p130Cas interacts with estrogen receptor α and modulates non-genomic estrogen signaling in breast cancer cells

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Summary

Steroid hormones bind to their receptors and trans-activate target genes. Rapid non-genomic action of steroid hormones has been proposed in addition to the one at the genomic level. Estrogen has been described to activate c-Src kinase and this activation has been shown to be responsible for estrogen-dependent mitogenicity. A major substrate of c-Src kinase activity is the cytoskeletal protein p130Cas, originally identified in v-Src-transformed cells. We show that in the human breast carcinoma T47D cells, upon estrogen treatment, p130Cas rapidly and transiently associates with the estrogen receptor α in a multi-molecular complex containing the c-Src kinase and the p85 subunit of PI 3-kinase. Association of p130Cas with the estrogen receptor α occurs within 3 minutes of estrogen treatment and is dependent on c-Src kinase activation. Transient over-expression of p130Cas in T47D cells increases estrogen-dependent Src kinase and Erk1/2 MAPKs activities and accelerates their kinetics of stimulation. A similar effect was detected on estrogen-dependent cyclin D1 expression, suggesting a role for p130Cas in regulating estrogen-dependent cell cycle progression. Double-stranded small RNA interference (siRNA) by silencing endogenous p130Cas protein, was sufficient to inhibit estrogen-dependent Erk1/2 MAPKs activity and cyclin D1 induction, demonstrating the requirement of p130Cas in such events. Therefore, our data show that the adaptor protein p130Cas associates with the estrogen receptor transducing complex, regulating estrogen-dependent activation of c-Src kinase and downstream signaling pathways.

Key words: p130Cas, Estrogen, Estrogen receptor, c-Src, Erk1/2 MAPK, Cyclin D1

Introduction

The steroid hormone 17β-estradiol (E2) plays an important role in regulating a wide variety of physiological and pathological processes such as development, homeostasis and breast cancer progression (McDonnell and Norris, 2002). Estrogen receptors upon hormone binding dimerize and interact with DNA sequences to regulate gene transcription (Beato et al., 1995; Mangelsdorf et al., 1995). Alternatively, estrogen receptors can bind to DNA-associated transcription factors stimulating or repressing transcription (Hermanson et al., 2002). However transcriptional activity of the estrogen receptor is not sufficient by itself to fully explain the activity of steroid hormones on different events, such as regulation of cell proliferation. Recently estrogen has been reported to induce multiple cytosolic signaling processes, such as activation of Src, Ras, Raf, PKC, PKA, potassium channels, intracellular calcium levels and nitric oxide (for reviews, see Cato et al., 2002; Kelly and Levin, 2001; Migliaccio et al., 1996; Segars and Driggers, 2002). Since activation of these signaling molecules depends on cell types studied and the conditions used, the precise non-genomic signaling pathways of estrogen and their functional significance are not yet well understood (Cato et al., 2002; Collins and Webb, 1999; Falkenstein and Wehling, 2000; Foster and Wimalasena, 1996; Revelli et al., 1998)

c-Src kinase plays a pivotal role in non genomic signaling of estrogen receptor α (Cato et al., 2002). The p130Cas (Crk-associated substrate) adaptor protein (for reviews see Bouton et al., 1997; O'Neill et al., 2000) is a major substrate of the c-Src kinase (Burnham et al., 1996; Hamasaki et al., 1996; Vuori et al., 1996) and is an essential component for cell transformation by this oncogene as demonstrated by the inability of constitutively active c-Src to transform fibroblasts isolated from *p130Cas*$^{-/-}$ mouse embryos (Honda et al., 1998).

p130Cas is a signaling molecule involved in the linkage of actin cytoskeleton to the extracellular matrix during cell migration, cell invasion and cell transformation (for reviews, see Bouton et al., 2001; Defilippi et al., 1997; Giancotti and Tarone, 2003; O'Neill et al., 2000). p130Cas cellular function relies on its modular structure, characterized by an amino-terminal Src homology 3 domain (SH3), a proline rich region, a large substrate binding domain containing 15 repeats of the YXXP sequence, a serine rich region and a carboxy-terminal domain

with an additional proline-rich sequence. Several signaling proteins such as p125Fak (focal adhesion kinase), the Fak-related protein Pyk2, Src family kinases, the adaptor Crk and the phosphatases PTP1B and PTP-PEST have been demonstrated to interact with distinct domains of p130Cas both in vivo and in vitro (Garton et al., 1996; Liu et al., 1996). While p130Cas binds to p125FAK through its SH3 domain (Harte et al., 1996), additional protein interaction sites are present in the carboxy-terminal part of p130Cas, where a canonical proline-rich region can bind to the SH3 domain of Src family kinases (Manie et al., 1997). How these interactions contribute to the role of p130Cas in cell proliferation, migration and survival is still poorly understood (Bouton et al., 2001; O'Neill et al., 2000).

So far, little is known about the role of p130Cas in breast cancer cells. Here we show that in human breast carcinoma T47D cells, p130Cas transiently associates with the estrogen receptor α in a macromolecular complex together with c-Src. We also show that complex formation is estrogen dependent and that the association regulates estrogen-dependent activation of c-Src and Erk1/2 MAPK and cyclin D1 expression.

Materials and Methods

Reagents and antibodies

Monoclonal antibody (mAb) 1H9 to p130Cas was prepared in our laboratory, by immunizing mice with a recombinant protein encompassing amino acids 360-685 of mouse p130Cas cDNA sequence (a kind gift from Dr S. Hanks, Nashville, USA). 1H9 mAb specificity was tested by using, as a negative control, fibroblasts derived from p130Cas null mice (a kind gift from Dr H. Hirai and T. Nakamoto, Tokyo, Japan). 1H9 recognizes p130Cas by immunoprecipitation and western blotting and its reactivity was identical to that obtained with the p130Cas mAb from Transduction Laboratories (BD Biosciences Pharmingen, San Diego, CA, USA) (unpublished results). mAbs to estrogen receptor α, c-Src, p85 and polyclonal antibodies to Erk-1/2 MAP kinase and cyclin D1 were from Santa Cruz Biotechnology, Palo Alto, CA, USA. Polyclonal antibodies to phospho-Erk-1/2 MAPK were from Cell Signaling Technology, Beverly, MA, USA. Human 17β-estradiol (E2) was obtained from Tocris (Ellisville, MO, USA). [γ-^{32}P]ATP (6000 Ci/mmole), protein A-sepharose, nitrocellulose, the ECL reagents and films were all from Amersham-Pharmacia, Buckinghamshire, UK. Culture media, sera and antibiotics were from Invitrogen, Germany. Non-radioactive ATP and all the remaining chemicals were from Sigma, St Louis, MO, USA. Lipofectamine 2000 was purchased from Invitrogen, Germany. PP1 and PD98059 were from Calbiochem.

Cell culture and transfection

Human breast epithelial carcinoma T47D cells were purchased from ATCC (Manassas, VA, USA) and grown in RPMI-1640 medium supplemented with phenol red, 0.02% insulin, 10% fetal calf serum (FCS) and antibiotics. Prior to experiments, cells were made quiescent for rapid estrogen signaling studies by incubating them for 24 hours in phenol red-free RPMI-1640 in the presence of 10% charcoal-treated serum.

T47D cells were transfected with the pCDNA3 empty vector (Invitrogen) or human myc-tagged p130Cas cDNA inserted in the pCDNA3 vector (kind gift from Dr A. Bouton, Charlottesville, VA, USA). Cells were cultured at 80% confluence and transfected by the Lipofectamine 2000 (Invitrogen) method, according to manufacturer's instructions. Medium was changed, 24 hours after transfection, to phenol red-free RPMI-1640 supplemented with charcoal-treated serum and experiments were performed 48 hours after transfection.

Cell lysis, immunoprecipitation and immunoblotting

Cells were extracted with 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8,5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 Unit/ml aprotinin). Cell lysates were centrifuged at 13,000 ***g*** for 10 minutes and the supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method (Biorad, Hercules, CA, USA). Proteins were run on SDS-PAGE under reducing conditions. For co-immunoprecipitation experiments, 2 mg of proteins were immunoprecipitated with antibodies to estrogen receptor α for 1 hour at 4°C in the presence of 50 μl protein A-Sepharose beads. Following SDS-PAGE, proteins were transferred to nitrocellulose, incubated with specific antibodies and then detected with peroxidase-conjugated secondary antibodies and chemiluminescent ECL reagent. When appropriate, the nitrocellulose membranes were stripped according to manufacturers' recommendations and re-probed. Densitometric analysis was performed using the GS 250 Molecular Imager (Biorad).

For analysis of cyclin D1 expression, cells were extracted in RIPA buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl pH 7, 0.4 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg /ml pepstatin and 0.1 Units/ml aprotinin) and analyzed as indicated above.

c-Src kinase assay

T47D cells, either untransfected or transfected and treated with estrogen for different times, were washed twice on ice with 1 mM Na_3VO_4 and then lysed in 1 ml of RIPA buffer. c-Src kinase assay was performed as previously described (Cabodi et al., 2000). Briefly, 1.5 mg of cell lysates was pre-cleared using protein G-Sepharose beads (Invitrogen) and then immunoprecipitated using 5 μg of monoclonal anti-Src antibody for 2-3 hours at 4°C under rotation. Protein G was added to the immunocomplexes for 1 hour, immunocomplexes were then washed four times with RIPA buffer and then twice with kinase buffer (50 mM Hepes, pH 7.4, 5mM $MgCl_2$, 3 mM $MnCl_2$). Immunocomplexes were resuspended in 45 μl of kinase buffer and incubated for 10 minutes at 30°C with 1 nM non-radioactive ATP and 5 μCi [γ-^{32}P]ATP. Reactions were stopped in 4× Laemmli buffer and half were loaded on a 8% SDS-PAGE. The gel was fixed in 10% acetic acid and 10% methanol for 20 minutes, rehydrated in water for 20 minutes, dried and exposed. The other half of the reaction was used to run a parallel gel for immunoblotting and control of equal level of immunoprecipitation.

siRNA

T47D cells were depleted of p130Cas using siRNA corresponding to nucleotides 2005-2023 of human p130Cas (Xeragon, Quiagen). The cells were exposed to p130Cas siRNA in the presence of TransMessenger Transfection Reagent (Quiagen) as described by the manufacturer. As a control, cells were exposed to non-silencing fluorescence-labeled control siRNA provided by Quiagen. After 4 hours of incubation with the siRNAs, cells were washed in PBS and incubated in phenol red-free RPMI-1640 supplemented with charcoal-treated serum for an additional 48 hours. Cells were then treated with 10 nM estrogen for different times, extracted in 150 mM NaCl, 50 mM Tris pH 7.4, 1% SDS and analyzed by western blotting as reported above.

Results

p130Cas associates with c-Src and the estrogen receptor α in an estrogen-dependent complex.

Estrogen receptors have recently been shown to associate with c-Src and with other transducing molecules to build up

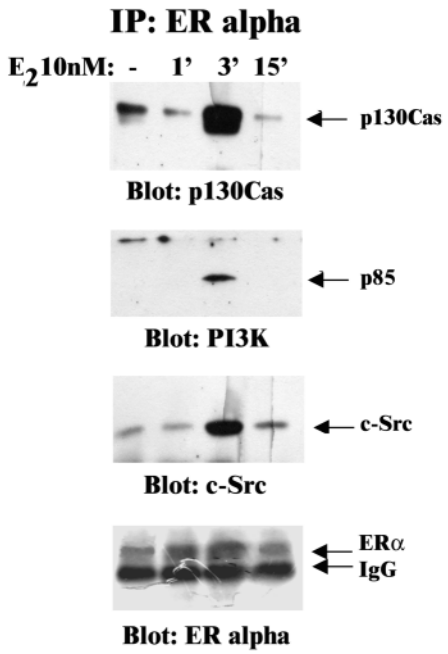


Fig. 1. p130Cas transiently associates with the estrogen receptor α, c-Src and the p85 subunit of PI 3-kinase (PI3K). T47D cells grown to confluence were made quiescent for rapid estrogen signaling by incubating them in phenol red-free RPMI-1640 medium supplemented with charcoal-treated serum for 24 hours. Cells were then left untreated or treated with 10 nM E2 for the indicated times (in hours) and detergent extracted. Cell lysates were immunoprecipitated with antibody to estrogen receptor α. Material co-immunoprecipitated with estrogen receptor were run on an 8% SDS-PAGE gel and immunoblotted with antibodies to p130Cas, c-Src, estrogen receptor α and p85 subunit of PI 3-kinase. The data reported here are a representative experiment out of five independent experiments.

signaling complexes (Castoria et al., 1999; Migliaccio et al., 2000). Since the p130Cas adaptor molecule is a selective substrate of c-Src kinase activity (Vuori et al., 1996) we investigated its possible role in estrogen signaling. Quiescent T47D cells were stimulated for different times with 10 nM E2. Cell lysates were then immunoprecipitated with antibodies to estrogen receptor α and the immunoprecipitates were analyzed by immunoblotting with antibodies to c-Src and the p85 subunit of phosphatidylinositol 3 kinase (PI 3-kinase), two known interactors of the estrogen receptor (Castoria et al., 2001; Migliaccio et al., 2000), and to p130Cas. As shown in Fig. 1, estrogen receptor α co-immunoprecipitated with p130Cas and c-Src. Although a basal level of association between estrogen receptor α and c-Src was detected in untreated cells, estrogen treatment strongly induced the co-immunoprecipitation of p130Cas, c-Src and the estrogen receptor α. In addition to p130Cas and c-Src, the p85 subunit of the PI 3-kinase was found in the same conditions (Fig. 1). In contrast p125FAK, a kinase known to bind p130Cas (Harte et al., 1996), was not detected, demonstrating the specificity of the observed interactions (data not shown). These data indicate that, upon hormone treatment, p130Cas associates with estrogen receptor α in a multi-molecular complex enriched in signaling molecules. Interestingly, estrogen receptor beta was also present in the complex (data not shown). Moreover, as shown in Fig. 1, we demonstrated that the timing of estrogen receptor α complex assembly is carefully regulated, occurring mostly within 3 minutes of E2 treatment, and is down-regulated to the basal level after 15 minutes of stimulation, indicating that the multi-molecular complex, including the estrogen receptor α, the c-Src kinase and the p85 PI 3-kinase subunit but not p125Fak, is the result of an early and transient event following E2 treatment.

Estrogen-induced c-Src kinase activity is required for multi-molecular complex assembly

17β-estradiol has been reported to activate c-Src in breast cancer cells (Migliaccio et al., 1996). To analyze whether c-Src kinase activity is required for assembling the p130Cas/c-Src/estrogen receptor α complex, we investigated c-Src kinase activation in T47D cells. Cells were treated for different times with 10 nM E2 and kinase assay was performed on c-Src immunoprecipitates. As shown in Fig. 2A, estrogen treatment induced a strong and rapid activation of c-Src kinase activity, measured as autophosphorylation of the c-Src protein. Activation was already detected 10 seconds after treatment, was maximal within 2 minutes, and decreased to basal level at 5 minutes. To further assess the relevance of c-Src kinase activity in recruiting the complex, T47D cells were treated with E2 for 3 minutes in the presence of 5 μM PP1, a specific c-Src kinase inhibitor. As shown in Fig. 2B, upon PP1 treatment, p130Cas was not detected in the immunoprecipitates of estrogen receptor α, showing that inhibition of c-Src activity prevents macromolecular complex assembly. These data indicate that E2 stimulates c-Src kinase activity, which is required for the association of estrogen receptor α to p130Cas. Moreover, to determine whether c-Src and p130Cas were both present in the same complex or mutually exclusive in their association with estrogen receptor α, T47D cells were treated with E2 for 3 minutes and cell extracts were immunoprecipitated with estrogen receptor α directly or after depletion of c-Src by sequential immunoprecipitations. As shown in Fig. 2C, estrogen receptor α did not co-immunoprecipitate p130Cas after depletion of c-Src in the cell extracts, indicating that p130Cas and c-Src are simultaneously associated with the estrogen receptor α.

Over-expression of p130Cas in T47D cells increases c-Src kinase activity

Since the association between c-Src and p130Cas has been shown to enhance phosphorylation of Src kinase substrates, such as cortactin and paxillin (Burnham et al., 2000; Riggins et al., 2003b), we tested whether binding of p130Cas modulates estrogen-dependent c-Src activity in T47D cells. Cells were transiently transfected with an empty vector or a myc-tagged human p130Cas cDNA, and treated with E2 for different times. Over-expression of p130Cas led to increased co-immunoprecipitation of p130Cas with the estrogen receptor α and c-Src (data not shown), indicating that the association of these molecules might be modulated by the expression level of p130Cas. Densitometric analysis showed

that, in cells transfected with p130Cas cDNA, c-Src kinase activity was already increased twofold (statistically significant) compared to empty vector-transfected cells (Fig. 3). Even though the basal level of c-Src kinase activity was increased by p130Cas over-expression, E2 treatment was able to further produce a statistically significant twofold induction of c-Src kinase activity. Interestingly, overexpression of p130Cas enhances and accelerates the kinetics of induction of c-Src kinase activity in response to estrogen treatment (Fig. 3).

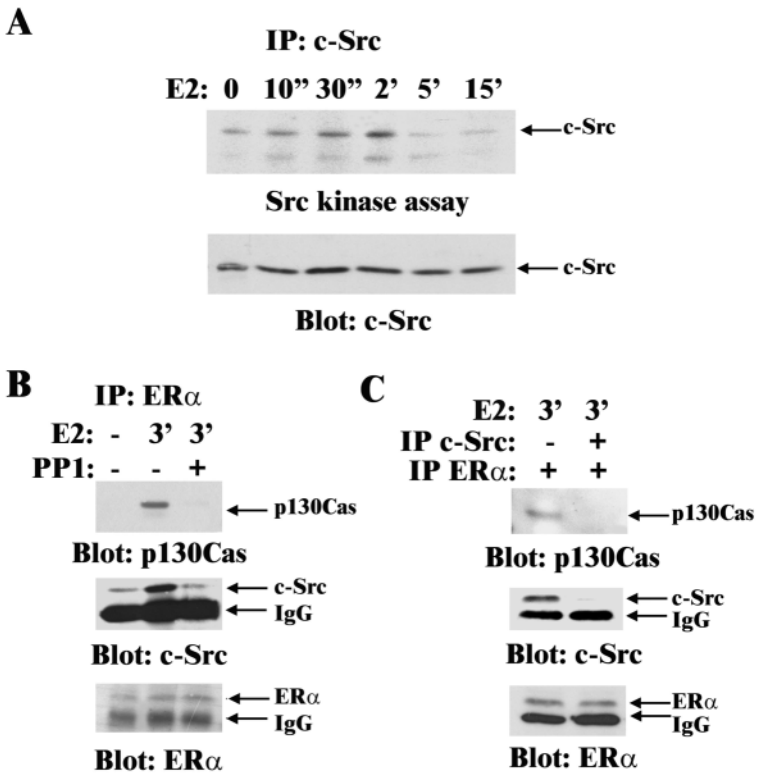
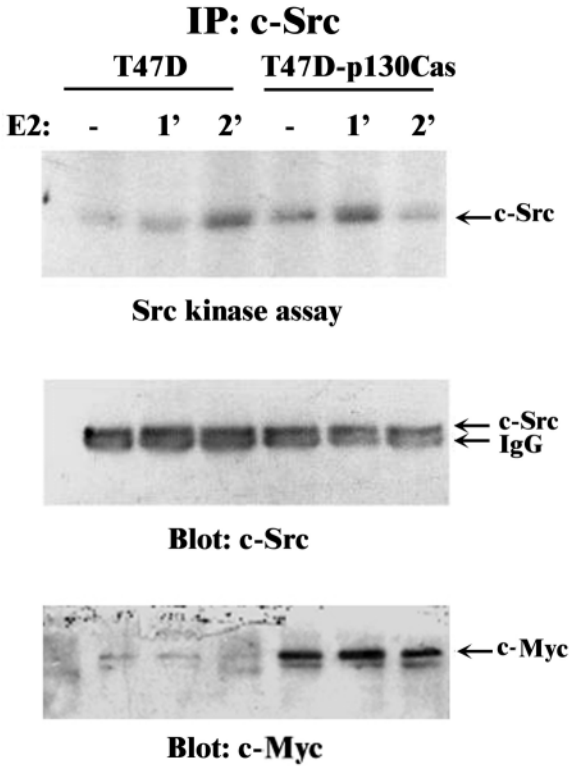


Fig. 2. Estrogen-dependent c-Src activation is required for complex formation. (A) T47D cells grown to confluence and made quiescent as indicated (in Fig. 1), were left untreated or treated with 10 nM E2 for the indicated times (in seconds and minutes) and then detergent extracted. Cell extracts were immunoprecipitated with c-Src antibodies and immunoprecipitates subjected to kinase assay. Immunoprecipitates were run on 6% SDS-PAGE gel, dried and exposed for 3 hours at –80°C. Half of the immunoprecipitates was run in a parallel gel, transferred and blotted with antibodies to c-Src. (B) T47D cells in the same conditions as in A were treated with 10 nM estrogen in the presence of 5 μM PP1 for the indicated minutes. Cell lysates were immunoprecipitated with antibodies to estrogen receptor α. Material co-immunoprecipitated with estrogen receptor were run on an 8% SDS-PAGE gel and immunoblotted with antibodies to p130Cas, c-Src and estrogen receptor α. The data reported here are of a representative experiment out of three separate experiments. (C) T47D were treated for 3 minutes with 10 nM estrogen and cell extracts were immunoprecipitated either with antibodies to estrogen receptor α or first with antibodies to Src and then to estrogen receptor α. The immunoprecipitates were processed as described in B.



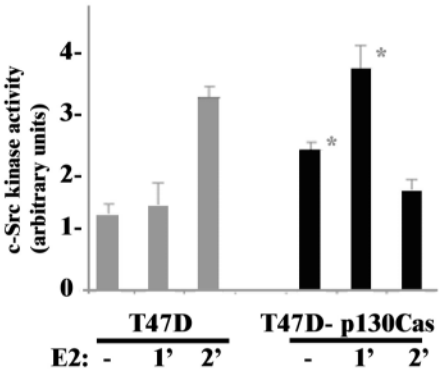


Fig. 3. p130Cas over-expression modulates estrogen-dependent c-Src activation. T47D cells were transiently transfected with empty vector or the p130Cas cDNA by the Lipofectamine 2000 method. 24 hours post-transfection, cells grown to confluence and made quiescent (as indicated in Fig. 1) for an additional 24 hours, were left untreated or treated with 10 nM E2 for the indicated times and then detergent extracted. Cell extracts were immunoprecipitated with c-Src antibodies and immunoprecipitates subjected to kinase assay. Immunoprecipitates were run on a 6% SDS-PAGE gel, dried and exposed at –80°C. Half of the immunoprecipitates was run in a parallel gel, transferred and blotted to antibodies with c-Src and re-blotted with polyclonal antibodies to myc epitope (lower panel). The relative amount of c-Src autophosphorylation was determined by densitometric analysis (on the right) of the autoradiographs. In each case, the autophosphorylation signal was normalized for the corresponding amounts of c-Src immunoprecipitated and expressed as arbitrary units relative to the untransfected and untreated control. The statistical significance of the different values was calculated using Student's *t*-test (*) ($P<0.05$). Similar results were obtained in two other experiments.

Over-expression of p130Cas modulates E2-induced c-Src-dependent Erk1/2 MAPK kinase phosphorylation in T47D cells

Several recent reports demonstrate that estrogen rapidly activates p44/p42 Erk (Erk1/2) MAPKs in human breast cancer cells (Migliaccio et al., 1996; Song et al., 2002). In quiescent T47D cells, E2 treatment induced activation and phosphorylation of Erk1/2 MAPKs in a time-dependent manner (Fig. 4A-C, left). In response to estrogen, Erk1/2 MAPKs activation was biphasic, with one peak at 30 seconds and the other at 15 minutes after treatment. Interestingly, phosphorylation of Erk1/2 MAPKs, occurring already 10 seconds after estrogen treatment, correlated with the earliest time of c-Src activation (see Fig. 2A), suggesting that Src kinase activity modulated MAPKs phosphorylation. Indeed, as shown in Fig. 4B, concomitant treatment with E2 and the specific c-Src kinase inhibitor PP1, completely abolished Erk1/2 MAPKs phosphorylation, indicating that c-Src kinase activity is required for regulating Erk1/2 MAPKs activation in response to estrogens. Moreover, overexpression of p130Cas by transient transfection induced a fourfold increase of Erk1/2 MAPKs phosphorylation, already appreciable at 10 seconds after estrogen treatment and peaking at 1 minute as indicated by densitometric analysis (Fig. 4C). Interestingly, the second peak of Erk1/2 MAPKs activation observed in untransfected cells at 15 minutes after E2 treatment was missing in p130Cas over-expressing cells.

siRNA on p130Cas inhibits estrogen-dependent Erk1/2 MAPK

A new strategy to specifically down-regulate expression of genes in mammalian cells is the use of small interfering RNA, 'siRNA technology'. To evaluate whether endogenous p130Cas was required to modulate the effect induced by estrogen treatment on the activation of Erk1/2 MAPKs, the expression of p130Cas was knocked down by using double-stranded RNA (siRNA) in T47D cells. As shown in Fig. 5, the specific p130Cas siRNAs (indicated with + in Fig. 5) but not the control siRNAs (–) were able to down regulate the expression of the endogenous protein. Either p130Cas or the control siRNAs did not change the level of expression of the related kinase p125Fak, indicating that the silencing observed for p130Cas was specific. The suppression of endogenous p130Cas protein, even though not complete (Fig. 5), was sufficient to abrogate the estrogen-dependent activation of Erk1/2 MAPKs, indicating the requirement of p130Cas in regulating this event. In conclusion, these results indicate that

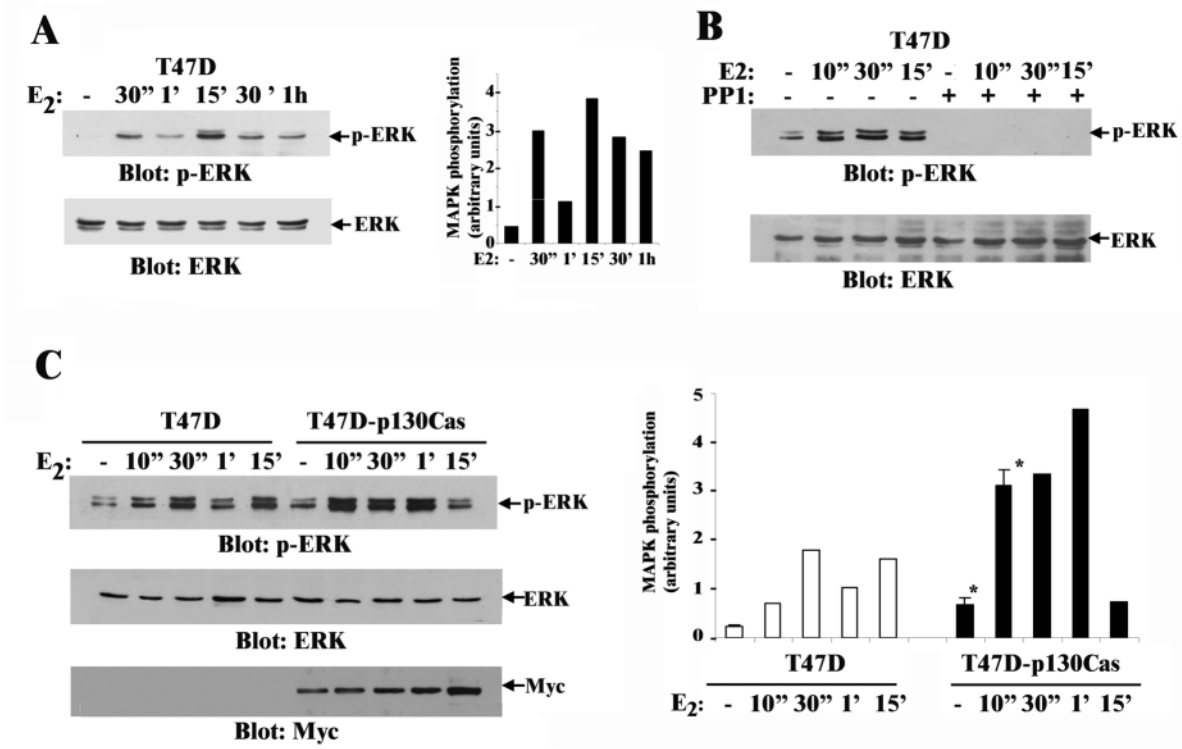


Fig. 4. p130Cas over-expression modulates estrogen-dependent Erk1/2 MAPK activation. (A) T47D cells were grown to confluence and made quiescent (as indicated in Fig. 1) for 24 hours, left untreated or treated with 10 nM E2 for the indicated times and detergent extracted. Erk1/2 MAPK activation was measured and the relative amount of MAPK activation was calculated by densitometric analysis (on the right) as explained in Fig. 3. (B) T47D cells in the same conditions as in A were treated with 10 nM estrogen in the presence of 5 μM PP1 for the indicated times. (C) Alternatively, T47D cells were transiently transfected either with empty vector or the p130Cas cDNA using Lipofectamine 2000. 24 hours post transfection, cells were made quiescent in phenol red-free RPMI-1640 medium supplemented with charcoal-treated serum for an additional 24 hours, left untreated or treated with 10 nM E2 for the indicated times and detergent extracted. Densitometric analysis (right) was performed as previously described and the statistical significance was calculated using Student's *t*-test (*) $P<0.05$. (A-C) Cell extracts were run on 10% SDS-PAGE, transferred to nitrocellulose, blotted with anti-phospho Erk1/2 antibodies (upper panels) and re-blotted with polyclonal antibodies to Erk1/2 (lower panels in A and B) and c-myc (lower panel in C). Similar results were obtained in four independent experiments.

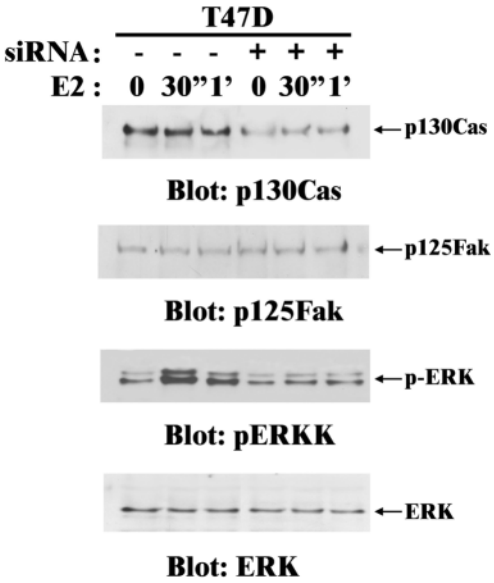


Fig. 5. p130Cas silencing by siRNAs inhibits estrogen-dependent Erk1/2 MAPK activation. T47D cells were transfected with p130Cas siRNAs (+) or with control siRNAs (–) as described in Materials and Methods. 48 hours after transfection cells were stimulated with 10 nM estrogen for different times and detergent extracted. Cell extracts were run on 8% SDS-PAGE and blotted with the indicated antibodies. The same results were obtained in two independent experiments.

the endogenous p130Cas is essential for T47D cells responses to non-genomic estrogen signaling.

Over-expression of p130Cas modulates estrogen-dependent cyclin D1 expression

c-Src kinase has been shown to be important in the activation of cyclin D1 transcription in response to 17β-estradiol (Castoria et al., 1999; Foster et al., 2001). In quiescent empty vector-transfected T47D cells, E2 treatment induced expression of cyclin D1 in a time-dependent manner, reaching a maximal level at 8 hours (Fig. 6A). In contrast, in cells transfected with p130Cas, and treated with E2 for different times, kinetic analysis showed that cyclin D1 was induced within 1-4 hours of E2 treatment, with an earlier increased expression compared to control cells. Interestingly, at 8 hours of treatment cyclin D1 expression was completely down-regulated, indicating that over-expression of p130Cas drastically modifies the kinetics of estrogen-dependent cyclin D1 expression. In addition the increased expression of cyclin D1 promoted by E2 treatment in quiescent cells was abolished by both PP1 and PD98059, indicating that the regulation of cyclin D1 expression accounted for both c-Src and MAPK activities (Fig. 6B). The silencing of the endogenous p130Cas by double-stranded RNA caused an inhibitory effect on cyclin D1 expression upon estrogen treatment, revealing that the endogenous level of p130Cas contributes to the onset of cyclin D1 expression (Fig. 6C).

Discussion

In this work we show that in human T47D breast cancer cells, upon estrogen treatment, the p130Cas adaptor protein associates in a transient macromolecular complex with the estrogen receptor α, c-Src and the p85 subunit of PI-3K. Moreover, over-expression of p130Cas increases and accelerates estrogen-dependent c-Src kinase and Erk1/2 MAPKs activities and cyclin D1 expression. Alternatively, the suppression of p130Cas expression by siRNA inhibits estrogen-dependent Erk1/2 MAPKs activation and affects cyclin D1 expression. Taken together these data demonstrate that the p130Cas protein is a new highly dynamic component of the estrogen receptor signaling complex and plays a crucial role in the early steps of estrogen-dependent non-genomic signaling.

p130Cas is a docking molecule containing distinct functional domains, which provide binding sites for several effector proteins, including Src and PI 3-kinase (Bouton et al., 2001; Riggins et al., 2003a). It has been previously demonstrated that estrogen receptor α interacts through its phosphotyrosine residue 537 with the SH2 domain of the c-Src kinase in breast cancer

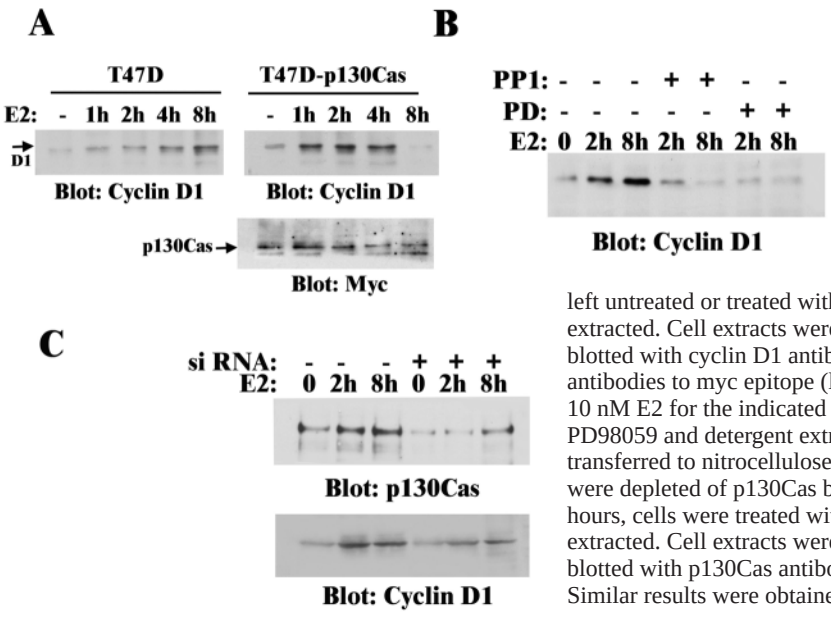


Fig. 6. p130Cas modulates estrogen-dependent cyclin D1 expression. (A) T47D cells were transiently transfected with empty vector or the myc-tagged p130Cas cDNA by the Lipofectamine 2000 method. 24 hours post transfection, cells made quiescent (as indicated in Fig. 1) for an additional 24 hours, were left untreated or treated with 10 nM E2 for the indicated times and detergent extracted. Cell extracts were run on 10% SDS-PAGE, transferred to nitrocellulose, blotted with cyclin D1 antibodies (upper panels) and re-blotted with polyclonal antibodies to myc epitope (lower right panel). (B) T47D cells were treated with 10 nM E2 for the indicated times in the presence of either 5 μM PP1 or 25 μM PD98059 and detergent extracted. Cell extracts were run on 10% SDS-PAGE, transferred to nitrocellulose and blotted with anti-cyclin D1 antibodies. (C) T47D were depleted of p130Cas by siRNA transfection as indicated in Fig. 5. After 48 hours, cells were treated with 10 nM estrogen for the indicated times and detergent extracted. Cell extracts were run on 10% SDS-PAGE, transferred to nitrocellulose, blotted with p130Cas antibodies (upper panel) or with cyclin D1 (lower panel). Similar results were obtained in three independent experiments.

cells. One possible mechanism of p130Cas recruitment to the estrogen receptor α/c-Src macromolecular complexes might involve the p85 subunit of the PI 3-kinase since this molecule has been recently reported to bind estrogen receptor (Castoria et al., 2001) as well as p130Cas (Riggins et al., 2003a). As we demonstrated in Fig. 2C, depletion of c-Src prior to immunoprecipitation with estrogen receptor α prevents the association of p130Cas with estrogen receptor α, suggesting that p130Cas can be recruited into the complex together with c-Src through the binding of its proline-rich region with the SH3 domain of c-Src (Burnham et al., 1996; Nakamoto et al., 1996; Sakai et al., 1994). Although the mechanistic events leading to the association of p130Cas with the estrogen receptor α are not yet completely defined, our results demonstrate that the formation of the macromolecular complex requires c-Src kinase activity. In fact p130Cas is not recruited to the complex in the presence of PP1, a specific c-Src kinase inhibitor. In addition the time course of c-Src-kinase activation correlates to the kinetics of p130Cas association to the estrogen receptor α. We thus propose that, following estrogen treatment, c-Src is activated and allows the dynamic association of p130Cas to the estrogen receptor α, further demonstrating a relevant and primary role of c-Src kinase in estrogen signaling (see model in Fig. 7).

To further investigate the relevance of p130Cas association to the c-Src/estrogen receptor α complex, we over-expressed p130Cas in T47D cells. Our data show that over-expression of p130Cas leads to an earlier and stronger activation of c-Src in response to estrogens, indicating a positive regulation of p130Cas on c-Src activity. It has been recently reported that over-expression of full length p130Cas in Cos-1 cells increases Src-dependent tyrosine phosphorylation of multiple endogenous cellular proteins, such as cortactin or paxillin (Burnham et al., 2000; Riggins et al., 2003b), suggesting a role of p130Cas in Src activation. Our results demonstrate that p130Cas expression is able to directly influence the extent of activation of c-Src kinase, both in unstimulated cells and in response to specific stimuli such as estrogen treatment. In addition, p130Cas modulates the estrogen-dependent kinetics of c-Src activation, leading to an earlier activation of c-Src and of its downstream signaling pathways. It is well known that Src can switch from an inactive 'closed' conformation to an 'open' active state (Superti-Furga, 1995). Our data show that p130Cas functions as a positive regulator of c-Src kinase activity, suggesting that p130Cas contributes to stabilize c-Src in its active conformation. In addition over-expression of p130Cas further enhances c-Src activity upon additional stimuli such as estrogen treatment. As already indicated, the full-length p130Cas molecule contains several potential binding sites for other effector proteins, such as p125FAK, Crk and tyrosine PTPases (Bouton et al., 2001; O'Neill et al., 2000). Although p125FAK has not been found in the estrogen receptor complex, the presence of additional p130Cas interacting molecules, relevant for the activation of c-Src kinase upon estrogen treatment, such as tyrosine PTPases, might not be excluded. Taken together these data show that p130Cas positively modulates c-Src kinase activity, which in turn allows the recruitment of p130Cas to the c-Src/estrogen receptor α complex. Therefore these results support the hypothesis that p130Cas might play a dual role of regulator and adaptor in estrogen-dependent physiological responses (Fig. 7).

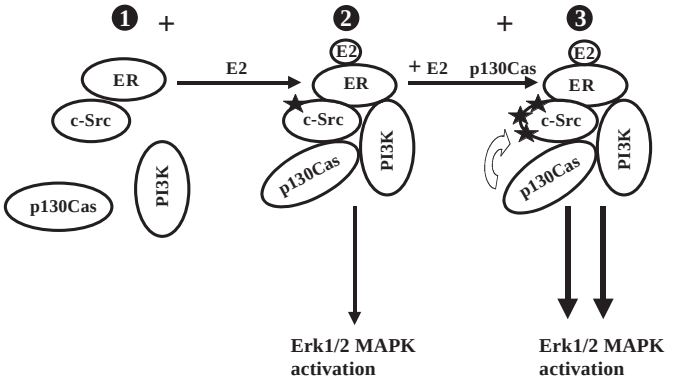


Fig. 7. Model of the assembly and function of the ER macromolecular complex. In the absence of hormone, ER α does not interact with p85 or p130Cas (1). Estradiol triggers association of ER α, c-Src, the p85 subunit of PI 3-kinase (PI3K) and p130Cas in a macromolecular complex (2) and activates the c-Src kinase leading to p130Cas-dependent Erk1/2 phosphorylation (see Fig. 5). Increased amount of cellular p130Cas (3) induces a further activation of the c-Src kinase and of the downstream Erk1/2 MAPK pathways.

In breast cancer cells, activation of Src kinase is a rapid event following estrogen treatment, leading to activation of Shc/Ras/Erk (Cato et al., 2002; Migliaccio et al., 1996; Song et al., 2002) and of PI-3K/AKT pathways (Castoria et al., 2001). In our experiments estrogen treatment induces a biphasic Src-dependent activation of Erk1/2 MAPK, which is strongly modulated by p130Cas. In fact in response to estrogens, in cell over-expressing p130Cas, Erk1/2 MAPK activation is enhanced and accelerated, since the second wave of activation was shifted from 15 minutes to 1 minute (see Fig. 4C). In addition silencing of endogenous p130Cas by siRNA, probably affecting c-Src kinase activity, was sufficient to abrogate estrogen-dependent Erk1/2 MAPK activity, demonstrating that the endogenous level of p130Cas in T47D cells is crucial for regulating the non genomic responses to estrogen treatment. An early event in estrogen-dependent cell cycle regulation is increased transcription, translation and/or stabilization of cyclin D1, either by a direct estrogen-dependent genomic effect or by a non-genomic signaling involving Src/Shc/Ras/Erk and PI 3-kinase activation (Altucci et al., 1996; Foster et al., 2001). Our data show that in T47D cells, estrogen treatment induces time-dependent expression of cyclin D1 within 4-8 hours of stimulation by a c-Src and Erk1/2-dependent mechanism. Over-expression of p130Cas, however, dramatically changes the kinetic and the extent of cyclin D1 expression with cyclin D1 being already detectable 1 hour after estrogen treatment, reaching maximal level within 2 hours and rapidly down-regulated at 8 hours. These data indicate that the over-expression of p130Cas, probably through increased c-Src and Erk1/2 MAP kinase activities, accelerates the onset of cyclin D1 expression and its down-regulation. Since cyclin D1 has been shown to be tightly regulated within the G1 phase and its correct timing of expression is required for progression to the S phase of cell cycle (Sherr, 1995; Welsh et al., 2001), the accelerated expression detected in our experiments suggests that the over-expression of p130Cas might lead to altered regulation of cell cycle progression. Moreover, the experiments involving suppression of

endogenous p130Cas, which results in a strong inhibition of cyclin D1 expression, indicate that p130Cas finely contributes to regulate cyclin D1 cellular levels.

Recently, Brinkmann et al. (Brinkman et al., 2000) isolated and characterized by retroviral-insertion mutagenesis, the BCAR1 (Breast Cancer Resistance 1) gene as a genetic factor that could lead to anti-estrogen resistance in breast cancer cells in vitro. Interestingly, sequence analysis revealed that the BCAR1 gene encodes for the human counterpart of the p130Cas (van der Flier et al., 2001). Transfection of p130Cas/BCAR1 cDNA into estrogen-dependent ZR-75-1 cells resulted in sustained cell proliferation in the presence of anti-estrogen treatment (Brinkman et al., 2000). Analysis of BCAR1 protein level in a large series of carcinomas indicated that patients with primary breast tumors, expressing a high level of BCAR1 protein, experience more rapid disease recurrence and are at a greater risk for intrinsic resistance to the anti-estrogen tamoxifen therapy (van der Flier et al., 2000; van der Flier et al., 2001). The molecular mechanisms underlying the occurrence of resistance to anti-estrogen therapy are completely unknown. The data presented here provide new hints on possible roles of p130Cas/BCAR1 in breast cancer cells. In fact, in vivo, the level of p130Cas finely regulates c-Src kinase and Erk1/2 MAPK activation, leading to a different kinetics of cyclin D1 expression that could ultimately affect cell cycle progression in response to estrogens. Whether these events might contribute to the onset of tamoxifen resistance in breast cancer cells should be investigated.

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