Transactivation of erbB2 by short and long isoforms of leptin receptors

Avital Eisenberg[a], Eva Biener[a], Madia Charlier[b], R.V. Krishnan[c], Jean Djiane[b], Brian Herman[c], Arieh Gertler[a,*]

[a]*Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel*
[b]*Unite de Neuroendocrinologie Moleculaire de la Prise Alimentaire, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France*
[c]*Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX 78229-3900, USA*

Received 27 February 2004; revised 28 March 2004; accepted 28 March 2004

First published online 9 April 2004

Edited by Veli-Pekka Lehto

Abstract **We generated kinase-positive and kinase-negative erbB2 tagged with YFP and the long form of leptin receptor (LEPRb) tagged with CFP. Both were as active as their untagged analogs. Both short and long isoforms of leptin receptor phosphorylated and thereby activated erbB2 upon leptin binding and enhanced MAPK activity. Our results unveil a novel route by which leptin may provoke erbB2's phosphorylation and thus enhance its oncogenic potential independently of HER family ligands or its overexpression. Using FRET technology in living cells, we found no evidence of complex formation between erbB2 and prolactin or leptin receptors, indicating that the transactivation occurs through an indirect interaction.**
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Keywords: erbB2; Leptin; Prolactin; Receptor; Fluorescence resonance energy transfer

1. Introduction

Identification of obesity as a risk factor for breast and prostate cancer types suggests that adipocyte-derived factors such as leptin, the level of which is known to increase in obesity, may play a role in tumor progression [1–4]. Two main isoforms of leptin receptor, resulting from an alternative splicing, have been identified: the short form (LEPRa) and the long form (LEPRb). Both isoforms have identical extracellular and transmembrane domains but differ in their intracellular domains [5]. Extensive studies have revealed that only LEPRb is capable of activating the Jak2/Stat3 pathway [6]. The ubiquitously expressed LEPRa lacks a Stat3 docking site but is still able to bind and activate Jak2 through box 1 and subsequently activate the MAPK pathway [7]. Both LEPRb and LEPRa are expressed and are functional in breast as well as prostate carcinoma [1,8]. However, the precise mechanism by which leptin mediates oncogenic transformation is not yet understood.

ErbB2, a member of the human epidermal growth factor receptor (HER) family, has been implicated in oncogenic transformation through its overexpression or the formation of long-lived signaling heterodimers with other HER family receptors [9,10]. Exogenous prolactin (PRL), acting through its receptors and activating Jak2 are capable of erbB2 phosphorylation, leads to subsequent activation of MAPK [11]. In the present study, we asked whether LEPR-mediated Jak2 phosphorylation can also lead to phosphorylation and subsequent activation of erbB2. We used fluorescence resonance energy transfer (FRET) technology in living cells to elucidate the putative mechanism of PRLR/erbB2 and LEPR/erbB2 cross-talk.

2. Materials and methods

2.1. Materials

Expression plasmids encoding cyan and yellow fluorescent proteins (pECFP-N1, pEYFP-N1, respectively) were from Clontech Inc. (Palo Alto, CA), the STAT3-responsive pAH32 luciferase-encoding plasmid was from Merck Research Laboratories (Rahway, NJ). Mouse LEPRa, LEPRb and LEPRb-GFP (green fluorescent protein) constructs were a gift from Dr. C. Bjorbaek and erbB2 constructs were from Dr. Y. Yarden. Restriction enzymes were from New England-Biolabs (Saint Quentin en Yvelines, France), monoclonal antibodies (mAbs) against di-phosphor-MAPK (8159) were from Sigma (St. Louis, MO), mAb against GFP and fetal bovine serum were from Boehringer (Manheim, Germany) and mAb anti-phospho-tyrosine (PY20) was from Transduction Laboratories (Lexington, KY). Anti-serum (Ab 220) against recombinant human leptin-binding domain [12] was prepared in our laboratory. ECL-Western blotting detection reagent was from Amersham Biosciences (Buckinghamshire, UK). Recombinant ovine leptin and PRLR-CFP construct were prepared as described previously [13,14]. OPTIMEM was purchased from Gibco-BRL (Bethesda, MD), Fugene 6 from Roche Diagnostics Co. (Indianapolis, IN) and Vectashield mounting medium from Vector Laboratories (Burlingame, CA). Molecular weight markers for SDS–PAGE, Dulbecco's modified Eagle's medium (DMEM) and DMEM-HAM F12 medium were obtained from Life Technologies, Inc. (Rockville, MD) and SDS–PAGE reagents were from Bio-Rad (Hercules, CA). Other materials were from Sigma.

2.2. Preparation of mLEPRb-CFP, erbB2-YFP and kinase-negative (KN) erbB2-YFP

LEPRb cDNA inserted into the pEGFP-N1 was digested with *Age*I and *Bsr*GI, isolated from an agarose gel after removal of a 722 bp insert encoding for GFP, dephosphorylated and ligated into a 722 bp fragment encoding YFP, prepared by digesting the pEYFP-N1 plasmid with the same enzymes, using the Rapid DNA Ligation kit (Roche Molecular Biochemicals, France). The fused plasmid was prepared by transforming DH5α *Escherichia coli* bacteria (Invitrogen, France). Proper exchange of the GFP fragment with the YFP fragment was confirmed by the appearance of a new *Pst*I site (position 888), which exists only in YFP, and not in GFP or CFP. The fragment encoding erbB2 was isolated from the pcDNA3 vector by digestion with *Hin*dIII and *Xba*I. Mutagenesis introduced by PCR was performed on the last 700 bp of erbB2 (generated by cleavage of *Kpn*I) to replace the stop codon by a *Hin*dIII site. Following further cleavage the two fragments were ligated into pEYFP-N1 cleaved by *Xho*I and *Hin*dIII. To create the KN erbB2-YFP expression vector, KN erbB2 was cleaved with

* Corresponding author. Fax: +972-8-936-3208.
E-mail address: gertler@agri.huji.ac.il (A. Gertler).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.03.089

*Kpn*21 and *Sgr*AI to generate the fragment between amino acids 681 and 895 which contains the mutant ATP-binding site. Then, an erbB2-YFP cleaved with the same enzymes and the mutant fragments were ligated generating KN erbB2-YFP.

2.3. Total cell-protein extracts, immunoprecipitation and immunoblotting

HEK 293T cells were cultured as described before [14]. The cells were transiently transfected with 5 μg KN erbB2-YFP, 4 μg Jak2 and 5 μg LEPRa, LEPRb or PRLR. Following an overnight incubation in starvation media, the cells were treated with 400 ng/ml of leptin or PRL or vehicle for 0–10 min (37 °C), washed in cold PBS and lysed [15]. Following 15-min centrifugation at $12\,000 \times g$ (4 °C), 1 mg of total protein lysate was taken for immunoprecipitation (IP) by incubating with anti-GFP antibodies in rotation overnight (4 °C); 50 μl of protein A + G agarose was added for 2 h and the immunoprecipitates were collected by centrifuging for 5 min at $5000 \times g$, washed in lysis buffer (2×), PBS (2×), boiled in reducing sample buffer, separated by 10% SDS–PAGE, immunoblotted with anti-phospho-tyrosine and following stripping reprobed with anti GFP.

2.4. Determination of biological activity of LEPRb-CFP

Chinese hamster ovary (CHO) cells, grown in HAM-F12 medium as described previously [14], were co-transfected with the pCH110, with STAT3-responsive pAH32 luciferase-encoding plasmid, and with LEPRb or LEPRb CFP-encoding plasmid, using ExGen 500 (Euromedex, France) according to the manufacturer's protocol. To test the biological activity, transfected cells were incubated for 24 h with different concentrations of ovine leptin in a serum free medium of (1/1: v/v) Dulbecco's modified Eagle's medium (DMEM) and HAM-F12. The plates were washed with PBS and the enzymatic activity was determined as described previously [16]. The results were expressed as fold induction, after the luciferase activity was corrected for β-galactosidase activity.

2.5. Determination of MAPK activity

HEK 293T cells were transfected with 650 ng LEPRb or LEPRa, 650 ng Jak2 and either 650 ng or no KN erbB2-YFP. After 36 h, the cells were washed with PBS and transferred to starvation media for 16 h. Then, the cells were treated with 400 ng/ml ovine leptin or vehicle for either 0, 2.5, 5 or 10 min (37 °C) and lysed with 150 μl lysis buffer [15]. Following centrifugation for 15 min at $12\,000 \times g$ (4 °C), 75 μg of the lysate's protein was separated by SDS–PAGE and transferred to nitrocellulose. Activated MAPK was detected using anti-di-P MAPK antibodies.

2.6. Fluorescence resonance energy transfer microscopy

FRET microscopy is an optical assay to detect protein interactions in the range of a few nanometers in individual cells [17]. Molecular proximity between the two fluorophores, LEPRb-CFP (donor) and erbB2-YFP (acceptor), can be detected by exploiting one of the different manifestations of energy transfer between these fluorescent species [18]. In the present case, we employed the acceptor photobleaching approach, which involves measuring the changes in donor emission intensity before and after photobleaching (depleting) the acceptor population [19]. Data acquisition and analysis were performed in a Zeiss LSM510 laser-scanning microscope as described previously [20].

For transient transfections, HEK 293T cells were grown on coverslips in a 24-well culture dish (0.7×10^5 cells/well, 1 ml/well). Each well was transfected with 100 ng of erbB2-YFP, 150 ng Jak2 and 400 ng LEPRb-CFP or 50 ng PRLR-CFP using Fugene 6 according to the manufacturer's directions. Forty hours after transfection, the cells were washed with PBS and serum-free DMEM was added for 5 h. Then, 400 ng of the respective hormone (diluted in 0.1% BSA in PBS) was added to each well for different periods of incubation. Cells were then washed, fixed with 4% paraformaldehyde and mounted using Vectashield mounting medium.

3. Results

3.1. Expression of YFP-tagged erbB2 and CFP-tagged short and long leptin receptors

Expression of YFP-tagged erbB2 and KN erbB2 in HEK 293T cells was visualized by fluorescence microscopy (Figs. 1 and 4A); the localization of the expressed fusion protein is confined to cell membranes. Expression of CFP-tagged LEPRa and LEPRb in transiently transfected HEK 293T cells was verified by IP with Ab 220, followed by immunoblotting with anti-GFP Ab, and the expected bands were revealed with the respective moleculer masses of 205 and 125 kDa (not shown). The presence of LEPRb and LEPRa was also validated by binding of ^{125}I ovine leptin, yielding a respective specific binding of 55 ± 7 and 86 ± 5 fmol (mean ± S.E.M., $n = 3$) ^{125}I ovine leptin/well containing an average of $\sim 10^6$ cells.

3.2. Biological activity of CFP-tagged and untagged short and long leptin receptors

The biological activities of LEPRb-CFP and non-tagged LEPRb were tested in CHO cells. Leptin-induced luciferase activity (50 and 200 ng/ml, respectively) in cells transfected with LEPRb-CFP (7.5 ± 1.1- and 15.6 ± 2.0-fold induction) was similar to cells transfected with untagged LEPRb (7.0 ± 1.2- and 11.6 ± 2.3-fold induction). Both tagged (not shown) and untagged LEPRa and LEPRb (Figs. 2 and 3) were also capable of phosphorylating erbB2 and activating MAPK.

3.3. Leptin or PRL-receptor-enhanced phosphorylation of KN erbB2-YFP

In cells co-transfected with DNA encoding LEPRa or LEPRb, Jak2 and KN erbB2-YFP, 5 min exposure to 25 nM leptin resulted in significant and specific phosphorylation of the latter. The phosphorylation could already be seen in the total cell lysate (TCL) and was verified by IP with α-GFP (Fig. 2A and B). No such phosphorylation was seen in cells not exposed to leptin or in cells transfected with only Jak2 and KN erbB2-YFP (not shown). Similar results were obtained in cells

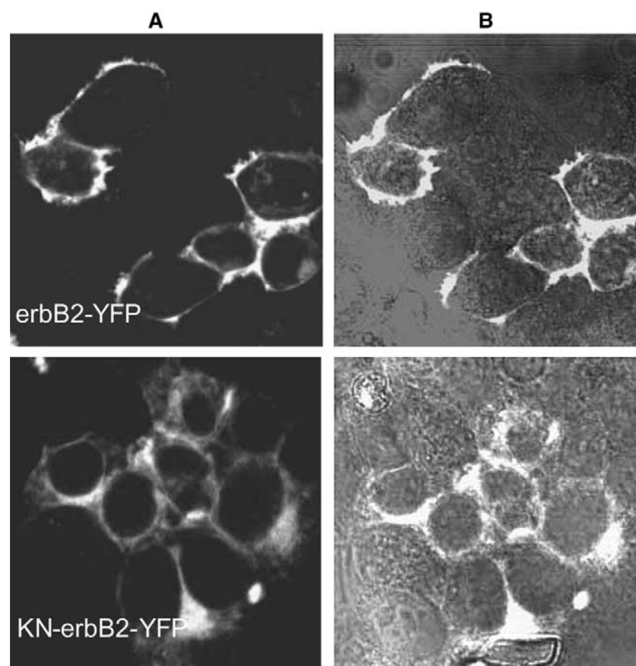

Fig. 1. Expression of erbB2-YFP (upper) or KN erbB2-YFP (lower). HEK 293T cells were transfected with the respective constructs and grown for 48 h. Cells were then fixed and analyzed by confocal microscope. (A) Confocal images; (B) the corresponding overlayed transmitted-light images. For details see text.

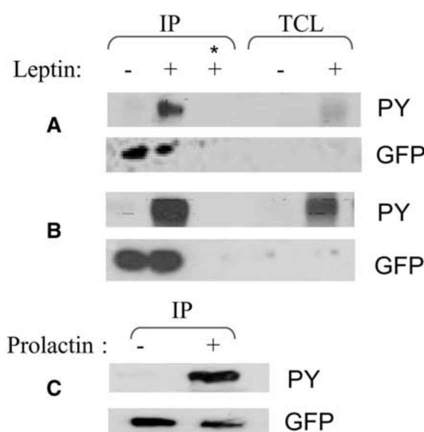

Fig. 2. Leptin- or prolactin-enhanced phosphorylation of KN erbB2-YFP. HEK 293T cells transfected with KN erbB2-YFP, Jak2 and either LEPRb (A) or LEPRa (B) were treated with 400 ng/ml leptin as indicated. Lysates were subjected to IP using anti-GFP antibodies or no antibody as a control (*), separated by SDS–PAGE along with samples from the TCL and immunoblotted using anti-phospho-tyrosine (PY) and reprobed with anti-GFP. (C) Immunoprecipitates from cells transfected with KN erbB2-YFP, Jak2 and PRLR, which were treated with 400 ng/ml PRL. Other details as in (A) or (B).

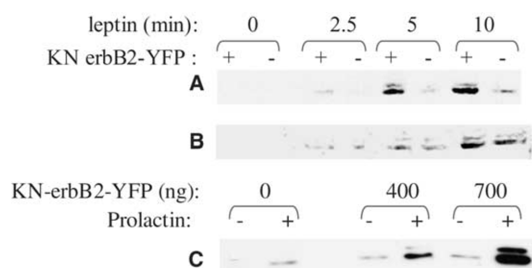

Fig. 3. MAPK activation in cells transfected with Jak2, LEPRa or LEPRb and KN erbB2-YFP. HEK 293T cells were transfected with either LEPRb (A) or LEPRa (B), Jak2 and either with or without KN-erbB2-YFP as indicated. Following overnight starvation cells were treated with 400 ng/ml leptin for up to 10 min. The lysates were separated by SDS–PAGE and immunoblotted with anti-di-phosphorylated MAPK antibodies. (C) Cells transfected with PRLR, Jak2 and increasing amounts of KN-erbB2-YFP were starved, treated with 400 or 700 ng/ml prolactin for 5 min and checked for MAPK activation as above.

co-transfected with ovine PRLR and stimulated with ovine PRL (Fig. 2C), confirming others' results [11], and showing that tagging the KN erbB2 with YFP does not interfere with its ability to be phosphorylated.

3.4. MAPK activation in cells transfected with Jak2, LEPRa or LEPRb and KN-erbB2-YFP

Exposure to leptin in HEK 293T cells transfected with either LEPRa or LEPRb resulted in time-dependent activation of MAPK, the effect being stronger in the latter. The results cannot be attributed to differential expression of MAPK, as the amount of total MAPK was similar in cells transfected with LEPRa and LEPRb (not shown). However, when the cells were co-transfected with KN erbB2-YFP, MAPK activation was both stronger and faster (Fig. 3A and B), in contrast to the relatively weaker response in cells transfected with LEPRa in the absence of KN-erbB2-YFP. Similar results were obtained in cells transfected with PRLR and KN erbB2-YFP (Fig. 3C), indicating that tagging of KN erbB2 with YFP does not affect its ability to activate MAPK.

3.5. FRET in HEK 293T cells co-transfected with KN erbB2-YFP and LEPRb-CFP or PRLR-CFP

In order to elucidate the possible mechanism of KN erbB2-YFP activation by leptin or PRL receptors tagged with CFP, we employed acceptor photobleaching FRET microscopy to detect an interaction between the receptor types. To ensure the proper experimental conditions, only cells expressing similar amounts of expression of CFP- and YFP-tagged proteins were chosen (Fig. 4A). In each case, two independent experiments were carried out in cells exposed to the respective hormone for up to 30 min. The results shown in Fig. 4B, clearly indicate that there is no significant FRET signal and hence, there is no interaction between the receptors during the tested period. Minor variations in FRET efficiency reflect experimental error only. In the case of co-transfection of KN erbB2-YFP and PRLR-CFP, a decrease in FRET signal was observed after 30

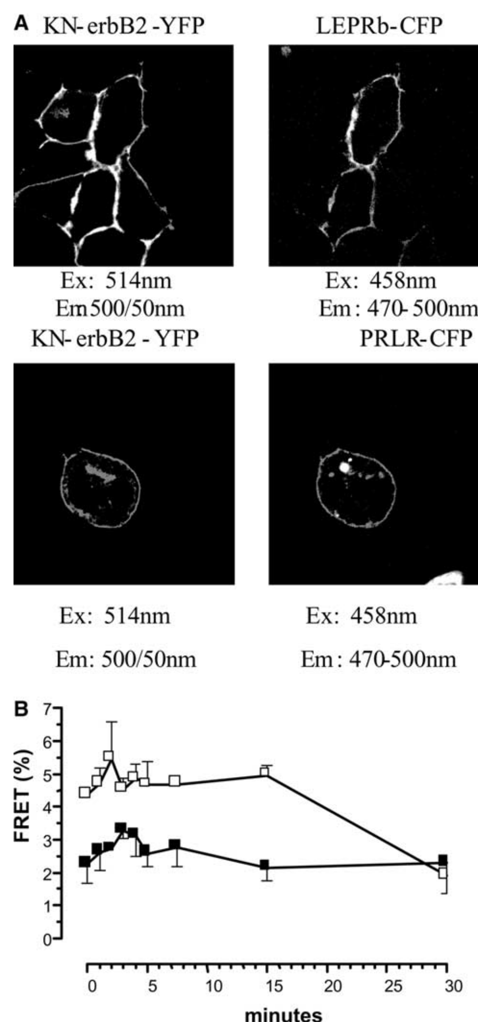

Fig. 4. FRET microscopy. (A) Multitrack imaging of HEK 293T cells transfected with LEPRb-CFP (upper) or PRLR-CFP (lower) along with erbB2-YFP. (B) Time-dependent leptin- or PRL-induced FRET signal in cells transfected with LEPRb-CFP/erbB2-YFP (■) or PRLR-CFP/erbB2-YFP (□). For details see text.

min of exposure to the hormone. It should be noted that a significant FRET was measured using similar technique in a different system [14], as well as by measuring LEPRb-YFP and LEPPRb-CFP interaction (unpublished data) and thus excluding the possibility of technical failure. An attempt to co-immunoprecipitate LEPRb and erbB2-YFP using anti GFP Abs was also unsuccessful. Though a band of 205 kDa corresponding to LEPRb was observed in the supernatant after IP, no such band was seen in the immunoprecipitated erbB2-YFP using commercially available anti-LEPR Ab.

4. Discussion

We demonstrate that KN erbB2-YFP is as active as its untagged analog and show transactivation of KN erbB2-YFP by both LEPRa and LEPRb, since both LEPRs phosphorylated KN erbB2 and subsequently promoted MAPK activation. Despite the fact that LEPRa has a lesser ability to activate MAPK (unpublished data), in our case its capacity to enhance phosphorylation of KN erbB2 and activation of MAPK through transactivation of KN erbB2 was stronger than that of LEPRb. Though it could be partially attributed to its higher expression (see reblot with α-GFP in Fig. 3), this finding may be of physiological or pathological significance as LEPRa is more abundant in peripheral tissues. Our present results show that not only PRL, but also leptin and perhaps other cytokines such as IL6 [21], oncostatin M [22] and tumor necrosis factor [23], may transactivate erbB2; as such they may unveil a novel route by which cytokines promote erbB2 phosphorylation and enhance its oncogenic potential independently of HER family ligands or its overexpression. The case of leptin is of particular interest as both the hormone and obesity have been linked to increased risk of carcinogenesis [1–4,8]. Therefore, a hypothetical situation, in which the same cell overexpresses erbB2 and leptin or PRL receptors along with high circulating levels of those hormones, makes it potentially more vulnerable to oncogenic transformation and tumor promotion.

In the present work, we also tried to elucidate the molecular mechanism underlying the putative cross-talk between the LEPRs or PRLR and erbB2 by measuring their appearance under FRET microscopy. As no hormone-dependent FRET signal was observed, two alternative interpretations of our results can be suggested: (a) PRLR/LEPR and erbB2 do not interact and Jak2, activated by PRLR or LEPR translocates to activate erbB2; or (b) PRLR/LEPR and erbB2 do form a complex through binding to Jak2 but the cytosolic domains of these receptors are not close enough to be observed by FRET. The present data do not enable a determination of which interpretation is correct. However, further experiments involving FP-tagged Jak2 or anisotropy experiments to be executed in the near future will likely resolve this question.

Acknowledgements: This work was supported by the Binational USA – Israel Science Foundation (Grant No. 2000115) to AG and BH. We thank Dr. Y. Yarden from the Weizmann Institute, Rehovot, Israel for the plasmids encoding erbB2 and KN erbB2 and reading this manuscript, Dr. C. Bjorbaek from Harvard Medical School, Boston, MA for the plasmids encoding mouse LEPRs and Ms. Nathalie Daniel for the help in bioassay of LEPRb-CFP.

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