Disabled-2 (Dab2) is a putative tumor suppressor in breast and ovarian cancers. Its expression is lost in a majority of tumors, and homozygous deletions have been identified in a small percentage of tumors. Dab2 expression is absent or very low in the majority of breast and ovarian cancer cell lines, including MCF-7 and SK-Br-3 breast cancer cells. Transfection and expression of Dab2 in MCF-7 and SK-Br-3 cells suppress tumorigenicity. The cells reach a much lower saturation density and have reduced ability to form colonies on agar plates. In examining the signal transduction pathway of Dab2-transfected cells, we found that serum-stimulated c-Fos expression was greatly suppressed; however, the effects of Dab2 on MAPK family kinases were not as consistent. In MCF-7 and SK-Br-3 cells, although c-Fos expression was suppressed, the Erk1/2, JNK, and p38 MAPK activities were unchanged or even increased. Serum-stimulated c-Fos expression is dependent on MAPK/Erk activity because the MEK inhibitor PD98059 suppresses Erk activity and c-Fos expression. Therefore, Dab2 appears to uncouple MAPK activation and c-fos transcription. Thus, we conclude that Dab2 re-expression suppresses tumorigenicity by reducing c-Fos expression at a site downstream of the activation of MAPK family kinases. Because Dab2 is frequently lost in cancer, the uncoupling of MAPK activation and c-Fos expression may be a favored target for inactivation in tumorigenesis.

Mitogen-activated kinases (MAPK), also known as extracellular signal-regulated kinases (Erk), are the key downstream targets of the Ras pathway (1–3). The MAPK pathway is used in numerous signaling systems involved in cell growth, differentiation, and development (1–5). The Ras/MAPK pathway has the potential for oncogenic transformation of cells (6, 7), as revealed by the discoveries of viral oncogenes such as v-Ras, v-Raf, v-Jun, and v-Fos. The cellular components of these oncogenes function in the Ras/MAPK pathway. The pathway is also a key target for cell transformation in tumor development in that about 50% of cancers harbor an activating mutation of Ras (6, 8). The cell regulatory system has developed an intricate network for the fine regulation of the Ras/MAPK pathway to counter cell transformation. For example, normal human fibroblasts will undergo senescence or programmed cell death when an activated Ras is introduced (9, 10). Growth factor-stimulated Ras signals are also feedback regulated/inhibited following growth factor binding by receptor degradation (11), dissociation of Sos and Grb2 upon phosphorylation of Sos by activated MAPK (12–15), by the enzymatic actions of Ras GAP, and by the actions of phosphatases specific for the kinases in the pathway. Additionally, the surrounding environment of the cells, such as contact with the extracellular matrix, can modify the effects of growth factors on the activation of Ras/MAPK pathway (16, 17). In breast and ovarian cancers, mutations of Ras are rare, and activating mutations of other components in the Ras/MAPK pathway are also uncommon (18). It is believed that regulators in the fine-tuning of the Ras/MAPK pathway are lost, resulting in aberrant activation of the pathway.

The conservation of the Ras/MAPK pathway in yeast, Cae
norhabditis elegans and Drosophila has helped to delineate the components and regulation of the pathway. In mammalian cells, the growth factor binds to its tyrosine kinase receptor and stimulates its autophosphorylation on its tyrosine residues. The phosphotyrosine residues on the receptor act as a docking site for assembling critical intracellular signaling molecules at the cell membrane to initiate a signal cascade (19, 20). The adapter molecule Grb2 binds to the tyrosine receptor through Shc or directly to the phosphotyrosine residue, bringing associated Sos to activate Ras on the plasma membranes. Ras is activated upon conversion to the GTP bound form and initiates the Raf-1/MEK/MAPK kinase cascade. An established target for MAPK is Elk-1, a transcription factor required for transcription of c-Fos (21–23). c-Fos was first identified as a cellular counterpart of the viral oncogene capable of cell transformation (24), and its expression is the target of regulation in cell growth control (25). c-fos is an immediate early gene whose transcription is activated by serum and growth factors, and its expression is a key switch in cellular regulation (24, 25). c-Fos, together with c-Jun, form the AP-1 transcriptional complex required for the transcription of many genes important for cell growth, differentiation, and transformation (26, 27).

We now report that the expression of c-Fos is a target for the regulatory function of Disabled-2 (Dab2), a candidate tumor suppressor of breast and ovarian tumors (28–30). Dab2, a mammalian ortholog of the Drosophila Abi kinase-interacting protein Disabled (31), was isolated as a mitogen responsive phosphoprotein (32). Dab2 was identified by differential displaying to be a gene whose expression was absent in ovarian...
cancer cells but present in normal ovarian epithelial cells (28). We have previously found that Dab2 is expressed in breast and ovarian epithelial cells, but its expression is lost in the majority (about 85%) of breast and ovarian tumor cells (28, 29, 30). Dab2 contains a phosphotyrosine-interacting domain (PID, or PTB) in its N terminus and a proline-rich, SH3-binding domain in its C terminus, resembling an adapter molecule (32). Its binding to Grb2, competing with Sos, leads to the hypothesis that Dab2 is a Ras/MAPK pathway regulator that is lost in cancer (33). We found that re-expression of Dab2 in breast cancer cells leads to suppression of c-Fos expression and cell growth inhibition. Surprisingly, Dab2 does not inhibit MAPK activity. Thus, a regulatory step in the Ras/MAPK pathway is the uncoupling of the activation of MAPK and transcriptional activation of c-Fos, mediated by Dab2. Tumor cells likely abrogate the essential regulation of the Ras/MAPK pathway in normal cells by the elimination of Dab2, which might contribute to cell transformation.

EXPERIMENTAL PROCEDURES

Materials—Kinase inhibitors, PD98059 and SB202190, were purchased from Calbiochem (San Diego, CA). Tissue culture plastic wares were obtained from Fisher Scientific Inc. (Springfield, NJ). DMEM medium was purchased from Mediatech (Herndon, VA); fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA); antibiotic-antimycotic (100×) solution, LipofectAMINE, and serum-free Opti-MEM 1 medium were purchased from Life Technologies, Inc. (Grand Island, NY). The ECL Western blot detection kit was purchased from Pierce (Rockford, IL); Hybrisol I hybridization solution was from Intergen Inc. (Purchase, NY); positively charged nylon membranes were from Roche Molecular Biochemicals; general chemicals and solvents including Me$_2$SO, ethanol, isopropyl alcohol, and agarose were from Sigma or Fisher and were of reagent grade or higher.

Cell Culture—MCF-7 and SK-Br-3 human breast cancer cells were purchased from ATCC. The cells were cultured in DMEM with 10% FBS supplemented with 1% non-essential amino acid mix and antibiotic-antimycotic solution.

Antibodies and Western Blot—Anti-Dab2 antibodies were characterized previously (30–32). Anti-p96 antibodies were purchased from Transduction Labs. (Lexington, KY); anti-c-Fos was from UpState Biotechnology (Lake Placid, NY); anti-β-actin was from Sigma; anti-Erk1/2 and phospho-Erk1/2 were from BioLegend and Cell Signaling Technology Inc. (Beverly, MA); anti-Erk1 and anti-phospho-Erk1 were from Promega and Santa Cruz Biotechnology (Santa Cruz, CA). Western blotting was performed according to standard procedures, as described previously (33). In some cases, after gaining experience with usage of a single antibody, two or more antibodies were used in the same incubation to detect various molecular weight proteins simultaneously.

Cell Transfection—The full-length human DAB2 cDNA (GenBank™ accession number AF188298) was inserted into the pcDNA/zeo eukaryotic expression vector (Invitrogen, La Jolla, CA). Plasmid DNA was purified using the Qiagen Maxiprep column, and LipofectAMINE reagent was used for transfection. Briefly, 2 µg of Dab2 expression construct or vector control plasmid DNA was mixed with 20 µl of LipofectAMINE in 1 ml of Opti-MEM and was added to cells for 16 h. The transfection mix was removed, and fresh DMEM containing 10% FBS was added. After 12 h, selection medium (DMEM with 10% FBS and 300 ng/ml of Zeomycin) was added to the cells. Following a 10–12 day selection with change of medium every 2 days to remove dead cells, selected clones were isolated and collected by cloning rings, expanded by further culturing, and examined for Dab2 expression by Western blotting.

Cell Growth Analysis—Cell growth was determined by counting untransfected Dab2, and the three high expressing clones 49, 50, and 51 of 64 Sk-Br-3 clones selected retained some expression of the transfection mix was removed, and fresh DMEM containing 10% FBS was added. After 12 h, selection medium (DMEM with 10% FBS and 300 ng/ml of Zeomycin) was added to the cells. Following a 10–12 day selection with change of medium every 2 days to remove dead cells, selected clones were isolated and collected by cloning rings, expanded by further culturing, and examined for Dab2 expression by Western blotting.

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Cell Cycle Analysis by Flow Cytometry—Cells on 100-mm plates were harvested with trypsin-EDTA solution and pelleted by centrifugation. The cells were then fixed with 70% ethanol, pelleted, and resuspended in propidium iodine staining solution for 30 min at 4 °C. The stained cells were analyzed by flow cytometry.

RESULTS

Establishment of Dab2 Expression in MCF-7 and SK-Br-3 Breast Cancer Cells—We have previously found that Dab2 is expressed in breast and ovarian epithelial cells, but its expression is lost in the majority (about 85%) of breast and ovarian tumor cells tested (28, 29, 30). Forced expression of Dab2 in tumor cells reduces cell growth, induces cell death, and suppresses tumorigenicity in the nude mouse xenograft model (29, 34). To further determine the biological consequence of Dab2 loss for the tumor cells and to examine the signal transduction pathway affected, we have transfected and established Dab2 expression in MCF-7 and SK-Br-3 breast tumor cells. Dab2 expression is absent in these two breast carcinoma cell lines (30).

Following transfection of the human Dab2 cDNA in the pcDNA expression vector into MCF-7 breast cancer cells, 26 zeomycin-resistant clones were selected. Of these clones, only two clones were found to express Dab2 as detected by Western blot, and a single clone (clone 8) still retained Dab2 expression upon expansion of the cells in culture (Fig. 1A). In contrast, 22 of SK-Br-3 clones selected retained some expression of the transfected Dab2, and the three high expressing clones 49, 50, and 51 were chosen for further analysis (Fig. 1B). Three randomly selected vector transfected clones of each cell line were expanded for use as controls.

Transfection and Expression of Dab2 Inhibit Cell Growth and Transformation in MCF-7 and SK-Br-3 Breast Cancer Cells—Upon establishment of the Dab2-expressing cells, we first characterized the growth properties of the cells. Transfected MCF-7 cells (clone 8) were found to grow more slowly in either low (0.1%) or high (10%) serum compared with vector-transfected controls (Fig. 2A). A similar growth retardation
was found in the three Dab2-expressing SK-Br-3 clones compared to three vector-transfected clones (Fig. 2B). The Dab2-transfected MCF-7 cells were found to have a reduced ability to form colonies on agar plates; MCF-7-Dab2 clone 8 cells formed fewer (about 20% of control) and smaller colonies than vector-transfected clones (data not shown). All three clones of Dab2-transfected Sk-Br-3 cells also had a reduced ability to form colonies on agar plates (not shown). All four selected Dab2-expressing tumor cell clones were analyzed for cell cycle parameters using flow cytometry (Table I). Compared with vector-transfected and non-transfected cells, both MCF-7 and Sk-Br-3 cells expressing Dab2 have about a 50% lower percentage of cells in S phase and about 25% lower percentage of cells in G2/M phase, suggesting a prolonged G1 phase. These results are consistent with previous reports with different tumor cell lines on the negative cell growth regulatory properties and tumor suppressive activity of Dab2 (29, 35). Our goal is to analyze the effects on the cellular signal transduction pathways by the Dab2 protein in these cells.

Dab2 Transfection and Expression Inhibits Serum-stimulated c-Fos Expression—Next, we examined the possible changes in mitogenic signaling in Dab2-expressing cells compared to vector-transfected cells. We observed by both Western and Northern blots a reduction of c-Fos expression upon serum stimulation in Dab2-expressing cells. For both MCF-7 cells and Sk-Br-3, c-Fos is induced by serum at 30 min and is maximal at 60 min in vector-transfected cells (and also in non-transfected cells). In Dab2-expressing MCF-7 cells (clone 8) (Fig. 3) and a representative clone 49 of Dab2-expressing SK-Br-3 cells (Fig. 5B), little c-Fos expression is induced by serum. The same effect of Dab2 expression on c-Fos expression was also observed in transfection of tumor cells with Dab2 using an adenoviral vector in our previous investigation (34). Thus, the suppression of c-Fos expression is not because of the particular properties of the selected cell clones but the result of Dab2 expression.

Effect of Dab2 Expression on the Activation of Erk1/2, JNK, and p38 MAPK Kinases following Serum Stimulation—It has been established that MAPK activation leads to c-Fos transcription and increases in AP-1 activity (21–23). To our surprise, however, no reduction in MAPK activity was observed in Dab2-expressing MCF-7 or SK-Br-3 cells although c-Fos expression was suppressed. To eliminate possible artifacts and to confirm this observation, we performed Western blot analysis to determine c-Fos expression and MAPK activation simultaneously on the same blot using an anti-phospho-MAPK (activated) antibody and p38 MAPK Kinase Antibody. Expression of c-Fos was determined simultaneously on the same blot with anti-c-Fos antibody in the same Western blot. Western blot results showed that c-Fos expression was reduced to nearly undetectable levels in Dab2-expressing MCF-7 and Sk-Br-3 cells expressing Dab2, whereas the activation of Erk1/2, JNK, and p38 MAPK kinases remained unchanged. These results indicate that the suppression of c-Fos expression is not due to the inhibition of MAPK activation but rather to a specific effect of Dab2 on c-Fos expression.

TABLE I
Cell cycle parameters of the transfected cell clones

| Cell clone | G0/G1 | S | G2/M |
|------------|------|---|------|
| MCF-7      | 66   | 16 | 18   |
| MCF-7-Vector | 64 | 15 | 21   |
| MCF-7-Dab2 (no. 8) | 76 | 9  | 15   |
| SK-Br-3    | 44   | 8  | 48   |
| SK-Br-3-vector | 52 | 10 | 38   |
| SK-Br-3-Dab2 (no. 49) | 71 | 3  | 26   |
| SK-Br-3-Dab2 (no. 50) | 63 | 3  | 34   |
| SK-Br-3-Dab2 (no. 57) | 69 | 4  | 27   |

FIG. 2. Characterization of MCF-7 and Sk-Br-3 cell clones transfected with Dab2. Growth curve for MCF-7 vector-transfected or Dab2-transfected clone 8 (A) and SK-Br-3 vector-transfected or Dab2-transfected clone 49 (B) cells. Cells on 35-mm plates were cultured in medium with 1 or 10% serum. Cell numbers were determined by counting (A) or MTT assay (B). Error bars indicate S.D. from measurement of triplicate plates. Data shown are representative of five or more independent experiments using either cell counting or the MTT assay.

FIG. 3. Effect of Dab2 expression on activation of Erk1/2, JNK, and p38 MAPK kinases following serum stimulation. MCF-7 cells transfected with vector or Dab2 (clone 8) were seeded on 35-mm plates. The cells were cultured without serum for 18 h and then stimulated with serum for 0, 15, 30, 60, and 120 min. Cells were immediately washed twice with cold phosphate-buffered saline, lysed with SDS gel loading buffer and boiled for 5 min. The cell lysates were analyzed by Western blotting for MAPK activation with anti-phosphopeptide antibodies for Erk1/2, JNK, and p38 MAPK. Expression of c-Fos was determined simultaneously on the same blot as MAPK activation. β-Actin was used as a loading control.
Dab2 Suppresses c-Fos Expression in Breast Cancer Cells

FIG. 4. Inhibition of serum-stimulated c-Fos expression by PD98059 and SB202190 in MCF-7 cells. MCF-7 cells were seeded onto 35-mm plates and grown to 80% confluency and were then cultured without serum for 18 h. By the end of the 18-h incubation, serial concentrations of PD98059 (A) or SB202190 (B) were added and incubated for 30 min. The cells were then stimulated with 10% serum in the presence of the same concentration of the compounds. Cell lysates were prepared at 0, 15 (or 30), and 60 min time points for Western blot analysis using antibodies for c-Fos and phospho-Erk1/2 to determine c-Fos expression and MAPK activation simultaneously.

serum-stimulated c-Fos expression is greatly reduced compared with vector-transfected control, MAPK activity is enhanced as detected by phosphopeptide antibodies in this experiment (Fig. 3). Similar effects of Dab2 expression in SK-Br-3 on c-Fos expression and MAPK activation were found in Dab2-expressing clones (not shown). We also investigated and found no effect of Dab2 expression on Ras and Raf-1 activation (not shown). Thus, restoration of Dab2 expression appears to dissociate MAPK activation and c-Fos expression in tumor cells.

The effect of Dab2 expression on other MAPK family kinases including JNK and p38MAPK kinases was also investigated. Dab2 expression appears to have no significant and consistent effect on serum-stimulated JNK activity as detected by Western blot with JNK-phosphopeptide specific antibodies (Fig. 3). In either Dab2-expressing or vector-transfected MCF-7 and SK-Br-3 cells, serum stimulation did not notably activate p38MAPK as detected by anti-p38MAPK phosphopeptide antibodies (not shown), though in the same experiment, strong activation was observed in a positive control using anisomycin as a stimulating agent.

Effect of Kinase Inhibitors on c-Fos Expression—The effect of MAPK family kinase activation on c-Fos expression was further explored in MCF-7 and Sk-Br-3 cells using kinase inhibitors PD98059 and SB202190. In MCF-7 cells, the MEK inhibitor PD98059 inhibited MAPK activation and c-Fos expression in a dose-dependent manner (Fig. 4A), indicating PD98059-inhibitable MAPK activity is necessary for serum to activate c-Fos expression. In contrast, the p38MAPK inhibitor SB202190, although it appears to reduce the basal state of MAPK activity, had no inhibitory effect on serum-stimulated MAPK (Erk) activation and c-Fos expression (Fig. 4B). In comparison, inhibition of c-Fos expression by MEK inhibitor PD98059 (Fig. 5A) mechanistically differed from inhibition of c-Fos expression by Dab2 expression (Fig. 5B) in SK-Br-3 cells. Thus, Erk1/2, but not p38MAPK, is required for serum-stimulated c-Fos expression. Unlike PD98059, Dab2 represses c-Fos expression at a step between MAPK activation and c-Fos expression without inhibiting MAPK activity (Fig. 6).

Effect of Dab2 Expression on the Phosphorylation/Activation of Elk-1—It has been established that MAPK phosphorylates/activates the transcription factor Elk-1, and activated Elk-1 binds to the c-Fos promoter and activates expression of c-Fos (21–23). We found that Dab2 expression reduced the serum-stimulated phosphorylation of Elk-1 in MCF-7 cells and in Sk-Br-3 cells (not shown). To explore the mechanism for the effects of Dab2 on MAPK and Elk-1, we examined the physical interaction between Erk1/2, Elk-1, and Dab2. In co-immunoprecipitation experiments, we found that Dab2 is not associated in any significant way with Erk1/2 or Elk-1, either the phosphorylated or unphosphorylated proteins. Thus, through an indirect but unclear mechanism, Dab2 uncouples MAPK activation and Elk-1 phosphorylation.

Conclusion—Dab2 is frequently lost in breast and ovarian tumors (30). We have shown here in MCF-7 and SK-Br-3 breast cancer cells, and others have shown in additional tumor cells (29, 35), that transfection and expression of Dab2 suppresses tumorigenicity; the cells reach a much lower saturation density, have reduced ability to form colonies on agar plates, and have suppressed ability to develop tumors in nude mice. In analysis of signal transduction pathways affected, we have found that serum-stimulated c-Fos expression is greatly sup-

FIG. 5. Comparison of Dab2 expression and PD98059 on the inhibition of serum-stimulated c-Fos expression in SK-Br-3 cells. A, SK-Br-3 cells (untransfected) were seeded onto 35-mm plates and were cultured without serum for 18 h. By the end of the 18-h incubation, PD98059 (100 μM) was added to one set of cells for 30 min. The cells were then stimulated with 10% serum, with or without addition of PD98059 (100 μM) for 0, 15, 30, and 60 min. The cell lysates were harvested at 0, 15, 30, and 60 min and were assayed for c-Fos expression and MAPK activation simultaneously by Western blot analysis.

FIG. 6. Schematic model presentation of Dab2 regulation of c-Fos expression. Serum and growth factor activates Ras and MAP kinase, which is inhibited upon addition of the MEK inhibitor PD98059. Phosphorylation of Elk-1 by MAPK is required for serum-stimulated c-Fos transcription. The pathway is regulated by Dab2 by uncoupling MAPK activation and c-Fos expression. This regulatory mechanism is often absent in tumor cells because of the loss of Dab2.
pressed. Expression of c-Fos is achieved through the action of MAP kinase phosphorylation (21–23). Surprisingly, the Erk1/2, JNK kinase, and p38MAPK activities were unchanged or even increased upon serum stimulation in transfected Dab2-expressing cells compared with vector-transfected cells. Thus, we conclude that Dab2 re-expression suppresses tumorigenicity by uncoupling MAPK activation and c-Fos expression. Although Dab2 could have additional effects on the cells, the suppression of c-Fos expression may be sufficient to suppress cell growth and transformation.

It is well established that the Ras pathway through a cascade of kinases, results in activating the expression of immediate early genes such as c-Fos (21–23). Normally, Ras/MAPK activity is well correlated with c-Fos expression. MAP kinases, Erk1 and Erk2, upon activation will phosphorylate Elk-1, an ETS family transcription factor (21). Phosphorylation of Elk-1 at serine 383 activates its ability to participate in the transcription complex that transcribes c-Fos (21–23). Two recent studies report that MAPK activation and Elk-1 phosphorylation/activation are uncoupled (36, 39). KSR, a mammalian ortholog of the Drosophila kinase suppressor of Ras (KSR), can inhibit Elk-1 phosphorylation without affecting MAPK activation (36). The affect of KSR on inhibition of Elk-1 phosphorylation is believed to act through the activation of the Ca2+ and calmodulin-regulated PP2B (calcineurin), the major phosphatase for Elk-1 (37, 38). The mechanism for the activation of PP2B by KSR is still unknown. Another report shows that adapter protein Gab2, the probable ortholog of the protein Gab1, can inhibit Elk-1, though no mechanism is yet known (39). It is interesting that both Dab2 and Gab2 are Grb2-binding proteins (32, 39), which may provide some common mechanism in uncoupling MAPK and Elk-1. In normal cells that are Dab2-positive, the ability to uncouple MAPK activation and c-Fos expression will enable the cells to achieve precise control of biological processes, because the Ras/MAPK pathway is widely used for cell growth, differentiation, and development (1–5).

There are several possible mechanisms for Dab2 to uncouple MAPK and Elk-1. First, Dab2 may act to dephosphorylate Elk-1, similar to KSR in activating PP2B. Dab2 may do so by inducing calcium influx or recruiting PP2B to a particular cellular location. Alternatively, Dab2 may inhibit phosphorylation of Elk-1 by MAPK. We have found no physical association between Dab2 and Erk1/2 or Elk-1. Dab2 could still prevent the phosphorylation of Elk-1 by blocking nuclear entry of the activated MAPK or sequestration of Elk-1 from being phosphorylated by the activated MAPK. We are currently investigating these possibilities.

In summary, we have uncovered a regulatory site in the Ras pathway by the candidate tumor suppressor Dab2: the uncoupling of MAPK activation and c-Fos expression (Fig. 6). Suppression of c-Fos expression is consistent with the finding that Dab2 expression retards the progression of the cells through G1 phase (Table I). Dab2 is lost in the majority of breast and ovarian cancer cells and tissues. Thus, in non-tumorigenic normal cells, the Ras pathway is regulated by Dab2-mediated uncoupling of MAPK activation and c-Fos expression. This appears to be a favored target for inactivation during tumorigenicity; tumor cells eliminate Dab2 and thus a regulatory site in the Ras pathway. The loss of Dab2-mediated regulation of c-Fos expression likely contributes to malignancy.

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Disabled-2 Exerts Its Tumor Suppressor Activity by Uncoupling c-Fos Expression and MAP Kinase Activation

Junqi He, Elizabeth R. Smith and Xiang-Xi Xu

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