Estrogen Receptor α, a Molecular Switch Converting Transforming Growth Factor-α-mediated Proliferation into Differentiation in Neuroblastoma Cells*

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Transforming growth factor-α (TGF-α) is known to promote both proliferation and differentiation of neural cell progenitors. Using the human neuroblastoma cell line SK-N-BE that is induced to proliferate by TGF-α, we demonstrated that the expression of a single transcription factor, the estrogen receptor-α (ERα), can reroute the TGF-α mitogenic signaling toward a path leading to differentiation. With selected mutations in ERα and signal transducer and activator of transcription 3 (Stat3), we demonstrated that the blockade of TGF-α mitogenic potential was not dependent on ERα DNA binding activity but required a transcriptionally active Stat3. In neuroblastoma cells, 17β-estradiol treatment induced a transient increase in the transcription of estrogen-responsive element-containing promoters including those regulating TGF-α and prothymosin α synthesis. Based on the data presented, we hypothesized that in the presence of prothymosin α, ERα activates its direct target genes and increases cell proliferation, whereas in the presence of high levels of TGF-α, ERα preferentially interacts with Stat3 and causes cell differentiation. Our results reveal a novel form of “end-product” regulation of an intracellular receptor that occurs through recruitment of membrane receptors and their signaling effector system. Cross-coupling between membrane and intracellular receptors has been described by several laboratories. This study proves the relevance of these interactions in cellular responses to growth factors.

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§ The abbreviations used are: TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor; ER, estrogen receptor; RTK, receptor tyrosine kinase; ProTα, prothymosin α; E2, 17β-estradiol; Stat, signal transducer and activator of transcription.
proliferation and induces phenotypic differentiation. This is in contrast with the marked mitotic effect of this hormone in the ER-negative parental cell line. We here investigate on the involvement of ERα in TGF-α signaling and demonstrate that ERα converts the mitotic potential of TGF-α into a stimulus to differentiate. Our results indicate that this effect does not require ERα DNA binding activity, is mediated by Stat3, and is blocked by prothymosin α (ProTα), a co-regulator of ERα transcripational activity of which expression is positively associated with cell proliferation.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Plasmids—SK-N-BE and SK-ER3 cells were grown as previously described (15). Unless otherwise specified, chemicals were purchased from Merck KGaA (Darmstadt, Germany) and culture media and additives were from Invitrogen. 17β-estradiol (E2) and TGF-α were from Sigma, and ICI 182,780 was kindly donated by AstraZeneica (London, United Kingdom). TGF-α was at 10 ng/ml, E2 was at 10−8 M, and ICI 182,780 at 10−7 M was administered 1 h before hormone. pCMVProTα CDNA (16) and the variants obtained through mutagenesis were subcloned into the XhoI/XbaI sites of the expression vector pCDNA3.1A (Invitrogen). The internal control pTK-luciferase was generated in our laboratory (17). pCMVShERα was kindly provided by B. Katzenellenbogen (University of Illinois, Urbana, IL), pBStGF-α was provided by L. Beguinot (DIBIT Hospital San Raffaele, Milan, Italy), gGAS-luc was provided by E. Liboi (University of Verona, Verona, Italy), and RcCMVStat3, RCMVStat3F, and RCMVStat3D were provided by M. Greenberg (Harvard Medical School, Boston, MA).

Reporter and Morphometric Assays—The calcium phosphate precipitation procedure and reporter assays were described previously (8). Luciferase counts normalized versus protein content are expressed as fold induction with respect to untreated sample. For morphometric assays (18), cells were transfected in a 12-well plate with 0.2 μg/well pCMV-lacZ (Promega, Madison, WI) alone or with 0.4 μg/well of the expression vectors containing for transcription factors and/or Bas variants as specified in the figures or figure legends. Morphometric analysis was carried out 96 h after treatments, measuring the neurite length of β-galactosidase-stained cells (18). For each experimental group, a minimum of 50 cells/dish was evaluated.

Cell Counting—SK-N-BE and SK-ER3 were seeded in RPMI 1640 medium without Phenol Red + 0.5% dextran-coated charcoal in 24-well plates, and after the specified treatments, vital cells were counted in triplicate wells. For transfected SK-N-BE, β-galactosidase-positive cells were counted in 25 fields/well chosen at random for each experimental group. Transfection efficiency was normalized by including 0.2 μg of pTK-luciferase in three additional wells not subjected to transfection vectors containing for transcription factors and/or Bas variants as specified in the figures or figure legends. Morphometric analysis was carried out 96 h after treatments, measuring the neurite length of β-galactosidase-stained cells (18). For each experimental group, a minimum of 50 cells/dish was evaluated.

Cell Counting—SK-N-BE and SK-ER3 were seeded in RPMI 1640 medium without Phenol Red + 0.5% dextran-coated charcoal in 24-well plates, and after the specified treatments, vital cells were counted in triplicate wells. For transfected SK-N-BE, β-galactosidase-positive cells were counted in 25 fields/well chosen at random for each experimental group. Transfection efficiency was normalized by including 0.2 μg of pTK-luciferase in three additional wells not subjected to β-galactosidase staining and transfected in parallel. The number of β-galactosidase-expressing cells was finally referred to 105 luciferase counts.

[3H]Thymidine Incorporation Assay—106 SK-ER3 cells/well (plated the day before the assay in a 6-well dish) were incubated for 3 h with 1 μCi/well [3H]thymidine (Amersham Biosciences). Cells were washed twice with phosphate-buffered saline containing 2 mM cold thymidine and lysed, and the trichloroacetic acid precipitates were assayed in a scintillation counter (TRI-CARB 2100TR, Packard).

Western Analysis and Immunoprecipitation—Whole cell extracts were prepared with Western blot or immunoprecipitation as previously reported (8) using as primary antibody anti-ERα (H-20, kindly provided by G. Greene, University of Chicago, IL), anti-ProTα (Calbiochem), anti-Stat3, or anti-Phospho-Tyr 705-Stat3 (New England Biolabs, Beverly, MA). For immunoprecipitation analysis, whole cell extracts from SK-N-BE cells expressing ERα were set at the final concentration of 150 μM NaCl.

Northern Analysis—20 μg of total RNA were loaded onto 1% denaturing agarose gel containing 2.2% formaldehyde and blotted on nylon membrane (Hybond-N+, Amersham Biosciences). Pre-hybridization and hybridization were performed at 68 °C in Quick-Hyb solution (Stratagene, La Jolla, CA), and two washes were carried out in 0.1× saline/sodium phosphate/EDTA, 0.1% SDS at 50°C for 30 min. Membranes were exposed to autoradiographic films (Hyperfilm, Amersham Biosciences) for 2 (ProTα) or 7 days (TGF-α).

Nuclear Run-on Assay—50 × 106 cells were suspended in a lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 0.5% Nonidet P-40, and 10 mM NaCl), and nuclei were pelleted by Microfuge centrifugation. Nuclear run-on assays were performed as described previously (20). 4-h pre-hybridization and 24-h hybridization were carried on in

FIG. 1. TGF-α action on proliferation and differentiation of neuroblastoma cells. A, cells were grown in low serum (0.5% FBS) and counted with a Burker’s chamber in the presence of a vital staining at the indicated time points. Data represent the mean ± S.E. of three separate experiments made in triplicates in the absence (white bars) or in the presence (filled bars) of TGF-α. *, p < 0.05, †, p < 0.01 versus vehicle calculated by two-way analysis of variance followed by Scheffe’s test. B, microphotographs are representative of the morphology of cells treated for 96 h with vehicle (–), E2, or TGF-α (×200 magnification).
cells transfected with the pCMVLacZ alone, TGF-α treatment induced a 2.1-fold increase of the blue-labeled cells with respect to untreated cells. Co-transfection of increasing concentrations of pCMVERα progressively reduced the effect of TGF-α to reach control values (Fig. 2A). Morphological analysis indicates that treatment with TGF-α or E2 induced a differentiated morphology characterized by a decreased size of the soma and long neurites (Fig. 2B). Computer-assisted morphometric analysis, based on the measurement of neurite length, demonstrated that both E2 and TGF-α caused a significant increase in neurite outgrowth in the presence but not in the absence of ERα (Fig. 2C). This analysis is used to assess differentiation of cells of neuronal origin and has been previously shown as applicable to study the effects of ER ligands in the SK-N-BE cell system (21). Fig. 2C shows that TGF-α and E2 induce a similar increase in neurite length (3.5- and 4.0-fold, respectively). The observation that the pure ER antagonist, ICI 182,780 (ICI), blocked TGF-α-dependent neuritogenesis further demonstrated that ERα was indispensable to the differentiating activity of TGF-α.

**ERα-dependent Neurite Outgrowth Is Mediated by the Ras Pathway and Occurs through a DNA-binding Independent Mechanism**—Several studies reported that RTRs induce the transcriptional activation of unliganded ERα on ERE promoters via Ras/MAPKs enzymatic cascade, (8, 22–25). To investigate the role of Ras in the TGF-α effects above described, plasmids expressing the Ras dominant positive p21(Leu61)Hras (Ras+) or negative p21(Asn17)Hras (Ras−) mutants were co-transfected with pCMVERα and pCMVLacZ in SK-N-BE cells. The effects on cell number and neurite outgrowth were evaluated at 96 h after transfection. In the absence of exogenous E2, Ras(+) induced an increase in the number of β-galactosidase-
positive cells that was not further augmented by TGF-α. This effect was blocked by the presence of unliganded ERα (Fig. 3A).

With regard to neuroblastoma differentiation, Ras(+) induced neurite outgrowth only in the presence of ERα. Its effect was not significantly augmented by estrogen or TGF-α and was blocked by ICI 182,780. On the contrary, the dominant negative Ras(−) completely prevented both TGF-α- and E2-induced differentiation (Fig. 3B). These data suggested that ERα is sufficient to redirect Ras pro-mitotic activity toward a program causing growth arrest and differentiation.

The current model on RTKs-ERα cross-coupling implies that selective phosphorylations of the receptor (26) and/or its co-activators (27, 28) trigger ERα activity on ERα promoters. However, ERα mutated in the sites shown to be indispensable for the cross-coupling to RTKs (Ser-122 in mouse corresponding to Ser-118 in human ERα) (8, 24, 25, 29, 30) or Src (Tyr-541 in mouse) (31, 32) could still interfere with TGF-α (Fig. 3C) or Ras(+)-proliferative action (data not shown). Most importantly, the use of C241A/C244A ERα variant impaired in its ability to bind DNA (Ref. 33 and data not shown) demonstrated that ERα-mediated neuroblastoma differentiation does not require binding to the canonical estrogen-responsive element and the transcriptional regulation of its primary target genes. This last observation led us to hypothesize that ERα-described activities in neuroblastoma were associated to its functional interactions with other nuclear transcription factors.

**Stat3 Is Indispensable to ERα-dependent Neuroblastoma Differentiation—**ERα is able to modulate the transcriptional activity of several transcription factors that are also downstream target of RTKs, including AP-1 (34), NF-kB (35), and STATs (36–39). This function does not require ERα direct binding to DNA. Therefore, we tested whether any of these factors were
able to induce SK-N-BE differentiation. Transfection of plasmids encoding NF-kB (NF-kB1/p50 and RelA/p65) and AP-1 (c-Jun and c-Fos) did not affect SK-N-BE morphology, nor did these factors alter TGF-α and E2 activity in the presence or absence of ERα (data not shown). Stat3 did not have any effect by itself, but when co-transfected with ERα, it induced a significant increase in neurite length, an activity that was completely prevented by the treatment with ICI 182,780 (Fig. 4A). The extent of Stat3 effect was only slightly modified by TGF-α and E2 treatments. The observation that the two Stat3 dominant negative mutants (40), Stat3F (Y705F) and Stat3D (E434A/E435A), blocked E2 and TGF-α activity suggested that Stat3 has a role in ERα-mediated differentiation of SK-N-BE. Dominant negative mutants of Stat3 also blocked the activity of ERα mutated in the DNA binding domain C241A/C244A (data not shown). On the other hand, cell count showed that Stat3 wild type opposed the proliferation of SK-N-BE cells, even in the absence of ERα. Conversely, the two Stat3 dominant negative mutants promoted cell proliferation. In the presence of ERα, Stat3 completely blocked TGF-α-induced proliferation (Fig. 4B). It is important to point out that transient transfection lead to the overexpression of the Stat3 variants in accordance with the dominant negative action hypothesized for Stat3F and Stat3D (Fig. 4C). These results therefore suggested a functional interaction between Stat3 and ERα. Interestingly, the two transcription factors appeared to interact also in the absence of E2 or TGF-α (Fig. 4A). Indeed the constitutive expression of ERα was sufficient to modify the basal levels of Stat3 tyrosine phosphorylation as shown by Western blot analysis of SK-ER3 and SK-N-BE cell extracts (Fig. 4D). Consistently with a functional interaction, immunoprecipitation experiments reported in Fig. 4E demonstrate that in neuroblastoma cells a physical interaction between Stat3 and ERα occurs. Interestingly, this interaction was better detected when the immunoprecipitation was performed with the anti-Stat3 antibody (Fig. 4E, upper panel) instead of anti-ERα (H222) (Fig. 4E, lower panel), thus suggesting that the H222 antibody might recognize an epitope in the vicinity of the interaction site. Finally, the interaction between ERα and Stat3 was also investigated at the level of Stat3 transcriptional activity by transient transfection assay using a luciferase reporter driven by the GAS sequence, a known Stat3-responsive element (pGAS-luciferase) (Fig. 4F). Treatment with TGF-α alone did not affect Stat3 transcriptional activity significantly; however, in the presence of increasing concentration of ERα, we observed a dose-dependent augmentation of luciferase transcription (7.9- and 14.4-fold stimulation versus Stat3 alone). Similar results were obtained when ERα was activated by E2 (6.7- and 9.4-fold stimulation). Consistent with our findings on neurite elongation, the ERα mutants C241A/C244A, Y541A, and S122A, had the same effect on the wild type receptor. The ability of activated ERα to physically interact with Stat3 family of transcription factors has been revealed also in other cell systems (36, 38, 39), and in accordance with our observation in neuroblastoma cells, this interaction has been shown to increase Stat3-dependent transcription (39). These experiments demonstrated the hypothesized transcriptional interaction between ERα and Stat3 and its relevance for TGF-α-induced differentiation of SK-N-BE cells.

**TGF-α- and E2-mediated Differentiation Is Blocked by the Constitutive Expression of ProTa**—We previously reported that ProTa and TGF-α are positively regulated by E2 in the SK-ER3 cell line (16). Other laboratories have shown that both genes are directly regulated by ERα through binding to EREs present in their promoters (41, 42). Run-on analysis using SK-ER3 nuclei demonstrated that the E2-mediated increase in ProTa and TGF-α mRNA is at least in part due to the activation of gene transcription (Fig. 5A). This effect was not detected in the ERα-negative SK-N-BE cell line (data not shown). The prolonged hormonal effect observed on TGF-α and ProTa with respect to progesterone receptor gene transcription (Fig. 5A) suggested that factors other than ERα intervened to strengthen the effect triggered by the hormone. Northern blot analysis revealed that after E2 treatment, TGF-α and ProTa mRNAs slowly accumulated to reach a peak level at 48 h but with a different kinetics. ProTa reached the highest level of expression at 24 h while TGF-α reached at 48 h (Fig. 5B). The accumulation of TGF-α transcript was expected as a component of SK-ER3 differentiation. More puzzling was the prolonged high level of ProTa expression that generally is associated with proliferation rather than differentiation (43). Therefore, we investigated proliferative activity of SK-ER3 upon E2 treatment by studying [3H]thymidine incorporation. In SK-ER3 cells, E2 induced a significant increase in the synthesis of nucleic acids up to 48 h (Fig. 5C). At 72 h, when cells visibly started to change their morphology, the [3H]thymidine incorporation dropped below control levels. Thus, the decrease in ProTa mRNA content temporally correlated with the decreased
[3H]thymidine incorporation and the onset of E2-dependent differentiation. We then tested the effect of constitutive expression of ProTα by transfection of pCMVProTα in SK-N-BE cells. Fig. 6A shows that maintaining high levels of ProTα expression the morphological differentiation mediated by ERα was completely prevented. To identify motifs relevant to ProTα function, we compared its amino acid sequence with other proteins implicated in ERα signaling. ProTα does not contain the se-
sequence motif LXXL (44); however, a computational analysis using the Match-Box website server (45) identified a conserved motif (EKK) present in ProTα, steroid receptor coactivator-1, and ribosomal protein large subunit-7 (Fig. 6B). The program assigned a high score (=4) in terms of calculated match index corresponding to a 75–100% likelihood of correctly predicting the residue alignment. Searching the EsPASY protein databases, SWISS-PROT and TR | EMBL, for the identified box by means of PATTIN| PROT program at NPS| server (46), we obtained only the three above mentioned co- regulators. On the basis of this analysis we devised two different mutated sequences to replace the EKK pattern: QQQ and IKI (Fig. 6D). The former was chosen to completely remove the positive charge present in the wild type sequence without gross alteration of hydrophilicity, the latter to drastically reduce this parameter. Both ProTα mutations were unable to prevent ERα-mediated neurite elongation (Fig. 6A), even though the mutated variants were correctly expressed at a concentration comparable to wild type ProTα as demonstrated by reverse transcription-PCR assays on total RNA samples from transfected cells (Fig. 6C) and by Western blot on whole cell extracts shown in Fig. 6D. These experiments suggest that the effect of constitutive expression of ProTα on neuroblastoma differentiation is mediated by the EKK motif. The experiments reported in Fig. 4 led us to hypothesize that TGF-α induces neuroblastoma differentiation via ERα/Stat3 interaction. Therefore, we tested whether ProTα effect on differentiation correlated to a blockade of ERα activity on a GAS-containing promoter, ProTα wild type, but not the EKK-mutated variants, abolished the transcriptional response of the GAS promoter to Stat3 and ERα in a concentration-dependent manner (Fig. 6E). Co-transfection of the co-regulator activity-modulating protein REA (47) with ERα had no effect on GAS-luc transcription and on neuroblastoma differentiation induced by Stat3/ERα (data not shown); this suggest that REA does not play a role in the interaction between Stat3 and ERα, leading to neuroblastoma differentiation.

**DISCUSSION**

The molecular events inducing a proliferating cell to stop dividing and undertaking a differentiation program are still largely unknown. Using a neuroblastoma model, we studied how TGF-α may induce the same cell type to proliferate or differentiate. We here demonstrate that the molecular switch between the SK-N- BE proliferation and differentiation programs may be a single transcription factor, ERα. TGF-α induces SK-N-BE to proliferate or differentiate depending on the absence or presence of ERα. Because E2 increases the transcription of TGF-α gene in SK-ER3 cells, it might be argued that E2-dependent differentiation of SK-ER3 cells is due to a mechanism of signal amplification (48, 49). We believe that this is not the case because exogenous administration of TGF-α (thus the persistent stimulation of its receptor) or the constitutive expression of Ras(+) into the ERα-negative SK-N-BE cells causes an increase in cell growth but is not associated with any morphological alteration (Fig. 1 and 3, respectively).

The classical view of ERα activity involves the binding of the hormone receptor complex to specific sequences in the promoter of responsive genes, thereby favoring their transcription (50). More recent studies have shown that the unliganded receptor may also be transcriptionally activated by molecules of the RTK signaling pathways (8, 22, 24, 25, 50). This study shows that mutations of ERα impairing its ability to bind ERE or to be the target of RTK-dependent kinases still allow neuroblastoma differentiation induced by TGF-α. It is well known that in the absence of DNA binding function through protein/protein interaction, ERα can still modulate the activity of other transcription factors including AP-1, NF-kB, or Stats (34–36). Little is known with regard to the functional relevance of these interactions. We here demonstrate that ERα/Stat3 functionally interacts to regulate a GAS-containing promoter and that this interaction is necessary and sufficient to prevent TGF-α-induced proliferation. This study underscores a novel mechanism of cross-coupling between growth factors and intracellular receptors that does not require the phosphorylation of the AF-1 domain and that does not result in ERα activation on ERE promoters but facilitates its functional interaction with other transcription factors, namely Stat3.

Several lines of evidence demonstrate that ProTα is associated to cell proliferation (43), and its transcription is positively regulated by estrogen (16, 42). Indeed, E2 treatment of SK-ER3 cells induces an increase in ProTα mRNA and thymidine incorporation (Fig. 5). Furthermore, ProTα has been shown to facilitate ERα-mediated transcription on its responsive element ERE by removing REA (47). We speculate that the initial synthesis of ProTα helps to release REA from ERα, thus increasing TGF-α synthesis. Indeed, here we show that the initial response of SK-ER3 to E2 is increased proliferation. However, after 48 h, ProTα mRNA levels decline and cells strongly decrease their proliferation rate and start differentiating. This raises the question regarding the mechanism interrupting the receptor activity on ERE promoters and initiating the regulation of genes associated with cell differentiation that are not primary targets for ER. On the basis of our data, it might be speculated that the initial production of TGF-α triggers, through the EGFR signaling cascade, ligand-independent activation of ERα, resulting in its association with intranuclear factors abundant in SK-ER3 such as Stat3. This protein/protein interaction subtracts ER from the binding to the ERE-containing promoters, shifting the cell program toward differentiation (Fig. 5). The fact that REA cannot modify cell differentiation suggests that this factor is not implicated in Stat3/ERα interaction.

We here propose that ERα might have proliferative or anti-proliferative action depending on the relative content of the nuclear factors capable of interaction with the receptor. Our model predicts that ProTα strengthens the action of ERα on the ERE promoters. In its absence, ERα is free to interact with other transcription factors, such as Stat3, with opposite physiological consequences. Indeed, we here show that ProTα prevents ERα-dependent transcriptional activation of Stat3 on its responsive element and that, conversely, high expression of Stat3 induces ERα-dependent differentiation of SK-N-BE cells (Fig. 7). Supporting this view is the fact that in SK-ER3 neuroblastoma cells estrogen acts as a differentiation factor, whereas in other cell types, E2 induces proliferation. Interestingly, high levels of ProTα are associated with high proliferative ER-positive neoplasia (19). The results of this study might be of relevance also for the understanding of the activity of ER in other tissues and might provide novel target for limiting ERα-dependent proliferation in ER-positive neoplasia.

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