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Cloning of a Novel Phosphoprotein Regulated by Colony-stimulating Factor 1 Shares a Domain with the *Drosophila disabled* Gene Product*

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A unique protein with an apparent molecular mass of 96 kilodaltons (p96) was detected in the murine macrophage cell line, BAC1.2F5. The murine cDNA encoding p96 was cloned and sequenced, along with cDNAs representing two alternatively spliced forms of the protein. All three proteins possessed identical amino-terminal domains with significant similarity to the amino-terminal domain of the *Drosophila disabled* gene product and carboxyl-terminal domains containing proline-rich sequences characteristic of *src* homology region (domain 3) binding regions. BAC1.2F5 cells predominately expressed the p96 protein, although mRNA and protein corresponding to the p67 splice variant were also detected. Electrophoretic gel retardation of p96 in response to stimulation of the cells with colony-stimulating factor 1 was noticeable within 5 min after growth factor addition and reached a maximum at 60 min. Metabolic labeling experiments showed that the gel retardation of p96 was associated with increased phosphorylation of the protein exclusively on serine residues. These data identify a novel protein that is phosphorylated in response to mitogenic growth factor stimulation.

CSF-1[1] is a growth factor that is required for the differentiation, proliferation, and survival of cells of the mononuclear phagocyte lineage (for review, see Stanley (1985)). The biological effects of CSF-1 are mediated by its binding to a single high affinity receptor (Guilbert *et al.*, 1986) encoded by the c-*fms* proto-oncogene (Sherr *et al.*, 1985). The CSF-1 receptor is similar to the platelet-derived growth factor receptor and the c-*kit* proto-oncogene in that the extracellular domain consists of five immunoglobin-like loops linked by a single transmembrane helix to the cytoplasmic tyrosine kinase domain that is interrupted by a unique "kinase insert" sequence (Ullrich and Schlessinger, 1990). CSF-1 binding triggers dimerization of the receptor (Lee and Nienhuis, 1990; Li and Stanley, 1991; Ohtsuka *et al.*, 1990), autophosphorylation on tyrosine (Rettenmier *et al.*, 1985), and stimulation of its tyrosine kinase activity (Yeung *et al.*, 1987; Downing *et al.*, 1989).

Autophosphorylation of the CSF-1 receptor promotes its interaction with cytoplasmic proteins that activate multiple signal transduction pathways and culminate in a wave of immediate early gene expression (for reviews, see Sherr (1991); Vairo and Hamilton (1991)). In addition, CSF-1 is required throughout the G_1 stage of the cell cycle (Tushinski and Stanley, 1985), indicating that CSF-1-dependent signaling pathways later in G_1 are required for the production or activation of key proteins that ultimately determine the commitment to DNA synthesis. The response to CSF-1 is very complex, and describing the components in this signal transduction network is a major challenge. CSF-1 elevates intracellular concentrations of diglyceride derived from phosphatidylcholine in human monocytes (Imamura *et al.*, 1990), bone marrow-derived macrophages (Veis and Hamilton, 1991), a fibroblast cell line expressing the CSF-1 receptor (Choudhury *et al.*, 1991), and BAC1.2F5 cells (Xu *et al.*, 1993). The addition of exogenous phosphatidylcholine-specific phospholipase C (PC-PLC) to BAC1.2F5 cells mimics the mitogenic effects of CSF-1 pointing to a role for phosphatidylcholine hydrolysis in CSF-1 signal transduction (Xu *et al.*, 1993). PC-PLC activates a signal transduction pathway that triggers the transcription of the c-*fos* and *jun*B genes independent of the activation of protein kinase C and Ras and that collaborates with c-*myc* in the mitogenic stimulation of BAC1.2F5 cells.

As part of our investigation of proteins involved in the signal transduction pathways in a murine macrophage cell line, BAC1.2F5, initiated by PC-PLC and CSF-1, we have cloned the cDNA for a unique 96-kDa protein related to the *Drosophila disabled* gene. The 96-kDa protein, termed p96, is phosphorylated on serine residues following mitogenic stimulation in the BAC1.2F5 cell line identifying it as a component in the CSF-1 signal transduction cascade.

EXPERIMENTAL PROCEDURES

Materials—Sources of supplies were as follows: DMEM, Whittaker Bioproducts; fetal calf serum, HyClone Laboratories; ECL kit for the detection of proteins in immunoblots, Amersham Corp.; [^{35}S]methionine (specific activity, 1,000 Ci/mmol), [α-^{32}P]dCTP (specific activity, 3000 Ci/mmol), and [^{32}P]orthophosphate (carrier-free), DuPont NEN; goat antirabbit IgG conjugated to alkaline phosphatase, Jackson Laboratories; phosphatidylcholine-specific phospholipase C and sphingomyelinase from *Bacillus cereus*, Boehringer Mannheim; mouse brain 5′-stretch λgt10 cDNA library, mouse brain λgt11 cDNA library, mouse macrophage λgt11 cDNA library, and PCR primers flanking the λ phage insert, Clontech; random primed DNA labeling kit, Boehringer Mann-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U18869.

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[1] The abbreviations used are: CSF-1, colony-stimulating factor 1; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; bp, base pair(s); kb, kilobase(s); DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; ERK, extracellular regulated kinase; SH3, *src* homology region, domain 3; PC-PLC phosphatidylcholine-specific phospholipase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MOPS, morpholinepropanesulfonic acid.

heim; Immobilon-P membrane, Millipore; *in vitro* transcription/translation assay kit and restriction endonucleases, Promega; plasmid purification kits, Qiagen, Inc.; and Bradford protein assay reagent, Bio-Rad. Homogeneous CSF-1 was kindly provided by Genetics Institute. L-cell conditioned medium was used as the source of CSF-1 for the routine maintenance of BAC1.2F5 cells (Stanley and Heard, 1977). Polyclonal rabbit antisera were produced by Rockland Laboratories, Inc. using synthetic peptides coupled to keyhole limpet hemocyanin. The αM15 sera was raised against a synthetic peptide (RTQNGVSERENQNGFHIKSS) corresponding to amino acids 376–394 of p96. The αM2 antisera was raised against a synthetic peptide (IDEKTGVIEHEHPVNKIS) corresponding to amino acid residues 105–122 of p96. The αM15 sera was raised against a sequence unique to p96 and p93, whereas the epitope in αM2 was a sequence that was present in p96, p93, and p67. The rabbit antisera (αERK1pep) was raised against a synthetic peptide (LKELIFQETARFQPGAPEAP) corresponding to the carboxyl-terminal region of ERK1 and was a generous gift from Dr. J. Downing (St. Jude Children's Research Hospital). All other chemicals were reagent grade or better.

Cell Culture—The BAC1.2F5 cell line was a CSF-1-dependent clone (Schwarzbaum *et al.*, 1984; Morgan *et al.*, 1987) that exhibits many of the properties of macrophages. BAC1.2F5 cells were routinely maintained in DMEM supplemented with 15% fetal calf serum, 25% L-cell conditioned medium, and 20 mM HEPES, pH 7.4. BAC1.2F5 cells were growth-arrested in G_1 phase of the cell cycle by the removal of CSF-1 for 18 h (Rock *et al.*, 1992) and restimulated by the addition of either human recombinant CSF-1 (90 ng/ml), PC-PLC (1 unit/ml), sphingomyelinase (0.1 unit/ml), or TPA (10^{-7} M). Experiments were replicated at least three times unless otherwise indicated.

Cloning, Sequence, and in Vitro Transcription / Translation of p96, p93, and p67—A protein with an apparent molecular mass of 96 kDa determined from its mobility in SDS-gel electrophoresis whose electrophoretic gel mobility was retarded following CSF-1 stimulation was detected with the αERK1pep antisera directed against a peptide present in the carboxyl-terminal domain of ERK1. This 96-kDa protein was designated p96, and the unfractionated αERK1pep antisera was used to screen a λgt11 murine brain cDNA expression library. Immunological screening for the presence of p96 was carried out by incubating the phage plus *Escherichia coli* host strain Y1090r (10,000 plaque-forming units/150-mm plate) at 42 °C for 2 h. The plate was overlaid with a nitrocellulose membrane soaked in Xgal. The proteins were adsorbed to the nitrocellulose by incubating at 37 °C for 7–10 h. Positive clones were identified by reaction of the nitrocellulose membranes with αERK1pep antibody essentially as described in the Clontech manual except that the primary antibody was detected by reaction with goat antirabbit IgG conjugated with alkaline phosphatase (Harlow and Lane, 1988). The positive plaques were excised from the agar and subjected to another 3–5 rounds of amplification and plaque purification using immunoscreening to detect the positive plaques. DNA was isolated from the plaque-purified phage, and the cDNA inserts were amplified by PCR using flanking primers purchased from Clontech and were cloned into a PCR cloning vector (pCRII, Invitrogen). Plasmid pBluescript KS (Stratagene) was used as the vector for further subcloning and DNA sequencing.

A second immunoscreen was used to identify the λ phages from the initial screen that specifically expressed p96. The purified phages were plated with cells, and nitrocellulose membranes containing adsorbed phage fusion proteins were prepared as described above. The nitrocellulose membranes were incubated with 2% dry milk in TBS for 1 h and then incubated in a 1/1000 dilution of αERK1pep antisera in TBS containing 2% dry milk. The membranes were washed three times with 100 ml of TBS containing 0.05% Triton X-100. The bound antibodies were eluted by soaking the membrane in 10 ml of 100 μM glycine, pH 2.5, for 10 min. The pH was neutralized by the addition of 1 ml of 1 M Tris-HCl, pH 8.8. The resulting solutions were used as the primary antibodies to test for reactivity against BAC1.2F5 cell proteins on immunoblots. Most of the 24 clones reacted with ERK1, and DNA sequence analysis of three of these clones verified that they expressed a fusion protein that contained ERK1 carboxyl-terminal sequences. One clone, N13, expressed a fusion protein that absorbed antibodies that recognized p96 (but not ERK1) when used as the primary antibody in immunoblotting total cell extracts of BAC1.2F5 cells. Antibodies were affinity purified against the recombinant phage N13 fusion protein and were used in a second immunoscreen of a macrophage λgt11 library. Two clones (M15 and M7) were isolated. Sequence analysis of the insert showed that the M7 and M15 clones were identical and that their sequences overlapped with the N13 clone. Clone M15 was used to synthesize a labeled probe to screen both the murine macrophage λgt11 and murine brain λgt10 cDNA libraries for clones that hybridized with M15 sequences according to the methods outlined in Sambrook *et al.* (1989). These experiments resulted in the isolation of clones N10–1, N1–1, and N10–2 from the macrophage λgt11 library and clones B4, B7, B14, and B21 from the brain λgt10 library.

Plasmids were isolated using columns purchased from Qiagen, and restriction enzyme digestions and agarose gel electrophoresis were performed as described (Sambrook *et al.*, 1989). At least three independent plasmids derived from the inserts of positive λ clones were mapped and their DNA sequence determined on both strands. A combination of the universal M13 primers or one of several dozen primers complementary to the predetermined sequence were used to extend the sequence using the automated sequencing instrumentation (Applied Biosystems Inc.) provided by the St. Jude Molecular Resource Center.

Expression vectors were constructed by cloning the p67 coding sequence from clone N10–2 into pBluescript downstream of the T7 promoter. The p93 and p96 vectors were constructed by substituting the *BclI-Ppu*MI fragments from clones B21 and B7, respectively, for the *BclI-Ppu*MI fragment in N10–2. Plasmid DNA was isolated, transcribed, translated, and labeled with [^{35}S]methionine using the Promega T7-coupled transcription/translation kit according to the manufacturer's instructions. The labeled proteins were analyzed by SDS-gel electrophoresis either before or after immunoprecipitation with αM15 and αM2 antisera. The labeled proteins were electroblotted onto nitrocellulose membrane and visualized by autoradiography.

RNA Analysis—BAC1.2F5 cell cultures were harvested by centrifugation, and total RNA was isolated by a guanidine isothiocyanate lysis procedure followed by pelleting RNA by CsCl gradient centrifugation (Chirgwin *et al.*, 1977). RNA pellets dissolved in 10 mM Tris-HCl, pH 7.5, 5% β-mercaptoethanol, 0.5% Sarkosyl, 0.5% SDS, and 5 mM EDTA were extracted with phenol:chloroform:isoamyl alcohol (24:24:1) and precipitated with 2 volumes of ethanol. Poly(A)$^+$ RNA was isolated by passing the total RNA through an oligo(dT) spin column (Clontech) as described by the manufacturer. Poly(A)$^+$ RNA (10 μg, determined from A_{260}) was denatured for 10 min at 55 °C in electrophoresis buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 6% formaldehyde) containing 50% deionized formamide. The samples were then quickly chilled on ice and fractionated by electrophoresis in 1.0% agarose gels. Blotting, prehybridization, hybridization with ^{32}P probes, and washing of blots was performed as described by Thomas (1980). ^{32}P-Labeled probes used for analysis of RNA levels were prepared by random priming using restriction enzyme fragments isolated by agarose gel electrophoresis.

RT-PCR was performed on total RNA isolated from 1×10^7 BAC1.2F5 cells. The cell pellet was lysed by adding 450 μl of 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40 and incubated on ice for 5 min. The cell debris was removed by centrifugation at 12,000 × *g* for 2 min. The cell lysate (450 μl) was transferred into a clean eppendorf tube and mixed with 8 μl of 10% SDS and 3 μl of proteinase K (20 mg/ml), and the mixture was incubated at 37 °C for 15 min. The RNA was extracted by adding an equal volume of phenol/chloroform (1:1, pH 8.0), and the extraction was repeated twice. Total RNA was precipitated with ethanol. The RNA pellet was rinsed once with 70% ethanol and resuspended in 10 μl of diethylpyrocarbonate-treated H_2O. The final concentration of total RNA was 0.76 μg/μl. The Stratascript RT-PCR kit (Stratagene) was used for reverse transcription to synthesize the first strand of cDNA with random primers. The procedure was performed as described in the manufacturer's manual except that 3 μg of total RNA was used in each reaction. 15 μl of the first strand cDNA synthesis mixture was used for PCR amplification of p96 sequences. The forward primer was 5′-GCTGGTCGCTCTCAGGGACAA-3′ corresponding to 460–480 bp of the p96 sequence, and the reverse primer was 5′-AAGGACTGTAGACAACAGGCG-3′ corresponding to 1750–1771 bp of the p96 sequence (see Fig. 1). The PCR was performed in a 100-μl reaction volume with 40 thermocycles at 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 2 min. The PCR products were precipitated with ethanol and separated by agarose-gel electrophoresis. The bands were excised from the gel, purified individually, and cloned into the PCR cloning vector, pCRII (Invitrogen).

Immunoblotting and Immunoprecipitation—Cells were washed with cold phosphate-buffered saline, scraped into 0.5 ml of SDS sample buffer, and immediately boiled for 5 min. The amount of protein present was determined by the method of Bradford (1976). The cell lysate (100 μg) was separated by SDS-gel electrophoresis on 8% polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were then blocked with 1% dry milk for 1 h, washed with Tris-buffered saline (150 mM NaCl, 10 mM Tris-HCl, pH 8.0), and exposed to the primary antibody for 2 h. The dilutions of the primary antibodies were αM2, 1/500; αM15, 1/5000. The blots were washed with Tris-buffered saline containing 0.05% Triton X-100 and incubated with the second

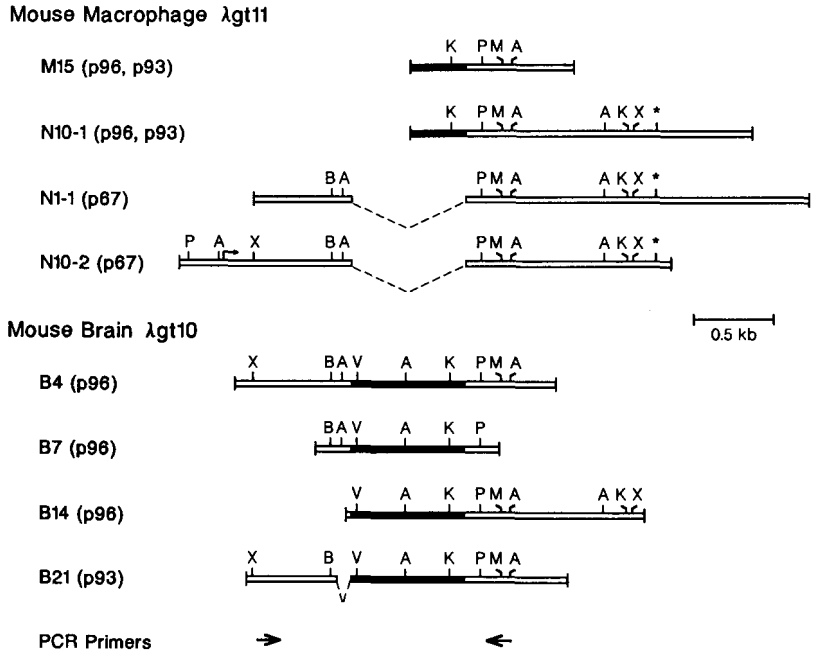

FIG. 1. **Schematic representation of the eight completely sequenced clones isolated from mouse macrophage and brain libraries.** The M15 clone was isolated using the αERK1pep antisera to detect fusion protein production. The remaining clones were isolated by hybridization with a labeled oligonucleotide probe derived from M15. The designations in *parentheses* indicate the proteins that were predicted to be encoded by these cDNAs. In the case of M15 and N10–1, the sequences isolated were common to both the p96 and p93 isoforms. The *solid bar* indicates the sequences common to p96 and p93 that were absent from p67. The *two arrows* indicate the position of the PCR primers used in the RT-PCR experiment described in Fig. 5*C*. The abbreviations for the restriction enzymes are: *A*, *Acc*I; *B*, *Bcl*I; *K*, *Kpn*I; *M*, *Pvu*MI; *P*, *Pst*I; *V*, *Eco*RV; and *X*, *Xho*I.

antibody conjugated with peroxidase for 1 h. After five rinses with Tris-buffered saline containing 0.05% Triton X-100, the Amersham ECL detection kit was used to locate the secondary antibody.

BAC1.2F5 cells were pelleted and resuspended in 200 μl of 0.6% SDS and heated at 95 °C for 5 min to denature the proteins. The lysate was diluted with 1 ml of Tris-buffered saline (pH 7.4), and the antibody (either αM15 or αM2) was added. The immune complexes were isolated by the addition of protein A-Sepharose beads (50 μl of a 50% solution), which were collected by a brief centrifugation and washed twice with Tris-buffered saline. The beads were boiled in SDS sample buffer and separated by electrophoresis using an 8% separating gel. The proteins were transferred onto Immobilon-P membranes and visualized by autoradiography following metabolic labeling or detected by immunoblotting using the ECL assay kit.

Metabolic Labeling—To measure the incorporation of phosphate into p96, 100-mm dishes of BAC1.2F5 cells were deprived of CSF-1 for 18 h in phosphate-free DMEM plus dialyzed FBS, and during the last 2 h, 0.1 mCi/ml [^{32}P]orthophosphate was added. Cells were harvested at zero time or after a 30-min stimulation with 90 ng/ml recombinant CSF-1. The cells were then washed with cold phosphate-buffered saline and lysed in 1 ml of 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 0.2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was immunoprecipitated, and the immune complexes were separated by SDS-gel electrophoresis on 8% polyacrylamide gels. The ^{32}P-labeled p96 band was located by autoradiography, excised, and hydrolyzed with 5.7 N HCl for 1 h at 110 °C. The hydrolyzate was lyophilized and was resuspended in 5–10 μl of pH 1.9 buffer (88% formic acid/glacial acetic acid/H_2O; 50:156:1794 (v/v)) containing 0.2 mg/ml of each phosphoserine, phosphothreonine, and phosphotyrosine and then spotted onto a cellulose thin layer plate. The phosphoamino acids were separated by two-dimensional electrophoresis according to Cooper *et al.* (1983). The first dimension was developed in pH 1.9 buffer at 1500 V for 20 min and the second dimension in pH 3.5 buffer (pyridine/glacial acetic acid/H_2O; 10:100:1890 (v/v)) at 1300 V for 25 min. The standard phosphoamino acids were visualized by staining with 0.2% ninhydrin. The ^{32}P-labeled phosphoamino acids were visualized by autoradiography and identified by comparing their locations with the standard phosphoamino acids.

RESULTS

Isolation of p96 cDNA Clones—An αERK1pep antiserum raised against a peptide corresponding to the carboxyl-terminal region of ERK1 also detected a protein with an apparent molecular mass of 96 kDa determined from SDS-gel electrophoresis of total lysates of BAC1.2F5 cells and that exhibited slower electrophoretic mobility following CSF-1 stimulation. This 96-kDa protein was designated p96, and the unfractionated αERK1pep antisera was used to screen a brain λgt11 expression library. 24 immunoreactive clones were obtained and plaque purified. Plate lysates of the purified clones were adsorbed onto nitrocellulose membranes, and the membranes were used to affinity purify antibodies from the αERK1pep antisera pool (see "Experimental Procedures"). Antibodies eluted from the individual discs were used to immunoblot BAC1.2F5 cell lysates. Most of the clones adsorbed antibodies that detected a 44-kDa protein, but not a 96-kDa protein, on the immunoblots, and several of these clones were confirmed to encode murine ERK1 by DNA sequence analysis. The antibodies absorbed by a single clone (N13) detected the p96 protein on immunoblots but did not detect the 44-kDa ERK1 protein. This clone was used to prepare affinity-purified antisera using the phage fusion protein as the ligand, and the purified antisera were then used to screen a macrophage λgt11 expression library. Two positive clones (M15 and M7) were obtained and were found to be identical by DNA sequence analysis. The M15 clone was between 1224 and 1987 bp of the p96 sequence, and the original N13 clone extended from 1347 to 2136 bp of the p96 sequence. Radiolabeled DNA probes were synthesized from the M15 clone and used to screen both a macrophage λgt11 and a brain λgt10 library for complementary sequences. Clone M15 and the seven largest clones arising from these screens were sequenced completely and are schematically shown in Fig. 1. The sequence analysis of the cDNA fragments suggested the existence of three alternately spliced forms of the protein.

Analysis of the p96 Sequence—The DNA sequences from the clones in Fig. 1 were aligned, and the composite sequence is shown in Fig. 2. The *boxed areas* in Fig. 2 indicate the sequences that were absent in the p93 (residues 209–229) and p67 (residues 230–447) spliced forms of p96. The longest open reading frame corresponding to the p96 protein detected in BAC1.2F5 cell lysates was predicted to encode a protein with 837 amino acids and a molecular weight of 90,764 and a pI of 6.62. The AUG at 217 bp was preceded by stop codons in all three reading frames and flanked by bases consistent with its function as an initiator codon (Kozak, 1987). The p93 protein was predicted to contain 816 amino acids with a molecular weight of 88,545 and a pI of 7.95. The p67 protein was predicted to have 619 amino acids with a molecular weight of 67,232 and

FIG. 2. **DNA and predicted amino acid sequence of p96.** The complete cDNA sequence was assembled by bidirectional sequence analysis of multiple clones isolated from both a murine macrophage λgt11 library and a murine brain λgt10 library as outlined in Fig. 1 and described under "Experimental Procedures." The two ATG start codons in the open reading frame were located at 217 and 418 bp and are highlighted in *bold*. The *first box* indicates the sequences that were not present in the clone encoding p93 (amino acids 209–230), and the *second box* indicates the sequences absent from the clone encoding p67 (amino acids 230–447).

a pI of 7.25. In all cases, the most abundant amino acid residues in all of the splice variants were proline comprising between 10.9 and 11.5 mol % and serine, which comprised between 10.4 and 11.6 mol %. The p96 protein was hydrophilic, and there was no indication of hydrophobic domains in p96 that would suggest interaction with membranes.

Analysis of the p96 predicted amino acid sequence revealed three areas with similarities to known protein structural motifs (Fig. 3*A*). First, there was a sequence beginning at residue 25 (KKEK) that was identical to the sequence in villin that has been identified as critical for the interaction of villin with actin (Friedrich *et al.*, 1992). The second domain in the amino terminus had a high degree of similarity to the amino-terminal domain of the *Drosophila disabled* gene product (Fig. 3*B*). These two proteins were 46% identical and 66% similar in this region. The third region was a domain rich in proline and serine residues that contained five proline-rich sequences (Fig. 3*A*) that represent potential binding sites for proteins containing SH3 domains (Viguera *et al.*, 1994; Yu *et al.*, 1994; Feng *et al.*, 1994; Lim *et al.*, 1994).

Expression of p96 Isoforms in BAC1.2F5 Cells—The full-length coding sequences for all three p96 isoforms were cloned into pBluescript KS and expressed *in vitro* (Fig. 4). The largest proteins synthesized by each of these clones corresponded to the predicted size of the protein according to the DNA sequence. Two peptide antisera were generated in rabbits to characterize the expression of p96 isoforms in BAC1.2F5 cells. The αM15 antiserum was raised against a peptide corresponding to residues 376–394, which were within the sequences present in p96 and p93 but were absent from p67. The αM2 antiserum was raised against residues 105–122 that were common to p96, p93, and p67. The αM15 antiserum immunoprecipitated the p96 and p93 proteins expressed *in vitro* but did not precipitate the p67 protein (Fig. 4). The αM2 antiserum precipitated all three proteins expressed *in vitro*. We have also expressed the p96 cDNA clone in COS-7 cells and detected a single protein band with the αM15 antiserum at the correct molecular weight (data not shown). The *in vitro* expression

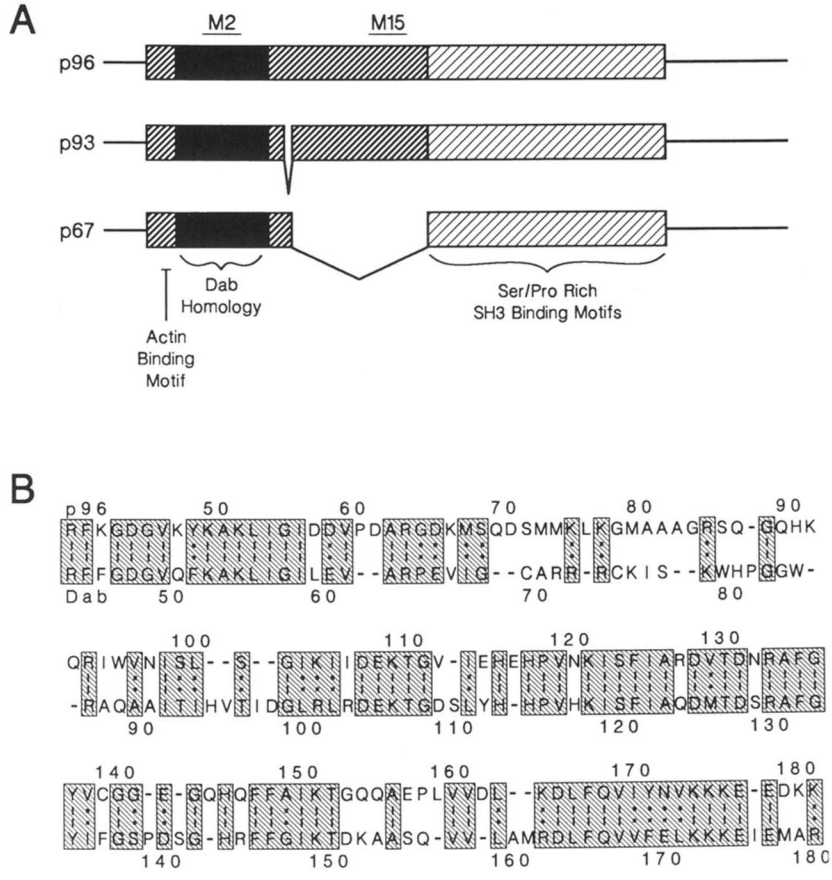

FIG. 3. **Analysis of the p96 protein sequence.** *Panel A*, a schematic representation of the domain structure of p96, p93, and p67. The predicted open reading frames (*bars*) contained an actin-binding motif (KKEK) located between residues 25 and 29 of p96, a region between residues 40 and 180 of p96 with similarity to the *Drosophila disabled* gene product (Dab), and a serine/proline-rich region between residues 447 and 837 of p96. The proline-rich sequences that could potentially associate with SH3 domains were: residues 502–510, PVTPPQAGP; residues 559–567, PASPPPP; residues 617–625, PPQPPP; residues 661–669, PPLVP; and residues 712–720, PPKPAP. The location of the peptides used to generate the αM2 and αM15 antisera are indicated above the p96 protein. *Panel B*, the similarity between the predicted amino acid sequence of p96 and the *Drosophila disabled* gene product. Alignment of the amino-terminal regions of p96 (residues 40–180) and Dab (residues 43–179) showed that the two proteins were 46% identical and 66% similar in this region. Identical residues are indicated by |, and similar residues are indicated by :. Amino acid groups are defined as P, A, G, S, T; Q, N, E, D; H, K, R, C; V, L, I, M; and F, Y, W.

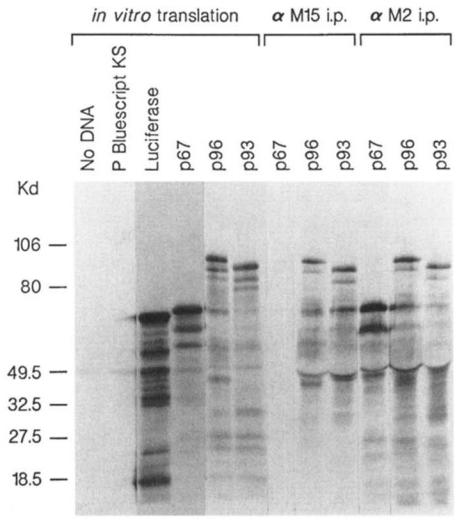

FIG. 4. **Translation of p96, p93, and p67 cDNAs *in vitro*.** The assembled p96, p93, and p67 coding sequences were cloned into pBluescript KS downstream of the T7 promoter. The proteins expressed by these constructs were identified using a coupled transcription/translation reticulocyte lysate system (Promega) containing 40 μCi/50 μl [^{35}S]methionine (1,000 Ci/mmol). The products were analyzed by SDS-gel electrophoresis either before (*left lanes*) or after (*right lanes*) immunoprecipitation (intraperitoneal) with either αM15 or αM2 antisera, and the bands were visualized by autoradiography. A plasmid expressing luciferase was used as a positive control.

results confirmed the isolation of cDNAs encoding proteins of the predicted sizes and showed that the proteins reacted with the αM15 and αM2 antibodies with the anticipated specificity.

The expression of p96 isoforms in BAC1.2F5 cells was examined at the protein and mRNA level. BAC1.2F5 cells expressed p96 protein that was electrophoretically retarded following CSF-1 stimulation (Fig. 5*A*). Both antisera also faintly detected another protein of approximately 86 kDa. This faint band did not correspond to any of the cDNAs and was not investigated further. It is possible that the 86-kDa protein was an undetected splice variant of p96, a translation product derived from the second initiator methionine in the sequence, or perhaps a degradation product, although protease inhibitors were present in all the samples. These data indicated that BAC1.2F5 cells expressed p96 (or p93) and p67. Northern blots were used to analyze mRNA expression in BAC1.2F5 cells (Fig. 5*B*). Using a labeled probe derived from a sequence unique to p96 and p93, a single 3.7-kb mRNA was detected (Fig. 5*B*, *lane 1*). Northern analysis with a labeled probe containing cDNA sequences common to all three splicing variants revealed two mRNA species of 3.7 and 2.9 kb. These data supported the idea that BAC1.2F5 cells expressed both the p96 (or p93) protein and the p67 protein. We performed a reverse transcriptase PCR analysis (Fig. 5*C*) since analysis of either protein or mRNA expression could not unambiguously distinguish between the expression of p96 or p93 in BAC1.2F5 cells. Primers located outside of the splice junctions were synthesized (Fig. 1), and PCR products derived from random priming of mRNA from BAC1.2F5 cells were amplified and cloned. Two PCR reaction products of 1.3 and 0.66 kb were detected consistent with the expression of p96 and p67. To conclusively establish the identity of these bands, they were cloned and digested with *Acc*I (Fig. 5*C*). Digestion with this restriction enzyme distinguished between p96 and p93 because p96 had an *Acc*I site at nucleotide 873 (Fig. 2) that was missing in p93. Taken together, these expression data showed that p96 was the most abundant form of the protein expressed in BAC1.2F5 cells, although the expression of p67 was also significant. There was no indication that the p93 isoform was expressed in BAC1.2F5 cells.

Phosphorylation of p96 in CSF-1-stimulated Cells—The pre-

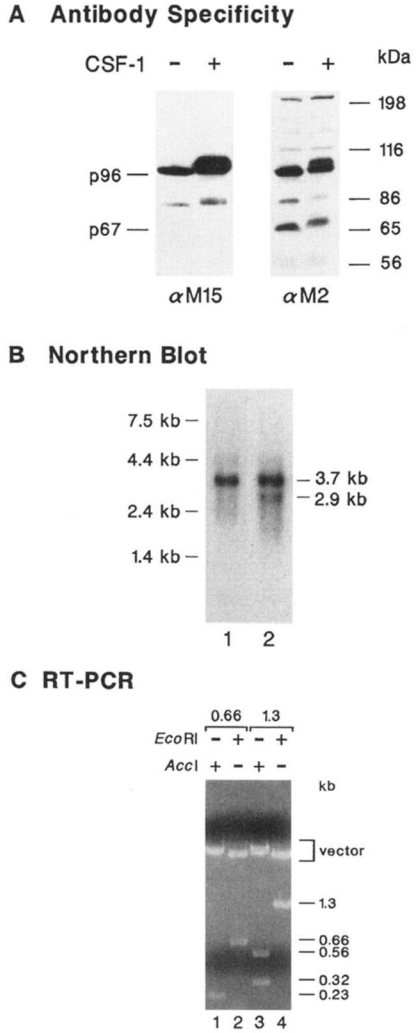

FIG. 5. **Expression of both p96 and p67 isoforms in BAC1.2F5 cells.** *Panel A*, antibody specificity and detection of protein expression in BAC1.2F5 cells by immunoblotting. BAC1.2F5 cells were starved for CSF-1 for 18 h and either harvested or stimulated for 30 min with CSF-1. A sample of the total cell lysate (150 μg of protein) was loaded into each *lane*, and the blots were probed with either the αM15 antibody (*left lanes*) or the αM2 antibody (*right lanes*) as described under "Experimental Procedures." *Panel B*, analysis of p96/p67 mRNA expression by Northern blotting. Poly(A)$^+$ mRNA was isolated from exponentially growing BAC1.2F5 cells and, 10 μg of mRNA per lane was fractionated on a 1.0% agarose gel, transferred to nitrocellulose, and probed as described under "Experimental Procedures." *Lane 1*, the mRNA was detected with a labeled probe derived from DNA between 1760 and 2136 bp of the p96 sequence, which was present in the p96 and p93 isoforms but not the p67 isoform. *Lane 2*, the mRNA was detected with a labeled probe derived from DNA between 1225 and 1440 bp of the p96 sequence. This probe contained sequences common to p96, p93, and p67. The sizes of the detected bands were calculated from a standard curve generated from an RNA ladder run on the same gel. *Panel C*, RT-PCR was used to amplify p96 sequences from the mRNA pool from BAC1.2F5 cells as described under "Experimental Procedures." The two products (1.3 and 0.66 kb) were cloned and analyzed by restriction enzyme digestion. Digestion with *Eco*RI confirmed the presence of the appropriate insert in the clones. Digestion of the 0.66-kb clone with *Acc*I yielded a single 0.23-kb band diagnostic for p67, whereas digestion of the 1.3-kb clone yielded 0.32- and 0.56-kb fragments diagnostic for p96.

liminary immunoblotting experiments with αM15 showed that p96 was retarded in its electrophoretic mobility following the stimulation of cells with CSF-1 (Fig. 5*A*). To determine if this change could be accounted for by phosphorylation, BAC1.2F5 cells were labeled with [^{32}P]orthophosphate and stimulated with CSF-1 (Fig. 6). The p96 protein was immunoprecipitated and separated by SDS-gel electrophoresis. There was a pronounced increase in the labeling of the protein following CSF-1 stimulation concomitant with a slower electrophoretic mobility. Two-dimensional phosphoamino acid analysis before and after CSF-1 stimulation showed that p96 was exclusively phosphorylated on serine residues. The kinetics of p96 modification following CSF-1 stimulation was examined by immunoblotting (Fig. 7). The electrophoretic gel shift was detected at 2 min and was clearly evident by 5 min after CSF-1 stimulation. The electrophoretic retardation of p96 continued to increase up to 30 min after CSF-1 stimulation and remained at this level through 120 min (not shown). Mitogenic stimulation with PC-PLC, sphingomyelinase, and TPA also triggered a retardation in the electrophoretic mobility of p96 (Fig. 7). Analysis of p96 electrophoretic mobility in asynchronously growing BAC1.2F5 cell populations showed that the protein was detected in the slower migrating, phosphorylated molecular form in growing cells continuously exposed to CSF-1 (data not shown).

DISCUSSION

Our results identify a novel protein, p96, that is rapidly phosphorylated in macrophages in response to mitogenic stimulation of CSF-1. The dephosphorylated form of the p96 protein accumulates in the absence of the growth factor as cells arrest in the G_1 stage of the cell cycle and then becomes progressively phosphorylated following incubation of the cultures with CSF-1, PC-PLC, sphingomyelinase, or TPA. Both PC-PLC and TPA, and also sphingomyelinase to a lesser extent, are capable of initiating progression through the first cell cycle (Xu *et al.*, 1993), and these agents elicit phosphorylation of p96 with the accompanying gel mobility shift (Figs. 6 and 7). The detection of increased p96 phosphorylation within 5 min after the addition of CSF-1 and the exclusive phosphorylation of the protein on serine residues indicates that p96 is an early, but not an immediate, component in CSF-1 signal transduction. There are three sites in the carboxyl terminus of p96 that are potential ERK kinase phosphorylation sites (Pro-*X*-Ser/Thr-Pro) (Thomas, 1992) beginning at residues 502, 510, and 559, suggesting that p96 may be an ERK kinase substrate. The protein also contains several proline-rich sequences that are potential binding sites for proteins containing SH3 domains. The rules for the interaction between SH3 domain proteins and proline-rich sequences (Yu *et al.*, 1994; Viguera *et al.*, 1994; Feng *et al.*, 1994; Lim *et al.*, 1994; Sparks *et al.*, 1994) indicate that all five of the sequences found in p96 (Fig. 3*A*) possess the minimum structural features required to interact with SH3 domain proteins, although p96 does not possess a proline-rich sequence that will bind with high affinity to well characterized SH3 domains. However, the idea that p96 interacts with proteins possessing SH3 domains should not be discarded since the SH3 domain is a common structural motif found in many cytoskeletal and cytoplasmic proteins (Musacchio *et al.*, 1992). The p96 protein was found primarily in the soluble fraction of detergent cell lysates, although up to 15% was associated with the cytoskeletal fraction.[2]

The p96 protein is most similar to the *Drosophila disabled* gene product, the Dab protein (Fig. 3*B*) (Gertler *et al.*, 1989; Bennett and Hoffmann, 1992; Gertler *et al.*, 1993). The *disabled* gene is a second-site modifier of the *abl* mutant phenotype. Flies defective in *abl* and *disabled* have an embryonic lethal phenotype and fail to form proper axonal connections in the central nervous system. Expression of either the *abl* or *disabled* gene restores the embryonic axonal architecture, illustrating the functional redundancy of the two gene products. Furthermore, the ability of *disabled* to suppress the *abl* mutant phenotype is dosage dependent, indicating that the two pro-

[2] X.-X. Xu, unpublished observations.

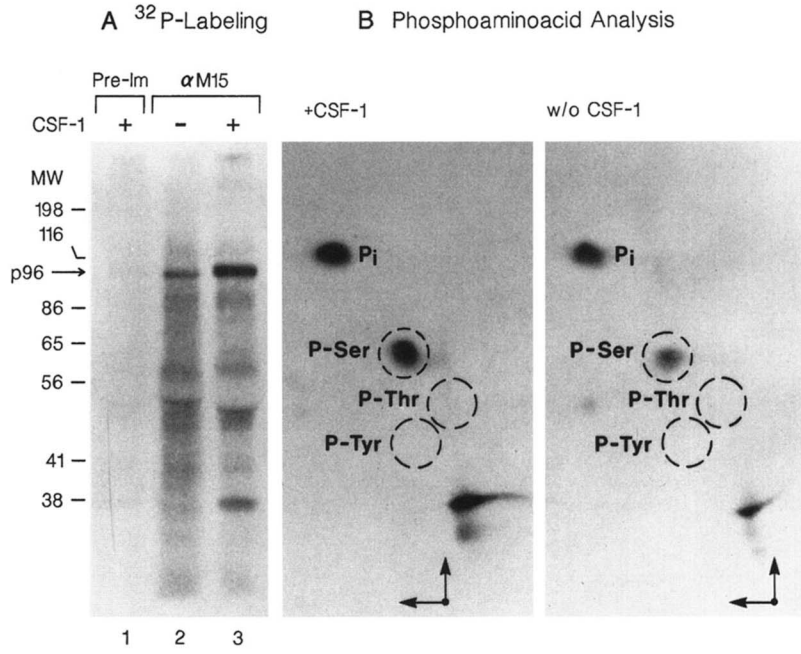

FIG. 6. **Correlation between retarded electrophoretic mobility and serine phosphorylation of p96.** BAC1.2F5 cells were deprived of CSF-1 for 18 h and metabolically labeled with [^{32}P]orthophosphate (0.166 mCi/ml) in phosphate-free DMEM for the last 2 h of the CSF-1 starvation period. The cells were then stimulated by the addition of 90 ng/ml CSF-1 for 30 min. The control and stimulated cells were harvested, lysed, and immunoprecipitated with αM15 antisera as described under "Experimental Procedures." The immune complexes were separated by SDS-gel electrophoresis and transferred to an Immobilon-P membrane. The labeled proteins were visualized by autoradiography (*panel A*). The p96 bands were excised from the membrane, and the phosphoamino acid composition of the hydrolyzed protein was determined by two-dimensional electrophoresis (*panel B*).

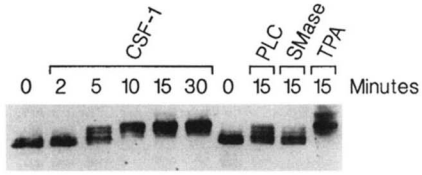

FIG. 7. **Phosphorylation of p96 phosphorylation following mitogenic stimulation.** BAC1.2F5 cells were arrested in G_1 by the removal of CSF-1 for 18 h. The cells were then stimulated with CSF-1 (90 ng/ml) for the indicated times or stimulated with either PC-PLC (1 unit/ml), sphingomyelinase (0.1 unit/ml), or TPA (10^{-7} M) for 15 min. The cells were lysed, and 150 μg of total cellular protein per lane was separated by SDS-gel electrophoresis; the p96 protein was detected by immunoblotting with αM15 antiserum as described under "Experimental Procedures."

teins may be involved in a multimeric structure whose function is sensitive to the stoichiometry of the individual components. The expression of *abl* and *disabled* are closely associated. The proteins are primarily localized in central nervous system axon bundles but are also present in peripheral nervous system cell clusters and the body wall musculature. The p96 and Dab proteins have a common 140-amino acid domain in the amino terminus that is 46% identical and 66% similar (Fig. 3*B*). Both proteins also are expressed as two alternatively spliced forms with the lower molecular weight form lacking an exon (221 residues in p96 or 214 residues in Dab) located in the center of the molecule. However, there are also several significant differences between p96 and Dab. The Dab protein consists of 2411 amino acids and is therefore much larger (264 kDa) than p96. Dab is phosphorylated on tyrosine, whereas there is no indication that p96 is tyrosine phosphorylated in the murine macrophage cell line (Fig. 6). Nonetheless, these data raise the possibility that p96 is the mammalian homolog of the *Drosophila disabled* gene product; however, functional complementation experiments in the *Drosophila* system are required to determine if this idea is correct.

One clue to the function of p96 is that the *disabled* homology domain may represent a phosphotyrosine interaction domain. This new protein motif was suggested from the findings that the amino-terminal domains of Shc (Blaikie *et al.*, 1994) and a related protein, Sck (Kavanaugh and Williams, 1994), mediate phosphotyrosine-dependent interactions. These regions were subjected to data base search methods to identify proteins that might contain homologous domains (Bork and Margolis, 1995). This search identified a region of homology between p96 and Dab (Fig. 3*B*) that was similar to residues 46–209 of Shc and thus constitutes a potential phosphotyrosine interaction domain. These data suggest that the Dab homology domain may mediate the interaction between p96 and other proteins phosphorylated on tyrosine, an exciting hypothesis that is currently being evaluated experimentally.

Mok *et al.* (1994) isolated a cDNA fragment that is the human homolog of the murine cDNA encoding p96. These investigators used a DNA-fingerprinting approach to detect candidates for tumor suppressor genes that are differentially expressed in human ovarian cancer cells. They cloned a 767-bp cDNA (called DOC-2) that was highly expressed in normal ovarian surface epithelial cells but consistently lacking in all ovarian cancer lines examined. The DNA sequence of DOC-2 was 89% identical to the p96 sequence between 346 and 1116 bp, and the amino acid sequence predicted from the DOC-2 sequence was 94.5% identical and 96% similar to the predicted p96 protein sequence between residues 49 and 300. Like p96, two mRNAs that differed by approximately 500 bp were detected by Northern analysis of normal ovarian epithelial cells. Detection of p96 expression in human ovary by *in situ* hybridization using a ^{35}S-labeled DOC-2 antisense riboprobe shows that the hybridization signal is restricted to the surface epithelial cells of the ovary, the cell type thought to give rise to ovarian carcinomas. Mok *et al.* (1994) suggest that the expression pattern of p96 (DOC-2) indicates that this gene may be involved in the development of ovarian carcinomas; however, considerably more information on the function and expression of p96 is required to substantiate this interesting idea.

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REFERENCES

Bennett, R. L., and Hoffmann, F. M. (1992) *Development* **116,** 953–966
Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., and Margolis, B. (1994) *J. Biol. Chem.* **269,** 32031–32034
Bork, P., and Margolis, B. (1995) *Cell* **80,** 693–694
Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299
Choudhury, G. G., Sylvia, V. L., Wang, L.-M., Pierce, J., and Sakaguchi, A. Y. (1991) *FEBS Lett.* **282,** 351–354

Cooper, J. A., Sefton, B. M., and Hunter, T. (1983) *Methods Enzymol.* **99,** 387–403
Downing, J. R., Margolis, B. L., Zilberstein, A., Ashmun, R. A., Ullrich, A., Sherr, C. J., and Schlessinger, J. (1989) *EMBO J.* **8,** 3345–3350
Feng, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994) *Science* **266,** 1241–1247
Friedrich, E., Vancompernolle, K., Huet, C., Goethals, M., Finidori, J., Vandekerckhove, J., and Louvard, D. (1992) *Cell* **70,** 81–92
Gertler, F. B., Bennett, R. L., Clark, M. J., and Hoffmann, F. M. (1989) *Cell* **58,** 103–113
Gertler, F. B., Hill, K. K., Clark, M. J., and Hoffmann, F. M. (1993) *Genes & Dev.* **7,** 441–453
Guilbert, L. J., Tynan, P. W., and Stanley, E. R. (1986) *J. Cell. Biochem.* **31,** 203–216
Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Imamura, K., Dianoux, A., Nakamura, T., and Kufe, D. (1990) *EMBO J.* **9,** 2423–2429
Kavanaugh, W. M., and Williams, L. T. (1994) *Science* **266,** 1862–1865
Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8148
Lee, A. W., and Nienhuis, A. W. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7270–7274
Li, W., and Stanley, E. R. (1991) *EMBO J.* **10,** 277–288
Lim, W. A., Richards, F. M., and Fox, R. O. (1994) *Nature* **372,** 375–379
Mok, S. C., Wong, K., Chan, R. K. W., Lau, C. C., Tsao, S., Knapp, R. C., and Berkowitz, R. S. (1994) *Gynecol. Oncol.* **52,** 247–252
Morgan, C., Pollard, J. W., and Stanley, E. R. (1987) *J. Cell. Physiol.* **130,** 420–427
Musacchio, A., Gibson, T., Lehto, V.-P., and Saraste, M. (1992) *FEBS Lett.* **307,** 55–61
Ohtsuka, M., Roussel, M. F., Sherr, C. J., and Downing, J. R. (1990) *Mol. Cell. Biol.* **10,** 1664–1671
Rettenmier, C. W., Chen, J. H., Roussel, M. F., and Sherr, C. J. (1985) *Science* **228,** 320–322
Rock, C. O., Cleveland, J. L., and Jackowski, S. (1992) *Mol. Cell. Biol.* **12,** 2351–2358
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Schwarzbaum, S., Halpern, R., and Diamond, B. (1984) *J. Immunol.* **132,** 1158–1162
Sherr, C. J. (1991) *Trends Genet.* **7,** 398–402
Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T., and Stanley, E. R. (1985) *Cell* **41,** 665–676
Sparks, A. B., Quilliam, L. A., Thorn, J. M., Der, C. J., and Kay, B. K. (1994) *J. Biol. Chem.* **269,** 23853–23856
Stanley, E. R. (1985) *Methods. Enzymol.* **116,** 564–587
Stanley, E. R., and Heard, P. M. (1977) *J. Biol. Chem.* **252,** 4305–4312
Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5201–5205
Thomas, G. (1992) *Cell* **68,** 3–6
Tushinski, R. J., and Stanley, E. R. (1985) *J. Cell. Physiol.* **122,** 221–228
Ullrich, A., and Schlessinger, J. (1990) *Cell* **61,** 203–212
Vairo, G., and Hamilton, J. A. (1991) *Immunol. Today* **12,** 362–369
Veis, N., and Hamilton, J. A. (1991) *J. Cell. Physiol.* **147,** 298–305
Viguera, A. R., Arrondo, J. L. R., Musacchio, A., Saraste, M., and Serrano, L. (1994) *Biochemistry* **33,** 10925–10933
Xu, X.-X., Tessner, T. G., Rock, C. O., and Jackowski, S. (1993) *Mol. Cell. Biol.* **13,** 1522–1533
Yeung, Y. G., Jubinsky, P. T., Sengupta, A., Yeung, D. C., and Stanley, E. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1268–1271
Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994) *Cell* **76,** 933–945