; erbB1, erbB2 or erbB3) (10-12), cytokine receptors (IL-1\beta and IL-2) (13) and even ion channels (CFTR) (14) have soluble forms capable of ligand binding, frequently detected in cultured tumor cells, conditioned media and in biological fluids, including blood and urine. These soluble forms are produced by limited proteolysis of full membrane receptors (15-18) or by alternative splicing (10,11,19). Their transcripts code for proteins that retain all, a part or nothing from the transmembrane or cytoplasmic domains. Their widespread presence indicates that these soluble forms may have important physiological functions, since the relative abundance of these mRNAs usually changes with the developmental stage of morphogenesis. Given that 3T3-L1 cells differentiate from preadipocytes (fibroblasts) to adipocytes, a mechanism of this type is to be expected. The occurrence of these receptor isoforms in 3T3-L1 cells may be of interest since they have not been previously described in adipocytes and their presence may explain the modulation that erbB2 and EGFR exhibit at the protein level during and after the differentiation induction phase (20).
Previously, we studied erbB1/EGFR, erbB2, erbB3 and erbB4 as well as ligands, heregulin and EGF, in terms of expression, activation and modulation during the adipogenic process in 3T3-L1 cells (20,21). In this study, the presence of full-length erbB mRNAs and, of splice or proteolytic variants were examined using RT-PCR.

Materials and methods

Cell culture. Swiss 3T3-L1 preadipocytes (fibroblasts, embryo, mouse: ATCC CCL 92.1), purchased from the Asociación Banco Argentino de Células, were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone) with 4 mM L-glutamine, 4.5 g/l glucose, 1x10⁻⁴ M biotin and 0.11 g/l sodium pyruvate, and supplemented with 10% fetal bovine serum (FBS) (Gen SA, Bs, As.) plus antibiotics (Gibco, Life Technologies).

RNA extraction, RT-PCR and primer sets. Total cellular RNA was isolated by the Chomczynski and Sacchi method (22). Briefly, 1x10⁵ 3T3-L1 cells were harvested during the proliferative phase. Cell monolayers were washed twice with Ca²⁺ and Mg²⁺ free-cold phosphate buffered saline (PBS), and cells were collected with a rubber policeman in 1 ml/dish of D Solution [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% w/v sarkosyl, 0.1 M β-mercaptoethanol], finishing lysis by passing cells 5 times using a 21G needle. Considering a total final volume of 10 ml, 1 ml 2 M sodium acetate (pH 4), 10 ml water-saturated phenol (pH 5) and 2 ml 49:1 chloroform/isoamyl alcohol were added, mixing thoroughly each time, with vortex agitation twice for 30 sec with an intermediate rest time of 1 min on ice. After 15 min of incubation on ice, the suspension was centrifuged at 10,000 rpm at 4°C for 20 min and the aequous phase was transferred to a fresh tube and mixed with an equal volume of isopropyl alcohol. Samples were maintained for at least 3 h at -20°C. After another centrifugation at 10,000 rpm at 4°C for 20 min, the supernatant was discarded and the RNA pellet was dissolved in 2 ml D Solution. RNA was again precipitated with 1 ml isopropyl alcohol and maintained at -20°C for at least 3 h. Following centrifugation at 10,000 rpm at 4°C for 20 min, the supernatant was discarded and the RNA pellet was washed with 70% ethanol, air-dried and resuspended in 100 μl RNAse-free water. RNA purity and concentration were evaluated spectrophotometrically (ADN/ARN absorbance, 1.8) and visually (in 1% agarose gels, stained with ethidium bromide, presence of 2 bands corresponding to 18S and 28S).

RT-PCR. The Promega Corp. System and a Mastercycler gradient thermocycler (Eppendorf, Hauppauge, NY, USA) were used to transcribe and amplify RNA. Briefly, RNA was incubated for 10 min at 70°C to destabilize secondary structures and after a brief centrifugation it was rapidly cooled on ice. cDNA was obtained through reverse transcription (RT) in the following mix reaction: 5 mM MgCl₂, buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% v/v Triton X-100], 1 mM dNTP, 15 U/μg Taq DNA polymerase and 0.5 μM (5’ and 3’) specific primers were used.

Samples were subjected to a single pre-denaturing cycle at 94°C for 5 min, followed by 30 cycles, each with a denaturing step at 94°C for 30 sec, annealing at a specific temperature for each set of primers for 1 min and elongation at 72°C for 2 min, ending with a final elongation at 72°C for 7 min and polymerase inactivation at 4°C.

Primers. All sets of primers, with the exception of those for EGFR/erbB1 (Genosys, Canada), were acquired from Tecnolab SA, Argentina (for Operon, a Qiagen company) and desalted in a 50-nM scale (Table I).

After amplification, specific fragments were separated and visualized in 1% agarose (Seakem ME) gels in buffer TBE [0.9 M Tris, 0.9 M boric acid and 20 mM EDTA (pH 7.8)] stained with ethidium bromide, at 70 V for 30 min.

Restriction enzyme analysis. PCR product specificity was analyzed by endonuclease restriction, with enzymes that cleave DNA inside specific sequences, generally palindromes. Basically, we searched for enzymes restriction sites in the amplified segment sequence and then, which one of them cut only once and finally, between these ones, which ones produce segments of a proper size to be observed in an agarose gel. Enzymatic digestion was performed using the reaction mixture: 5 μl PCR product, 0.5 μl endonuclease, 1 μl buffer and 3.5 μl water. The reaction was incubated at 37°C for 3 h and stopped with loading buffer [0.03% bromophenol blue, 0.03% cyline cyanol, 0.4% orange G, 15% Ficoll 400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA]. The PCR products digested in this manner were analyzed together with the undigested fragments in agarose gels to visualize the reaction and check for cleavage efficiency according to the expected length (pb).

Rsal. Pseudomonas sphaeroides (Biolabs). 5’…GTCA…3’, 3’…CAATG…5’; Eco RI 5’…GATAATTC…3’, 3’…CTTAAAG…5’.

Results

erbB2 mRNA expression in 3T3-L1 cells. ErbB2 expression in a variety of human cancers, such as breast, ovary and lung cancer, has been extensively documented in terms of overexpression and amplification. However, erbB2 expression or the presence of ErbB in general have not been frequently described in fibroblasts, with the exception of decidual cells (23,24), mammary gland fibroblasts (25) and the Rat-1 cell line (26). Therefore, erbB expression was evaluated in the 3T3-L1 model of adipogenic differentiation.

Of note is that when these experiments were first started, the complete murine erbB2 sequence was unknown (at present, the complete v-erbB2 murine sequence BC046811 is known to present an 83-87% homology with the human c-erbB2). Only a partial sequence was available (2,083 bp)
corresponding to the 3' end of human c-erbB2 (4,473 bp; 87% homology). The primers were designed, based on this sequence, to detect erbB2 mRNA expression in 3T3-L1 cells through RT-PCR. This erbB2 mRNA portion has the lowest homology with EGFR (<20%). The primers included wobble (X/Y) positions to increase possibilities of amplifying the erbB2 sequence in mouse. This set of primers provided an RT-PCR product of ~300 bp (Fig. 1A), which was confirmed as erbB2 by Rsa cleavage (328 bp = 237 + 91 bp).

Additionally, we amplified a larger segment using this forward primer designed against the human sequence (10), whereas the primer that had previously been designed against the mouse sequence was used as the reverse primer. This combination of primers yielded a segment of ~1,900 bp (Fig. 1B), confirmed as erbB2 by EcoRI cleavage (1,917 bp = 1,269 + 648 bp).

To the best of our knowledge, this is the first observation of erbB2 mRNA expression in the 3T3-L1 cell line in the literature.

### Table I. List of PCR primers used.

<table>
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<tr>
<th>Receptor</th>
<th>Genbank accession no.</th>
<th>Primer Sequence (5' to 3')</th>
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<td>erbB2</td>
<td>Human (31997) X03363</td>
<td>F: CT GGC TCC GAT GT(A/G) TTT 3GAT GG</td>
</tr>
<tr>
<td></td>
<td>Mouse (200711980) AK031099</td>
<td>R: (A/G)GC ACC CCC AAA GGC AAA AAC G</td>
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<tr>
<td></td>
<td>Human 3,723→3,744</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse 1,343→1,364</td>
<td></td>
</tr>
<tr>
<td>HER2-ECD</td>
<td>Mouse (200711980) AK031099</td>
<td>F: AGG GAG TAT GTG A(A/G)(G/T) G(C/G)G</td>
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<tr>
<td></td>
<td>Mouse 1,343→1,364</td>
<td>R: CCT GAA AGA AAG TCC TCC</td>
</tr>
<tr>
<td>Herstatin</td>
<td>Genbank (10181232) AF177761</td>
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<td></td>
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<td>R: TC CGG CA(A/G) AAA TGC CAG GCT CC</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td>EGFR</td>
<td>Mouse (488830) X78987</td>
<td>F: CC AAA TGT GAT CCA AGC TGT CC</td>
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<td>R: TCC GAG GAG CAT AAA GGA TTG</td>
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<td></td>
<td>Mouse 1,343→1,364</td>
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<tr>
<td>erbB3</td>
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<td>F: CTG GGC GTG TCT ACA TAA GTG C</td>
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<td>R: A TGG CAG GAG AAG CAA TGA GCC</td>
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<td>erbB4</td>
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<td>Mouse (23622469) XM_136679</td>
<td>R: AAA CAT CTC AGC CGT TGC A</td>
</tr>
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<td>Mouse 1,343→1,364</td>
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![Figure 1](image_url)
PAGANO et al: ErbB mRNAs IN PREADIPOCYTES

958

erbB2 splicing variants in 3T3-L1 cells

HER2-ECD expression in 3T3-L1 cells. We evaluated the possibility that 3T3-L1 cells express an erbB2 variant that provides a truncated protein of 100 kDa, the human erbB2-extracellular domain (HER2-ECD), which has an antiproliferative activity in tumor cells with a tendency to disappear in more advanced or aggressive tumor phenotypes. This splicing variant is derived from a 2.3 kb-mRNA, almost half the wild-type length, which retains the four extracellular subdomains, but loses the transmembrane and the cytoplasmic domains (from the 633 aa it embraces, the last 25 from the 3' end differ from the wild-type).

Another set of primers (Table I) (10) was used to identify this alternative splice variant. As shown in Fig. 2A a specific fragment was positively amplified with the expected size for HER2-ECD in 3T3-L1 cells, by the lack of any other bands. HER2-ECD expression was low in comparison with that shown by breast carcinoma.

Herstatin expression in 3T3-L1 cells. Herstatin (AF177761) is a 68-kDa protein described as an erbB2 auto-inhibitor, which, upon binding to the receptor itself, interferes with dimer formation and reduces receptor tyrosine phosphorylation (19). It is produced by an alternative truncated mRNA whose protein contains the first 340 aa of the erbB2 protein (ECD subdomains I and II) fused to a unique stretch of 79 aa that arises from the insertion of an intronic sequence.

Using the primers previously described (Materials and methods and Table I), we obtained an RT-PCR product of the expected length (1,143 bp) positively amplified in 3T3-L1 cells as in epithelial cells from murine breast cancer (Fig. 2B), although it appears evident that the expression of each one is relatively low.

As for HER2-ECD, the meaning or function of a low herstatin expression in 3T3-L1 cells, as in any other normal cells, would require more attention. It is possible that the expression of these erbB2 variants as alternative mechanisms to control cell proliferation and/or erbB2 activity are not required under physiological circumstances, although they are ready to be activated in case of need.

EGFR/erbB1 expression in 3T3-L1 cells. The proliferative dependence of 3T3-L1 cells on EGF presence in the culture medium was described at the beginning of this model characterization (27), whereas its receptor expression was described at the protein level (28). EGFR expression in adipocytes was demonstrated in in vivo studies (29) as well as in 3T3-L1 cell line cultures (28,30,31). In this study, EGFR expression was confirmed by RT-PCR in our 3T3-L1 cell system using specific primers designed in our lab from the complete murine sequence (gi: 488830, X78987) (Fig. 3).

erbB3 expression in 3T3-L1 cells. A set of primers matching the 5' end of this cDNA was designed from the 4,524 bp erbB3 sequence found in the NCBI database (gi: 25071684, XM_125954.4). These primers amplified several fragments by RT-PCR in 3T3-L1 cells, and one of these fragments had the expected size (308 bp) for erbB3. cDNA samples from a murine breast cancer and rat normal mammary acines were used as positive controls (Fig. 4A). The identity of erbB3 was confirmed by Rsai cleavage (Fig. 4B), which yielded two fragments of 104 and 204 bp. This is the first demonstration of erbB3 expression in preadipocytes in vivo as well as in vitro.

erbB4 expression in 3T3-L1 cells. Since the murine erbB4 sequence was incomplete (gi: 23622469, XM_136679), a combination of primers designed against the rat sequence were used (32) (gi: 4176734, AF041838). Homology of 95% was observed between the rat 4,060 bp and the mouse 1,284 bp.

Figure 2. erbB2 splicing variant expression in 3T3-L1 cells. (A) Using oligonucleotides designed to amplify a human truncated version of erbB2 (lacking cytoplasmic domain), HER2-ECD expression in 3T3-L1 cells was obtained (expected product size, 290 bp). It is evident that its expression is lower than that of a murine breast carcinoma (CaMa). It was used as the molecular weight marker (MW) Low DNA Mass Ladder. Subsequently, an evaluation of the mass bands was performed. While the full PCR reaction volume was loaded in two wells for 3T3-L1 cells, only half was loaded for CaMa (approximate ratio 3T3-L1/CaMa, 1/4). (B) Herstatin, another erbB2 soluble truncated variant, was amplified from the 3T3-L1 cell cDNA. Breast cancer cells (CaMa) as well as 3T3-L1 cells exhibit a very low fragment expression (1,143 bp).

Figure 3. EGFR expression in 3T3-L1 cells. Total RNA from 3T3-L1 cells underwent RT-PCR for EGFR mRNA. The product has the expected size (975 bp). Molecular weight markers (MW) Low DNA Mass Ladder.

Figure 4. erbB3 expression in 3T3-L1 cells. (A) erbB3 expression was confirmed by RT-PCR in our 3T3-L1 cell system using specific primers designed in our lab from the complete murine sequence (gi: 488830, X78987) (Fig. 3). (B) Rsai cleavage (Fig. 4B), which yielded two fragments of 104 and 204 bp. This is the first demonstration of erbB3 expression in preadipocytes in vivo as well as in vitro.
Homology was absolute (100%) between the primer binding regions. On the other hand, inside the region to be amplified there are only seven mismatches and none in the binding sites of the primers. Although we were able to amplify a specific fragment of the expected size (206 bp) in rat mammary acines by RT-PCR due to the satisfactory RsaI digestion (111 + 95 bp), none of the 3T3-L1 bands were erbB4 (Fig. 5).

Discussion

Since our concern was to evaluate the expression of not only the wild-type but also other mRNA variants of erbB2 in 3T3-L1 cells, we designed a set of primers and adapted and modified other primer sets obtained from the literature to achieve this target, based on a mouse sequence of only 2,083 bp with an 87% homology with its human counterpart (Fig. 1A). Primers recognized identical cDNA regions in both species with one base exception, allowing us to prepare wobble oligomers, which carry the murine or human base in that position in equal parts. In this manner, segment amplification was ensured despite this minor discrepancy. The amplified sequence was shown to match erbB2. Additionally, we were able to amplify a larger fragment using a 5' human (10) and a 3' murine primer that were previously used (Fig. 1B). In each case, enzymatic digestion allowed us to obtain a positive match with erbB2 mRNA, since cleavage sites remained intact in the two species. This result shows the high degree of homology that murine and human erbB2 share.

HER2-ECD has been described as one of these splicing variants (11). HER2-ECD is expressed in numerous tumor cell lines that also express erbB2/HER2, and its relative abundance is variable. In gastric cancer cells (MKN7) it is higher than, in decreasing order, ovarian (SK-OV-3) and breast cancer cells (BT-474 y SK-BR-3). HER2-ECD has also been observed in the sera of HER2-carrying tumor patients (33), in ganglia micrometastasis, in bone marrow (34) and in conditioned media from cell lines mostly produced by limited proteolysis as opposed to alternative splicing. However, HER2-ECD remained in the cytoplasm in the latter case. HER2-ECD function in tumor cells is not currently well understood. The role of HER2-ECD mRNA loss in advanced
gastric tumors indicates a potential role for erbB2 truncated forms in controlling tumor proliferation in humans. It is also likely that HER2-ECD function depends on the tumoral type under study, since high levels in the sera of oncologic patients inversely correlate with chemotherapy results (33). Since this variant is devoid of the kinase domain and is therefore unable to transduce intracellular signals, some authors have proposed that it may aggregate with complete receptors of the erbB family to modulate their activity (34), in a similar fashion to the inactivation that follows normal and truncated EGFR interaction (35,36). Although it was possible to amplify HER2-ECD in 3T3-L1 cells with specific primers (Fig. 2A), its expression was found to be lower than that of a breast carcinoma. Despite this, it is possible that future studies may demonstrate the existence of erbB2 variants in mice and their possible function in adipogenesis, whether similar or not to their human counterparts, since no previous studies are currently available on this subject.

Herstatin, another erbB2 splicing variant, is produced after a truncated mRNA (2.6 kb) that codifies for the first two extracellular subdomains (19). Herstatin is present in human fetal kidney and liver and in the non-tumorigenic cell lines ISEOVAR (epithelial ovarian cells) and HBL-100 (mammary cells), while its expression is lower in cells where erbB2 is amplified, such as BT474 or SKOV-3 (19). In this study, it was possible to obtain the expected size fragment in 3T3-L1 cells using primers described by Doherty et al (19), although its expression was found to be extremely low, not only in these cells, but also in breast carcinoma ones (Fig. 2B). Therefore, more studies are required to assess the expression of erbB2 variants in 3T3-L1 cells and any connection that may exist with adipogenic differentiation.

ErbB3 is another member of this family of receptors, occasionally referred to as ‘the dumb’, as well as erbB2, ‘the deaf’. This is due to the fact that erbB3 has a deficient kinase that prevents it from transducing an intracellular signal, and because erbB2 has no known ligand to date; hence, it is unable to ‘listen’ (37). For these reasons, the receptors are compelled to dimerize with a different dimerization partner, unless in case of a receptor overexpression. The erbB2-erbB3 heterodimer is able to transduce one of the more potent mitogenic signals in this family and to cause neoplastic transformation (38). Therefore, it was crucial for our differentiation model to test erbB3 mRNA existence in 3T3-L1 cells. erbB3 also has different isoforms, of which p85-serB3 (soluble) is capable of inhibiting hereregulin-mediated erbB2, erbB3 and erbB4 activation (39,40). However, in this study, only the wild-type receptor was investigated [erbB3 expression in 3T3-L1 cells at the protein level, previously published (20)]. Based on the erbB3 murine sequence, we designed a pair of primers corresponding to mRNA 5' end that amplified a fragment of the expected size (308 pb) plus two more fragments of variable size (Fig. 4A). A murine breast carcinoma and normal rat mammary acines were used as a positive control, since they present high erbB3 expression (25,41). The fragment was shown to be specific by restriction analysis (Fig. 4B). The observation of erbB3 expression in 3T3-L1 or adipocytic cells for the first time in the literature indicates a possibility for erbB3 function inside the adipogenic process.

ErbB4 is the last member of this family to be described. It mediates neuregulins and other EGF-related growth factor responses. It has a fundamental role in the regulation of cardiac, neural and mammary development and, as with erbB2, it is also related to neoplastic transformation and cardiac disorders. Chemotaxis and cellular survival are regulated by the expression of erbB4 isoforms that differ in their ability to couple PI3-K signaling (42). Four isoforms have been identified as structurally and functionally different from erbB4, which are sensitive or resistant to the proteolytic cleavage that releases a soluble extracellular domain and which are able or unable to activate MAPK or PI3-K/Akt pathways, explaining the different cellular outputs transduced through each of them (43).

ErbB4 expression has not previously been investigated before in this model of adipogenic differentiation. The murine sequence was not complete, resulting in rat sequence-based designed primers being used (32). Homology of 95% was observed between the 4,060 bp rat sequence and the 1,084 bp mouse sequence. Homology was absolute (100%) in the binding region of the primers. erbB4 was positively amplified in rat mammary acines, but none of the bands were revealed to be erbB4 in 3T3-L1 cells (Fig. 5). We have already discussed the presence of receptor isoforms at the protein level in a previous study (20).

Supporting these results, it is notable that the full presence of all members of the erbB family would be redundant in a single cell type, particularly if normal. Usually, a cell expresses one or two erbBs, and no more than three. For example, NIH-3T3 cells lack endogenous detectable levels of EGFR, erbB3 and erbB4 and exhibit only extremely low levels of erbB2 (3), while only EGFR and erbB2 are present in Rat-1 fibroblasts (26). Therefore, the mRNA expression of erbB2, erbB1/EGFR and erbB3 was confirmed in 3T3-L1 cells in this study.

Expression of erbB2 at the protein level in 3T3-L1 cells (20) and its involvement in the proliferation/differentiation processes of these cells were previously observed (21). The present study completes our understanding of the expression, modulation and activation of the erbB receptors in the 3T3-L1 cell line and poses new questions regarding possible roles for erbB soluble forms in this adipogenic model.

Acknowledgements
The authors thank Mr. M. Fourcade for the assistance in the preparation of the figures. This study was partially supported by a grant from ANPCyT (PICT 05-00000-00206) and UBACYT (X031).

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