The G Protein-coupled Receptor GPR30 Mediates c-fos Up-regulation by 17β-Estradiol and Phytoestrogens in Breast Cancer Cells*

Marcello Maggiolini, Adele Vivacqua, Giovanna Fasanella, Anna Grazia Recchia, Diego Sisci, Vincenzo Pezzi, Daniela Montanaro, Anna Maria Musti, Didier Picard¶, and Sebastiano Ando§¶

From the Departments of Pharmaco-Biology and Cellular Biology, University of Calabria, 87030 Rende (CS), Italy and the ¶Département de Biologie Cellulaire, Université de Genève, Sciences III, CH-1211 Genève 4, Switzerland

A growing body of evidence concerning estrogen effects cannot be explained by the classic model of hormone action, which involves the binding to estrogen receptors (ERs) α and ERβ and the interaction of the steroid-receptor complex with specific DNA sequences associated with target genes. Using c-fos proto-oncogene expression as an early molecular marker of estrogen action in ERα-positive MCF7 and ER-negative SKBR3 breast cancer cells, we have discovered that 17β-estradiol (E2), and the two major phytoestrogens, genistein and quercetin, stimulate c-fos expression through ERα as well as through an ER-independent manner via the G protein-coupled receptor homologue GPR30. The c-fos response is repressed in GPR30-expressing SKBR3 cells transfected with an antisense oligonucleotide against GPR30 and reconstituted in GPR30-deficient MDA-MB 231 and BT-20 breast cancer cells transfected with a GPR30 expression vector. GPR30-dependent activation of ERK1/2 by E2 and phytoestrogens occurs via a Gβγ-associated pertussis toxin-sensitive pathway that requires both Src-related and EGF receptor tyrosine kinase activities. The ability of E2 and phytoestrogens to regulate the expression of growth-related genes such as c-fos even in the absence of ER has interesting implications for understanding breast cancer progression.

Estradiol (E2)1 and natural estrogen-like compounds, such as genistein and quercetin, bind to and activate estrogen receptors (ER) α and β, which in turn regulate the expression of target genes directly and/or indirectly via protein-protein interactions with other transcription factors (1–7). Although it is often, but not always, straightforward to link the physiological effects of estrogens to the genomic model of the ligand-receptor complex activity, considerable controversy still exists on the ability of E2 to elicit transcriptional responses independently of ERα and ERβ.

A variety of molecules located close to the plasma membrane, such as the G protein-coupled receptors (GPRs) (8–11), Src, Ras, and Raf (12–14), have recently been recognized to mediate multiple extracellular stimuli, such as those induced by E2, in a cell context-specific manner (15–18). For instance, it has been proposed that in breast cancer cells, E2 induces ERK phosphorylation via the orphan GPR, named GPR30 (9). Interestingly, such responses elicited by E2 do not require ER expression and/or localization at the membrane level, as shown on the contrary by other investigations in various cell types (8, 14, 19–23). In this respect, the potential of E2 to trigger different pathways that integrate cell surface signaling with gene transcription has attracted an increased interest toward the identification of agonistic or antagonistic compounds.

The proto-oncogene c-fos plays a relevant role in the regulation of normal cell growth, differentiation, and cellular transformation processes (24–27). Besides, c-fos represents a prototypical “immediate early” gene since its expression is rapidly induced by different extracellular stimuli including mitogens and hormones (28–43). The nuclear protein encoded by c-fos interacts with c-jun family members to form the heterodimeric activating protein-1 transcription factor complex (24–27). The fos-jun heterodimers binding to activating protein-1 sites located within mammalian gene promoters regulates gene expression in a specific manner depending on cellular and promoter context as well as interacting proteins (44–49). Moreover, c-fos and other members of the fos family, such as fosB, fra-1, and fra-2, bind to sites identified in the regulatory region of target genes modulating the late response expression of critical factors for cell cycle re-entry, such as cyclin D1 (Ref. 50 and references therein).

The transcription of c-fos is controlled by multiple cis-elements present in the gene promoter: the cAMP-response element that binds to cAMP-response element-binding protein (51), the Sis-inducible enhancer that is recognized by the signal transducers and activators of transcription (STAT) group of transcription factors (52), the serum-response element that mediates c-fos induction by growth factors, and other extracellular stimuli leading to activation of MAPK pathways (53–57). The serum-response element initially binds a dimer of the serum-response factor, the binding of which recruits the ternary complex factors including Elk-1 and serum-response factor accessory protein 1 and 2 (54). Several studies have shown that ERα also activates c-fos expression in breast cancer cells and that the hormone-sensitive site is localized to a 240-bp region (~1300 to ~1060) (30, 32, 36, 38, 39, 43, 45). Further analysis demonstrated an imperfect palindromic estrogen-re-
Fig. 1. Transcriptional activation of c-fos and Gal4-Elk1 reporters by E2, genistein (G), and quercetin (Q) in MCF7 cells. A, the luciferase reporter plasmid c-fos encoding a −2.2-kb-long upstream fragment of human c-fos is activated by 1 μM E2, genistein, and quercetin. A concentration of 10 μM PD 98059 (PD) and the presence of an expression vector carrying the menin gene inhibited the response, which was not modified by 10 μM wortmannin (WM). The deletion mutant c-fosΔERE encodes a −1172-bp upstream fragment of human c-fos. Although lacking the ERE sequence, the above reporter is still activated by 1 μM E2, genistein, and quercetin. A concentration of 10 μM PD 98059 and the expression vector for the menin gene reversed the response. Luciferase activities were normalized to the internal transfection control, and the relative light unit values of untreated cells were set as 100%, upon which the activity induced by treatments was calculated. Each data point represents the mean ± S.D. of three independent experiments performed in triplicate. (□), (■), (○), and (●) each indicate p < 0.05 for untreated cells (−) versus treatments.

B, the luciferase reporter plasmid for the fusion protein consisting of Elk1 and the Gal4 DNA binding domain is activated by 1 μM E2, genistein, and quercetin. A concentration of 10 μM PD 98059 and the expression vector for the menin gene reversed the response. Luciferase activities were normalized to the internal transfection control, and the relative light unit values of untreated cells were set as 100%, upon which the activity induced by treatments was calculated. Each data point represents the mean ± S.D. of three independent experiments performed in triplicate. (□), (■), (○), and (●) each indicate p < 0.05 for untreated cells (−) versus treatments.

RESEARCH DESIGN AND METHODS

GPR30-mediated Signals in Breast Cancer Cells

Responding element (ERE) within this sequence that is able to bind ERα in gel mobility shift assays but is not sufficient for transactivation (58). Thereafter, it was shown that ERα must interact with Sp1 at a GC-rich site downstream from the imperfect palindromic ERE (58). In a subsequent study (59), both growth factors and E2 seem to converge on the serum-response element. In the latter case, however, the activation by E2 involved non-genomic signaling of ERα through the MAPK signaling pathway and phosphorylation and binding of Elk-1 to the serum-response element.

Herein, we show that early c-fos expression induced by E2 as well as the phytoestrogens genistein and quercetin is mediated at least in part by ERα in MCF7 breast cancer cells. However, the same agents are able to stimulate c-fos expression independently of ER expression in ER-negative breast cancer cells via GPR30.

EXPERIMENTAL PROCEDURES

Reagents—17β-estradiol, genistein, quercetin, 4-hydroxytamoxifen, cycloheximide, Wortmannin, pertussis toxin, PD 98059, LY 294002, dexamethasone, progesterone were purchased from Sigma. R1881, ICI 182780, tyrphostin AG 1478, and PP2 were obtained from AstraZeneca (Milan, Italy), Tocris Chemicals (Bristol, UK), Biomol Research Laboratories, Inc (DBA, Milan, Italy), and Calbiochem, respectively. All compounds were solubilized in dimethyl sulfoxide, except E2, hydroxytamoxifen (OHT), PD 98059, and LY 294002, which were dissolved in ethanol.

Plasmids—The firefly luciferase reporter plasmids were c-fos and the deletion mutant c-fosΔERE (which lacks the ERE sequence) encoding −2.2-kb and −1172-bp 5′ upstream fragments of human c-fos, respectively (gifts from K. Nose, Tokyo, Japan) (60) and Gal4-luc for the Gal4-Elk1 fusion protein. The latter two plasmids were described together with menin in our previous study (61). GPR30 and dominant negative ERK2 (DN/ERK2) expression vectors were kindly provided by R. Weigel (Philadelphia, PA) and M. Cobb (Dallas, TX). The Renilla luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as a transfection standard.

Cell Culture—MCF7, MDA-MB-231, BT-20 human breast cancer cells were a gift from E. Surname (Philadelphia, PA), whereas SKBR3 human breast cancer cells were from Picard’s laboratory (Geneve, Switzerland). MCF7 and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium/F12 without phenol red supplemented with 10% fetal bovine serum (Invitrogen). MCF7 and MDA-MB-231 cells were plated in Dulbecco’s modified Eagle’s medium/F12 without phenol red supplemented with 10% fetal bovine serum (Invitrogen). Cells were switched to medium without serum 24 h before transfections. 48 h before RT-PCR or immunoblot, 72 h before the evaluation of ERK1/ERK2 phosphorylation.

Transfections and Luciferase Assays—A total of 100,000 MCF7 cells were plated into 24-well dishes with 500 μl of regular growth medium/well the day before transfection. The medium was replaced with Dulbecco’s modified Eagle’s medium/F12 lacking phenol red and serum on the day of transfection, which was performed using FuGENE 6 reagent as recommended by the manufacturer (Roche Diagnostics) with a mixture containing 0.5 μg of reporter plasmid, 0.1 μg of effector plasmid where applicable, and 2 ng of pRLCMV. After 4 h, the medium was replaced again with serum-free Dulbecco’s modified Eagle’s medium lacking phenol red and containing 1 μM of each treatment, and then cells were incubated for 5 h. Luciferase activity was measured with the dual luciferase kit (Promega) according to the manufacturer’s recommendations. Firefly luciferase values were normalized to the internal transfection control provided by the Renilla luciferase activity. The normalized relative light unit values obtained from untreated cells were set as 100%, upon which the activity induced by treatments was calculated.

RT-PCR—The evaluation of gene expression was performed by semiquantitative RT-PCR as we have previously described (62). For c-fos, pS2, cathepsin D, and GPR30, with 36B4 used as a control gene, the primers were: 5′-CCCACTTTACCTCCACGCTC-3′ (c-fos forward) and 5′-TGCCAATCTCGGCTCTGCAA-3′ (c-fos reverse); 5′-TTCTATCTGTC-3′ (pS2 forward) and 5′-TTTGAGTAGTCAAA-3′ (pS2 reverse).
GTCAGAGC-3’ (pS2 reverse); 5’-AACACAGGGTGAGCTTC-3’ (cathepsin D forward) and 5’-ATGCAGGAACAGATCCTGTCTG-3’ (cathepsin D reverse); 5’-CTGGGAGTTTCTGTCTGA-3’ (GPR30 forward) and 5’-GCTTGGGAAGTCACATCCAT-3’ (GPR30 reverse); 5’-CTCAACATCTCCCTGCTTCCT-3’ (36B4 forward) and 5’-CAAATCCGCCATCTCGTC-3’ (36B4 reverse), to yield products of 381, 303, 210, 155 and 408 bp, respectively, with 20, 13, 20, 20, and 13 PCR cycles, respectively.

Western Blotting—Cells were grown in 10-cm dishes and exposed to ligands for 2 or 12 h before lysis in 500 μl of 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, a mixture of protease inhibitors containing 1 mM aprotinin, 20 mM phenylmethylsulfonyl fluoride, and 0.2 M sodium orthovanadate. Protein concentration was determined by Bradford reagent according to the manufacturer’s recommendations (Sigma). Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Biosciences), probed overnight at 4 °C with the antibodies against c-fos, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and pERK1/2, ERK2 (Cell Signaling Technology, Inc, Cellbio, Milan, Italy) and then revealed using the ECL system (Amersham Biosciences).

Antisense Oligodeoxynucleotide Experiments—Antisense oligonucleotides were synthesized as described (63–65) and purchased from MWG (Florence, Italy). The oligonucleotides used were: 5’-TTGGAAGGTCA-CATCCAT-3’ for GPR30; and 5’-GATCTCAGCACGGCAAAT-3’ for the scrambled control (66). For antisense experiments, a concentration of 200 nM of the indicated oligonucleotide was transfected following the procedure described above.

Statistical Analysis—Statistical analysis was performed using analysis of variance followed by Newman-Keuls testing to determine differences in means. p < 0.05 was considered as statistically significant.

RESULTS

E2, Genistein, and Quercetin Transactivate c-fos Promoter Constructs in MCF7 Cells—In our previous study (4), we demonstrated that an ERE reporter gene transfected in MCF7 cells responds to genistein and quercetin, like E2, through an ERα-mediated mechanism. Thus, in the present study, we first examined whether transiently transfected full-length human c-fos promoter (~2.2 kb) could respond to these phytoestrogens as reported previously for E2 (67). Fig. 1A demonstrates that genistein and quercetin mirror E2 activity. Activation by all three compounds is sensitive to the MAPK inhibitor PD 98059 and is unaffected by the PI3K inhibitor wortmannin (or LY 294002, not shown). In our previous investigation (61), we showed that the nuclear protein encoded by the tumor suppressor gene MEN1, named menin, inhibits ERK-dependent phosphorylation and activation of the ternary complex factor member Elk-1, which regulates c-fos gene expression. Therefore, we evaluated the action of menin on the transactivation property of estrogenic compounds using the above mentioned c-fos promoter assay and a corresponding reporter gene assay for monitoring the activation of Gal4-Elk-1. As expected, luciferase activity of both constructs was reversed in the presence of menin (Fig. 1, A and B). The transactivation of Elk-1 by E2 and phytoestrogens confirmed its involvement in c-fos expression, whereas the inhibitory effects of PD 98059 indicated that a MAPK-dependent mechanism is required for such a response (Fig. 1B). Interestingly, the transcriptional potential of these compounds was maintained using a mutant of the c-fos promoter lacking the ERE region (~1172 bp), suggesting that other pathways, not requiring an ERα-ERE interaction, contribute to c-fos transactivation.

E2, Genistein, and Quercetin Rapidly Induce c-fos mRNA Expression in ERα-positive MCF7 Cells—It has been largely reported (28–33,35–43,45,69–76) that c-fos is promptly stimulated by E2 and various agents. To evaluate whether phytoestrogens mimic E2 in this regard and in the activation of the two well known target genes pS2 and cathepsin D (77), we performed a semiquantitative RT-PCR comparing the mRNA levels after standardization on the housekeeping gene encoding the ribosomal protein 36B4. A treatment for only 1 h with E2 and phytoestrogens up-regulated c-fos expression in ERα-positive MCF7 cells (Fig. 2A). The same results were obtained for pS2 and cathepsin D with a 12-h exposure, whereas c-fos returned to levels resembling those of untreated cells (Fig. 2B).

E2, Genistein, and Quercetin Up-regulate c-fos Protein Levels in MCF7 Cells—The aforementioned results prompted us to evaluate whether c-fos protein levels follow the mRNA increase
observed after a short (2-h) treatment of ERα-positive MCF7 cells. A similar exposure with E2, genistein, and quercetin also resulted in a significant c-fos protein induction (Fig. 3A) that was dependent on protein synthesis, as ascertained by pretreating cells with the protein inhibitor cycloheximide (Fig. 3B). To verify the involvement of ERα in this process, we used the two ER antagonists ICI 182780 and OHT, which both partially reversed c-fos stimulation (Fig. 3, C and D). c-fos induction was also inhibited by the MAPK inhibitor PD 98059 and by transfection of an expression vector encoding DN/ERK2 (Fig. 3, E and F). Thus, both ERα and MAPK pathways may contribute to c-fos protein expression, although these two path-
Fig. 4. Immunoblots of c-fos from ER-negative SKBR3 cells treated with 1 μM E2, genistein (G), and quercetin (Q) for the indicated times. β-actin was used as a loading control. E2, genistein, and quercetin rapidly induced c-fos protein expression (A), which was blocked by pretreatment (6 h) with 50 μM protein synthesis inhibitor cycloheximide (Cx) (B). A concentration of 10 μM of the antiestrogens ICI 182780 (ICI) (C) and OHT (D) or PI3K inhibitor wortmannin (WM) (G) did not modify stimulations observed upon treatments, which were completely inhibited by 10 μM of the MAPK inhibitor PD 98059 (PD) (E) or in the presence of DN/ERK2 (F). Pretreatments (30 min) with 100 ng/ml of a G protein inhibitor pertussin toxin (PT), 10 μM Src family tyrosine kinase inhibitor PP2, 10 μM EGF receptor kinase inhibitor tyrphostin AG 1478 abrogated the up-regulation of c-fos by treatments (H–J, respectively). 1 μM dexamethasone (Dex), progesterone (Prog), or R1881 did not induce changes in c-fos protein levels (K), which were also similar to controls after a 12-h exposure to E2, genistein, and quercetin (L). The side panels show the quantitative representations of data (mean ± S.D.) of three independent experiments performed for each condition. ■ indicates p < 0.05 for untreated cells (−) versus treatments.
ways cannot be considered as mutually exclusive (see “Discussion”). The inhibitors wortmannin (Fig. 3G) and LY 294002 (data not shown) did not alter the c-fos stimulation observed with the compounds used, suggesting that PI3K is not involved in this process. After a 12-h exposure with E2, genistein, and quercetin, the levels of c-fos were similar to those of untreated cells (Fig. 3H) paralleling the mRNA levels (Fig. 2B).

E2, Genistein, and Quercetin Stimulate c-fos Protein Expression in ER-negative SKBR3 Cells—Are E2 and phytoestrogens able to trigger c-fos stimulation independently of ER in breast cancer cells, and if so, what is the signal transduction pathway involved in such a response? The partial inhibitory effects exerted by ICI 182780 and OHT in ERα-positive MCF7 cells (Fig. 3, C and D) prompted us to further investigate possible mechanisms regulating c-fos expression in ER-negative SKBR3 cells. Surprisingly, E2 as well as genistein and quercetin induced c-fos protein expression in SKBR3 cells as in MCF7 cells (Fig. 4, A and B). The ER antagonists ICI 182780 and OHT, which by themselves had no effect on c-fos stimulation (data not shown), did not alter the response to these treatments (Fig. 4, C and D), whereas the MAPK inhibitor PD 98059 and DN/ERK2 (see above) completely reversed the response (Fig. 4, E and F). Next, as observed in MCF7 cells, the PI3K inhibitors wortmannin (Fig. 4G) and LY 294002 (data not shown) did not modify c-fos induction. Considering that a large variety of extracellular ligands signal through GPRs and result in ERK1/2 activation (9, 78, 79), we examined whether GPR mediates c-fos induction by E2, genistein, and quercetin in ER-negative SKBR3 cells. These cells express higher levels of this orphan receptor as compared with other breast cancer cell lines as observed in the present study (data not shown) and a previous study (80). After ligand-receptor interaction, the Gα-subunit protein dissociates from the heterotrimeric Gαβγ complex, leading to MAPK activation through a mechanism sensitive to pertussis toxin and tyrosine kinase inhibitors. Interestingly, pretreatment with either pertussis toxin or the Src family tyrosine kinase inhibitor PP2 completely abrogated the ability of the tested compounds to stimulate c-fos expression in SKBR3 cells (Fig. 4, H and I). A previous study (9) reported that GPR30 can promote the tyrosine phosphorylation of the EGF receptor (EGFR), which in turn leads to ERK1/2 activation. It is worth noting that the EGFR kinase inhibitor, tyrphostin AG-1478, blocked c-fos induction by E2 and phytoestrogens (Fig. 4J), confirming an involvement of the EGFR in this pathway. To analyze the ligand specificity of the rapid c-fos response observed in SKBR3 cells, we performed treatments with other steroids, including dexamethasone, progesterone, and R1881. These all failed to induce c-fos expression (Fig. 4K), as did 17α-estradiol (data not shown). After treatment of cells with E2, genistein, and quercetin for 12 h, the rapid increase of c-fos was no longer noticeable (Fig. 4L).

To provide evidence that c-fos stimulation depends on MAPK in both ERα-positive MCF7 cells and ER-negative SKBR3 cells, we evaluated ERK1/2 phosphorylation after a very short treatment (5 min) with E2, genistein, and quercetin. Interestingly, all compounds were able to activate ERK1/2 in both breast cancer cell lines without changes in total ERK2 (Fig. 5, A and B), suggesting that c-fos stimulation can occur via MAPK independently of ER expression.

Furthermore, to investigate the involvement of GPR30 in the up-regulation of c-fos by E2, genistein, and quercetin, we transfected a specific GPR30 antisense oligonucleotide, which abrogated the response to agents used, whereas a control oligonucleotide had no effect (Fig. 6). This result clearly suggested that GPR30 mediates c-fos induction by E2 and phytoestrogens in SKBR3 cells.

**DISCUSSION**

The genomic response to the binding of ERα with its cognate ligand E2 is a relatively long term effect, which involves an appropriate fit of the hormone in the receptor binding pocket to produce a complex of sufficient stability and lifetime (81). As a consequence, multiple protein-protein and protein-nucleic acid interactions trigger the enzymatic reactions required for the remodeling of the chromatin architecture and changes in the rate of gene transcription (81). Nonetheless, a variety of cell types respond to estrogens in a very short time, making the classical ER-mediated mechanism of action unlikely (82). Thus, the ability of estrogens to interact with a putative membrane-associated ER has been associated with many rapid effects (83), although it is uncertain whether these activities are mediated by proteins similar or distinct from those involved in the well known transcriptional events occurring after E2 stimulation. Furthermore, the potential for signaling cross-talk between the classical ER-mediated mechanism of action and alternative signal transduction pathways triggered by E2 still remains to be explored.

We have demonstrated for the first time to our knowledge that E2 and the two major phytoestrogens genistein and quercetin are able to induce rapid c-fos up-regulation in breast cancer cells by a GPR30-mediated mechanism. In addition, the results obtained in MCF7 cells indicate that ERα contributes to this response, showing its potential to regulate the expression of both early and late genes. Collectively, these findings sug-
gest that endogenous as well as environmental estrogens control relevant genomic events through diverse signaling pathways in both ER-positive and ER-negative breast cancer cells (Fig. 8).

Of note, using the SKBR3 cells as a model system, we have determined that the G protein-coupled orphan receptor GPR30, via rapid ERK1/ERK2 activation, mediates an early c-fos up-regulation by E2 and even genistein and quercetin without the involvement of ERα. Moreover, the GPR30-deficient MDA-MB 231 and BT-20 breast cancer cells can be converted to cells that up-regulate c-fos in response to E2 and phytoestrogens upon heterologous expression of GPR30.

Consistent with the above observations, agents able to block G protein signaling such as pertussis toxin or the Src family-specific tyrosine kinase inhibitor PP2, as well the MAPK inhibitor PD 98059 or DN/ERK2, prevent c-fos induction by treatments. Considering that EGFR tyrosine kinase activity was required for GPR30-dependent ERK1/ERK2 activation by E2 (9), we also ascertained its role in the up-regulated expression of c-fos in SKBR3 cells. Indeed, the EGFR kinase inhibitor tyrphostin AG-1478 abrogated the response to E2 and phytoestrogens.

Given the above described potential of estrogen-like compounds on c-fos modulation, we wanted to evaluate an eventual response to other classes of hormones. Steroid receptor agonists tested, such as dexamethasone, progesterone, R1881, or...
even 17α-estradiol, did not promote an enhancement of c-fos, clearly suggesting that the GPR30-mediated cascade is specifically activated by E2 and phytoestrogens. This result obtained in SKBR3 cells was in agreement with a previous study (30), which demonstrated the lack of c-fos sensitivity to treatment with the same agents in MCF7 cells.

Could the pure antiestrogen ICI 182780 or the mixed agonist/antagonist OHT modify the c-erbB2 expression in breast cancer cells. In the absence of ERα expression in breast cancer cells.

**Model of the potential signal transduction pathways triggered by E2, genistein (**G**), and quercetin (**Q**) for c-fos expression in breast cancer cells. SRE, serum-response element; SRF, serum-response factor.

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