Deletion of the COOH Terminus Converts the ST5 p70 Protein from an Inhibitor of RAS Signaling to an Activator with Transforming Activity in NIH-3T3 Cells

(Received for publication, September 21, 1999, and in revised form, November 3, 1999)

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Expression of the human protein ST5-p70 correlates with reduced tumorigenic phenotype in mammalian cells, reverts their transformed phenotype, and restores their contact-dependent growth. Furthermore, expression of p70 in COS-7 cells suppresses activation of mitogen activated protein kinase MAPK/ERK2 by the largest ST5 product, p126, in response to epidermal growth factor stimulation. Here we show that deletions of the COOH-terminal region of p70 transform NIH3T3 cells and induce their anchorage-independent growth. Analysis of signaling leading to MAPK/ERK2 stimulation revealed that in COS-7 cells, expression of either p70-AC1 or p70-AC2 markedly enhanced ERK2 activity in a growth factor-independent manner. Whereas wild-type p70 slightly inhibited ERK2 activation by RAS and MEK2, co-expression or p70-AC1 or p70-AC2 with either protein stimulated ERK2 cooperatively. This activity was completely blocked by the dominant negative mutants RAS17N or MEKAA, suggesting that p70 functions upstream of RAS. Unlike wild-type p70, expression of p70-AC1 or p70-AC2 mutant did not interfere with the ability of ST5-p126 to stimulate ERK2. Taken together, the data suggest that the COOH-terminal tail, residues 489–609, contains some of the critical determinants for the function of p70. Loss of this region converts the protein from an inhibitor to a constitutive activator of the RAS-ERK2 pathway.

The human ST5 protein, p70, is a new component of the growth regulatory signal transduction cascades that participate in cytoskeletal organization and regulation of the transformed phenotype (1). This protein is the smallest of three overlapping ST5 gene products which share common COOH-terminal sequences but differ in the amino-terminal segments of the proteins. Whereas the two larger proteins, p126 and p82, are the products of alternative splicing, the 2.8-kilobase mRNA species encoding p70 derives from a distinct promoter located within an intron of the ST5 gene (2, 3). The p70 protein consists of 609 amino acids, shared in common with the other two isoforms. The sequence of p70 contains a region of homology to a group of GDP/GTP exchange proteins specific for the ras3 family of small GTP-binding proteins (5) and to the MAP1 kinase-activating death domain protein, a protein identified as a binding partner of the tumor necrosis factor α receptor (4). In HeLa/fibroblast somatic cell hybrids and a variety of other cell lines, p70 expression correlates with reduced tumorigenic phenotype, a feature that is not displayed by p126 or p82. This differential expression of p70 suggests a regulatory function in tumor suppression (1). In support of this role, we previously reported that expression of p70 in transformed NIH3T3 cells could induce a flattened, nontransformed morphology and reorganization of the actin cytoskeleton (6).

Based on previous observations, it appears likely that p70 exerts its effect on cytoskeletal organization and cell growth regulation through its participation in cellular signaling. In COS-7 cells, the largest ST5 isofrom, p126, stimulates the activation of the MAP kinase ERK2 in response to epidermal growth factor (7). Co-expression of p70 blocks this activity. The amino acid sequence present only in the p126 isofrom contains two proline-rich regions, with motifs identical to Src homology 3 (SH3) binding domains and to MAP kinase phosphorylation sites (8–13). Many signaling molecules, including Sos, AAP1, R1N1, and P13K, share such structural features (14–17). One of the proline-rich regions in p126 interacts strongly with c-Abl in vitro and allows p126 to cooperate with c-Abl in activation of ERK2. Expression of p70 interferes with the ability of p126 to recruit the c-Abl protein to the ERK2 signaling pathway, again supporting a negative regulatory function for p70 in the MAPK/ERK2 cascade (7).

To fully understand the signaling role of p70 in cell growth and morphology, it will be important to elucidate the structure-function relationships at the cellular as well as biochemical levels. In this report, we show that deletion of the C-terminal tail, residues 489–609, converts the protein from an inhibitor of signaling to an activator of the pathway. Furthermore, in contrast to p126, these deletion mutants activated MAP kinase even in the absence of growth factor stimulation. Stable expression of these p70 mutants resulted in morphological transformation of NIH3T3 cells and induction of anchorage-independent growth.

EXPERIMENTAL PROCEDURES

Cell Lines and Tissue Culture—COS-7 and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 100 units of penicillin, and 100 μg of streptomycin/ml.

Plasmid Constructions and Mutagenesis—Deletion mutations were introduced directly into the 2.8-kilobase cDNA/pcDNA3 plasmid using the quickchange Site-directed Mutagenesis Kit (Stratagene) to insert stop codons followed by an XbaI site at various sites in the p126 cDNA.

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The cDNA segment downstream of the stop codon was then excised from the plasmid by XbaI digestion and religation. The following mutagenic oligonucleotides were used, with the inserted sequence marked by slashes: deletion (489, 609), upper strand, 5'-GCT CCG GAG AGG AAG/TAA CTG GAT GCT CAG TCA CTC TCT CCG GAG C-3'; deletion (309, 609), upper strand, 5'-CTT TTC ATG AGA AGT/ TAA CTC TAG A/CT CAT GGA GTC GCG C-3'; the lower strand, 5'-GGG CGA CTC GAG GAG/CT GTG CTG AGG G-3'.

The deletions were confirmed by sequencing.

Transformation Assay—DNA transfections were performed by the calcium phosphate precipitation method as described previously (18). NIH3T3 cells were seeded (5 x 10^5 cells per plate) one day prior to transfection with 4 μg of the expression vector. After 48 h, the cells were split into medium containing 800 μg/ml of the antibiotic G418 (Calbiochem) to generate stable transfectants. All plates were fed at 4-day intervals thereafter. Multiple isolated 3T3 cell clones derived from each expression vector were randomly picked, then expanded and passaged in the presence of 800 μg/ml G418 for subsequent analysis.

Anchorage-independent Growth Assay—From the clonal NIH3T3 cell lines, either 5000 or 10,000 cells were plated in 60-mm diameter plate in 2.5 ml of medium containing 0.3% agar, which was overlaid on 7 ml of solidified medium containing 0.5% agar. The medium used for soft agar was Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum. The cells were fed weekly with 2 ml of a mixture of 33% medium and 67% soft agar. Eighteen days post-transfection, the number of colonies (with more than 20 cells) were counted to determine the efficiency of colony formation.

MAP Kinase/ERK2 Assay—COS-7 cells were plated at 2 x 10^5 cells per 10-cm diameter plate and grown for 24 h. The cells were transfected with 2 μg of HA-tagged ERK2 and 4 μg of expression vector by the calcium phosphate method. The total amount of DNA was adjusted with pcDNA3 vector to 20 μg per plate. Two days post-transfection, the cells were starved in serum-free medium for 24 h and were either stimulated with EGF (150 ng/ml) or left untreated. Then the cells were lysed in a buffer containing 20 mM HEPES, pH 7.5, 1 mM EDTA, 40 mM β-glycerophosphate, 7.5 mM MgCl2, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml protease inhibitor mixture (Sigma). After centrifugation, the epitope-tagged ERK2 was immunoprecipitated from aliquots of the supernatant using monoclonal mouse anti-HA antibody 12CA5 (Roche Molecular Biochemicals) and protein G-SEPHAROSE beads (Amersham Pharmacia Biotech). The immune precipitates were washed three times in 1% NP40, 1% Nonidet P-40, 10 mM sodium orthovanadate, one time in 100 mM Tris, pH 7.5, 0.5 mM lithium chloride; and once in kinase buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM sodium fluoride, 0.5 mM sodium orthovanadate). The immune complexes were incubated in a 30-μl kinase reaction containing 40 μg of myelin basic protein (Sigma), 10 μM ATP, and 1 μCi of [γ-32P]ATP at 30 °C for 20 min. The reactions were quenched in 4 X Laemmli sample buffer and boiled for 5 min. The proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel, and the radioactivity incorporated into myelin basic protein was measured with a Molecular Dynamics Storm Imaging System. To verify approximately equal levels of expression of HA-ERK2 in all plates, the anti-HA precipitates were separated on SDS-polyacrylamide gel electrophoresis (12%), electroblotted electrophoretically transferred to a nitrocellulose membrane and probed with the epitope-specific monoclonal mouse antibody, 12CA5 (1:4000). After incubation with horseradish peroxidase coupled anti-mouse secondary antibody, bands were visualized by enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech).

RESULTS

Mutant p70 cDNAs were constructed in the expression vector pcDNA3. The wild-type 70 construct contains the full-length cDNA coding for the entire amino acid sequence. One construct, p70-C1, deleted the COOH-terminal tail, residues 489–609. Construct p70-C2 further truncated the 3′-end of the p70 cDNA removing 306 residues, including the GEF homology domain. The structures of the two mutants are diagrammed in Fig. 1.

p70-ΔC1 and ΔC2 Transform NIH3T3 Cells and Induce Anchorage-independent Growth—Wild-type p70, p70-ΔC1, or p70-ΔC2 mutants were transfected into NIH3T3 cells and the transfected cells selected for resistance to G418. An activated Ha-RAS cDNA (RAS12V) was used as positive control for transformation. After 2 weeks of drug selection, individual G418-resistant clones were screened for morphological transformation, expression of the ST5 proteins, and growth in soft agar.

Transfection of NIH3T3 cells with either empty vector or wild-type p70 resulted in uniformly flat colonies of contact inhibited cells (Fig. 2A and B). In contrast, transformed colonies were readily apparent on plates transfected with the p70 mutants, p70-ΔC1 or p70-ΔC2. To determine the fraction of G418 colonies that expressed the transfected cDNA and estimate the level of expression, 20 individual colonies per plasmid (screened from four plates per experiment) were subjected to Western blot analysis using an ST5 antisera. Of a total of 20 colonies examined from each transfection, five were found to express p70-ΔC1 and seven to express the p70-ΔC2 proteins. Whereas the nonexpressing colonies were not transformed, all of the expressing clones grew with a transformed morphology, forming colonies of densely packed refractile, fusiform cells (Fig. 2, C and D, and Fig. 3). As judged by apparent growth rate and saturation density, the transforming activity of the p70-ΔC2 mutant appeared to be more potent than that of p70-ΔC1.

Next, individual colonies expressing either wild-type or mutant p70 were tested for anchorage-independent growth. In soft agar, control cells or cells expressing the wild type protein failed to grow and persisted as a single cell, whereas cells expressing mutant p70-ΔC1 or p70-ΔC2 formed medium or large colonies, respectively (Fig. 4). This result contrasts with the approximately equal plating efficiencies of p70 wild-type and mutant expressing cells on plastic tissue culture plates. As reflected by colony size and number, the transformed phenotype induced by p70-ΔC2 again appeared to be more complete than that induced by p70-ΔC1. These findings demonstrate that expression of the two deletion mutants, p70-ΔC1 and p70-ΔC2, can transform NIH3T3 cells and induce anchorage independent-growth.
Cells were transfected with empty vector (D\textsubscript{A}), p70\textsubscript{C1} (C\textsubscript{B}), p70-\textsubscript{NIH3T3} cells were transfected with appropriate expression constructs for transformation. In both mutants, p70-\textsubscript{C2} markedly stimulated this activity (Fig. 5). Interestingly, and in contrast to the activity of p70\textsubscript{C1} and p70-\textsubscript{D}C\textsubscript{2}, these data support the conclusion that the COOH-terminal tail of p70 is critical for the ability of this protein to inhibit signaling through MEK.

**DISCUSSION**

Two deletions of the ST5 COOH terminus, a region shared in common by the three mRNA species derived from this gene, were studied for their ability to activate MAP kinase, inhibit the signaling activity of ST5 p126, and to transform NIH3T3 cells. The deletion end points were chosen so that one would lie upstream (p70-\textsubscript{D}C2) and the other downstream (p70-\textsubscript{D}C1) of the GEF homology domain. Both mutants were found to function similarly, although p70-\textsubscript{D}C2 appeared to be more potent at transforming NIH3T3 cells and promoting anchorage-independent growth. The results demonstrate that the COOH terminus of p70 plays a critical role in the ability of this protein to induce morphologic changes and to inhibit signaling through the RAS-MAP kinase pathway. In NIH3T3 cells, deletion of the COOH-terminal tail of p70, residues 489-609, contains elements that are critical for the negative regulatory function.

**ERK2 Activation by p70 Mutants**

Since RAS\textsubscript{17N} completely abrogated this stimulatory effect (Fig. 7), expression of p70\textsubscript{D}C\textsubscript{1} or p70-\textsubscript{D}C\textsubscript{2} constructs resulted in 17- and 20-fold increases, respectively, over basal levels. In a second series of experiments, the effect of the dominant negative mutant, RAS17N, on ERK2 activation mediated by the deletion mutants was analyzed. Whereas each mutant by itself resulted in a marked stimulation of ERK2 activity, co-expression of RAS17N completely abrogated this stimulatory effect (Fig. 7). These results imply that in the MAP/ERK signaling pathway, the p70 protein signals through RAS.

**p70-\textsubscript{D}C\textsubscript{1} and p70-\textsubscript{D}C\textsubscript{2} Act Synergistically with MEKEE to Activate ERK2**

To explore the effect of p70 expression on other components of the ERK2 pathway, COS-7 cells were transfected with the HA-ERK2 cDNA together with the activated dual specificity kinase MEK (19), MEKEE, and either wild-type p70, p70-\textsubscript{D}C\textsubscript{1}, or p70-\textsubscript{D}C\textsubscript{2} cDNAs. As expected, co-expression of p70 with either p70-\textsubscript{D}C\textsubscript{1} or p70-\textsubscript{D}C\textsubscript{2} cDNAs did not interfere with the ability of p126 to activate ERK2 (Fig. 10). Consistent with our above findings, these data support the conclusion that the COOH-terminal tail of p70, residues 489—609, contains elements that are critical for the negative regulatory function.
growth, one of the most fundamental differences between transformed and normal cells (20).

At the biochemical level, we found that in COS-7 cells deletion of the COOH-terminal tail of p70 converts the protein from an inhibitor of signaling to one that constitutively activates ERK2 (Fig. 5). Analysis of downstream effectors leading to the activation of ERK2 revealed a strong synergy between p70-D_C1 or p70-D_C2 mutants and activated mutants of RAS and MEK in the stimulation of ERK2 (Figs. 6 and 8). This activity of the two p70 mutants was completely abrogated when either of the dominant negative mutants RAS17N or MEKAA was expressed (Figs. 7 and 9), suggesting that the p70-D_C1 and p70-D_C2 mutants function upstream of RAS.
Several observations suggested that the p70-ΔC2 mutant possesses a more potent transforming activity than p70-ΔC1. In comparison with the p70-ΔC1 expressors, the p70-ΔC2 expressing clones grew to a higher saturation density, demonstrated a higher cloning efficiency in soft agar, and formed larger colonies. One possible explanation for these results is that quantitative differences in expression levels of the truncated proteins may be responsible for the different phenotypes observed. However, on Western blots of stable transfectants, the p70-ΔC2 protein was generally expressed at levels similar to those observed with p70-ΔC1, as illustrated in Fig. 3. A second possibility is that the greater apparent potency of p70-ΔC2 could reflect the complete loss of a binding site, only partly lost in p70-ΔC1, for a ligand critical for the negative regulatory properties of this protein.

The present results do not resolve the question of how precisely p70 signals to regulate the RAS-ERK2 pathway. However, it is clear that the COOH-terminal tail interferes with the ability to constitutively activate this pathway. These findings suggest that this COOH-terminal region might participate in macromolecular complex formation with other proteins, therefore blocking the stimulating effect of the p70 protein. One interaction currently being investigated is the possibility of heterodimer formation with another ST5 isoform, such as p126. We have noted that on Western blots with the ST5 antibody, clones stably expressing one of the p70 mutant proteins often overexpress a protein consistent in size with p126. Therefore it is possible that the transforming activity that we have observed results in part from the stabilization of endogenous ST5 p126.

The region between the two deletion endpoints examined in this study contains a segment of homology to GEFs for the Rab3 family of small GTP binding proteins. The presence of this domain raises the possibility that the ST5 proteins may function as GEFs in a manner analogous to that of other proteins with this activity, including Sos, Dbl, ARNO, and GRF/mCDC25 (21–24). It is interesting to note that many of the GEF proteins were discovered by virtue of their ability to transform NIH3T3 cells when overexpressed or when activated by truncation (25).

The results with the deletion mutants revealed unexpected constitutive activation of the RAS-ERK2 pathway. A search of GenBank™ with the COOH-terminal sequence downstream of the GEF homology region revealed no informative homologies and no recognizable protein motifs. However, we note that this deletion resulted in placing the sequence CVIS near the COOH terminus. This sequence is a CAXX motif (where C indicates a cysteine, A an aliphatic amino acid, and X is any amino acid). This motif is present at the COOH terminus of RAS proteins, where it signals RAS to undergo a series of posttranslational modifications including truncation, farnesylation, and carboxymethylation (26–28). These modifications are required for RAS proteins to become associated with plasma membrane. It is possible that the CAXX motif near the COOH terminus of the p70-ΔC2 mutant serves as a membrane localization signal that permits the truncated protein to interact with effectors that the wild-type p70 would not normally contact due to its cytoplasmic...
localization. This motif is present in p70-ΔC1 but lies further from the COOH terminus of the protein, possibly accounting for the difference in potency of the two mutants in the transfection assays. Further studies are required to test this hypothesis.

The results presented demonstrate that the COOH-terminal tail of the p70 protein contains elements that are critical to the negative regulatory role of this protein in signal transduction pathways as well as in the regulation of cell growth and morphology. Further studies will be aimed at obtaining additional information regarding the mechanisms by which ST5 proteins regulate signaling pathways and the transformed phenotype.

Acknowledgments—We thank Alan Hubbs, Ronald Przygodzki, and Andrew Louden for technical assistance. We also thank Thomas Fan ning for critical reading of the manuscript.

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J. Biol. Chem. 2000, 275:6560-6565. doi: 10.1074/jbc.275.9.6560

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