The c-Jun amino-terminal kinases (JNKs) are a subfamily of mitogen-activated protein kinases that phospho-}y}late c-Jun and ATF2, and it has been postulated that phosphorylated c-Jun enhances its own expression through AP-1 sites on the c-Jun promoter. In this study, we asked whether signals activating JNK regulate the c-Jun promoter. Using NIH 3T3 cells expressing G protein-coupled m1 acetylcholine receptors as an experimental model, we have recently shown that the cholinergic agonist carbachol, but not platelet-derived growth factor, increased the activity of a c-Jun promoter-driven reporter gene (for chloramphenicol acetyltransferase). However, coexpression of JNK kinase (MEKK) effectively increased JNK activity, but resulted in surprisingly limited induction of the c-Jun promoter. This raised the possibility that pathway(s) distinct from JNK control the c-Jun promoter, and prompted us to explore which of its regulatory elements participate in transcriptional control. We observed that deletion of the 3′ AP-1 site diminished chloramphenicol acetyltransferase activity in response to carbachol, but only to a limited extent. In contrast, deletion of a MEF2 site dramatically reduced expression, and deletion of both the MEF2 and 3′ AP-1 sites abolished induction. Furthermore, cotransfection with MEF2C and MEF2D cDNAs potently enhanced the activity of the c-Jun promoter in response to carbachol, and stimulation of m1 receptors, but not direct JNK activation, induced expression of a MEF2-responsive plasmid. Taken together, these data strongly suggest that MEF2 mediates c-Jun promoter expression by G protein-coupled receptors through a yet to be identified pathway, distinct from that of JNK.

The jun and fos gene families are nuclear proto-oncogenes whose expression is induced in quiescent cells by the addition of serum or other growth promoting stimuli (1, 2). The expression of these genes is rapid and does not require newly synthe-
expressed in response to platelet-derived growth factor (PDGF) (3), a mitogen acting on endogenous tyrosine kinase receptors. In particular, carbachol caused a much greater induction of c-jun mRNA expression and AP-1 activity (3). Interestingly, we have observed that the m1 agonist, carbachol, but not PDGF, can induce a remarkable increase in JNK activity, following a temporal pattern distinct from that of MAPK activation (3).

In view of these observations, we set out to investigate whether signals activating JNK regulate expression from the c-jun promoter, using NIH 3T3 cells expressing m1 G protein-coupled receptors (NIH-m1.2 cells) as an experimental model. In these cells, we observed that carbachol, but not PDGF, potently increased the expression from a reporter plasmid containing the chloramphenicol acetyltransferase (CAT) gene under the control of the murine c-jun promoter. However, this effect was not mimicked by expression of activated molecules acting downstream from m1 receptors in the JNK pathway, although they activated JNK to an extent much greater than that caused by the m1 agonist alone. This prompted us to explore the existence of JNK-independent signaling pathways regulating the expression from the c-jun promoter. Using deletion and point mutational analysis of the c-jun promoter, we found a critical role for a DNA sequence that binds the MEF2 family of transcription factors. Furthermore, cotransfection with plasmids carrying the MEF2C and MEF2D cDNAs potently enhanced expression from the c-jun promoter in response to carbachol. In addition, we found that signaling from m1 receptors, but not direct JNK activation, induced expression from a MEF2-responsive plasmid. Taken together, these findings strongly suggest that signaling from G protein-coupled receptors to the c-jun promoter involves a novel, JNK-independent pathway acting on the MEF2 family of transcription factors.

MATERIALS AND METHODS

Cell Lines and Transfection—NIH 3T3 fibroblasts expressing approximately 20,000 human m1 mACHR/cell, designated NIH-m1.2 cells (11), were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf bovine serum. Transfections were performed by the calcium phosphate precipitation technique, adjusting the total amount of plasmid DNA to 5–10 μg/plate with vector alone.

Reporter Gene Assays—NIH-m1.2 cells were transfected with different expression plasmids together with 1 μg of pCMV-βgal, a plasmid expressing the enzyme β-galactosidase, and 1 μg of each of the reporter plasmids. After overnight incubation, the cells were washed with serum-free DMEM, and kept for 24 h in DMEM supplemented with 0.5% fetal bovine serum. Cells were then stimulated with agonists for an additional 6 h and lysed using reporter lysis buffer (Promega).

CAT activity was assayed in the cell extracts by incubation for 6–16 h in the presence of 0.25 μCi of [14C]chloramphenicol (100 μCi/mmol) and 200 μg/ml butyryl-CoA in 0.25 μl Tris-HCl, pH 7.4. Labeled butyrylated products were extracted using a mixture of xylene (Aldrich) and absolute ethanol (Merck). The radioactivity of the resulting precipitate was measured using a Packard Tri-Carb 1900CA liquid scintillation spectrometer.

Kinesin Assays—Phosphorylating activity of endogenous MAPK, JNK, and p38, or of transfected, epitope-tagged MAPK, JNK, and p38 were determined as follows. Cells were seeded at 10% confluence, and the following day cells were left untreated for endogenous activity, or transfected with pcDNA3-HA-MAPK, pcDNA3-HA-JNK, and pcDNA3-HA-p38 and additional DNAs by the LipofectAMINE technique (Life Technologies, Inc.). Transfection efficiencies were determined using the chloramphenicol acetyltransferase (CAT) gene as substrate for MAPK or 1 μg of purified, bacterially expressed, GST-ATF2 protein as substrate for JNK and p38. Samples were analyzed by SDS-gel electrophoresis on 12% acrylamide gels, and autoradiography was performed with the aid of an intensifying screen.

DNA Constructs—A reporter plasmid containing the MEF2 DNA binding sequence (pG5L53) was engineered by inserting the sequence 5′-TCGACGGCTATTTTAGGCGC-3′ in the Sall site of the pGL4 plasmid DNA (Promega), upstream of a SV40 minimal promoter and a luciferase gene. The mutant sequence 5′-TCGACGCGGTAGTTTCGGCC-3′, that does not bind MEF2 proteins (3), was used to generate pGL3MEF2mut. pJC6, pJC9, pJC9, pJTX, pJSTX, and pJSTX have been described previously (14). MEF2C and MEF2D cDNAs were kindly provided by Dr. Bruce Paterson (National Cancer Institute, National Institutes of Health, Bethesda, MD) and subcloned in the pcDNAIIIIB expression vector (Invitrogen). Other DNA expression vectors have already been described (15).

RESULTS

In previous studies, we have observed that stimulation of G protein-coupled receptors of the m1 class by the cholinergic agonist, carbachol, potently elevates JNK activity (3, 16). In turn, JNK has been shown to phosphorylate the NH2-terminal transactivating domain of c-Jun, thereby stimulating AP-1-mediated transcription (17). We have also reported that, consistent with these observations, stimulation of quiescent NIH-m1.2 cells with carbachol induces AP-1 activity and greatly enhances the expression of c-jun mRNA (3). As an approach to explore the relationship between the enzymatic activity of JNK and the regulation of c-jun expression, we transfected NIH-m1.2 cells with a reporter construct containing murine c-jun promoter sequences from −225 to +150 fused to the CAT gene (pJC6) (14) (see below). In agreement with our previous observations (3), we show in Fig. 1 that, whereas carbachol, the tyrosine kinase-receptor ligand PDGF, and the phorbol ester TPA induced MAPK activity to similar extents, only carbachol elevated the phosphorylating activity of JNK in these cells. When reporter gene assays were performed in parallel, we observed that c-jun promoter activity was strongly enhanced by addition of carbachol, whereas treatment with PDGF and TPA did not result in demonstrable activation. Thus, these data suggest that the activity of the c-jun promoter correlated with JNK, but not MAPK activation.

To further explore the roles of JNK and MAPK in the regulation of expression from the c-jun promoter, we employed a constitutively active mutant of MEK, MEKEE, which results in a remarkable increase in MAPK activity (15, 18), and a truncated JNK kinase kinase, MEK, which potently activates JNK (19, 20). Accordingly, we cotransfected expression plasmids for MEKEE and MEK together with plasmids expressing epitope-tagged MAPK and epitope-tagged JNK, or the c-jun promoter containing reporter plasmid. As shown in Fig. 2, whereas MEKEE strongly induced MAPK activity, it had no effect on JNK or on the expression of the c-jun promoter-driven reporter plasmid. On the other hand, MEK did not elevate MAPK activity but potently stimulated JNK-phosphorylating activity, to an extent much greater than even that elicited by m1 receptor stimulation. Surprisingly, however, activation of CAT transcription was more responsive to stimulation through the MAPK pathway than that induced by MEK (Fig. 2). These observations raised the possibility that signaling pathways in addition to JNK, may participate in the activation of the c-jun promoter by m1 G protein-coupled receptors.

The lack of a strict correlation between JNK activation and the expression from the c-jun promoter-containing reporter

an antibody against ERK-2 (SC-154, Santa Cruz Biotechnology) for endogenous MAPK, with an antibody against JNK-1 (15701A, Phar- mingen) for endogenous JNK, and with the anti-HA monoclonal antibody (HA.11, Berkeley Antibody Co.) for the epitope-tagged MAPK, JNK, and p38. The immunoprecipitates were washed, and in vitro kinase assays were performed using 1.3 μg/ml myelin basic protein (Sigma) as substrate for MAPK or 1 μg of purified, bacterially expressed, GST-ATF2 protein as substrate for JNK and p38. Samples were analyzed by SDS-gel electrophoresis on 12% acrylamide gels, and autoradiography was performed with the aid of an intensifying screen.

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The lack of a strict correlation between JNK activation and the expression from the c-jun promoter-containing reporter
plasmid prompted us to search for those regulatory elements in the c-jun promoter that participate in the m1-induced response. In this regard, binding sites for Sp1, AP-1, CTF, and MEF2 transcription factors have been identified in the c-jun promoter at nucleotides −117, −92, −72, and −59, respectively, and two GATAA elements are found at −33 and −11 (Fig. 3A) (14). In addition, a putative site for AP-1, referred to here as AP-1-like, is located at −180 (4). To assess the contribution of the AP-1-like and Sp1 sites, we used two deletion mutants, pJC8 and pJC9, containing fragments −115 to +150 and −80 to +150 of the c-jun promoter, respectively (Fig. 3A). As shown in Fig. 3B, carbachol addition to cells transfected with pJC8 or pJC9 caused an even higher level of CAT expression than that observed for pJC6. These data suggest that the AP-1-like and the Sp1 sites that are absent in both constructs, as well as the CAAT box that is deleted in pJC9, are dispensable for signaling from m1 receptors to the c-jun promoter.

To explore the role of the AP-1 and MEF2 sites, we used a plasmid, pJTX, that harbors four point mutations in the TRE element located at −72 thereby abolishing AP-1 binding (5, 14); a plasmid, pJSX, containing two point mutations, at nucleotides −51 and −58, that have been shown to abolish MEF2 binding (13, 14); and a double mutant, pJSTX, that lacks both AP-1 and MEF2 binding sites (14) (Fig. 3A). As expected, in control experiments we observed that mutation of the AP-1 site, but not of the MEF2 site, nearly abolishes the expression from the reporter plasmid elicited by cotransfection of the

**FIG. 2. Effects of the activation of the MAPK and JNK cascades on the c-jun promoter activity.** NIH-m1.2 cells were cotransfected with pJC6 (1 μg/plate) and pCMV-β-gal (1 μg/plate) plasmid DNAs by the calcium phosphate technique. Expression vectors for MEKK or the constitutively activated mutant of MEK (MEKEE) were included in the transfection mixtures, as indicated, as an approach to activate the JNK or MAPK pathway, respectively. 48 h later, cells were left untreated or exposed for 4 h to 100 μM carbachol, as indicated. The cells were collected, and the lysates were assayed for CAT and β-galactosidase activity. The data represent CAT activity normalized by the β-galactosidase activity present in each sample, and are the average ± S.E. of triplicate samples from a typical experiment. Similar results were obtained in five independent experiments. The autoradiograms correspond to representative experiments for MAPK and JNK activities, respectively. In vitro kinase reactions were performed in immunoprecipitates from cellular lysates, and the reaction mixtures were fractionated by 12% SDS-polyacrylamide gel electrophoresis. Positions of the labeled products are indicated by arrows. Similar results were obtained in three independent experiments. MBP, myelin basic protein; n-but-14C-chl., 14C-labeled chloramphenicol.
activated JNK kinase kinase, MEKK (data not shown). However, the TRE-deficient mutant, pJTX, showed only a relatively small reduction in the response to m1 stimulation (Fig. 3). In contrast, the MEF2-binding mutant, which contains an intact TRE site (pJSX), showed a drastic reduction in the m1-induced response (70–80%). Furthermore, as shown in Fig. 3, mutation of both MEF2 and TRE sites in the c-jun promoter (pJSTX) nearly abolished the expression of CAT elicited by carbachol in m1-expressing cells. These results suggest that both the MEF2 and AP-1 regulatory sites are critical for the regulation of expression from the c-jun promoter in response to intracellular signals transmitted by G protein-coupled receptors. In addition, these data suggest that, in these cells, the majority of the stimulatory response on the c-jun promoter-containing reporter plasmid is exerted through the MEF2 binding site and not through the TRE, as we had initially hypothesized.

MEF2 proteins bind the consensus sequence motif CTA(A/T)4TAG (13, 14), which is present in the c-jun promoter between positions −50 and −59. As an attempt to further investigate if that sequence participates in the transcriptional response triggered by stimulation of m1 receptors, we engineered a reporter construct by introducing oligonucleotides that match exactly the sequence CTATTTTTAG found in the c-jun promoter, upstream of a minimal promoter in the experiment. Similar results were obtained in four independent experiments. The autoradiogram corresponds to a typical experiment resolved on a thin layer chromatography (TLC) plate. The arrows indicate the position of the butyrylated forms of [14C]chloramphenicol.
pGL3promoter plasmid (Promega). The resulting construct expresses a luciferase reporter gene and was designated pGL3MEF2. As a control, we generated a similar reporter plasmid containing the mutant sequence CGATTTTTCG, which does not bind MEF2 (13, 14) and therefore is not able to respond to MEF2 (pGL3MEF2mut). Fig. 4 shows that upon transfection in NIH-m1.2 cells with pGL3MEF2, carbachol induced a remarkable increase in the luciferase activity when compared with the unstimulated control cells. Furthermore, coexpression of MEF2C (Fig. 4) or MEF2D (data not shown) also elevated the reporter gene activity. However, no demonstrable response was observed upon transfection with MEKK, thus suggesting that the MEF2 binding site is insensitive to the activation of the JNK pathway. On the other hand, cells transfected with the construct pGL3MEF2mut exhibited the same level of luciferase activity in each of these conditions, supporting that an intact MEF2 binding site is required to elicit a transcriptional response. Taken together, these findings strongly suggest that the MEF2 binding site could be the target for signaling pathways linking G protein-coupled receptors to the c-jun promoter.

To address directly whether proteins of the MEF2 family could regulate the activity of the c-jun promoter, we next cotransfected pJC6 with expression plasmids harboring the cDNAs for MEF2C or c-Jun in NIH-m1.2 cells. As shown in Fig. 5, CAT expression driven by the c-jun promoter was elevated by cotransfection with the construct pGL3MEF2mut exhibited the same level of luciferase activity in each of these conditions, supporting that an intact MEF2 binding site is required to elicit a transcriptional response. Taken together, these findings strongly suggest that the MEF2 binding site could be the target for signaling pathways linking G protein-coupled receptors to the c-jun promoter.

FIG. 5. Coexpression of MEF2 or c-Jun can potentiate the induction of the c-jun promoter by carbachol. NIH-m1.2 cells were cotransfected with pJC6 alone or together with expression vectors for c-Jun or MEF2C, as indicated. In each case, 1 μg of pCMV-β-gal reporter plasmid DNA was included in the transfection mixture. 48 h later, cells were left untreated or exposed for 4 h to 100 μM carbachol. The cells were collected, and the lysates were assayed for CAT and β-galactosidase activity. The data represent CAT activity normalized by the β-galactosidase activity present in each sample, and are the average ± S.E. of triplicate samples from a representative experiment. Similar results were obtained in three independent experiments.

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A recent study (29) showed that one of the members of the MEF2 family (MEF2C) is phosphorylated by a novel member of the MAPK superfamily, p38, in myeloid lineage cells. Therefore we explored whether p38, by regulating the activity of MEF2, might mediate in the induction of the c-jun promoter by G protein-coupled receptors. To that end, we transfected NIH-m1.2 cells with pJC6 and measured CAT activity upon carbachol stimulation in the presence or absence of the specific p38 inhibitor, SB203580 (30). As shown in Fig. 6, incubation with SB203580 nearly abolished p38 activation by anisomycin or carbachol but did not have any demonstrable effect on transcriptional activation by the c-jun promoter. Similar results were obtained using pJTX, the mutant of the c-jun promoter lacking the AP-1 site but containing a MEF2-responsive element (Fig. 6). These results suggest that p38 does not regulate MEF2 activation of the c-jun promoter in NIH-m1.2 cells.

DISCUSSION

Positive autoregulation has been considered the major regulatory event in the response of the c-jun promoter to proliferative stimuli. Two steps have been described as participating in that process: 1) post-translational modification of preexisting c-Jun proteins and 2) transcriptional activation of c-jun that leads to an increase in the pool of c-Jun protein present in the cell (1, 4, 5, 21). c-Jun is a constitutive part of the AP-1 transcription factor, and binding of such a factor to the TRE element in the c-jun promoter has been shown to result in the stimulation of transcription (5). Using NIH-3T3 cells expressing m1 G protein-coupled receptors, we had previously ob-
MEKK resulted in activation of the c-jun promoter activity and JNK, but not with MAPK. Surprisingly, we observed a positive correlation between the c-jun receptors to the promoter of c-jun and JNK, but not with MAPK. Indeed, we observed a positive correlation between the c-jun promoter activity by carbachol. NIH-m1.2 cells were transfected with 1 μg of pJC6 or pJTX as indicated. In each case, 1 μg of pCMV-β-gal reporter plasmid DNA was included in the transfection mixture. 48 h later, cells were left untreated or exposed for 2 h to 20 μM SB203580 or left untreated, followed by a subsequent incubation for 4 h in the presence or absence of 100 μM carbachol, as shown. Cells were collected, and the lysates were assayed for CAT and β-galactosidase activity. The data represent CAT activity normalized by the β-galactosidase activity present in each sample, and are the average ± S.E. of triplicate samples from a representative experiment. Similar results were obtained in two independent experiments. In parallel, NIH-m1.2 cells were transfected with pcDNA3-HA-p38 (1 μg/plate) for p38 kinase assays and treated with 20 μM SB203580 for 2 h or left untreated. Cells were then incubated with 100 μM carbachol or 10 μM amionycin for 15 min. as indicated. Kinase reactions were performed in anti-HA immunoprecipitates from the corresponding cellular lysates as described under “Materials and Methods.” 32P-Labeled products are indicated.

We found that deletion of the binding sites for Sp1 or CTF did not affect reporter gene activity. Furthermore, we found that mutations that abolish AP-1 binding prevent expression from the reporter plasmid when induced by MEKK, but display only a partial reduction in the response to carbachol. In contrast, a more dramatic decrease was observed when mutations that inhibit binding of MEF2 factors were introduced, thus suggesting a role for the MEF2 factors in regulating c-jun promoter activity, perhaps even more critical than that played by AP-1 itself. Furthermore, the fact that the double mutant that does not bind AP-1 or MEF2 fails to respond to carbachol, indicates that, for signals initiated at the level of m1 G protein-coupled receptors, the AP-1 and MEF2 sites are sufficient to account for the transcriptional response of the c-jun promoter.

While extensive studies have established the role of AP-1 complexes in transcriptional control, very limited information is available about the functions of MEF2 proteins. The myocyte enhancer family (MEF) of transcription factors comprises a group of transcriptional activators MEF2A, -B, -C, and -D (22–25). MEF2 binding sites have been found in the promoters and enhancers of many skeletal and cardiac muscle structural genes (26). Structurally, MEF2 proteins contain a common motif that was named the MEF2 domain, this feature being unique to all MEF2 family members described so far (26). Despite the importance of MEF2 factors in the regulation of muscle gene expression, very little is known about the mechanism by which these proteins promote transcription or the signal transduction pathways controlling their activity. Recently, MEF2C, but not the other members of the family, has been shown to be a substrate for the MAPK-related p38 (29). While the expression of MEF2A, -B, and -D proteins is ubiquitous, the expression of MEF2C seems to be restricted to cortical neurons, mononuclear and muscle cells (23, 24, 27). This wide distribution suggests that their function is not limited to the regulation of muscle specific gene expression (27). Interestingly, they share with other transcription factors, such as serum response factor, the presence of a common structural domain termed MADS box, and due to that characteristic they were originally named RSRFs (related to serum response factors) (13).

In this study, we provide evidence for a critical role for MEF2 in the regulation of c-jun promoter activity by stimulation of G protein-coupled receptors. In addition, we show that overexpression of the MEF2 protein potentiates carbachol-induced expression of the c-jun promoter, strengthening the concept of MEF2 proteins as the targets of a yet uncharacterized signal transduction pathway. We also report here that an isolated MEF2 binding site stimulates transcription of a reporter gene when m1-expressing cells are stimulated by carbachol, but not by cotransfection with the JNK-inducing construct MEKK. Taken together, our data support the existence of two distinct signaling pathways that initiating on the same receptor at the level of the plasma membrane converge on the c-jun promoter: a JNK-dependent pathway, acting on the AP-1 responsive element; and a JNK-independent pathway, acting on the MEF2 regulatory sequence. In this regard, a recent report showed that the transactivating activity of MEF2C can be regulated by p38 (29). However, we observed that SB205380, a potent p38 and p38β blocker, fails to inhibit c-jun promoter activity, thus ruling out these p38 proteins as mediators of the carbachol-induced signal to MEF2. Nevertheless, NIH 3T3 cells express MEF2A, -B, and -D but not MEF2C (23, 24, 27), and it has been recently shown that p38α and p38β are not inhibited by SB205380 (31). Thus, it is likely that these or other members of the MAPK superfamily might mediate MEF2 activity in these contexts.
The identity of such regulatory molecule acting on other members of the MEF2 family of transcription factors is under current investigation.

Our results assign a critical role for MEF2 in the regulation of c-jun expression by the G protein-linked class of cell surface receptors. Interestingly, Han et al. provided evidence that the MEF2 binding site in the c-jun promoter also participates in the activation of c-jun by epidermal growth factor receptors and serum in HeLa cells (14, 28). Thus, the elucidation of the molecular entities that participate in the transduction of signals from the membrane to MEF2 proteins, as well as of the mechanisms whereby those signaling molecules control the transcriptional activity of MEF2, is expected to be central for understanding the regulation of c-jun expression by growth promoting factors. Furthermore, considering the ubiquitous expression of MEF2 proteins and their resemblance to other transcription factors such as serum response factor, we can envision a growing interest for dissecting the signaling pathways that converge on MEF2.

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