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Thrombin Protease-activated Receptor-1 Signals through G_q- and G_{13}-initiated MAPK Cascades Regulating c-Jun Expression to Induce Cell Transformation*

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Although the ability of G protein-coupled receptors to stimulate normal and aberrant cell growth has been intensely investigated, the precise nature of the molecular mechanisms underlying their transforming potential are still not fully understood. In this study, we have taken advantage of the potent mitogenic effect of thrombin and the focus-forming activity of one of its receptors, protease-activated receptor-1, to dissect how this receptor coupled to $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ transduces signals from the membrane to the nucleus to initiate transcriptional events involved in cell transformation. Using endogenous and transfected thrombin receptors in NIH 3T3 cells, ectopic expression of muscarinic receptors coupled to $G\alpha_q$ and $G\alpha_i$, and chimeric G protein α subunits and murine fibroblasts deficient in $G\alpha_{q/11}$, and $G\alpha_{12/13}$, we show here that, although coupling to $G\alpha_i$ is sufficient to induce ERK activation, the ability to couple to $G\alpha_q$ and/or $G\alpha_{13}$ is necessary to induce c-*jun* expression and cell transformation. Furthermore, we show that $G\alpha_q$ and $G\alpha_{13}$ can initiate the activation of MAPK cascades, including JNK, p38, and ERK5, which in turn regulate the activity of transcription factors controlling expression from the c-*jun* promoter. We also present evidence that c-Jun and the kinases regulating its expression are integral components of the transforming pathway initiated by protease-activated receptor-1.

Growth factors acting on cell-surface receptors possessing an intrinsic tyrosine kinase activity can initiate the activation of multiple intracellular signaling pathways, which in turn control key biological processes, including cell proliferation, differentiation, adhesion, and migration and cell fate decisions (reviewed in Ref. 1). Subtle alterations in the normal activity of these tyrosine kinase receptors or their intracellular downstream targets can have dramatic biological consequences, as they may promote the aberrant growth and survival of tumor cells (for review, see Ref. 2). The discovery of the *mas* oncogene, the predicted structure of which resembles that of the G protein-coupled receptors (GPCRs)[1] rather than a tyrosine kinase receptor (3), provided the first evidence that heptahelical receptors can also harbor transforming potential. GPCRs represent the largest family of cell-surface receptors, and they regulate intracellular signaling pathways primarily by interacting with heterotrimeric G proteins composed of α, β, and γ subunits. Upon receptor activation, there is a conformational change that promotes the exchange of GDP bound to the α subunit for GTP and the release of $\beta\gamma$ dimers, thereby initiating a series of signaling events that culminate in a wide variety of cellular responses (4, 5). Constitutively activated mutant receptors (6) and receptors persistently activated by agonists (7, 8) were found to cause cell transformation. Furthermore, paracrine and autocrine stimulation of GPCRs by tumor-released agonists has been implicated in different types of neoplasias such as small cell lung carcinoma and prostate and gastric cancer (for review, see Refs. 9 and 10), thus highlighting a role for the large GPCR family in carcinogenesis. However, the molecular mechanisms underlying the transforming potential of GPCRs are still not fully understood.

Using a retroviral expression library approach to identify novel oncogenes from a mouse myeloid progenitor cell line, Whitehead *et al.* (11) identified several independent cDNAs encoding murine PAR-1, a thrombin-stimulated GPCR. Thrombin is a serine protease that exerts multiple physiological effects (12). Among them, the best known function of thrombin is its key role in blood coagulation. In addition, thrombin can act on many cell types, eliciting a large variety of cellular responses, including the regulation of cell proliferation and invasion and tumor growth (13, 14). At least three receptors for thrombin, PAR-1, PAR-3, and PAR-4, have been cloned thus far and found to belong to the GPCR superfamily (12). Rather than being a direct agonist for these receptors, thrombin acts by cleaving an Arg–Ser bond in their N-terminal extracellular domain, thereby generating a new N terminus that functions as a tethered agonist. The potent transforming potential of PAR-1 suggests that its deregulated expression can promote the aberrant activation of growth-promoting pathways. This

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[1] The abbreviations used are: GPCRs, G protein-coupled receptors; PAR, protease-activated receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; CAT, chloramphenicol acetyltransferase; MEF2, myocyte enhancer factor-2; HA, hemagglutinin; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MKK, mitogen-activated protein kinase kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; GST, glutathione *S*-transferase; ATF2, activating transcription factor-2; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; JIP-1, JNK-interacting protein-1; jAP1, c-*jun* AP1-like response element.

receptor is known to couple effectively to the $G\alpha_i$, $G\alpha_q$, and $G\alpha_{13}$ families of G protein α subunits (reviewed in Ref. 12). Indeed, activated forms of the $G\alpha_q$ and $G\alpha_{13}$ families can themselves transform NIH 3T3 cells (15, 16), supporting that at least these G proteins and their coupled receptors can promote cell-transforming pathways.

Of interest, thrombin can potently induce the nuclear expression of members of the AP1 transcription factor family (17), which is composed of members of the Jun and Fos families of nuclear proteins that bind as Jun dimers or Jun-Fos heterodimers to DNA sequences known as 12-*O*-tetradecanoylphorbol-13-acetate response elements on the regulatory region of target genes, thereby enhancing or inhibiting their expression (18, 19). In particular, thrombin can induce the rapid expression of the c-*jun* proto-oncogene (17), which is a critical molecule in the regulation of cell proliferation and neoplastic transformation (18–20). However, the nature of the intracellular signaling route by which thrombin stimulates c-*jun* expression and whether its protein product, c-Jun, contributes to the transforming ability of PAR-1 are still unknown.

In this study, we have explored the molecular mechanisms by which endogenously expressed or overexpressed thrombin receptors can transduce signals from the membrane into nuclear events participating in cell transformation. For this work, we have used endogenous and transfected thrombin receptors, ectopic expression of muscarinic receptors coupled to $G\alpha_q$ and $G\alpha_i$ (m1 and m2, respectively), and chimeric G protein α subunits and murine fibroblasts deficient in $G\alpha_{q/11}$ and $G\alpha_{12/13}$. We show here that, although coupling to $G\alpha_i$ is sufficient to induce ERK activation, the ability to couple to $G\alpha_q$ and/or $G\alpha_{13}$ is necessary to induce c-*jun* expression and cell transformation. Furthermore, we show that $G\alpha_q$ and $G\alpha_{13}$ can initiate the activation of MAPK cascades regulating the activity of transcription factors controlling the activity of the c-*jun* promoter. We also present evidence that c-Jun and kinases regulating its expression are integral components of the transforming pathways initiated by PAR-1.

MATERIALS AND METHODS

Cell Lines

NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% calf serum. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Mouse embryonic fibroblasts from wild-type and $G\alpha_{q/11}$ and $G\alpha_{12/13}$ knockout animals were kept in the same media supplemented with 1 mM sodium pyruvate and nonessential amino acids.

DNA Constructs

A plasmid encoding a luciferase gene driven by a murine wild-type c-*jun* promoter, pJLuc, was kindly provided by R. Prywes (21). Plasmids pJC6, pJC9, pJTX, pJSX, and pJSTX are pBLCAT3-based reporter constructs carrying a chloramphenicol acetyltransferase (CAT) gene controlled by the murine full-length c-*jun* promoter and its mutants, as previously described (22). A pGL3 reporter plasmid (Promega) containing the jAP1 (TGACATCA) and MEF2 (CTATTTTTAG) sites from the murine c-*jun* promoter, designated pjAP1-MEF2, was engineered by inserting the oligonucleotide sequence 5′-GTACCGTCGACTCGGGGT-GACATCATGGGCTATTTTTAGGGAGATC-3′ as an Asp718/BglII fragment upstream of an SV40 minimal promoter and a luciferase gene. Reporter plasmids with mutations in the jAP1 (pjAP1m-MEF2) or MEF2 (pjAP1-MEF2m) site and a double mutant (pjAP1m-MEF2m) and a plasmid carrying two jAP1 sites were prepared following the same strategy. A similar reporter plasmid carrying an MEF2 site has been previously reported (23). Expression vectors for HA-ERK2, HA-JNK, HA-ERK5, HA-p38α, HA-p38γ (ERK6), pCEFL-MEK5DD, pCEFL-MEK5AA, pCEV29-MEKEE, pCEFL-MEKAA, pCEFL-GST-MKK6, pCEFL-GST-MKK6KR, pCEFL-MEKK, and constitutively activated small G proteins Ras, RhoA, Rac1, and Cdc42 have also been described (23–26). H-Ras^{V12} and a dominant-negative mutant of RhoA, $RhoA^{N19}$ have been described (24). Gal4 fusion proteins, including the transactivating domains of ATF2 (amino acids 1–96) and MEF2A (amino acids 266–360) and a TATA-Gal4-driven luciferase reporter plasmid (pGal4-Luc), and bacterially expressed GST-ATF2 and GST-MEF2C fusion proteins were described previously (25). pcDNAIII-MKK3b-WT and its constitutively activated (EE) and dominant-negative (AA) mutants were kindly provided by J. Han (27). pCEFL-AU5-JunTAM67 has been described (28). PAR-1, kindly provided by Dr. L. F. Brass, was subcloned into the pCEFL vector as an EcoRI fragment. DNA encoding a $G\alpha_{13i5}$ chimera, in which five amino acids at the C terminus of $G\alpha_q$ were replaced with the corresponding sequence of $G\alpha_{i2}$, was prepared by PCR amplification using pcDNA3-HA-$G\alpha_{13}$ (29) as a template, and the resulting DNA was subcloned into the pCEFL-HA vector (25) as a BglII/EcoRI fragment. A DNA plasmid encoding a $G\alpha_{qi5}$ chimeric protein, in which five amino acids at the C terminus of $G\alpha_q$ were replaced with the corresponding sequence of $G\alpha_{i2}$, was a gift from Dr. B. R. Conklin (30). Expression plasmids for constitutively activated forms of $G\alpha_q$, $G\alpha_{i2}$, $G\alpha_s$, $G\alpha_{12}$, and $G\alpha_{13}$; G protein β and γ subunits; and m1 and m2 muscarinic receptors were described previously (24, 25, 29, 31).

Transfections

Transient transfections of NIH 3T3 and human embryonic kidney 293T cells cultured in 6-well plates were performed using the LipofectAMINE Plus reagent (Invitrogen) following the manufacturer's instructions. Stable transfections of NIH 3T3 cells expressing the m1 or m2 receptor (NIH-m1 and NIH-m2 cells, respectively; each expressing ~100,000 receptors/cell) (8, 32) and the m2 receptor plus the $G\alpha_{13i5}$ or $G\alpha_{qi5}$ chimera (NIH-m2$G\alpha_{13i5}$ and NIH-m2$G\alpha_{qi5}$ cells, respectively) were performed using the same protocol as described above, and cells were selected in culture medium containing Geneticin (750 μg/ml).

Northern Blotting

Cells were grown to 70% confluence in 10-cm plates and serum-starved for 20 h. They were left untreated (controls) or were treated with 1 mM carbachol or 5 units/ml thrombin for different times. After treatment, they were washed with cold PBS, and total RNA was extracted by homogenization in TRIzol (Invitrogen) according to the manufacturer's specifications. For Northern blotting, 10–20 μg of total RNA was fractionated on 2% formaldehyde-agarose gels, transferred to nylon membranes, and hybridized with murine full-length ^{32}P-labeled c-*jun* cDNA probe prepared using a Prime-a-Gene labeling system (Promega). Accuracy in gel loading and transfer was confirmed by fluorescence under UV light upon ethidium bromide staining.

Reporter Gene Assays

Luciferase Assays—Cells were transfected with different expression plasmids together with 0.1 μg of each reporter plasmid and 0.01 μg of pRL-null (a plasmid expressing luciferase from *Renilla reniformis*) as an internal control. In all cases, the total amount of plasmid DNA was adjusted with pcDNAIII-β-gal (a plasmid expressing β-galactosidase). Firefly and *Renilla* luciferase activities present in cell lysates were assayed using a dual-luciferase reporter system (Promega), and light emission was quantitated using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) as specified by the manufacturer.

CAT Assays—NIH 3T3 cells were transfected with different expression plasmids together with 0.1 μg of each reporter plasmid and 0.5 μg of pcDNAIII-β-gal. After a 24-h incubation, cells were washed and lysed using reporter lysis buffer (Promega). CAT activity was assayed in cell extracts by incubation for 1 h in the presence of 0.25 μCi of [^{14}C]chloramphenicol (100 mCi/mmol) and 200 μg/ml butyryl-CoA in 0.25 M Tris-HCl (pH 7.4). Labeled butyrylated products were extracted using a 1:2 mixture of xylenes and 2,6,10,14-tetramethylpentadecane (Sigma), and incorporated radioactivity was counted by liquid scintillation.

Kinase Assays

Cells were seeded at 70–80% confluence and transfected with expression vectors for HA-tagged kinases alone or in combination with different upstream molecules. After transfection, cells were cultured for 24 h and incubated in serum-free medium overnight for ERK2 and ERK5 and for 2 h for JNK, p38α, and p38γ. Cells were washed with cold PBS and lysed at 4 °C in buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 1 mM vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Cleared lysates containing HA-tagged kinases were immunoprecipitated at 4 °C for 2 h with anti-HA monoclonal antibody HA.11 (Berkeley Antibody Co.). Immunocomplexes were recovered with protein G-Sepharose (Amersham Biosciences). Beads were washed three times

with PBS containing 1% Nonidet P-40 and 2 mM vanadate, once with 100 mM Tris (pH 7.5) and 0.5 M LiCl, and once with kinase reaction buffer (12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, and 0.5 mM vanadate). Samples were resuspended in 30 μl of kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP/reaction and 20 μM unlabeled ATP. After 20 min at 30 °C, the reactions were terminated by addition of 10 μl of 5× Laemmli buffer. *In vitro* kinase assays were performed using 1.5 μg/μl myelin basic protein (Sigma) for ERK2 and 1 μg of purified, bacterially expressed GST-ATF2 for JNK, p38α, and p38γ and GST-MEF2C for ERK5 as substrates, as indicated. Samples were analyzed by SDS-gel electrophoresis on 12% (or 15% for myelin basic protein) acrylamide gels, and autoradiography was performed with the aid of an intensifying screen.

Western Blotting

HA-tagged immunoprecipitates from transiently transfected NIH 3T3 cells carrying HA-MAPK, HA-JNK, HA-ERK5, HA-p38α, and HA-p38γ cDNAs were analyzed by Western blotting after SDS-PAGE using anti-HA monoclonal antibody HA.11. $G\alpha_q$ and $G\alpha_{11}$ were detected by rabbit anti-$G\alpha_{q/11}$ antibody (Santa Cruz Biotechnology, Inc.). $G\alpha_{12}$ and $G\alpha_{13}$ were detected by a mixture of anti-$G\alpha_{12}$ antibody (Santa Cruz Biotechnology, Inc.) and anti-$G\alpha_{13}$ antibody (33). Proteins were visualized by enhanced chemiluminescence detection (Amersham Biosciences) using horseradish peroxidase-coupled goat anti-mouse and anti-rabbit IgGs as the secondary antibodies (Cappel).

Indirect Immunofluorescence

NIH 3T3 cells and these cells stably transfected with the m1 or m2 receptor were seeded on glass coverslips and transfected using LipofectAMINE Plus reagent as described above. 24-h serum-starved cells were treated with 1 mM carbachol and 5 units/ml thrombin, washed twice with 1× PBS, and then fixed and permeabilized with 4% formaldehyde and 0.5% Triton X-100 in 1× PBS for 10 min. After washing with PBS, cells were blocked with 1% bovine serum albumin and incubated with the indicated primary antibodies for 1 h. c-Jun was detected using rabbit anti-c-Jun antibody (Santa Cruz Biotechnology, Inc.). Following incubation, cells were washed three times with 1× PBS and incubated with the corresponding fluorescein isothiocyanate-conjugated secondary antibodies (1:200 dilution; Jackson ImmunoResearch Laboratories, Inc.). Coverslips were washed three times and mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Inc.) and viewed using a Zeiss Axiophot photomicroscope equipped with epifluorescence. Immunofluorescence was photographed using Eastman Kodak TMAX 3200 film.

Focus Forming Assays

NIH 3T3 cells were transfected by the calcium phosphate precipitation technique with different expression plasmids together with 1 μg of pcDNAIII-β-gal, adjusting the total amount of plasmid DNA with empty vector. The day after transfection, cells were washed with medium supplemented with 5% calf serum and then maintained in the same medium until foci were scored, 2–3 weeks later. Duplicate plates were fixed with 1× PBS containing 2% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde and stained at 37 °C for β-galactosidase activity with 1× PBS containing 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to evaluate the transfection efficiency.

RESULTS

Human PAR-1 and m1, but Not m2, Harbor Oncogenic Potential—PAR-1, a GPCR activated by thrombin and other proteases (34, 35) that is linked to $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ subunits (12), was cloned as an oncogene using an expression library approach (11). Indeed, as previously reported for the murine PAR-1 gene, human PAR-1 readily induced the appearance of foci of transformation after 2–3 weeks of culture, as shown in Fig. 1. Interestingly, PAR-1 was even more potent than a G_q-coupled receptor, the m1 muscarinic receptor, which transforms NIH 3T3 effectively when cells are cultured in the presence of the cholinergic agonist carbachol (8). In contrast, m2 receptors that are coupled to G_i proteins do not transform cells in culture, thus suggesting that $G\alpha_q$ and $G\alpha_{13}$, but not $G\alpha_i$, can stimulate transforming pathways in these murine fibroblasts.

c-jun Expression Is Stimulated by Transforming GPCRs, and a Dominant Inhibitory Mutant of c-Jun Prevents Their Focus-forming Activity—To begin addressing the molecular mechanism underlying the transforming ability of these GPCRs, we first examined whether they could stimulate the ERK signaling route, a key component of cell growth-promoting pathways (36), using wild-type NIH 3T3 cells, which express PAR-1 endogenously, and the same cells stably transfected with the m1 or m2 receptor (NIH-m1 and NIH-m2 cells, respectively). As shown in Fig. 2, agonist addition to NIH 3T3 cells resulted in the potent activation of ERK. However, in repeated experiments, there were no remarkable differences in the strength and duration of the ERK signal elicited by transforming and non-transforming GPCRs, suggesting that the ability to stimulate ERK does not correlate with their transforming activity. In the search for the molecular mechanisms underlying the distinct biological activities of these GPCRs, we focused our attention on nuclear responses, in particular on the expression of the transcription factor c-Jun, the function of which has often been associated with malignant conversion (reviewed in Ref. 19). As shown in Fig. 2 (*lower panels*), activation of PAR-1 and m1 receptors induced the rapid accumulation of c-*jun* mRNA. Consistently, the expression of the c-Jun protein was also increased as revealed by the nuclear c-Jun immunostaining of thrombin- and carbachol-stimulated cells (Fig. 2*B*). Of interest, carbachol did not induce c-*jun* message or c-Jun protein expression in NIH-m2 cells, suggesting that only transforming GPCRs can stimulate this particular nuclear response.

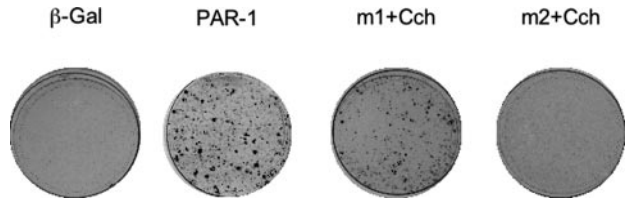

FIG. 1. **Human PAR-1 induces focus formation in NIH 3T3 cells.** NIH 3T3 cells were transfected by the calcium phosphate technique with pcDNAIII-β-gal, pCEFL-PAR-1, pCEFL-m1, and pCEFL-m2 (1 μg each), as indicated. Cells were cultured in 5% calf serum, and those cells transfected with the m1 and m2 receptors were maintained in the same medium supplemented with 100 μM carbachol (*Cch*). After 3 weeks, the plates were fixed and stained. The plates shown are representative of three to five different experiments.

We next investigated whether the ability to trigger c-Jun expression and transformation by PAR-1 and the m1 receptor is two functionally related events. As shown in Fig. 3, transformation induced by these receptors was potently inhibited by the coexpression of a dominant-negative mutant form of c-Jun, c-Jun TAM67 (37), even at concentrations that displayed a much more limited effect on Ras^{V12}-induced transformation (28, 38) and MEK1EE-induced transformation (Fig. 3 and data not shown). Together, these findings indicate that the functional activity of c-Jun proteins is required for abnormal cell growth promotion in response to PAR-1.

$G\alpha_q$ and $G\alpha_{13}$, but Not $G\alpha_i$ or βγ Subunits, Stimulate the c-jun Promoter—The available results indicated that, in murine fibroblasts, receptors coupled to $G\alpha_{12/13}$ and/or $G\alpha_q$, but not to $G\alpha_i$, can transduce mitogenic signals that, in turn, promote the expression of genes involved in normal and abnormal cell growth. This prompted us to investigate which G protein subunits are able to induce the expression of c-*jun*. As an experimental approach, we first compared the effect of thrombin and carbachol acting on PAR-1 and the m1 and m2 receptors, respectively, on a murine c-*jun* promoter-driven luciferase reporter gene (pJLuc) (22). As shown in Fig. 4*A*, thrombin or PAR-1 expression alone induced the c-*jun* promoter by nearly 2-fold, and this was enhanced to nearly 3.5-fold when PAR-1-transfected cells were stimulated with thrombin, whereas car-

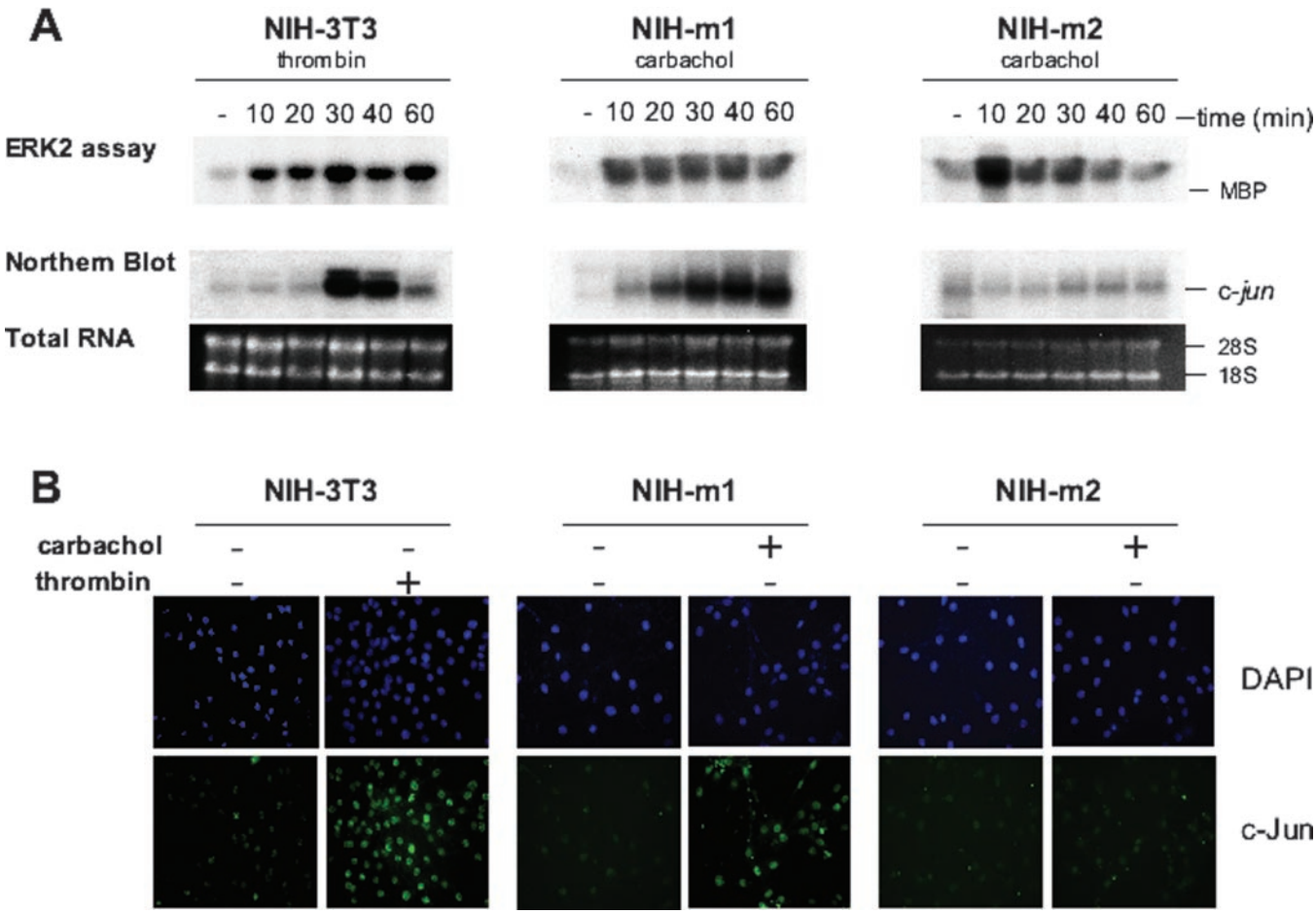

FIG. 2. **Thrombin and m1 receptors stimulate the expression of c-*jun* and its protein product, c-Jun, in NIH 3T3 cells.** *A*, NIH 3T3, NIH-m1, and NIH-m2 cells were cultured in serum-free medium for 24 h and left untreated or treated with 5 units/ml thrombin or 100 μM carbachol for the indicated time points. *Upper panels*, lysates were immunoprecipitated with anti-ERK2 antibody and used for kinase reactions. ^{32}P-Labeled myelin basic protein (*MBP*) used as a substrate is indicated. Data represent results from a typical experiment. Similar results were obtained in three additional experiments. *Middle* and *lower panels*, cells were seeded in 10-cm plates and, after 24 h of serum deprivation, were left untreated or treated with 5 units/ml thrombin or 100 μM carbachol for different time points, and total RNA was extracted. Samples containing 20 μg of total RNA were fractionated and analyzed by Northern blotting using murine ^{32}P-labeled c-*jun* cDNA as a probe. Total RNA present in each lane was assessed to be equivalent by ethidium bromide staining of rRNAs. The autoradiogram corresponds to a representative experiment. *B*, NIH 3T3, NIH-m1, and NIH-m2 cells were seeded on coverslips, cultured in serum-free medium for 24 h, and left untreated or treated with 5 units/ml thrombin or 100 μM carbachol for 4 h, as indicated. Cells were fixed and analyzed by immunofluorescence for c-Jun (*lower panels*) and nuclei labeled with 4,6-diamidino-2-phenylindole (*DAPI*; *upper panels*).

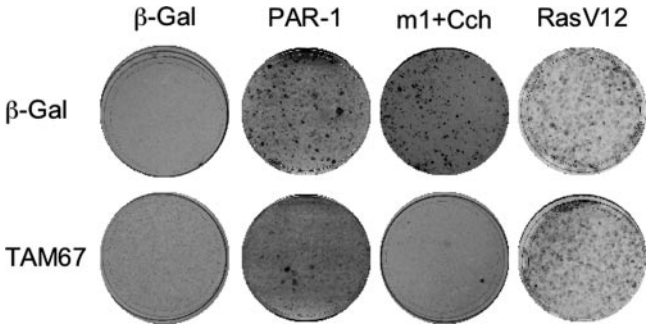

FIG. 3. **A dominant-negative c-Jun mutant inhibits the transforming activity of PAR-1.** NIH 3T3 cells were transfected by the calcium phosphate technique with pcDNAIII-β-gal, pCEFL-m1, pCEFL-PAR-1, or pCEFL-AU5-Ras^{V12} (1 μg) alone or in combination with c-Jun TAM67 (0.5 μg). Cells were cultured for 3 weeks in 5% calf serum and left untreated or treated with 100 μM carbachol (*Cch*), as indicated, and then fixed and stained. Representative plates for each transfection are shown.

bachol activated pJLuc by 5-fold, but only in cells transfected with the m1 receptors. In view of the correlation between c-*jun* mRNA expression and reporter activation, we next studied the effect of activated forms of G protein α subunits on the regulation of pJLuc. These forms express GTPase-deficient active subunits that can elicit effector pathways, obviating the need for receptor stimulation (39). Thus, we cotransfected pJLuc along with the activated forms of $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{13}$, which are representative members of the four Gα subunit families (40), as well as $\beta\gamma$ dimers. Under these conditions, $G\alpha_s$, $G\alpha_i$, and the $\beta\gamma$ dimers failed to stimulate the c-*jun* promoter. In contrast, the activated forms of $G\alpha_q$ and $G\alpha_{13}$ potently induced pJLuc by 10- and 6-fold, respectively (Fig. 4*B*), strongly suggesting that heterotrimeric G protein α subunits of the $G\alpha_q$ and $G\alpha_{12/13}$ families can mediate the effect of activated PAR-1 on the c-*jun* promoter.

Chimeric G Protein α Subunits from the $G\alpha_q$ and $G\alpha_{12/13}$ Families, Including C-terminal $G\alpha_i$ Sequences, Promote c-jun Expression in Response to Stimulation of G_i-coupled Receptors—The prolonged exposure of cells to the constitutively activated forms of $G\alpha_q$ and $G\alpha_{13}$ subunits could lead to secondary effects resulting in the indirect activation of the c-*jun* promoter. Thus, to confirm our previous results, we took advantage of the availability of the $G\alpha_{qi5}$ and $G\alpha_{13i5}$ chimeras, in which the C-terminal region of the α subunits was replaced with the corresponding region of $G\alpha_i$ (29, 30). In this case, coexpression of these chimeras along with the G_i-coupled m2 receptor gen-

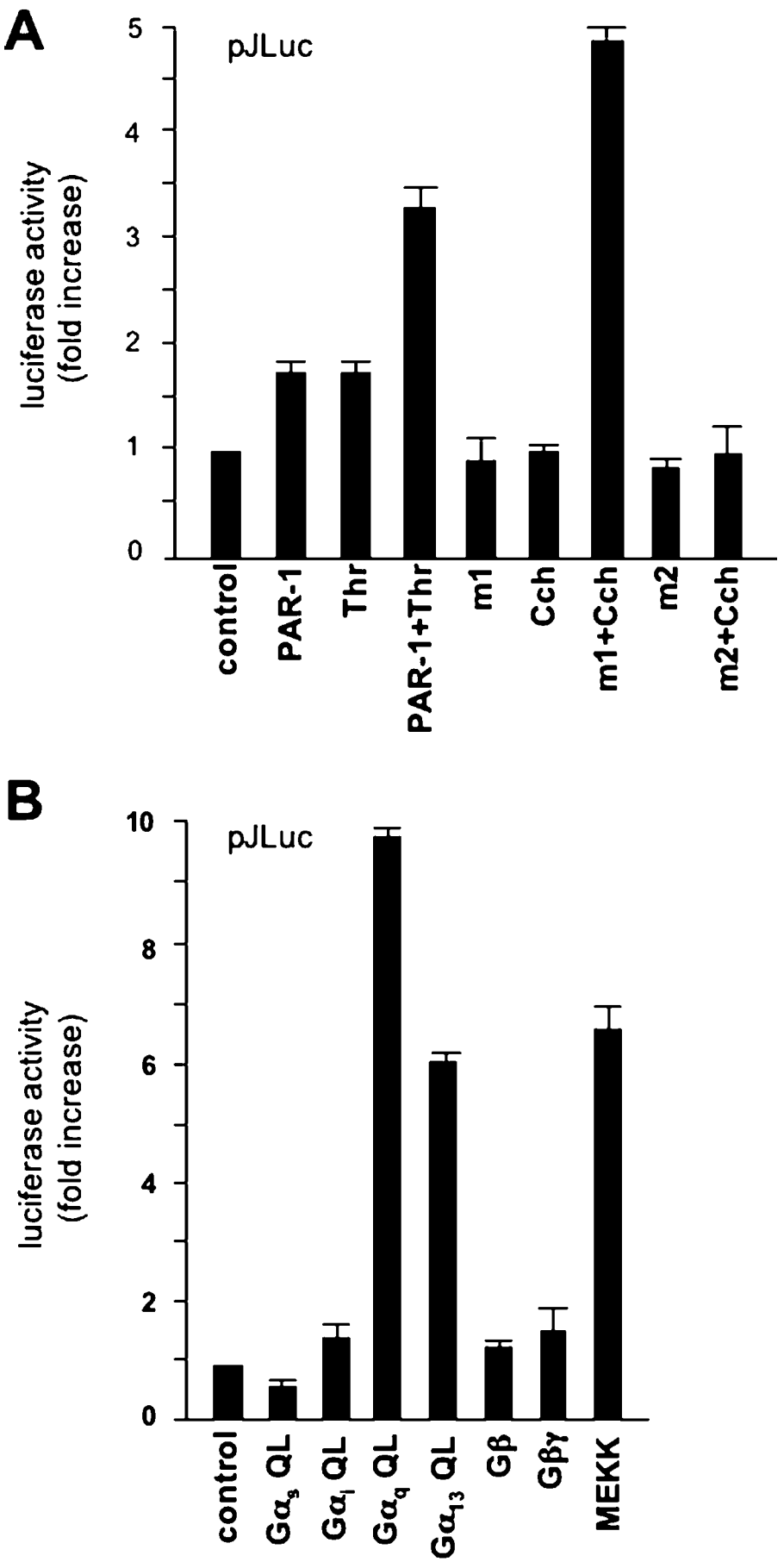

FIG. 4. **Stimulation of PAR-1 and activated mutants of heterotrimeric G protein α subunits of the $G\alpha_q$ and $G\alpha_{12/13}$ families enhances the activity of the c-*jun* promoter.** *A*, NIH 3T3 cells were cotransfected with pJLuc and pRL-null together with expression vectors for β-galactosidase (*control*), m1, m2, or PAR-1, as indicated. 24 h after serum starvation, cells were then exposed to vehicle (*control*), 100 μM carbachol (*Cch*), or 5 units/ml thrombin (*Thr*) for 4 h, and lysates were assayed for dual luciferase activity. *B*, cells were transfected as described for *A* with expression plasmids for β-galactosidase (*control*), MEKK, $G\alpha_s$QL, $G\alpha_i$QL, $G\alpha_q$QL, $G\alpha_{13}$QL, or G$\beta\gamma$ subunits. 24 h after serum starvation, cells were lysed and assayed for dual luciferase activity. In *A* and *B*, the data represent luciferase activity normalized to *R. reniformis* luciferase activity present in each cell lysate, expressed as -fold induction with respect to control cells, and are the means ± S.E. of triplicate samples from a typical experiment. Similar results were obtained in three separate experiments.

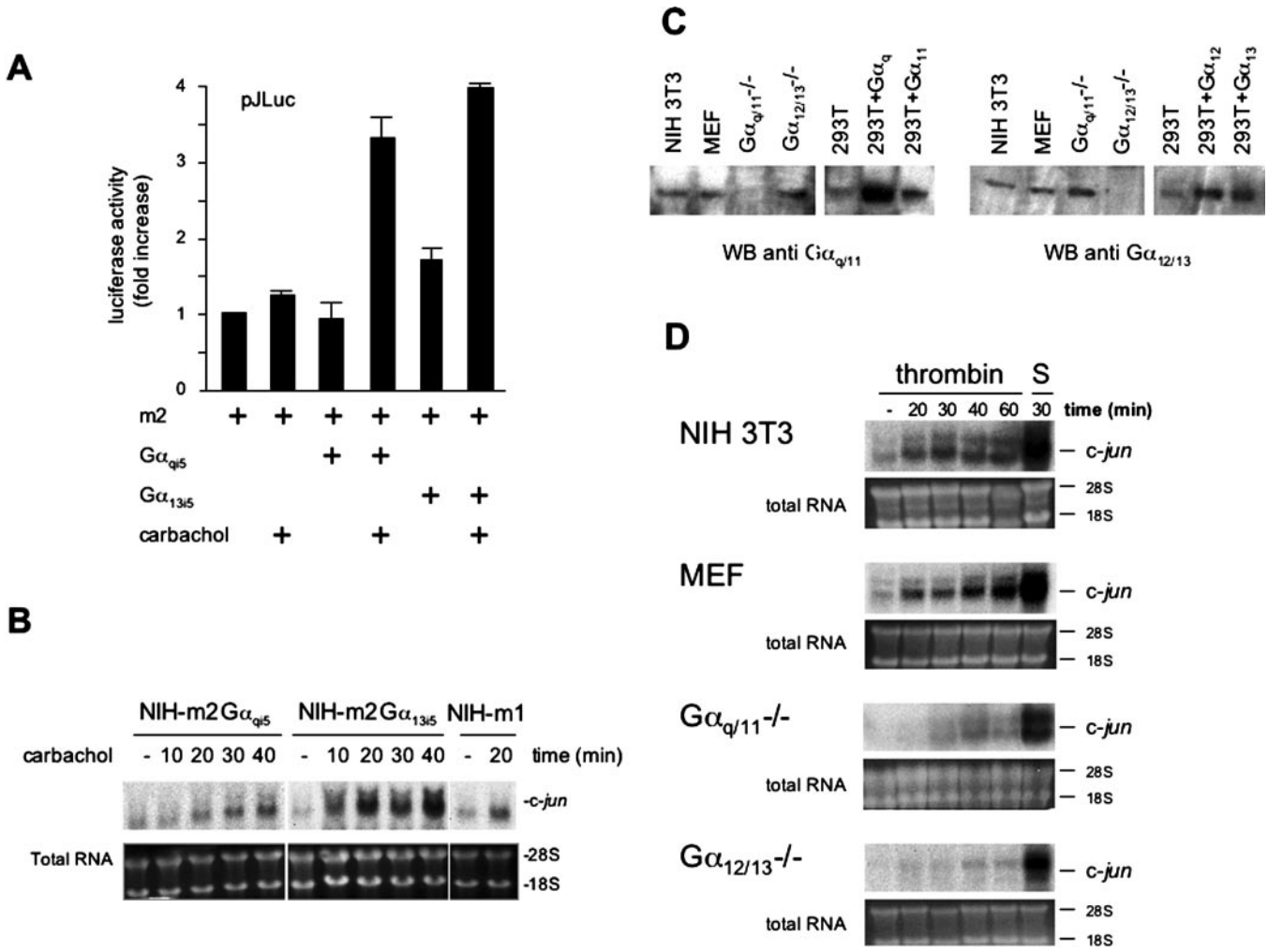

FIG. 5. **Activation of Gα$_q$ and Gα$_{13}$ leads to enhanced expression from the c-*jun* promoter.** *A*, NIH 3T3 cells were cotransfected with pJLuc and pR-null (0.1 and 0.01 μg/plate, respectively) plus expression vectors for β-galactosidase (control) or m2 along with chimeric forms of Gα$_q$ and Gα$_{13}$ (Gα$_{qi5}$ and Gα$_{13i5}$, respectively; 1 μg each). 24 h after transfection and serum starvation, cells were treated for 4 h with 100 μM carbachol and collected, and lysates were assayed for dual luciferase activities. The data represent firefly luciferase activity normalized to *Renilla* luciferase activity present in each sample, expressed as -fold induction relative to controls. Values are the means ± S.E. of triplicate samples from a typical experiment. Nearly identical results were obtained in four additional experiments. *B*, NIH 3T3 cells were stably transfected with expression vectors for m2 along with the Gα$_{qi5}$ or Gα$_{13i5}$ chimera (NIH-m2Gα$_{qi5}$ and NIH-m2Gα$_{13i5}$ cells, respectively). After 24 h of starvation, cells were treated with carbachol for the indicated periods. NIH-m1 cells treated for 20 min with carbachol were used as a positive control. After treatment, total RNA was extracted, and 20-μg samples were fractionated and analyzed by Northern blotting using murine ^{32}P-labeled c-*jun* cDNA as a probe. Total RNA present in each lane was assessed to be equivalent by ethidium bromide staining of rRNAs. The autoradiogram corresponds to a representative experiment. *C*, total lysates from NIH 3T3 cells; mouse embryonic fibroblasts (*MEF*); Gα$_{q/11}$ and Gα$_{12/13}$ knockout cells; and human embryonic kidney 293T cells untransfected or transfected with expression vectors for Gα$_q$, Gα$_{11}$, Gα$_{12}$, or Gα$_{13}$ were assayed by Western blotting (*WB*) to detect the presence of Gα$_{q/11}$ subunits (*left panels*) or Gα$_{12/13}$ subunits (*right panels*). *D*, NIH 3T3 cells, wild-type mouse embryonic fibroblasts, and Gα$_{q/11}$ and Gα$_{12/13}$ knockout cells were treated with 5 units/ml thrombin for the indicated times and with 10% serum as a positive control. The expression of c-*jun* mRNA was measured as described for *B*. The autoradiogram is representative of a typical experiment.

erates a system that can specifically signal downstream from Gα$_q$ and Gα$_{13}$ and that can be turned on by agonist addition. Because m2 receptors and Gα$_i$ subunits did not activate pJLuc, this system represents a useful approach to study the isolated effect of Gα$_q$ or Gα$_{13}$ on the c-*jun* promoter. As shown in Fig. 5*A*, the exposure of cells expressing the m2 receptor and either the Gα$_{qi5}$ or Gα$_{13i5}$ chimera to carbachol induced c-*jun*, whereas the expression of the Gα$_{qi5}$ or Gα$_{13i5}$ chimera alone did not have a major effect on pJLuc activity. To validate the specificity of this approach, we stably transfected NIH-m2 cells with the Gα$_{qi5}$ and Gα$_{13i5}$ chimeras (NIH-m2Gα$_{qi5}$ and NIH-m2Gα$_{13i5}$ cells, respectively) and measured the expression of c-*jun* mRNA upon carbachol treatment. As depicted in Fig. 5*B*, the cholinergic agonist induced c-*jun* in both cell lines, although the response was stronger in the NIH-m2Gα$_{13i5}$ cells. These data demonstrate that, in this setting, the Gα$_{qi5}$ and Gα$_{13i5}$ chimeras can be stimulated by m2 receptors and are therefore capable of transmitting Gα$_q$- and Gα$_{13}$-mediated signaling pathways promoting c-*jun* expression, thus mimicking the effect of activated PAR-1 and m1 receptors. Collectively, they also indicate that each of these G protein α subunits is sufficient to transmit signals from its coupled receptors to the nucleus.

Activation of c-jun Expression by Thrombin Is Reduced in Cells Derived from Gα$_{12/13}$ and, to a Lesser Extent, Gα$_{q/11}$ Double Knockout Embryos—To address which G proteins are required to signal to c-*jun* in response to thrombin, we used mouse embryonic fibroblasts lacking Gα$_q$ and Gα$_{11}$ subunits or Gα$_{12}$ and Gα$_{13}$ subunits (Fig. 5*C*), derived from Gα$_{q/11}$ and Gα$_{12/13}$ double knockout embryos, respectively (reviewed in Refs. 41 and 42). For these experiments, we evaluated the expression of c-*jun* upon thrombin addition using serum as a control. As shown in Fig. 5*D*, thrombin was able to induce c-*jun* expression in Gα$_{q/11}$ knockout cells, albeit to a more limited

extent than in murine wild-type embryonic or NIH 3T3 fibroblasts. In contrast, in the $G\alpha_{12/13}$ knockout cells, the induction of c-*jun* expression in response to thrombin was markedly reduced. As the response to serum (which served as an internal control) was nearly identical for all cells, these results suggest that thrombin can signal to the c-*jun* promoter using $G\alpha_{q/11}$ or $G\alpha_{12/13}$, although the majority of the signal is likely provided by the activation of $G\alpha_{12/13}$. These observations also suggest that, although both families of G protein α subunits can signal to the nucleus independently, both of them may be required for the full stimulation of c-*jun* expression by thrombin.

Distinct Response Elements on the c-jun Promoter Respond to $G\alpha_q$ and $G\alpha_{13}$ Subunits—The c-*jun* promoter exhibits a number of response elements that bind transcription factors, including SP1, CTF, AP1, and MEF2, along with two GATAA elements (Fig. 6*A*) (22). Among them, the jAP1 and MEF2 sites have been found to mediate the regulation of the c-*jun* promoter by m1 receptors (23, 25). Thus, we asked whether these sites contribute to $G\alpha_q$- and $G\alpha_{13}$-induced activation of this promoter. As shown in Fig. 6*B*, both subunits induced the expression of a CAT reporter gene controlled by the murine full-length c-*jun* promoter (pJC6). Mutations in the jAP1 or MEF2 site (pJTX and pJSX plasmids, respectively) only partially reduced the transcriptional response to the α subunits, suggesting that each of these elements can independently contribute to the activation of the c-*jun* promoter by the heterotrimeric G proteins. However, no activation was elicited when both sites were absent, suggesting that these sites are strictly required for G protein-dependent activation of the c-*jun* promoter. To further investigate whether $G\alpha_q$ and $G\alpha_{13}$ can in fact stimulate these response elements, a fragment containing nucleotides -71 to -50 from the c-*jun* promoter, including a single jAP1 and MEF2 site, was inserted into the pGL3 reporter plasmid (pjAP1-MEF2-Luc). As shown in Fig. 6*C*, pjAP1-MEF2-Luc was activated by both $G\alpha_q$ and $G\alpha_{13}$ by nearly 10-fold. Interestingly, $G\alpha_q$ and $G\alpha_{13}$ also activated reporter plasmids carrying only a single jAP1 or MEF2 site, confirming that both subunits can signal independently to each response element. Taken together, these results indicate that, downstream from GPCRs, both $G\alpha_q$ and $G\alpha_{13}$ can activate signaling pathways that regulate the c-*jun* promoter by acting specifically on the jAP1 and MEF2 sites.

$G\alpha_q$ and $G\alpha_{13}$ Subunits Can Signal to the c-jun Promoter through MAPK Cascades—Because the activity of the transcription factors bound to the jAP1 and MEF2 sites within the c-*jun* promoter is controlled by distinct MAPKs, including JNK, ERK5, p38α, and p38γ (25), we tested whether the inhibition of these pathways by dominant-negative mutants blocks the activation of pJLuc by $G\alpha_q$ and $G\alpha_{13}$. For these experiments, we transfected NIH 3T3 cells with pJLuc along with JIP-1, which, when overexpressed, blocks the nuclear translocation of JNK, thereby impeding JNK-dependent gene expression regulation (43), or with dominant-negative forms of MEK5 (MEK5AA) and MKK3 (MEK3AA) (44). As shown in Fig. 7*A*, JIP-1, MEK5AA, and MKK3AA partially inhibited the c-*jun* promoter-dependent gene expression induced by $G\alpha_q$ and $G\alpha_{13}$. Control experiments showing the specificity of these molecules in this cell setting have been previously described (25, 28). Based on these results, we explored whether $G\alpha_q$ and $G\alpha_{13}$ are indeed able to activate the MAPKs involved in the regulation of the c-*jun* promoter. We expressed HA epitope-tagged forms of JNK, ERK5, p38α, and p38γ along with the activated forms of $G\alpha_q$ and $G\alpha_{13}$ or control molecules in NIH 3T3 cells. As depicted in Fig. 7*B*, $G\alpha_q$QL and $G\alpha_{13}$QL were able to stimulate the activity of each of these kinases, as evidenced by an increased ability to phosphorylate their specific substrates compared with samples transfected with pcDNAIII-β-Gal as a negative control. the positive controls used were activation of JNK by MEKK, ERK5 by MEK5DD, and p38α and p38γ by MEK3EE. In contrast, the activated forms of these G protein α subunits failed to stimulate ERK2 under identical experimental conditions (data not shown), as previously reported in other cell types (45). These results indicate that expression of the activated forms of $G\alpha_q$ and $G\alpha_{13}$ can elevate the enzymatic activity of MAPKs involved in the stimulation of the c-*jun* promoter.

The Transactivating Activity of Transcription Factors Bound to the jAP1 and MEF2 Response Elements Can Be Stimulated by $G\alpha_q$ and $G\alpha_{13}$ Subunits—As previously described (9, 46, 47), supershift analysis showed that the c-Jun and ATF2 proteins are the most prominent nuclear proteins binding the jAP1 site in NIH 3T3 cells and other cell types. With regard to the MEF2 site, our previous results indicate that MEF2A and MEF2D are expressed in NIH 3T3 cells and that the activity of MEF2A is regulated by ERK5, p38α, and p38γ (25). To assay whether activated mutants of $G\alpha_q$ and $G\alpha_{13}$ are able to stimulate the transactivation domain of the Jun, ATF2, and MEF2A proteins, we fused these domains to the yeast transcription factor Gal4 (9) and assessed their ability to stimulate expression from a Gal4-regulated reporter plasmid, pGal4-Luc. Both $G\alpha_q$QL and $G\alpha_{13}$QL significantly enhanced the transcriptional activity of Gal4-c-Jun, Gal4-MEF2A, and Gal4-ATF2, as shown in Fig. 8. Cotransfection with MEK5DD + ERK5, which specifically activates MEF2A, and with MEKK, which stimulates ATF2 and c-Jun (44), served as a positive control. Collectively, these findings suggest that $G\alpha_q$ and $G\alpha_{13}$ and their coupled receptors, including PAR-1, promote c-*jun* expression through multiple MAPK cascades that converge to stimulate the activity of transcription factors regulating the activity of the c-*jun* promoter.

The JNK, p38, and ERK5 Pathways Are Integral Components of the Transforming Pathway Elicited by PAR-1—Based on the role of JNK, p38 isoforms, and ERK5 in the activation of the c-*jun* promoter by $G\alpha_q$ and $G\alpha_{13}$ and their coupled receptors, we next asked whether these kinases participate in the transforming ability of PAR-1 in NIH 3T3 cells. Thus, we assayed the focus-forming activity of PAR-1 in the presence of molecules interfering with the activation of each of these MAPK pathways, such as JIP-1, MKK3AA, and MEK5AA. As depicted in Fig. 9, these molecules, which limit the activation of the JNK, p38, and ERK5 pathways, respectively, were able to reduce the transforming activity of PAR-1. In contrast, none of these dominant-negative forms affected the focus-forming activity of an activated form of MEK, MEKEE, which was used as a specificity control. Parallel plates transfected with the same DNAs and β-galactosidase were fixed and stained for β-galactosidase and showed no difference in transfection efficiency (data not shown). Taken together, these results indicate that the activation of JNK, p38 isoforms, and ERK5 contributes to the transforming potential of PAR-1.

DISCUSSION

Although the ability of G protein-coupled receptors to stimulate normal and aberrant cell growth has been intensely investigated (for review, see Refs. 9 and 10), the precise nature of the molecular mechanisms underlying the transforming potential of GPCRs are still poorly understood. In this study, we have taken advantage of the potent growth-promoting activity of thrombin and the focus-forming potential of one of its receptors, PAR-1, in NIH 3T3 cells to begin dissecting how thrombin receptors transduce signals from the membrane to the nucleus, thereby initiating transcriptional events participating in cell transformation. We show here that, although $G\alpha_i$-coupled receptors stimulate ERK potently, only $G\alpha_q$ and/or $G\alpha_{13}$ and

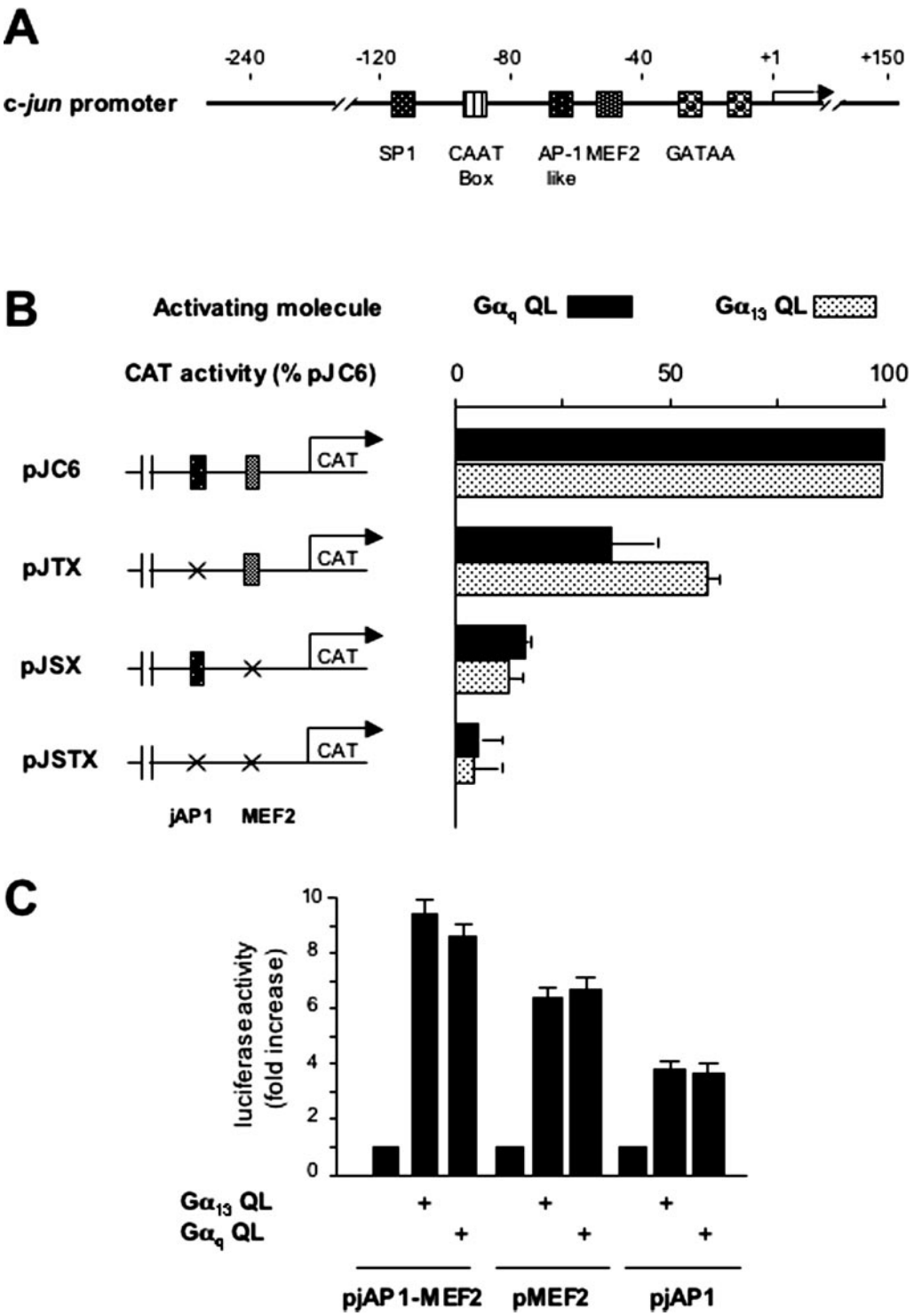

FIG. 6. **Similar regulatory elements mediate the stimulation of the c-*jun* promoter by activated $G\alpha_q$ and $G\alpha_{13}$.** *A*, shown is a schematic representation of the c-*jun* promoter depicting the relative positions of the response elements. *B*, NIH 3T3 cells were cotransfected with the reporter plasmid pcDNAIII-β-gal (0.5 μg) along with pJC6, pJTX, pJSX, or pJSTX (0.1 μg/plate), as indicated. *X* indicates the sites of point mutations in the jAP1-binding (pJTX) and MEF2-binding (pJSX) sites. Activated forms of $G\alpha_q$ and $G\alpha_{13}$ ($G\alpha_q$QL and $G\alpha_{13}$QL, respectively) or an empty vector (control) was included in each transfection (1 μg each). 24 h later, cells were collected, and lysates were assayed for CAT and β-galactosidase activities. The data represent CAT activity normalized to β-galactosidase activity present in each sample, expressed as the percentage of the pJC6 induction elicited by the activating molecules. *C*, cells were transfected with the reporter plasmid pjAP1-MEF2, pMEF2, or pjAP1 (0.05 μg) and pRL-null (0.01 μg/plate). pcDNAIII-β-gal (control) or activated forms of $G\alpha_q$ and $G\alpha_{13}$ (1 μg each) were added to the transfection mixture for each reporter. Dual luciferase activities were assayed as described in the legend to Fig. 4. The data represent firefly luciferase activity normalized to *Renilla* luciferase activity present in each sample, expressed as -fold induction relative to controls for each reporter, the values of which were taken as 1. All values are the means ± S.E. of triplicate samples from a typical experiment. In each case, similar results were obtained in three additional experiments.

their coupled receptors, including PAR-1, can induce c-*jun* expression through the activation of multiple MAPK cascades that converge in the nucleus to stimulate the transcriptional activity of the c-*jun* promoter. Furthermore, we provide evidence that these molecular events represent key components of the transforming pathway utilized by PAR-1 (Fig. 10).

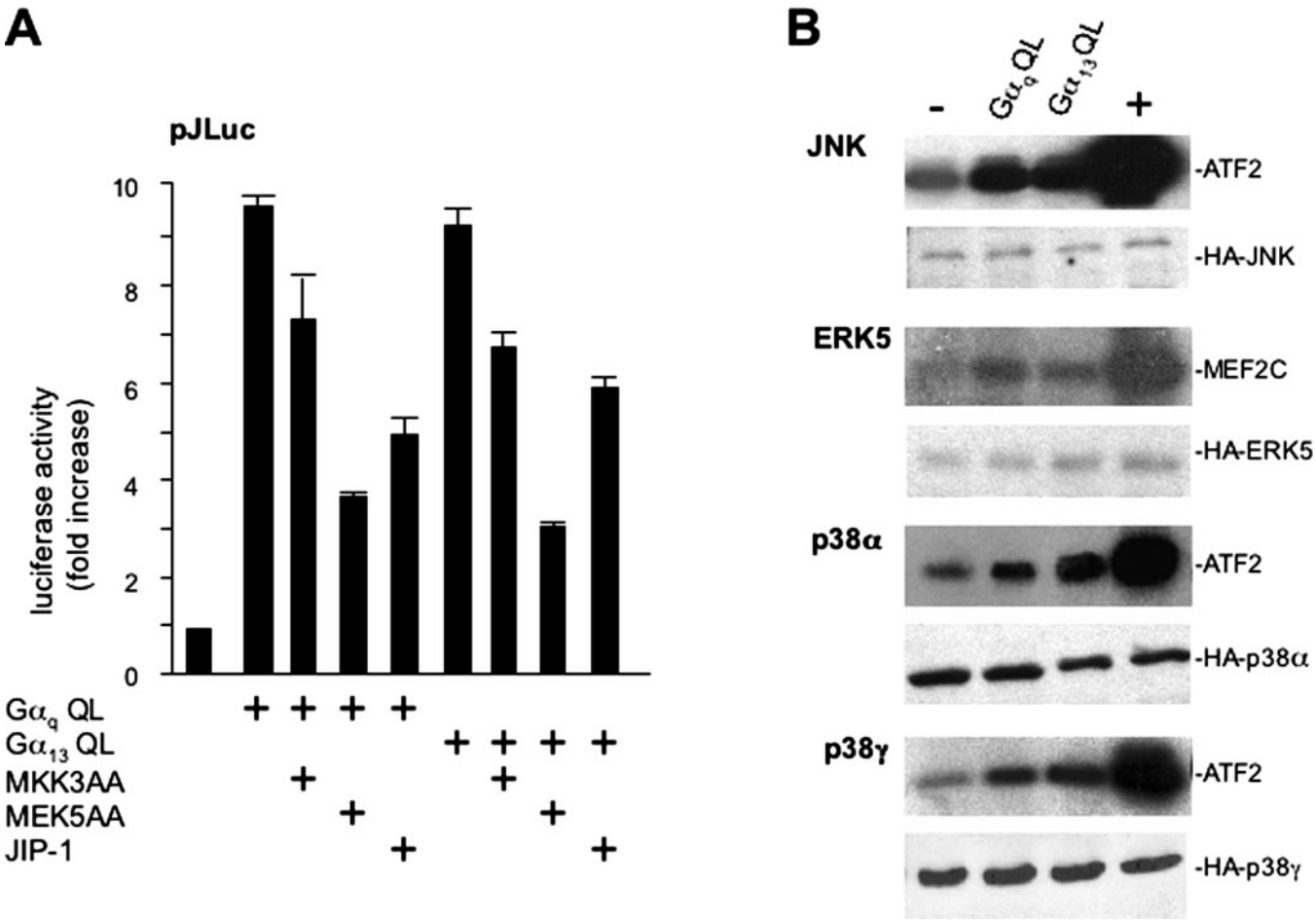

FIG. 7. **$G\alpha_q$ and $G\alpha_{13}$ stimulate the activity of the c-*jun* promoter through the JNK, p38, and ERK5 pathways.** *A*, NIH 3T3 cells were transfected with pJLuc, pcDNAIII-β-gal, and the activated form of $G\alpha_q$ or $G\alpha_{13}$ along with MKK3AA (1 μg), MEK5AA (1 μg), or JIP-1 (0.1 μg), as indicated. 24 h later, cells were collected, and lysates were assayed for dual luciferase activities. The data represent firefly luciferase activity normalized to *Renilla* luciferase activity present in each sample, expressed as -fold induction relative to the corresponding controls, the values of which were taken as 1. Results are the means ± S.E. of triplicate samples from a typical experiment. Similar results were obtained in three additional experiments. *B*, NIH 3T3 cells were transfected with expression vectors containing HA-tagged JNK, ERK5, p38α, or p38γ along with $G\alpha_q$QL and $G\alpha_{13}$QL and vector (−) or the corresponding positive controls (+) (MEKK for JNK, MEK5DD for ERK5, and MKK3 for p38α and p38γ). After serum starvation, lysates were immunoprecipitated with anti-HA antibody and used for kinase reactions. ^{32}P-Labeled substrates are indicated. Data represent results from a typical experiment. Similar results were obtained in three additional experiments. The *lower panels* of each pair of panels show expression of the HA-tagged kinases in lysates from each indicated transfectant upon analysis by Western blotting using a specific anti-HA antibody.

Once thrombin cleaves the N terminus of PAR-1, it exposes a tethered ligand, resulting in receptor activation and the consequent stimulation of G proteins of the $G\alpha_i$, $G\alpha_q$, and $G\alpha_{13}$ families (12). Of interest, treatment with pertussis toxin, which ADP-ribosylates a C-terminal threonine conserved in $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ and uncouples them from receptor activation, has been recently shown to inhibit the transforming activity of PAR-1 (34), suggesting a necessary role for G proteins of the $G\alpha_i$ family in transformation by this thrombin receptor. Treatment with pertussis toxin has also been shown to prevent DNA synthesis in response to thrombin, primarily by blocking its ability to stimulate the activation of ERK (14, 48). These observations suggest that thrombin receptors may utilize $G\alpha_i$ primarily to stimulate ERK. However, activation of G_i alone is not sufficient to cause malignant conversion of NIH 3T3 cells, as activated forms of $G\alpha_{i2}$ induce a hyperproliferative state without causing cell transformation (49). Moreover, $G\alpha_i$-coupled receptors such as m2 receptors do not induce cell growth or exhibit focus-forming potential (8), although they stimulate ERK effectively in a pertussis toxin-sensitive fashion (45). Thus, it is likely that signaling to ERK through $G\alpha_i$ may represent a necessary and yet not sufficient event to stimulate cell proliferation by certain GPCRs, including PAR-1.

In contrast to ERK stimulation, activation of c-*jun* expression requires the functional activity of $G\alpha_q$ and/or $G\alpha_{13}$ family members, as reflected by the reduced c-*jun* expression in response to thrombin in cells defective in $G\alpha_q/G\alpha_{11}$ and by the even more dramatically diminished c-*jun* expression in cells defective in $G\alpha_{12/13}$ compared with both wild-type mouse embryonic fibroblasts and NIH 3T3 cells, in agreement with the more selective coupling to $G\alpha_{12/13}$ by thrombin receptors (50). Although it is possible that these G protein double knockout cells express fewer thrombin receptors, hence exhibiting a limited response to thrombin, these findings were further supported by the fact that m2 receptors can effectively stimulate c-*jun* expression only when coexpressed with chimeric forms of $G\alpha_q$ and $G\alpha_{13}$ that can be activated by G_i-coupled receptors. In addition, the activated forms of $G\alpha_q$ and $G\alpha_{13}$ were alone sufficient to stimulate the transcriptional activity of the c-*jun* promoter, in contrast to $G\alpha_i$, $G\alpha_s$, or $\beta\gamma$ subunit overexpression, which did not elicit any demonstrable response. Together, these data suggest that, in NIH 3T3 cells, only $G\alpha_q$ and $G\alpha_{13}$ and their coupled receptors can promote the activation of signaling events leading to stimulation of the c-*jun* promoter and the consequent expression of c-*jun*.

In the search for the mechanism by which $G\alpha_q$ and $G\alpha_{13}$

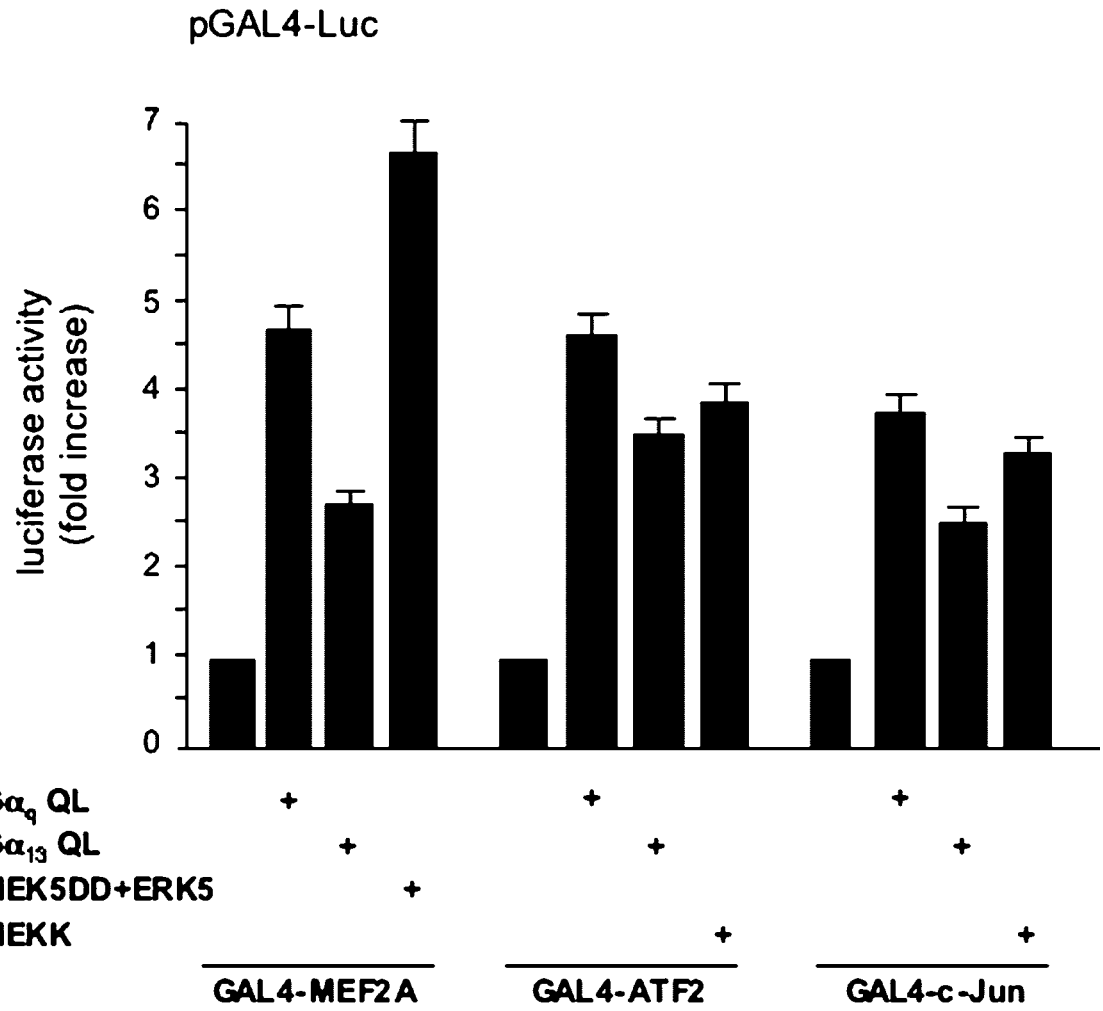

FIG. 8. **Activated mutants of $G\alpha_q$ and $G\alpha_{13}$ stimulate the transcriptional activity of MEF2A, ATF2, and c-Jun.** NIH 3T3 cells were cotransfected with pcDNAIII-Gal4-MEF2A, pcDNAIII-Gal4-ATF2, or pcDNAIII-Gal4-c-Jun (0.05 μg) along with pGal4-Luc and pRL-Null (0.1 and 0.01 μg/plate, respectively). Expression vectors for $G\alpha_q$QL and $G\alpha_{13}$QL (1 μg), MEKK (0.5 μg), and MEK5DD + ERK5 (0.5 μg each) were included in the transfection mixtures, as indicated. 24 h after transfections, cells were collected, and lysates were assayed for dual luciferase activities. The total amount of plasmid DNAs was adjusted with empty vector. The data represent firefly luciferase activity normalized to *Renilla* luciferase activity present in each sample, expressed as -fold induction relative to the respective controls (pcDNAIII-Gal4-MEF2A, pcDNAIII-Gal4-ATF2, or pcDNAIII-Gal4-c-Jun alone), the values of which were taken as 1. Values are the means ± S.E. of triplicate samples from a typical experiment. Nearly identical results were obtained in three additional experiments.

stimulate c-*jun* expression, we first examined which responsive elements within the c-*jun* promoter respond to signals originating from the activated forms of these heterotrimeric α subunits. Using c-*jun* promoter mutants and reporter plasmids containing each critical site individually or in combination, we obtained information supporting that the jAP1 and MEF2 sites are necessary and sufficient to stimulate transcription from the c-*jun* promoter. Furthermore, the use of chimeric molecules, including the DNA-binding domain of the yeast transcription factor Gal4 fused to the transactivating domain of c-Jun and ATF2, which bind jAP1, and MEF2A, the major MEF2 form present in NIH 3T3 cells (25), revealed that $G\alpha_q$ and $G\alpha_{13}$ can effectively stimulate the transcriptional activity of these factors binding the jAP1 and MEF2 response elements. This extends prior observations indicating the requirement of these two elements for the stimulation of the c-*jun* promoter by G_q-coupled receptors (25) and suggests that $G\alpha_{13}$ and its coupled receptors may utilize a mechanism either similar or functionally related to that used by $G\alpha_q$ to induce c-*jun* expression. As each of these two sites is known to respond to the activation of MAPK cascades, we next focused our attention on the contribution of distinct MAPK pathways that are known to act on transcription factors regulating the c-*jun* promoter. Indeed, we observed that activated forms of both $G\alpha_q$ and $G\alpha_{13}$ can stimulate the activity of JNK, p38α, p38γ, and ERK5. Thus, each of these GTPase-deficient G proteins can initiate the activity of multiple MAPK cascades. Consistent with these observations, we found that the use of molecules interfering with each of these pathways can alone diminish, albeit partially, the ability to stimulate the c-*jun* promoter by $G\alpha_q$ and $G\alpha_{13}$. Together, these data support the emerging notion that $G\alpha_q$ and $G\alpha_{13}$ and their coupled receptors can initiate the coordinated activation of multiple MAPK pathways that converge in the nucleus to control the activity of transcription factors regulating c-*jun* expression.

In turn, how $G\alpha_q$ and $G\alpha_{13}$ stimulate JNK, p38 isoforms, and ERK5 is not fully understood at present. Recent data indicate that PAR-1- and m1 receptor-induced cell transformation requires the activity of the small GTPase RhoA (34, 51) and that both $G\alpha_q$ and $G\alpha_{13}$ can potently stimulate Rho activation (52–54). In line with these findings, Rho can enhance the enzymatic activity of JNK and p38γ in certain cells (55, 56). However, many GPCRs are also able to activate Rac1 and Cdc42, both of which can stimulate JNK and c-*jun* expression (24, 56, 57) and even transformation in NIH 3T3 (58, 59). Thus, further work will be required to elucidate whether any of these small GT-

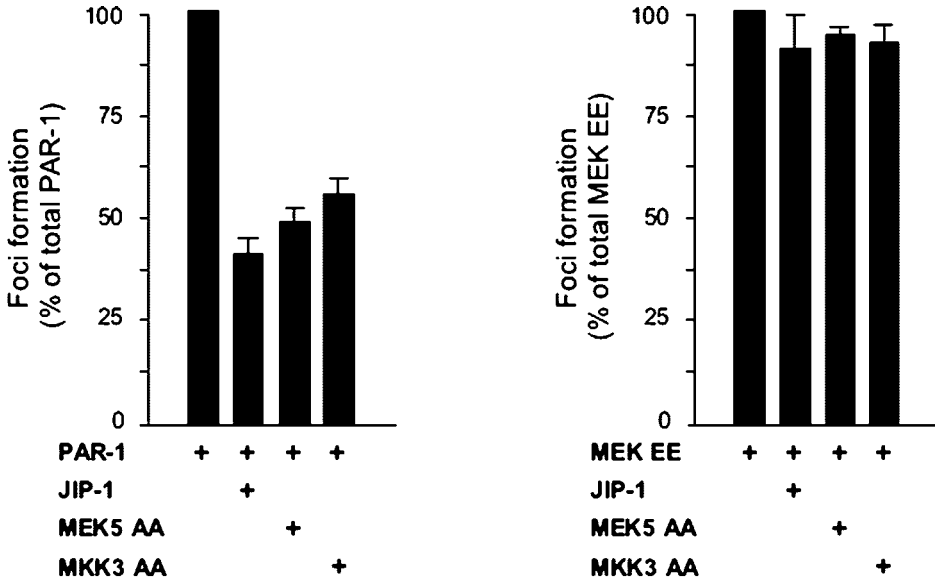

FIG. 9. **The transforming activity of PAR-1 is decreased by molecules interfering with the JNK, ERK5, and p38 pathways.** NIH 3T3 cells were transfected by the calcium phosphate technique with pcDNAIII-β-gal, pCEFL-PAR-1, or pcDNAIII-MEKEE (1 μg) alone or in combination with JIP-1, MEK5AA, or MKK3AA (1 μg). Plates were cultured for 3 weeks in 5% calf serum and then fixed and stained. The data represent the means ± S.E. of triplicate samples from a typical experiment. Similar results were obtained in three additional experiments.

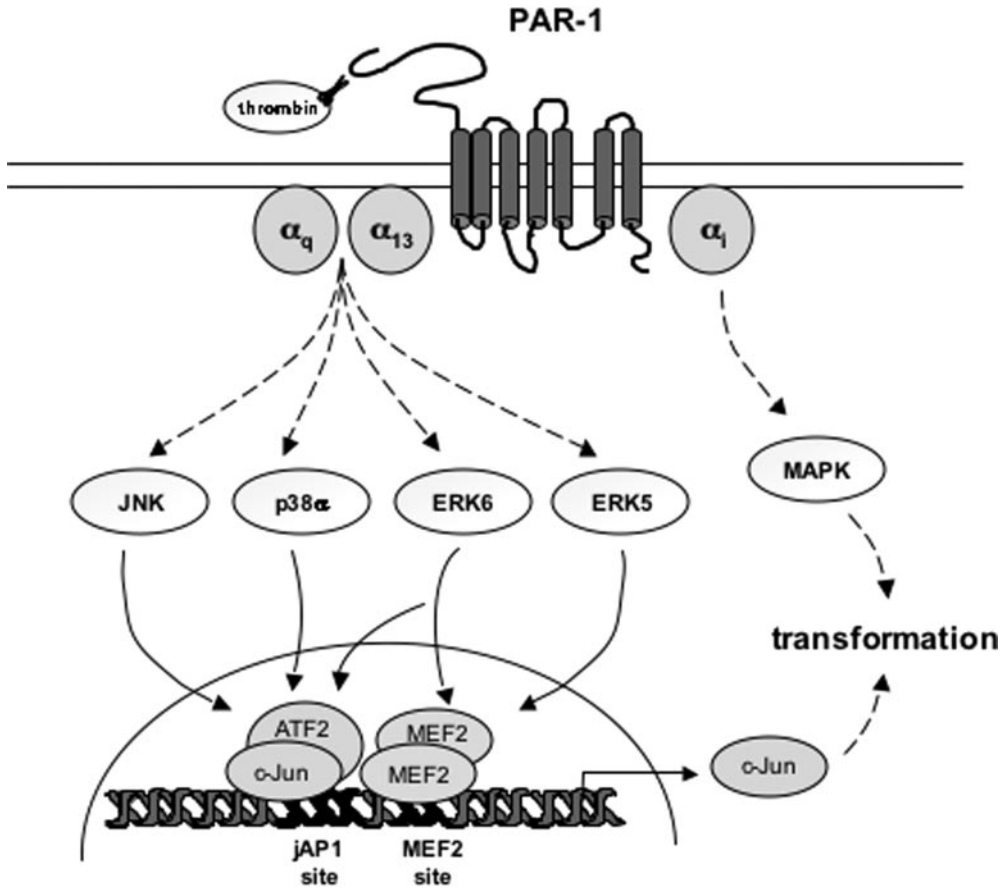

FIG. 10. **Proposed model for PAR-1 signaling to the c-*jun* promoter and transformation.** PAR-1 can initiate a signaling pathway that induces the $G\alpha_q$ and $G\alpha_{13}$ subunits of heterotrimeric G proteins. In turn, they activate, through several members of the MAPK family, the transcription factors bound to the jAP1 and MEF2 sites on the c-*jun* promoter (MEF2A, ATF2, and c-Jun), thereby enhancing its activity. Available data suggest that c-Jun is a critical component in the transforming phenotype induced by these GPCRs in NIH 3T3 cells. Parallel pathways are depicted and described under "Results."

Pases participate in the activation of each MAPK by $G\alpha_q$ and $G\alpha_{13}$ in NIH 3T3 cells and, if so, to elucidate fully the nature of the intervening mechanisms. In this regard, our present study may provide a molecular framework in which these or other related GTPases or signaling molecules might act, which is by controlling the activity of specific MAPKs regulating c-*jun* expression in response to the stimulation of PAR-1 and other receptors coupled to G_q and G_{13}.

On the other hand, the role of Rho GTPases in transformation by PAR-1 may not be solely dependent on their effect on c-*jun* regulation, as Rho proteins are also able to induce the expression of another member of the AP1 family of transcription factors, the c-*fos* proto-oncogene (60). Rather than through MAPKs, this particular effect is exerted through the serum

response element within the sequence of the c-*fos* promoter by a mechanism that is sensitive to changes in the actin cytoskeleton (60, 61). Thus, as dominant-negative c-Jun may prevent focus formation induced by PAR-1 by blocking the activity of both Jun-Jun homodimers and Jun-Fos heterodimers (37), it is still possible that Rho GTPases may act downstream from PAR-1 by enhancing c-Fos expression, thus cooperating with those pathways promoting c-Jun expression through MAPKs to stimulate AP1-dependent genes that are required for cell transformation.

In summary, our results suggest that the ability to stimulate c-*jun* expression can distinguish transforming from non-transforming GPCRs and that this transcriptional response is dependent on the stimulation of $G\alpha_q$ and/or $G\alpha_{13}$ and the subsequent stimulation of MAPK cascades regulating transcription factors that control the c-*jun* promoter. Together, these results define a signaling route by which thrombin receptors can signal to the nucleus to promote the expression of growth-regulating genes. Rather than being restricted to NIH 3T3 cells, these findings may have broad implication in cancer biology, as thrombin can stimulate c-*jun* expression in a variety of cell types such as lung fibroblasts, astrocytoma cells, and vascular smooth muscle cells, leading to cell growth or hypertrophy (17, 48, 62, 63). In addition, overexpression of GPCRs linked to $G\alpha_q$ and/or $G\alpha_{12/13}$ has been reported in numerous cancers, many of which also express their ligands, resulting in the activation of these GPCRs in an autocrine or paracrine fashion (10). Thus, it is likely that the biochemical route by which PAR-1 promotes c-*jun* expression may represent a common mechanism participating in the promotion of cell proliferation in numerous cell types, the contribution of which to normal and aberrant cell growth can now begin to be explored.

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