IQGAP1 Binds to Estrogen Receptor-α and Modulates Its Function*

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The estrogen receptor (ER)3 participates in a diverse range of biological functions, including cell proliferation, apoptosis, migration and differentiation (1). Humans have two isoforms of ER, termed α and β, which are expressed from different genes (2). ERα and ERβ contain analogous structural and functional domains, including the ligand-independent transcriptional activation function-1 located in regions A and B, a DNA-binding domain (DBD) in region C, a hinge region (region D), and hormone-binding domain/ligand-dependent activation function-2 (region E). Notwithstanding these similarities, the receptors differ in both their tissue distribution and functions (1, 2). 17β-Estradiol (E2) binding to the ligand-binding domain of ERα can produce distinct effects in the target cells by stimulat- ing two pathways. These are the “classic” pathway in the nucleus that enhances gene transcription and extranuclear actions that are initiated from ERα in the plasma membrane and result in rapid actions of E2 by activating signaling pathways (3). In the classic pathway, E2 induces a change in ERα conformation, resulting in dimerization of the receptor. In the nucleus, the E2-ERα complex binds either directly to estrogen response elements in the promoters of target genes or indirectly through protein-protein interactions with other transcription factor complexes like Fox-Jun (activator protein-1 response elements) and influences transcription of genes that lack estrogen response elements (2, 4). Multiple ERα target genes have been identified, including activator protein-1 (5), specific protein-1 (6), progesterone receptor (PR) (7), and pS2/trefoil factor 1 (8).

Distinct co-regulatory proteins are recruited to ERα and modulate ERα function by serving as co-repressors or co-activators (2). Accumulating evidence reveals that ERα associates with interconnected networks of proteins that maintain the structure and function of the receptor and influence estrogen-responsive gene expression (9). One of the proteins that interact with ERα is the Ca2+ signaling protein calmodulin. Prior work from our laboratory characterized the interaction between ERα and calmodulin. We demonstrated that calmodulin binds directly to ERα in a Ca2+-regulated manner (10). Calmodulin binding promotes the stability of ERα by sequestering it away from the ubiquitin-proteasome pathway (11). Interestingly, calmodulin is also necessary for E2-stimulated transcriptional activation of ERα (12). Thus, specific cellular proteins can influence ERα degradation and transcriptional activity.

IQGAP1 is a ubiquitously expressed scaffold protein that associates with a wide repertoire of binding partners. Almost 100 proteins have been identified that interact with IQGAP1 either directly or in a multiprotein complex (13, 14). These range from signaling proteins and small GTPases to cytoskeletal components and kinases (13–15). IQGAP1 also binds to and regulates the function of selected growth factor receptors, such as human epidermal growth factor receptor-2 (16), epi-
ERα target gene expression results from the coordinated action of ERα and its co-regulators (4). Most of these co-regulators contain an LXXL motif (L, leucine; X, any amino acid) that interacts with the ligand-binding domain of ERα (20). Inspection of the amino acid sequence of human IQGAP1 revealed three LXXL motifs, raising the possibility that IQGAP1 might bind ERα. In this study, we demonstrated that IQGAP1 and ERα associate in vitro and co-immunoprecipitate from cells. Binding was regulated by E2, and the ability of E2 to induce transcriptional activation was impaired in cells in which IQGAP1 was specifically knocked down by siRNA.

**EXPERIMENTAL PROCEDURES**

**Materials**—MCF-7, T47D, and HEK293 cells were obtained from the American Type Culture Collection. All reagents for tissue culture were bought from Invitrogen. Protein A-Sepharose and glutathione-Sepharose were purchased from GE Healthcare. Anti-FLAG affinity gel was from Sigma-Aldrich. PVDF membranes were purchased from Millipore Corp. Anti-ERα polyclonal and monoclonal antibodies, anti-ERβ polyclonal antibodies, anti-FLAG polyclonal antibody, rabbit IgG, and mouse IgG were obtained from Santa Cruz Biotechnology. Anti-IQGAP1 polyclonal antibodies have been characterized previously (21). Blocking buffer and infrared dye-conjugated (IRDye) antibodies, both anti-mouse and anti-rabbit, were obtained from LI-COR Biosciences. Recombinant human ERα and mouse IgG were obtained from Santa Cruz Biotechnology. Anti-FLAG affinity gel was from Sigma-Aldrich. Anti-FLAG monoclonal antibody and IRDye-conjugated anti-mouse antibody for 1 h, and antigen-antibody complexes were detected using the Odyssey imaging system (LI-COR Biosciences). The lower portion of the gel containing GST and ERα was stained with Coomassie Blue.

For GST-IQGAP1 pulldown, recombinant human ERα (1 μg) was incubated with 1 μg of GST or 1 μg of GST–IQGAP1 on glutathione-Sepharose beads in Buffer B for 3 h at 4 °C. After washing the beads five times with Buffer A, samples were resolved by SDS-PAGE. The gel was cut at the 100-kDa region. The top part of the gel was transferred to PVDF, blocked with Blocking Buffer (LI-COR Biosciences) for 1 h at 22 °C, and then probed with anti-IQGAP1 polyclonal antibodies overnight at 4 °C. The membrane was incubated with IRDye-conjugated anti-rabbit antibody for 1 h, and antigen-antibody complexes were detected using the Odyssey imaging system (LI-COR Biosciences). The lower portion of the gel was transferred to PVDF and processed for Western blotting with anti-ERα monoclonal antibody and IRDye-conjugated anti-mouse antibody. GST-IQGAP1 pulldown of recombinant human ERβ was performed essentially as described for ERα except that the blots were probed with anti-ERβ antibody.

**Cell Culture and Transfection**—HEK293 and MCF-7 cells were maintained in DMEM, and T47D cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium. Both media were supplemented with 10% FBS and 1% penicillin/streptomycin. HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transient overexpression of IQGAP1 increased the amount of the protein in cell lysates by 1.95 ± 0.23-fold (mean ± S.E., n = 5).
lysat were incubated with 4 µg of GST, GST-ERα, or GST-IQGAP1 for 3 h at 4 °C. After centrifugation, samples were washed five times with Buffer A and separated by SDS-PAGE. The gel was cut at the 100- and 37-kDa regions. For GST-IQGAP1 pulldown, the top and bottom portions containing GST-IQGAP1 and GST proteins, respectively, were stained with Coomassie Blue. The middle part of the gel was transferred to PVDF. For GST-ERα pulldown, the gel was cut at 100 kDa. The bottom portion of the gel was stained with Coomassie, and the top portion was transferred to PVDF. After transferring to PVDF, samples were processed by Western blotting.

Transcription and Translation (TnT) Product Production and Binding Analysis—[35S]Methionine-labeled TnT products were synthesized using the TnT Quick Coupled Transcription/Translation system (Promega) essentially as described previously (16). Briefly, 1 µg each of pcDNA3-IQGAP1, IQGAP1-N, IQGAP1-C, IQGAP1-IQ, IQGAP1-IQ, ERα, ERα(2-270), ERα(180-595), ERα(300-595), and ERα(353-595). ERαΔ185-240, ERαΔ240-311, ERαΔ240-270, ERαΔ270-311, or ERβ was incubated with 40 µl of TnT Quick Master Mix and 20 µCi of [35S]methionine (PerkinElmer Life Sciences) for 90 min at 30 °C. TnT products were diluted in Buffer A and used in pull-down assays.

Generation of Cell Lines with Stable IQGAP1 Knockdown—To obtain a control plasmid for knockdown experiments, siRNA sequences of Renilla luciferase, 5-AAAACAGCA-GAAACUGCU-3 (sense) and 5-CAGCAUUUUCUCAUGUU-3 (antisense), were inserted into the pRETRO-SUPER vector (OligoEngine). The following day, the medium was replaced with fresh medium containing 100 nM E2 or vehicle (ethanol). After 15 min, 30 min, 60 min, 120 min or 360 min, E2 stimulation was stopped by washing cells with PBS and lysing them with Buffer B. Immunoprecipitation with anti-IQGAP1 polyclonal antibodies and Western blotting were performed as described above.

Quantitative RT-PCR—To measure pS2 hnRNA, MCF-7 cells were incubated in phenol red-free medium for 24 h. Then vehicle (EtOH) or E2 (to obtain a final concentration of 100 nM) was added to the medium. After 6 h, cells were harvested, and total RNA was isolated from the cells using TRIzol (Invitrogen). 1 µg of RNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems) according to the manufacturer’s instructions. RT-PCR was performed on a StepOnePlus Real Time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM forward and reverse primers. The primers used were: pS2 forward primer, 5-TTGGAAGAAGGTGAGTGG-3; 60 °C then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3. RT-PCR enzyme activation was initiated for 10 min at 95 °C and then amplified by 40 cycles (15 s at 95 °C and 1 min at 60 °C). All samples were assayed in triplicate, and β-actin, forward primer, 5-GCTGGTATGTTGCTTCCA-3; reverse primer, 5-GGTTGTTGCTTCCA-3.
The specificity of binding was confirmed by the absence of ERα GST, and the purified protein was incubated with a GST fusion protein of full-length IQGAP1, and complexes were isolated and washed as described under “Experimental Procedures.” The samples were separated by SDS-PAGE, and the gel was cut at the 100-kDa region. The lower portion of the gel was transferred to PVDF, and the blot was probed with anti-IQGAP1 polyclonal antibodies (WB; top panel). The bottom part of the gel was stained with Coomassie Blue (Coomassie; lower panel). Input is pure IQGAP1. Data are representative of five independent experiments. WB, Western blot.

**RESULTS**

**IQGAP1 and ERα Bind Directly in Vitro**—Analysis was performed in vitro with pure proteins to ascertain whether IQGAP1 and ERα interact. Pure ERα was incubated with a GST fusion protein of full-length IQGAP1, and complexes were isolated with glutathione-Sepharose beads. Western blotting showed that ERα bound to IQGAP1 (Fig. 1A). By contrast, no ERα binding was detected to GST alone, validating the specificity of the association with IQGAP1. The interaction was confirmed by pulldown with GST-ERα. IQGAP1 was cleaved from GST, and the purified protein was incubated with a GST fusion protein of full-length ERα. Complexes were isolated, and Western blotting was performed. IQGAP1 bound GST-ERα (Fig. 1B). No IQGAP1 was detected with GST alone. Coomassie staining showed the expression of GST-tagged IQGAP1 and ERα proteins (Fig. 1).

**ERα and IQGAP1 Interact in Cells**—To determine whether IQGAP1 and ERα interact in a normal cell milieu, we used cultured human breast epithelial cell lines that express ERα. MCF-7 cells were lysed and incubated with GST-IQGAP1. Endogenous ERα bound to GST-IQGAP1 (Fig. 2A, left panel). The specificity of binding was confirmed by the absence of ERα from the samples incubated with GST alone. Essentially identical results were obtained with a second ERα-containing human breast epithelial cell line, T47D (Fig. 2A, right panel).

A reciprocal analysis was conducted with GST-ERα to investigate whether endogenous IQGAP1 binds to ERα. MCF-7 and T47D cell lysates were incubated with GST-ERα. Western blotting documented that IQGAP1 in both cell lines bound specifically to GST-ERα (Fig. 2B). Coomassie staining confirmed the expression of the GST-tagged proteins (Fig. 2).

**IQGAP1 and ERα Co-immunoprecipitate**—The interaction between IQGAP1 and ERα in intact cells was evaluated by immunoprecipitation. HEK293 cells were transiently co-transfected with ERα and IQGAP1. Immunoprecipitation with anti-ERα antibodies revealed that IQGAP1 binds to ERα in cells (Fig. 3A). No IQGAP1 was detected in the samples precipitated with rabbit IgG, validating the specificity of the interaction. Western blotting confirmed that HEK293 cells did not express ERα, and neither ERα nor IQGAP1 was detected in anti-ERα immunoprecipitates.
precipitates from cells transfected with vector only (Fig. 3A). The transfection was repeated, and lysates were immunoprecipitated with anti-IQGAP1 antibody. ERα co-immunoprecipitated with IQGAP1 (Fig. 3B). No ERα was detected in samples precipitated with IgG or from the cells immunoprecipitated with anti-IQGAP1 antibody but not transfected with ERα. These data confirm the specificity of the interaction. We used both MCF-7 and T47D cells to examine whether endogenous IQGAP1 binds to endogenous ERα. Cells were exposed to a cross-linker, paraformaldehyde, before lysis. Formaldehyde, which has short spacer arms, is widely used to stabilize protein-protein interactions. Immunoprecipitation with anti-ERα antibody revealed that IQGAP1 co-immunoprecipitated with ERα from MCF-7 (Fig. 3C, left panel) and T47D cells (Fig. 3C, right panel). Very little protein was seen in samples precipitated with IgG, indicating that endogenous ERα binds to endogenous IQGAP1 in both MCF-7 and T47D cells.

**IQGAP1 Binds Estrogen Receptors**

**FIGURE 3. ERα and IQGAP1 co-immunoprecipitate from cell lysates.** A, HEK293 cells were transiently transfected with both IQGAP1 and ERα plasmids or with empty vector alone. After lysis, equal amounts of protein were loaded directly onto the gel (lysates) or immunoprecipitated (IP) with either anti-ERα polyclonal antibodies (ERα) or rabbit IgG as a negative control. Immune complexes were isolated with protein A-Sepharose. Samples were processed by Western blotting for IQGAP1 (Fig. 3C, right panel) and ERα (Fig. 3A). B, HEK293 cells were transiently transfected with empty vector or with empty vector alone. After lysis, equal amounts of protein were loaded directly onto the gel (lysates) or immunoprecipitated (IP) with either anti-IQGAP1 polyclonal antibodies (IQGAP1) or rabbit IgG as a negative control. Equal amounts of protein lysate were immunoprecipitated (IP) with either anti-IQGAP1 polyclonal antibodies (IQGAP1) or rabbit IgG as a negative control. Samples were processed by Western blotting, E, empty lane. All data in A and B are representative of at least three independent experimental determinations. C, untransfected MCF-7 (left panel) and T47D (right panel) cells were incubated with 0.2% paraformaldehyde and processed as described under “Experimental Procedures.” Equal amounts of cell lysates were immunoprecipitated with anti-ERα antibody. Rabbit IgG was used as a negative control. Samples were processed by Western blotting for IQGAP1 and ERα. An aliquot of lysate was processed in parallel. All data are representative of six (for MCF-7 cells) or three (for T47D cells) independent experiments.

**FIGURE 4. ERα binds to the IQ region of IQGAP1.** A, schematic representation of IQGAP1 constructs. Full-length IQGAP1 and deletion mutants are depicted. The specific amino acid residues in each construct are indicated. CHD, calponin homology domain; WW, proline-binding domain; IQ, IQ domain containing four IQ motifs; GRD, Ras GTPase-activating protein-related domain; RGCT, Ras GTPrase-activating protein C terminus. FL, full-length IQGAP1; N, N-terminal half of IQGAP1; C, C-terminal half of IQGAP1; IQ, IQ region of IQGAP1; ΔIQ, full-length IQGAP1 that lacks the IQ region (amino acids 764–863). IQGAP1 construct binding to ERα is shown with binding status defined as follows: +, binding detected; −, no binding. B, [35S]methionine-labeled full-length IQGAP1 (FL), IQGAP1-N (N), IQGAP1-IQ (IQ), IQGAP1-C (C), or IQGAP1ΔIQ (ΔIQ) was incubated with equal amounts of GST-ERα (top panel). Each IQGAP1 construct was also incubated with GST alone (middle panel). Complexes were isolated with glutathione-Sepharose beads and washed. Samples were resolved by SDS-PAGE and transferred to PVDF, and blots were processed by autoradiography. In addition, an aliquot of each [35S]methionine-labeled TNT product (equivalent to 5% of the amount that was used in the pulldown) was resolved by SDS-PAGE, transferred to PVDF, and processed by autoradiography (input; bottom panel). The data are representative of three independent experiments.
but the C-terminal half of IQGAP1 did not bind. Specificity of binding to GST-ERα was confirmed by the absence of bands from the samples incubated with GST alone (Fig. 4B, middle panel). The level of expression of each TNT product was approximately equivalent (Fig. 4B, bottom panel).

Several proteins associate with the IQ region of IQGAP1 (13, 14). To ascertain whether ERα binds to the IQ region, a fragment comprising amino acids 717–916 of IQGAP1 was labeled with [35S]methionine and incubated with GST-ERα. Autoradiography demonstrated that the IQ region of IQGAP1 bound to ERαs (Fig. 4B, top panel). Consistent with these findings, deletion of the IQ domain (the construct is termed IQGAP1ΔIQ) abrogated the binding of IQGAP1 to ERα (Fig. 4B, top panel). These data reveal that the IQ region of IQGAP1 is necessary and sufficient for ERα binding.

**IQGAP1 Binds to the Hinge Region of ERα**—The TNT system was also used to identify the region of ERα with which IQGAP1 interacts. [35S]methionine-labeled full-length ERα and a series of ERα fragments and deletion mutants (Fig. 5A) were incubated with GST-IQGAP1. The complexes were isolated with glutathione-Sepharose beads and processed by SDS-PAGE and autoradiography. As observed with pure proteins and cell lysates (Figs. 1 and 2), full-length ERα expressed by the TNT system bound to GST-IQGAP1 (Fig. 5B, top panel). Although the N-terminal portion of ERαs bound to IQGAP1, the C-terminal portion (ERα300–595) did not bind. Extending the C-terminal portion proximally by adding the hinge region and DBD (ERα180–595)) restored binding equivalent to that of full-length ERαs (Fig. 5B, top panel). Similarly, ERα180–353, which comprises the DBD, hinge, and the proximal portion of the C-terminal of ERαs, bound IQGAP1 to essentially the same extent as full-length ERαs. Input data reveal that the amounts of each ERα fragment were essentially the same (Fig. 5B, bottom panel).

To further narrow the binding region, we deleted selected portions of ERαs from the DBD and hinge regions. Analysis of the deletion constructs revealed that removing amino acids 185–240 did not impair binding to IQGAP1 (Fig. 5C). By contrast, ERα constructs that lacked portions of the hinge region (namely ERα240–270 and ERα270–311) exhibited weaker binding, and removal of amino acids 240–311 abrogated binding. Collectively, these data strongly suggest that IQGAP1 binds to ERα between amino acids 240 and 311, and the whole hinge region of ERα is needed for effective binding.

**E2 Attenuates the Binding between IQGAP1 and ERαs**—The possible effect of E2 on the interaction between IQGAP1 and ERα was investigated. HEK293 cells transiently transfected with IQGAP1 and ERα were stimulated with E2 for different time intervals, and the association between the two proteins was examined by co-immunoprecipitation. Consistent with prior reports (10), we observed a decrease in the total ERαs in lysates of cells treated with E2 that became statistically significant at 360 min (Fig. 6, A and B). As anticipated, E2 did not significantly change the amount of IQGAP1 in the lysates or in the immunoprecipitates. By contrast, the amount of ERα that co-immunoprecipitated with IQGAP1 decreased in a time-dependent manner (Fig. 6, A and C). To correct for the reduction of total ERα produced by E2, we quantified both the total amount of ERα in the cells and the amount of ERα in anti-IQGAP1 immunoprecipitates. After correction for total ERαs in lysates, the amount of ERαs that co-immunoprecipitated with IQGAP1 from E2-stimulated cells was lower than that from control cells (Fig. 6D). Reduction was seen at the earliest time point (15 min) and became statistically significant at 60 min, and at 360 min of E2 treatment, the amount of ERαs that co-immunoprecipitated with IQGAP1 was reduced by 58 ± 6.5% (mean ± S.E., n = 3). These data reveal that E2 modulates the interaction between IQGAP1 and ERαs in cells.

**Knockdown of IQGAP1 Impairs ERα Function**—We evaluated the possible role of IQGAP1 in ERα function. ERα-positive MCF-7 cell lines were chosen because they express high levels of IQGAP1 (25, 27). We used two different MCF-7 cell lines with stable IQGAP1 knockdown by siRNA. These cells, termed MCF-siIQ8 and MCF-siIQ15, had IQGAP1 protein levels 65 ± 8 and 66 ± 4% (mean ± S.E., n = 4), respectively, lower than control cells (Fig. 7A, left panel, and B). E2-induced transcriptional activation of pS2 was measured. E2 produced a 7.2 ± 0.6-fold (mean ± S.E., n = 3) increase in pS2 hnRNA in MCF-7 cells stably expressing control siRNA (Fig. 7C). The ability of E2 to stimulate pS2 hnRNA was significantly attenuated when the amount of IQGAP1 was reduced. E2 stimulated pS2 hnRNA expression in MCF-siIQ8 and MCF-siIQ15 cells by only 2.7 ± 0.2- and 3.8 ± 0.5-fold (mean ± S.E.), respectively (Fig. 7C). Note that knockdown of IQGAP1 did not significantly reduce the amount of ERα in the MCF-7 cell lines (Fig. 7A). Moreover, the level of the ERαs in cells incubated with E2 was not significantly altered by IQGAP1 knockdown (Fig. 7A, right panel). E2 stimulation reduced the amount of ERαs as observed in Fig. 6A and described previously (10). Collectively, these data validate that IQGAP1 is required for maximal E2-stimulated transcriptional activation of pS2 in MCF-7 cells.

To extend these findings, we examined the effects of IQGAP1 on the ability of ERα to activate two other endogenous estrogen-responsive genes, namely PR and cyclin D1. Analysis revealed that E2 stimulation of PR hnRNA was significantly impaired in both MCF-siIQ8 and MCF-siIQ15 cells (Fig. 7D). Similarly, reducing the amount of IQGAP1 in MCF-7 human breast epithelial cells decreased stimulation of cyclin D1 hnRNA by E2 (Fig. 7E). These results support our observations with pS2 and confirm that endogenous IQGAP1 contributes to normal transcriptional function of ERα.

In addition to promoting transcription of selected genes, ERα mediates an increase in cell proliferation (1). We evaluated the possible role of IQGAP1 in this process. The ability of E2 to enhance proliferation of MCF-7 control cells was compared with cells with IQGAP1 knockdown, namely MCF-siIQ8 and MCF-siIQ15 cells. Reducing IQGAP1 levels in MCF-7 cells abrogated the stimulation of cell proliferation by E2 (Fig. 7F).

**ERβ Binds to IQGAP1**—We investigated the possible interaction between IQGAP1 and ERβ, which is another member of the ER family with a domain structure similar to that of ERα (Fig. 8A). Pure ERβ was incubated with a GST fusion protein of full-length IQGAP1, and complexes were isolated with glutathione-Sepharose beads. Western blotting showed that ERβ bound to IQGAP1 (Fig. 8B). By contrast, no ERβ binding was
detected to GST alone, validating the specificity of the association with IQGAP1. Coomassie staining showed GST-IQGAP1 and GST (Fig. 8B). ERβ, synthesized with TnT and labeled with [35S]methionine, bound specifically to GST-IQGAP1 (Fig. 8C). Thus, IQGAP1 and ERβ interact directly.

Immunoprecipitation was performed to ascertain whether IQGAP1 and ERβ associate in cells. HEK293 cells were transiently transfected with IQGAP1 and FLAG-tagged ERβ. Immunoprecipitation with anti-FLAG antibodies revealed that IQGAP1 bound to ERβ in cells (Fig. 9A). Analogous experiments were performed in which cells were transfected and lysates were immunoprecipitated with anti-IQGAP1 antibodies. ERβ co-immunoprecipitated with IQGAP1 (Fig. 9B). Neither IQGAP1 nor ERβ was seen in samples precipitated with IgG nor was ERβ seen in samples obtained from cells transfected with empty vector (Fig. 9, A and B). The blots were also probed with anti-ERβ antibodies, which confirmed that the bands detected with anti-FLAG antibodies are ERβ (data not shown). Collectively, these data reveal that ERβ and IQGAP1 interact in cells.

### FIGURE 5. IQGAP1 binds to the hinge region of ERα. A, schematic representation of ERα constructs. Full-length ERα (FL), truncated ERα fragments, and deletion mutants are depicted. Amino acid residues of each truncated fragment and the specific amino acids absent from each deletion construct (designated with Δ) are indicated. AF-1, activation function-1 (or region A/B); Hinge, hinge region (or region D); HBD/AF-2, hormone-binding domain/activation function-2 (or region E/F). Binding to IQGAP1 is defined as follows: +, binding; wk, weak binding; −, no binding. B, [35S]methionine-labeled full-length ERα (FL), ERα1–270, ERα180–353, and ERα180–333 were incubated with equal amounts of GST-IQGAP1 (top panel). Each ERα construct was also incubated with GST alone (middle panel). Complexes were isolated with glutathione-Sepharose beads and washed. Samples were resolved by SDS-PAGE and transferred to PVDF, and blots were processed by autoradiography. In addition, an aliquot of each [35S]methionine-labeled product (equivalent to 5% of the amount that was used for the pulldown) was processed by electrophoresis and blotting as described above (input; bottom panel). The data are representative of three independent experiments. C, [35S]methionine-labeled full-length ERα (FL), ERαΔ185–240, ERαΔ270–311, ERαΔ240–270, and ERαΔ240–311 were incubated with equal amounts of GST-IQGAP1 (top panel). Each ERα construct was also incubated with GST alone (middle panel). Complexes were isolated, and samples were processed as described for B. An aliquot of each [35S]methionine-labeled product was processed as described above (input; bottom panel). The data are representative of two independent experiments.
IQGAP1 interacts with numerous proteins, thereby coordinating the assembly of multiprotein complexes and modulating diverse signaling pathways (13). For example, IQGAP1 binds to several components of the epidermal growth factor receptor-Raf-MAPK cascade, regulating activation of ERK (13, 17, 28). Work from a number of groups has demonstrated that IQGAP1 associates with selected transmembrane receptors and participates in their normal function (13, 14). Here we show for the first time that IQGAP1 also interacts directly with nuclear receptors. We demonstrate that IQGAP1 binds to both ERα and ERβ and influences ERα transcriptional function.

**DISCUSSION**

IQGAP1 interacts with numerous proteins, thereby coordinating the assembly of multiprotein complexes and modulating diverse signaling pathways (13). For example, IQGAP1 binds to several components of the epidermal growth factor receptor-Raf-MAPK cascade, regulating activation of ERK (13, 17, 28). Work from a number of groups has demonstrated that IQGAP1 associates with selected transmembrane receptors and participates in their normal function (13, 14). Here we show for the first time that IQGAP1 also interacts directly with nuclear receptors. We demonstrate that IQGAP1 binds to both ERα and ERβ and influences ERα transcriptional function.

Based on the presence of three LXXLL motifs located at amino acid residues 347–351, 975–979, and 1646–1650 in IQGAP1, we predicted that ERα would bind one or more of these regions. The data did not validate this hypothesis. ERα did not associate with the C-terminal half of IQGAP1, which contains two of the LXXLL regions. Although ERα bound the N-terminal half of IQGAP1, which contains the other LXXLL motif, amino acids 717–916 of IQGAP1 were sufficient for binding ERα, indicating that the LXXLL motif at amino acids 347–351 does not mediate binding. Moreover, the observation that deletion of amino acids 763–864 from IQGAP1 eliminates its association with ERα confirms that the IQ region of IQGAP1
IQGAP1 Binds Estrogen Receptors

A

IQGAP1

ERα

β-actin

B

IQGAP1 (relative amount)

Control    silQ8    silQ15

C

ERα

D

PR

E

Cyclin D1

F

Cell proliferation

IQGAP1 Binds Estrogen Receptors
is the site to which ERα binds. Several other proteins, including calmodulin (29, 30), myosin light chain (31), Rap1 (32), human epidermal growth factor receptor-2 (16), epidermal growth factor receptor (17), and S100B (33), also bind to the IQ region of IQGAP1. Protein binding to IQGAP1 may be regulated. For example, Ca$^{2+}$ modulates the interaction of calmodulin with the IQ region of IQGAP1 (21, 29). Similarly, Zn$^{2+}$ promotes the association of S100B with the IQ motif of IQGAP1 (33). In addition, although there are no published data, it is likely that the different proteins bind the IQ domains of IQGAP1 with different affinities. Therefore, a cell may have several distinct IQGAP1 complexes with different proteins associated with the IQ region depending on signaling inputs, subcellular location, cell type, and other factors.

An important finding in our study is that E2 regulated the association between IQGAP1 and ERα. The effects of ligand binding on the interaction of receptors with IQGAP1 are diverse. For example, EGF has no effect on the association between IQGAP1 and epidermal growth factor receptor (17). By contrast, stimulation of Madin-Darby bovine kidney cells with FGF2 promotes binding of fibroblast growth factor recep-

**FIGURE 7. Knockdown of IQGAP1 alters ERα function.** A, MCF-7 cells were stably transfected with siRNA against Renilla luciferase (Control) or two distinct siRNAs directed against different regions of IQGAP1 (siIQ8 and siIQ15). Where indicated, cells were cultured in phenol red-free medium for 24 h followed by incubation with vehicle (EtOH) or 100 nM E$_2$ for 6 h (right panel). Cells were lysed, and equal amounts of protein lysate were analyzed by Western blotting. B, the amount of IQGAP1 in the Western blot was quantified with Image Studio 2.0 (LI-COR Biosciences) and corrected for the amount of actin in the corresponding sample. Data represent the means ± S.E. (error bars) of three independent experiments with control cells set as 1. ***, p < 0.001. C, MCF-7 cells stably expressing control siRNA, siIQ8, or siIQ15 were incubated in phenol red-free medium for 24 h followed by 6-h incubation with vehicle (EtOH; black bars) or 100 nM E$_2$ (white bars). Total RNA was extracted, and quantitative RT-PCR analysis was performed to measure p52 hnRNA. The amount of hnRNA in each sample was corrected for β-actin hnRNA in the same sample. Vehicle-treated cells were set as 1. The data represent the means ± S.E. (error bars) of three independent experiments, each performed in triplicate. *, p < 0.05; **, p < 0.01 compared with the control cell line treated with EtOH. D, MCF-7 cells expressing control siRNA, siIQ8, or siQ15 were incubated in phenol red-free medium with vehicle (EtOH; black bars) or 10 nM E$_2$ (white bars) for 4 h, and cyclin D1 hnRNA was measured by quantitative RT-PCR. Samples were analyzed as described for p52. A representative experiment of three independent determinations is shown. E, cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described under “Experimental Procedures.” Data are representative of three independent experiments, each performed in triplicate.

**FIGURE 8. ERβ binds to IQGAP1 in vitro.** A, schematic representation of ERα and ERβ. AF-1, activation function-1; Hinge, hinge region; HBD/AF-2, hormone-binding domain/activation function-2. The homology between the domains of the proteins is depicted below. The figure is adapted from Thomas and Gustafsson (1). B, 4 μg of GST-IQGAP1 (IQGAP1) or 4 μg of GST alone bound to glutathione-Sepharose was incubated with 1 μg of pure ERβ. Complexes were isolated and washed as described under “Experimental Procedures.” The samples were separated by SDS-PAGE, and the gel was cut at both the 100- and 37-kDa regions. The portion of the gel between 37 and 100 kDa was transferred to PVDF, and the blot was probed with anti-ERβ monoclonal antibody (WB, top panel). The top and bottom parts of the gel containing GST-IQGAP1 and GST, respectively, were stained with Coomassie Blue. Input is pure ERβ. C, [$^{35}$S]methionine-labeled ERβ was incubated with 4 μg of GST-IQGAP1 or GST alone. Complexes were isolated with glutathione-Sepharose beads and washed. Samples were resolved by SDS-PAGE and transferred to PVDF, and blots were processed by autoradiography. In addition, the [$^{35}$S]methionine-labeled product (equivalent to 5% of the amount that was used for the pulldown) was processed as described above (Input). Data are representative of at least two independent experiments. WB, Western blot.

**FIGURE 9. ERβ and IQGAP1 co-immunoprecipitate from cell lysates.** A, HEK293 cells were transiently transfected with both IQGAP1 and FLAG-tagged ERβ plasmids or with empty vector alone. After lysis, equal amounts of protein were loaded directly onto the gel (Lyates) or immunoprecipitated (IP) with either anti-FLAG affinity gel (Flag) or IgG (negative control). Samples were separated by SDS-PAGE and transferred to PVDF membrane. After blocking, the blot was probed with anti-IQGAP1 polyclonal (top panel) and anti-FLAG monoclonal antibodies (bottom panel). E, empty lane. B, HEK293 cells were transiently transfected as described for A. Equal amounts of protein lysate were immunoprecipitated (IP) with either anti-IQGAP1 polyclonal antibodies (IQ) or rabbit IgG (negative control). Aliquots of lysates not subjected to immunoprecipitation were processed in parallel (Lyates). Samples were processed by Western blotting and probed with anti-IQGAP1 polyclonal (top panel) and anti-FLAG monoclonal antibodies (bottom panel). E, empty lane. Data are representative of two independent experiments.
tor 1 to IQGAP1 (18). Similarly, VEGF enhances the interaction of vascular endothelial growth factor receptor-2 with IQGAP1 in human umbilical vein endothelial cells (19), and transforming growth factor β-1 (TGF-β1) increases the binding of IQGAP1 to TGF-β receptor II (34). In contrast to these data, we observed that E2 significantly attenuated the interaction between IQGAP1 and ERα in cells. To our knowledge, this is the first description of a ligand that reduces receptor binding to IQGAP1.

Prior publications have identified the participation of IQGAP1 in transcriptional activation. β-Catenin is an integral component of the cadherin cell adhesion complex, and it co-activates transcription factors in the nucleus (35). IQGAP1 binds β-catenin and regulates its function (36, 37). Overexpression of IQGAP1 significantly enhances β-catenin-mediated transcriptional co-activation in SW480 colon carcinoma cells (37). Although IQGAP1 was not detected in the nucleus, IQGAP1 overexpression increased the amount of β-catenin in the nucleus. Similarly, IQGAP1 regulates the nuclear translocation of nuclear factor of activated T cells (NFAT) and alters transcription of NFAT target genes (38). More recent studies have revealed that IQGAP1 regulates nuclear translocation and transcriptional activation of both nuclear factor-erythroid-related factor 2 (Nrf2) (39) and Dishevelled (40). Analogous to our observations with ERα transcriptional activation, knockdown of IQGAP1 impairs the transcriptional activation of target genes by Nrf2 and Dishevelled (39, 40). By contrast, CD8+ T cells isolated from IQGAP1-deficient mice exhibit increased expression of interferon-γ, an established target gene of NFAT (38). Thus, IQGAP1 exerts different effects on different gene transcription pathways.

For ERα, IQGAP1 is necessary for E2 to maximally stimulate transcription of the pS2, PR, and cyclin D1 genes. Knockdown of IQGAP1 by two distinct siRNAs directed against different regions of IQGAP1 significantly reduced the ability of E2 to stimulate transcription of pS2 and PR. This finding initially appears to contradict the observation that E2 attenuated the interaction between IQGAP1 and ERα in cells. The knockdown experiments imply that IQGAP1 is required for ERα to enhance pS2 transcription, whereas the co-immunoprecipitation experiments revealed that E2 reduced the interaction of ERα with IQGAP1 at the same time point (6 h) at which pS2 transcription was analyzed. Taken together, these data suggest that a direct interaction with IQGAP1 in the nucleus is not required for ERα to promote pS2 gene transcription. Although IQGAP1 has been reported in the nucleus, the pool is small (~5% of total IQGAP1) (41). Moreover, IQGAP1 accumulates in the nucleus at the G1/S phase of the cell cycle and appears to contribute to cell cycle progression. Although not impossible, it is unlikely that nuclear IQGAP1 makes a substantial contribution to ERα function. There are several possible ways in which IQGAP1 may modulate ERα function. 1) The binding may alter ERα degradation by the ubiquitin-proteasome system. The transcriptional activity of ERα is linked to receptor turnover. Inhibition of ERα degradation leads to its stabilization but also to loss of its transcriptional activity (42). However, there is no evidence that IQGAP1 alters ERα degradation as we observed that reducing the intracellular amount of IQGAP1 in MCF-7 cells did not significantly change the amount of ERα. Therefore, changing ERα degradation is probably not the mechanism by which IQGAP1 influences ERα transcription. 2) Numerous cofactors associate with ERα in interconnected networks of proteins (9). IQGAP1 is a scaffold that integrates signaling pathways and may coordinate the assembly of a multiprotein complex to regulate ERα function. 3) IQGAP1 may alter the ability of ERα to bind other proteins. IQGAP1 binds to the hinge (D) region of ERα. Initially believed to serve exclusively as a flexible linker between the DBD and ligand-binding domain, evidence accumulated over the last decade has identified important functional roles for the hinge region of ERα. For example, a recent study reveals that selective mutations in the D domain of ERα alter its nuclear translocation and its ability to activate its target genes activator protein-1, pS2, and specific protein-1 (43). One of the mutant ERα constructs generated in that study can translocate to the nucleus and bind to DNA in the presence of E2 but is unable to interact with the necessary tethering factors to activate activator protein-1 and specific protein-1 response elements. The hinge region is implicated in interconnections with some co-regulator molecules (42). It is possible that IQGAP1 sterically hinders the interaction of one (or more) co-regulators, thereby changing pS2, PR, and cyclin D1 transcription. 4) In addition, several post-translational modifications, including phosphorylation, acetylation, methylation, and ubiquitination, occur in the hinge region of ERα (44). These covalent additions, which affect receptor activity, could be modified by IQGAP1 binding. 5) A nuclear localization signal is contained in the hinge region, and IQGAP1 could conceivably regulate the nuclear translocation of ERα as it does for β-catenin, NFAT, and Nrf2 (37–39). 6) Finally, IQGAP1 is known to dimerize (22). Although the dimerization domain of ERα is outside the hinge region, IQGAP1 could enhance ERα dimerization, analogous to that produced by calmodulin. Detailed structural analysis reveals that binding of calmodulin to the hinge region of ERα promotes dimerization of ERα (45). These possible mechanisms are not mutually exclusive, and more than one may be responsible for the effects we observed with IQGAP1. Additional work is required to dissect these possibilities and elucidate the molecular mechanism(s) by which IQGAP1 regulates ERα transcriptional activation.

Association of IQGAP1 with members of the steroid receptor family is not confined to ERα. We observed a direct in vitro interaction of pure ERβ with pure IQGAP1. Importantly, the two proteins co-immunoprecipitated from cell lysates, revealing that they interact in cells. Further studies are needed to determine whether the interaction with IQGAP1 alters ERβ function. Moreover, several other nuclear receptors, including the androgen receptor, glucocorticoid receptor, progesterone receptor, thyroid receptor, and vitamin D receptor, have domain structures similar to that of ER (46). We are examining the possible interactions of these receptors with IQGAP1, and if binding is detected, we plan to investigate whether IQGAP1 has any effects on receptor function.

Our data identify a previously unrecognized interaction between the nuclear receptor ERα and IQGAP1. Experimental evidence supports the notion that IQGAP1 modulates ERα transcriptional activation of pS2, PR, and cyclin D1. These
observations enhance our comprehension of both ERα signaling and IQGAP1 function. Importantly, a large body of data implicates both ERα (47) and IQGAP1 (27, 48) in breast carcinoma. It seems feasible that therapeutic agents directed toward the interaction between ERα and IQGAP1 might be useful in the management of patients with breast carcinoma. 

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