A 50 Kilodalton Protein Associated with Raf and pp60^{v-src} Protein Kinases Is a Mammalian Homolog of the Cell Cycle Control Protein cdc37

Gary H. Perdew,* Heather Wiegand, John P. Vanden Heuvel, Carlton Mitchell, and Sheo S. Singh

Department of Veterinary Science, The Pennsylvania State University, University Park, Pennsylvania 16802

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ABSTRACT: Several oncogenic protein kinases including c-raf-1 and pp60^{v-src} are known to directly interact with the 90 kDa heat shock protein (hsp90)/p50 complexes. Using a monoclonal antibody to detect p50 during a purification scheme, p50 was purified to homogeneity. Internal amino acid sequence information was obtained and used to clone a partial cDNA. Comparison of the p50 sequence to other cloned proteins revealed 89% homology with a glycosaminoglycan-binding protein and 54% homology with *Drosophila* cell cycle control protein (cdc) 37. Monoclonal and polyclonal antibodies were produced against a cleaved fusion protein that recognizes p50 with a high level of specificity. These antibodies recognize the 50 kDa protein present in c-raf-1 and pp60^{v-src} complexes. No other proteins were recognized with these antibodies suggesting that p50 is a unique protein. Immunocytochemical visualization of p50 in NIH 3T3 cells indicates a primarily cytoplasmic localization around the nuclear membrane. A survey of p50 expression in murine tissues on a protein blot revealed the following relative levels of expression; thymus > spleen > brain > heart > kidney > liver > lung > skeletal muscle. These results link studies demonstrating complexation of certain kinases with hsp90/p50 in mammalian cells and a number of reports in yeast and *Drosophila*, demonstrating the importance of cdc37 in cell cycle and kinase function.

During heat and chemical stress, cells in culture induce the synthesis of a group of proteins referred to as heat shock proteins. The most highly expressed heat shock protein in unstressed cells is hsp90.[1] The actual function(s) of hsp90 is(are) not well understood. A number of regulatory proteins have been shown to interact with hsp90, including viral tyrosine kinases (Lipsich et al., 1982), c-raf-1 kinase (Stancato et al., 1993), steroid hormone receptors (Smith & Toft, 1993), casein kinase II (Miyata & Yahara, 1992), and the *Ah* receptor (Perdew, 1988). Each one of the proteins was found to interact with hsp90 during the study of these individual proteins. Studies in our laboratory, using a monoclonal antibody to immunoprecipitate hsp90/protein complexes, revealed several relatively abundant cytosolic proteins with molecular masses of 68, 63, 56,and 50 kDa bound to hsp90 in Hepa 1 cells (Perdew & Whitelaw, 1991). The 68 kDa protein was identified immunochemically as hsp70. Hsp70 actually refers to a family of proteins that are involved in facilitating protein folding and assembly, and are also required to transport at least some proteins across the nuclear membrane (Beckman et al., 1990; Shi & Thomas, 1992). The identity of p63 associated with hsp90 is not known. The 56 kDa protein associated with hsp90 was initially reported as a protein found as part of a steroid receptor bound to hsp90. In addition, p56 is a heat shock protein (hsp56) which has been cloned, and sequence analysis has revealed homology with proline isomerases (Sanchez, 1990; Lebeau et al., 1992). This result may indicate that hsp56 is involved in the regulation of hsp90 function through a rotamase activity. In addition, hsp56 has been identified as an immunophilin capable of binding the immunosuppressant FK-506 (Tai et al., 1992). Immunoprecipitation analysis of hsp56 from human IM-9 lymphocytes indicated that hsp90 and hsp70 are complexed with hsp56, but the actual arrangement of these complexes is not known (Sanchez et al., 1990). The 50 kDa protein has been found to interact transiently with pp60^{v-src} and other tyrosine protein kinases (Lipsich et al., 1982; Whitelaw et al., 1991).

The viral oncogenic tyrosine kinase pp60^{v-src} interacts with a hsp90/p50 complex after translation in the cytoplasm. Pulse−chase experiments have revealed that pp60^{v-src} transiently binds to hsp90/p50, followed by insertion of the kinase into the plasma membrane. Other retroviral transforming proteins with tyrosine kinase activity interact transiently with hsp90/p50 (Lipsich et al., 1982; Ziemiecki et al., 1986). The role of both p50 and hsp90 in this complex is unknown. It has been suggested that p50 may serve a targeting function, perhaps signaling transport to the plasma membrane. Alternatively, hsp90/p50 binding may confer solubility to, or aid in the folding of, newly translated pp60^{v-src}, or maintain some regulation over the activity of pp60^{v-src} (Brugge, et al. 1986).

The Ras oncoproteins are potent mitogenic polypeptides that are critical determinants of cellular differentiation. Ras is capable of relaying mitogenic signals initiated by cell surface receptors into the cytoplasm and nucleus. It has been established that Ser/Thr protein kinases play an important role in mitogenic signal transduction downstream from Ras. The Ser/Thr kinase that is directly activated by Ras has recently been established as Raf (Ahn, 1993). Activated Raf is capable of binding to and phosphorylating the MAP kinase kinase, Mek, which in turn phosphorylates and activates

* To whom correspondence should be addressed.

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[1] Abbreviations: hsp90, hsp70, and hsp56, 70, and 56 kDa heat shock proteins, respectively; *Ah* receptor, aryl hydrocarbon receptor; Hepa 1, mouse hepatoma cell line 1c1c7; pp60^{v-src}, 60 kDa avian sarcoma oncoprotein; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; M_r, relative molecular weight; GA, geldanamycin.

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MAP kinase. The activated MAP kinase then translocates to the nucleus where many of the targets for the MAP kinase signal transduction pathway are found. Although considerable progress toward understanding the role of Raf in the MAP kinase signal transduction pathway has been achieved, little is known about the mechanism(s) of regulation of the Raf protein kinase. Both v-Raf and its cellular analog c-Raf have been demonstrated to stably interact with hsp90/p50 (Stancato et al., 1993; Wartmann & Davis, 1994). A deletion mutant containing the catalytic domain is also capable of binding to hsp90/p50 (Stancato et al., 1993). Unlike $pp60^{v-src}$, Raf requires gentle conditions of isolation to maintain complexation with hsp90/p50; this includes using low levels of mild detergents during isolation of the cytosolic fraction and subsequent immunoprecipitation. Although it is predominantly cytosolic, c-Raf also undergoes trafficking to the plasma membrane where it is under tight control by growth factor receptors (e.g., epidermal growth factor). After translocation to membrane sites, Raf kinase activity is stimulated. Thus, the factor(s) that regulates this translocation process should have a key role in regulating Raf activity. Recently, Wartmann and Davis have demonstrated that, in both serum-starved and serum or phorbol ester stimulated cells, Raf is found in the cytosolic fraction complexed with hsp90/p50 (Wartmann & Davis, 1994). Upon the addition of serum to cells in culture, an increase in association of raf/hsp90/p50 with membranes is observed. The possible role of p50 in Raf activity and targeting to the plasma membrane is unknown.

In a previous report, we developed an IgM monoclonal antibody to a 50 kDa protein associated with hsp90 and transiently to $pp60^{v-src}$ (Whitelaw et al., 1991). In this report, we describe the purification, partial internal amino acid sequence analysis of p50, cloning of a partial cDNA for p50, and production of monoclonal antibodies.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Cytosolic Extracts. Hepa 1 cells were grown and harvested, and the cytosolic fraction was isolated as previously described (Perdew, 1988). NIH 3T3 cells obtained from the American Type Culture Collection (Rockville, MD) were maintained in α-minimum essential medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT).

Detection of p50 on Western Blots. Samples were solubilized in SDS sample buffer and applied to 7.5% SDS−polyacrylamide gels. After electrophoresis, the protein was transferred to a PVDF membrane (Immobilon P, Millipore Corp.) using a Genie blotter (Idea Scientific, Corvallis, OR) at 6 V for 8 h. The membranes were blocked with 3% BSA/0.5% Tween 20/PBS, and the blot was gently shaken for 1 h with a 1:1000 dilution of mAb 3M/1B5p50 (1B5p50) ascites in 0.1% BSA, 0.5% Tween 20, 10 mM NaH_2PO_4, pH 7.4, + 150 mM NaCl (buffer A). The blot was washed 3 × 5 min in buffer A, and then shaken for 1 h with GAM IgG+IgM peroxidase (1:1000 dilution in buffer B). After 3 × 5 min washes with buffer A, bound antibodies were detected by staining with 3,3-diaminobenzidine (0.3 mg/mL)/0.005% H_2O_2 in 50 mM Tris, pH 7.4.

Mono Q Chromatography. A Mono Q HR 5/5 FPLC column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) was equilibrated in 20 mM triethanolamine hydrochloride, 175 mM NaCl, pH 7.4. Hepa 1 cytosol (20 mg, 2 mg/mL) was applied, and the column was washed with starting buffer for 6 min at a flow rate of 1 mL/min, followed by a linear 175−500 mM NaCl gradient in 20 mM triethanolamine hydrochloride, pH 7.4, over 20 min. Fractions were collected, and aliquots of each fraction were subjected to SDS−PAGE, followed by electrophoretic transfer of the protein to a PVDF membrane. The presence of p50 in each fraction was visualized on protein blots.

Hydroxyapatite Chromatography. The pooled p50 fractions from 40 mg of Hepa 1 cytosol after Mono Q chromatography were mixed with SDS (final concentration, 2.5%) and β-mercaptoethanol (final concentration, 5%). The sample was heated at 100 °C for 3 min and directly applied to a Pentax HPLC hydroxyapatite 8 × 100 mm glass column (Asahi Optical Co., Tokyo, Japan). The gradient elution system consisted of buffer B (1% SDS, 0.1 mM $CaCl_2$, 5 mM DTT, 10 mM sodium phosphate, pH 6.5) and buffer C (1% SDS, 7.5 nM $CaCl_2$, 5 mM DTT, 500 mM sodium phosphate, pH 6.5). The column was equilibrated in 55% buffer C (45% buffer B), and the temperature of the room was maintained at 27 °C. The start buffer was continued for 20 min (0.5 mL/min), and the adsorbed protein was subsequently eluted using a 55−100% linear gradient of buffer C over 55 min. Each fraction was dialyzed against Milli Q water overnight and tested for the presence of p50 on Western blots as described above.

Final Purification of p50 by Electrophoresis. The fractions containing p50 were pooled and lyophilized, followed by solubilization in SDS sample buffer. The p50 samples were heated at 100 °C for 5 min, applied to 7.5% SDS−polyacrylamide gels and electrophoretically transferred to a PVDF membrane. The membranes were stained with 0.1% Ponceau S in 5% acetic acid for 10 min, and the background stain was reduced by rinsing the blot with water. The p50 band was excised for fragmentation and sequencing. The level of purity of p50 was further assessed by subjecting an aliquot of the post-hydroxyapatite fraction to two-dimensional gel electrophoresis as previously described except that 3.5% acrylamide isoelectric focusing gels were used (Perdew & Hollenback, 1990).

Protein Fragmentation and Sequencing. The protein on PVDF (4 μg) was cleaved by CNBr (Fluka) in 70% formic acid, and fragments were subsequently extracted from the membrane. Digestion of the material from the initial extraction and subsequent HPLC separation were essentially as described (Stone et al., 1989), with some minor changes. The extract was dissolved in 0.4 M NH_4HCO_3, 6 M urea, 10 mM EDTA, 20 mM methylamine, pH 8.5, and subjected to reduction/alkylation with DTT and iodoacetamide followed by digestion with 0.25 μg (2.5 μL) of endoproteinase Lys-C (Boehringer-Mannheim, sequencing grade) at 37 °C for 18 h. A control experiment in the absence of protein was conducted concurrently.

Separation of the digested peptides was performed on a Vydac C18 narrow-bore column (2.1 × 250 mm, 300 Å, 5 μm) at a flow rate of 150 μL/min using a Waters 625 HPLC system. The linear gradient used to elute peptide was 5−70% solvent B (solvent A, 0.06% TFA in water; solvent B, 0.052% TFA in 80% acetonitrile) over 60 min. Peptides were detected at 214 nm. The control digest was also run on the HPLC under the same conditions for direct comparison. Peptides chosen for sequence analysis were rechro-

matographed under the same conditions as above and collected manually to obtain effective purification.

Sequence analysis was performed on an Applied Biosystems (Model 477A) pulsed liquid instrument with an on-line, Model 120A, PTH-analyzer. The HPLC-purified peptides were applied directly to a polybrene-conditioned glass fiber disc. A polybrene-conditioned glass fiber disc was inserted below the electroblotted protein sample in the sequencer cartridge prior to analysis. Reagents and programs were as supplied by the manufacturer of the instrument with modifications made according to Tempst and Riviere (1989), to allow for injection of 100 μL of the PTH-amino acid solution at each cycle, and according to Speicher (1989), to provide optimized cycles for use with the sample electroblotted to PVDF.

Isolation of Partial p50 cDNA. Degenerate primers were made corresponding to the two internal p50 amino acid sequences obtained; the following primers were used in PCR: P50/1, TTYGTIGARAARTAYGARAA; P50/4, 5′-TCYTCICCYTCYTTIGCYTC. A Hepa 1c1c7 cell gt22A phage library was produced using a Life Technologies cDNA library kit as described by the manufacturer (Gaithersburg, MD). The phage library was amplified once and subsequently used as a template for PCR. The PCR reaction conditions were essentially as described (Friedman et al., 1990). Using primers P50/1 and P50/4, a PCR product was obtained and cloned into an Invitrogen pCR II T/A cloning vector, and the insert was sequenced at the Purdue Core Sequencing Facility.

Generation of a p50/MBP Fusion Protein. Primers were designed to add a start site and *Xba*I to the 5′ end and a *Hind*III site to the 3′ end of the p50 PCR product. After amplification, the PCR product was subcloned into the pMAL-c2 vector. The p50/PCR/MBP fusion protein was expressed in *E. coli* and purified on amylose resin using methods supplied by New England Biolabs (Beverly, MA). The purified fusion protein was cleaved with factor Xa and the p50 protein purified on a Mono Q column.

Monoclonal and Polyclonal Antibody Production. Purified cleaved p50 protein, estimated to be 95% pure, was oxidized with performic acid as previously described and mixed with complete Freund's adjuvant (Whitelaw et al., 1991). Immunizations and monoclonal antibody production followed standard techniques as described (Perdew & Whitelaw, 1991). The resulting hybridomas were screened using p50 protein in an ELISA assay system. Positive clones were tested on a Western blot of Hepa 1c1c7 cytosol; positive cells were cloned by limiting dilution. Two clones were isolated, C1 and D1; each was determined to be an IgG_1 antibody. Polyclonal ascites against p50 was induced in mice after a standard immunization schedule (Perdew, 1994).

Immunocytochemistry. NIH 3T3 cells were seeded onto poly(L-lysine)-coated HTC-printed microscope slides (Celline Associates, Newfeld, NJ) and grown to 50% confluency in α-MEM containing 10% fetal bovine serum and antibiotics. The visualization of p50 in fixed cells was performed with anti-p50 monoclonal and polyclonal antibodies using methods previously described (Hord & Perdew, 1994).

Immunoprecipitation of pp60^{v-src} and c-raf-1. LA90-3T3 cells were harvested by trypsinization and washed twice with PBS followed by lysis in MENG containing 1% NP-40 and 1 mM sodium orthovanadate (buffer D). Anti-pp60^{v-src} mAb Ab-1 (10 μg) was absorbed to 50 μL of goat anti-mouse IgG−Sepharose (Sigma) in buffer C for 1 h. After two washes with buffer C, 1 mL (3 mg/mL) of LA90-3T3 cytosol was added to the sepharose pellet and incubated with shaking for 1 h on ice. The sepharose was washed 3 times with buffer C. Anti-c-raf-1 rabbit polyclonal antibody C-12 (5 μg) was absorbed to 50 μL of goat anti-rabbit IgG−Sepharose (Sigma) and incubated in buffer D for 1 h. After two washes in buffer C, 0.5 mL (5 mg/mL) of NIH 3T3 cytosol was added and incubated for 1 h on ice with shaking. The Sepharose was washed 3 times with buffer D. All immunoprecipitations were mixed with an equal volume of 2× SDS sample buffer, heated, and subjected to SDS−PAGE.

Immunoprecipitation of p50/Flag. A murine cdc37 (p50) cDNA subcloned into pcDNA3 was obtained from Dr. Wade Harper (Baylor College of Medicine). Using standard PCR techniques, we amplified the entire cdc37 cDNA and added a nucleotide sequence, corresponding to the Flag peptide sequence, to the 3′ end of the gene; this PCR product was subcloned into pcDNA3. The pcDNA3/mcdc37/Flag vector was *in vitro* translated in the TNT-coupled transcription/translation system (Promega, Madison, WI). The translation mixture (100 μL) was mixed with 900 μL of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 150 mM NaCl, 10 mM Tris-HCl, pH 7.2) and incubated overnight with 25 μL of M2 affinity gel (Kodak Scientific Imaging Systems, New Haven, CT). The gel was washed 4 times with RIPA buffer and incubated with 200 μL of RIPA buffer plus 90 nmol of Flag peptide. After 4 h, the gel was centrifuged down and the supernatant transferred to a separate tube and mixed with Tricine 2× sample buffer.

RESULTS AND DISCUSSION

Purification of p50. In a previous report, we developed a monoclonal antibody to a 50 kDa protein associated with hsp90, pp60^{v-src}/hsp90, and c-raf-1/hsp90 (Stancato et al., 1993; Whitelaw et al., 1991). Monoclonal antibody 1B5p50 was used in this report to assess the presence of the p50 protein throughout the purification scheme. Hepa 1 cytosol was chosen as the source for p50 because these cells express a relatively high level of this protein.[2] Hepa 1 cytosol was first applied to a Mono Q column, and the bound protein was eluted with a linear salt gradient (Figure 1). An aliquot of each fraction was then subjected to SDS−PAGE, and the protein was transferred to a PVDF membrane. The presence of p50 in each fraction was detected by immunochemical staining. Based on the immunodetection results, the p50 protein was found to coelute with hsp90, which agrees with a previous study where p50 was demonstrated to be complexed with hsp90 in Hepa 1 cytosol (Whitelaw et al., 1991). Attempts to dissociate p50 from hsp90 under nondenaturing conditions, followed by chromatography, proved to be unsuccessful. Because of this stable interaction, the remaining purification steps were performed under denaturing conditions. The Mono Q fractions containing p50 were pooled, placed in SDS sample buffer, applied to a hydroxyapatite HPLC column, and eluted with a linear phosphate gradient in the presence of SDS (Figure 2). After dialysis against distilled water, each fraction was tested for the presence of p50. An aliquot of each purification step was

[2] Unpublished results.

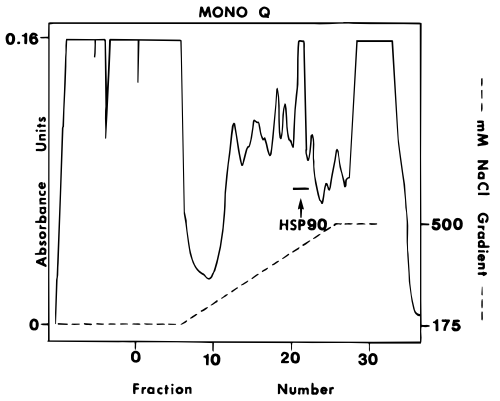

FIGURE 1: High-performance liquid chromatography on a Mono Q column. Cytosolic extracts from Hepa 1 cells (10 mL, 2 mg/ml) were applied to a Mono Q column as described under Experimental Procedures. After protein was applied and the absorbance at 254 nm returned to base line, a linear 175−500 mM NaCl gradient was applied. Fractions were collected and aliquots subjected to SDS−PAGE. The protein was electrophoretically transferred to a PVDF membrane, and the presence of p50 was detected with mAb 1B5p50. The p50 was found to comigrate with the hsp90 peak; the bar on the figure illustrates the fractions pooled for further purification.

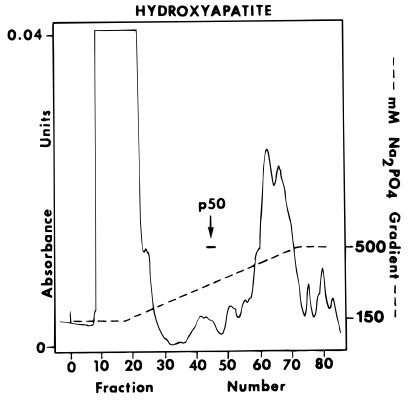

FIGURE 2: High-performance liquid chromatography on a hydroxyapatite column. The p50 fractions from two Mono Q chromatographic runs were pooled, mixed with SDS/β-mercaptoethanol, and heated. The protein was applied to a Pentax HPLC hydroxyapatite column and eluted with a linear sodium phosphate gradient. Fractions were collected and individually dialyzed against distilled water. An aliquot of each fraction was tested for the presence of p50; the bar marks the fractions containing p50. For more details of chromatography and analysis, see Experimental Procedures.

subjected to SDS−PAGE and transferred to membrane. The presence of p50 was immunochemically visualized with mAb 1B5p50, and compared with a duplicate blot stained with Coomassie Blue (Figure 3). A single band at 50 kDa was detected on the Coomassie-stained blot that comigrated with the 1B5p50-localized band in the p50 post-hydroxyapatite fractions. In addition, the post-hydroxyapatite p50 fraction was applied to two-dimensional gel electrophoresis to assess the level of purity of the p50 band and thus ensure that the p50 band used for amino acid sequencing was only one protein. The results in Figure 4 revealed that there was only one spot in the 50 kDa region of the blot. This would indicate that the p50 protein was purified to homogeneity and thus was suitable for sequencing. The post-hydroxyapatite fractions containing p50 were pooled, lyophilized, solubilized in SDS sample buffer, and subjected to SDS−PAGE. After electrophoresis, the protein on the gel was

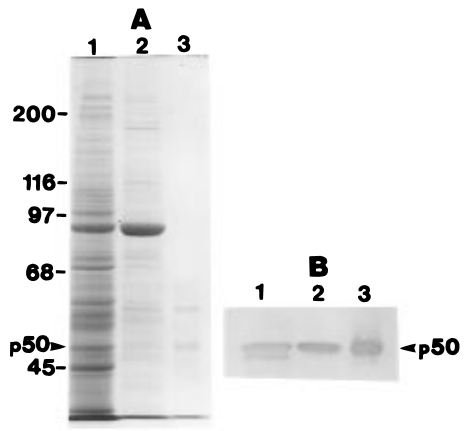

FIGURE 3: Presence of p50 compared to total protein after each chromatographic step. Total Hepa 1 cytosol (200 μg), pooled p50 fractions after Mono Q chromatography (~20 μg), and pooled p50 fractions after hydroxyapatite chromatography (~2 μg) were subjected in duplicate to SDS−PAGE. After electrophoresis, the protein was transferred to a PVDF membrane; one set of samples was stained with Coomassie Blue (panel A). The other set of samples was immunochemically stained using the mAb 1B5p50 (panel B).

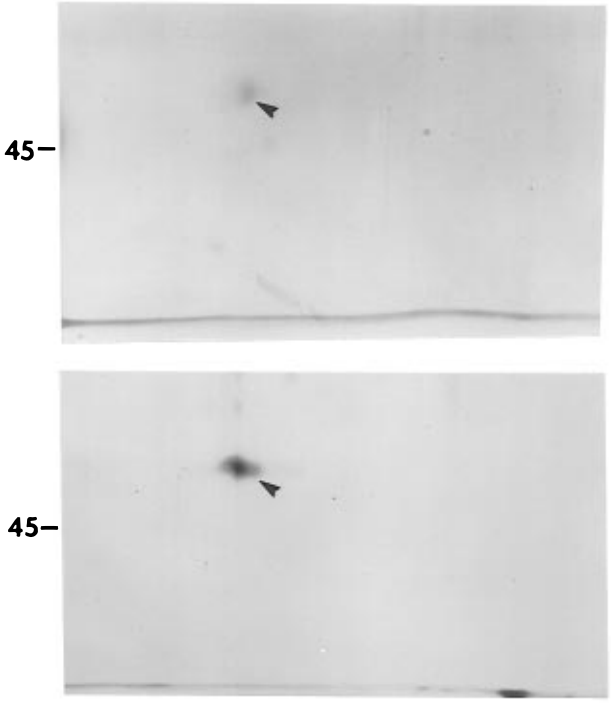

FIGURE 4: Two-dimensional gel electrophoresis of purified p50. The post-hydroxyapatite p50 fraction was subjected to two-dimensional gel electrophoresis to assess its purity. Two duplicate gels were run, each having approximately 1 μg of protein applied to the isoelectric focusing gel. After the second dimension was completed, the protein was transferred to a PVDF membrane. The upper panel is a membrane stained with Coomassie Blue, and the lower panel is a membrane immunochemically stained using mAb 1B5p50. The 45 kDa ovalbumin standard is shown; the arrow points to the p50 spot.

transferred to a PVDF membrane and stained with Ponceau S, and the p50 protein band was excised for sequencing.

Amino Acid Sequencing, Molecular Cloning, and Characterization. The N-terminus of p50 was determined to be blocked after Edman degradation failed to produce any sequence data from a sample of the protein electroblotted onto PVDF, thus necessitating access to the internal sequence by fragmentation. A piece of PVDF membrane containing

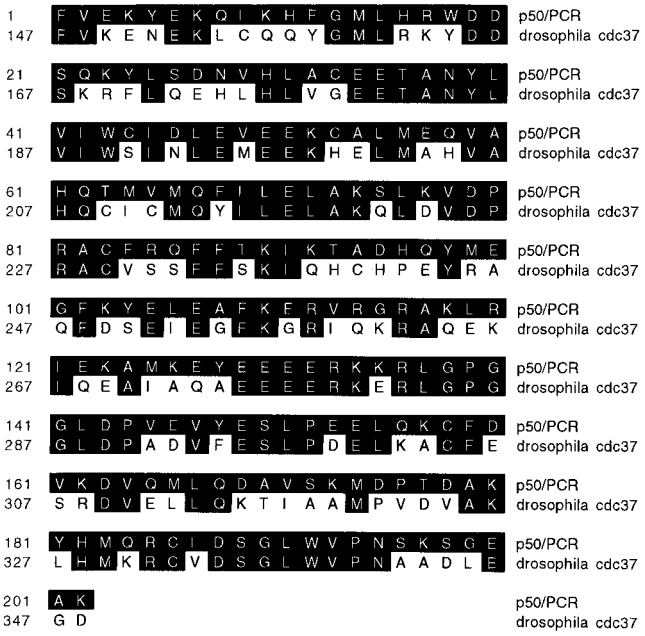

FIGURE 5: Amino acid sequence alignment of murine p50 PCR product with *Drosophila* cdc37. Amino acid residues of *Drosophila* cdc37 that match p50 are boxed in black.

p50 was subjected to cyanogen bromide cleavage and endoproteinase Lys-C digestion. The peptides obtained from this digestion were separated by reverse-phase HPLC. Two peptides were sequenced, and the resulting sequences are as follows: for peptide 1, Ser-Gly-Glu-Ala-Lys-Glu-Gly-Glu-Glu-Ala-Gly-Pro-Gly-Asp-Pro-(Leu-Leu-Glu-Ala-Val); for peptide 2, Thr-Phe-Val-Glu-Lys-Tyr-Glu-Lys.

Degenerate primers corresponding to each amino acid sequence shown above and a murine cDNA library were used to isolate a p50 cDNA. The translated cDNA indicated that the p50 clone has both amino acid sequences used for primer design. This result would indicate that we had isolated a cDNA corresponding to p50. A search of genebank revealed 54% sequence homology with *Drosophila* cdc37 and 89% with an avian glycosaminoglycan-binding (GAG) protein (Cutforth & Rubin, 1994; Grammatikakis et al., 1995). Sequence comparison of murine p50 and *Drosophila* cdc37 revealed homology throughout the sequence, suggesting that the p50 cDNA obtained appears to be the mammalian homolog of *Drosophila* cdc37 (Figure 5). While this work was in progress, we learned that Dr. Wade Harper's laboratory (Baylor College of Medicine) had cloned a full-length p50 cDNA. The p50 amino acid sequence reported here has the same sequence as the clone obtained by Dr. Harper's laboratory (Stepanova et al., 1996). The high level of sequence homology between *drosophila* cdc37 and p50 supports the concept that p50 is the mammalian homolog of cdc37. Examination of the sequence of the partial p50 clone reveals 55% sequence homology with *Drosophila* cdc37 between amino acid sequence F^{147} and A^{342}. Considering conserved amino acid substitutions, the degree of similarity rises to 78% between *Drosophila* cdc37 and p50. The assertion that p50 is mcdc37 is strengthened by the fact that *Drosophila* cdc37 and hsp90 are important for sevenless receptor function, which appears to be analogous to the importance of hsp90/p50 in mammalian $pp60^{v-src}$ function (Brugge, 1986; Cutforth & Rubin, 1994). The *Drosophila* cdc37 protein has a calculated molecular mass of 45 kDa and after *in vitro* translation has a mass of 58 kDa (Cutforth & Rubin, 1994). A full-length p50 clone isolated by Dr. Wade Harper's laboratory also has a calculated molecular mass of 45 kDa, suggesting that p50 is the homolog of cdc37 (Stepanova et al., 1996). Clearly, more functional studies are needed to firmly establish that p50 in mammalian cells is the functional homolog of yeast and *Drosophila* cdc37. The chick GAG protein after *in vitro* translation has a molecular mass of 31 kDa and has 246 amino acids, considerably smaller than the 389 amino acids in *Drosophila* cdc37. The GAG protein mRNA may undergo differential processing in chick embryo cardiocytes (Grammatikakis et al., 1995). The relationship of this protein to cdc37 or p50 will require further analysis. A GAG binding motif, corresponding to $B(X_7)B$, has been identified in a number of proteins (Yang et al., 1994). The p50 clone isolated here has at least one of these motifs, thus raising the possibility that GAG can interact with p50.

The cdc37 gene was isolated while screening temperature-sensitive mutants that exhibit a G_1 arrest. The role of cdc37 in the cell is largely unknown and has only been studied in yeast and *Drosophila* due to the lack of a mammalian cDNA clone. Studies in yeast have demonstrated that cdc37 is required for association of the protein kinase cdc28 with mitotic cyclins (Gerber et al., 1995). While, in *Drosophila,* cdc37 and hsp83 play a role in the proper signaling of the sevenless receptor tyrosine kinase (Cutforth & Rubin, 1994). Yet there has been no direct characterization of the biochemical properties of this protein and the various proteins that are capable of interacting with cdc37. Conversely, in mammalian cells, the ability of p50/hsp90 to interact with several kinases at the biochemical level has been extensively characterized (Brugge, 1986; Wartmann & Davis, 1994).

Antibody Production. The p50 PCR cDNA was subcloned into pMAL-c2 vector using standard PCR techniques. Fusion protein was isolated and cleaved with Xa protease. The 12 kDa p50 fusion protein fragment was subjected to protein blot analysis and was found to be recognized by mAb 1B5p50.[2] This demonstrates that the p50 cDNA clone is the same p50 originally detected in $pp60^{v-src}$ complexes. The purified p50 product was injected into a series of mice and polyclonal ascites, and two monoclonal antibodies (C1 and D1) were isolated (Figure 6). Each antibody was highly specific and binds to a 50 kDa protein that is recognized by previously generated IgM mAb against p50 (Whitelaw et al., 1991). There were no other proteins recognized by any of the antibody preparations used here, indicating that p50 is a unique protein and probably not a member of a family of proteins (Figure 6). MAb C1 is capable of immunoprecipitating p50 in the presence of detergent.[2] In the lower panel of Figure 6, we tested whether the mAb 1B5 and C1 are able to bind to mcdc37 cDNA obtained from Dr. Wade Harper's laboratory. The data indicate that both antibodies against p50 bind to *in vitro* translated mcdc37/Flag. In order to further demonstrate that these antibodies were raised against the 50 kDa protein associated with $pp60^{v-src}$ and c-raf-1 immunoprecipitated complexes, immunoprecipitations were probed with mAb C1 to detect the presence of p50 (Figure 7). The results reveal that p50 is indeed associated with these kinases as has been previously determined (Whitelaw et al., 1991; Stancato et al., 1993). Because these antibodies are highly specific on protein blots they should be suitable for immunocytochemical and immunohistochemical analysis of p50 distribution.

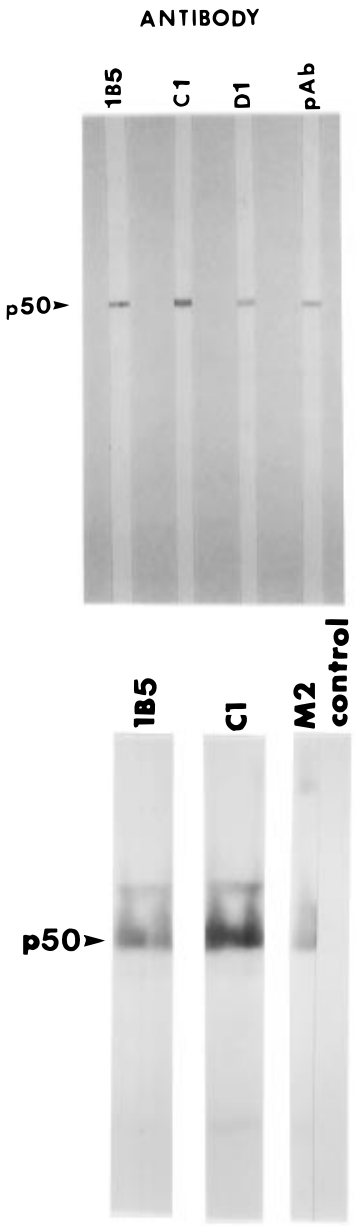

FIGURE 6: Protein blot analysis of antibodies specific to p50. (Upper panel) After SDS−PAGE, Hepa 1c1c7 cytosolic protein (0.5 mg) was transferred to an Immobilon membrane. The membrane was cut into strips and probed with mAb 1B5p50, mAb C1p50, MAb D1p50, and anti-p50 polyclonal ascites. (Lower panel) Murine cdc37/Flag was immunoprecipitated using M2 affinity gel from an *in vitro* translation, p50/Flag was displaced from the gel with Flag peptide, and subjected to SDS−PAGE followed by transfer of protein to membrane. The membrane was probed with either mAb 1B5p50, mAb C1p50, or mAb M2. The last lane was cut in half, and the control part of the lane was incubated with secondary antibody/peroxidase alone. Primary antibodies were visualized with goat anti-mouse peroxidase conjugate and 3,3′-diaminobenzidine.

Characterization of p50 Expression and Localization. The subcellular localization of p50 was examined in NIH 3T3 cells using indirect immunofluorescence techniques. Results indicated that p50 is predominantly localized in the cytoplasm, with the most intense staining around the nuclear membrane (Figure 8). Essentially the same staining pattern was obtained with both mAb C1 and the anti-p50 polyclonal antibody. We did not detect significant immunocytochemical staining of p50 along the plasma membrane, despite the apparent importance of hsp90/p50 complexes in the transport of pp60^{v-src} and raf-1 to the plasma membrane. Recently,

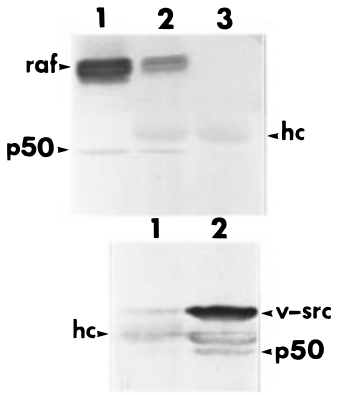

FIGURE 7: Monoclonal antibody C1p50 recognizes a 50 kDa protein coimmunoprecipitated with c-raf-1 or pp60^{v-src}. c-raf-1 (upper panel) and pp60^{v-src} (lower panel) were immunoprecipitated from NIH 3T3 and LA90-3T3 cellular extracts, respectively. The blots were cut in half across the IgH heavy-chain band; the upper portion was probed with either raf or pp60^{v-src} antibodies. The lower portion was probed with mAb C1p50. Upper panel: lane 1, NIH 3T3 cytosol; lane 2, raf immunoprecipitation; lane 3, control IgG immunoprecipitation. Lower panel: lane 2, pp60^{v-src} immunoprecipitation; lane 1, control IgG immunoprecipitation.

two investigators have utilized mAb 1B5p50 to visualize p50; the results also indicated a strong perinuclear staining pattern using similar methods described here (Owens-Grillo et al., 1996; Stepanova et al., 1996). Examination of previous reports on localization of c-raf-1 in NIH 3T3 cells also revealed strong cytoplasmic and perinuclear staining (Oláh et al., 1994; Rapp et al., 1988). In addition, immunocytochemical localization of pp60^{v-src} and and pp60^{c-src} in several cell lines has indicated a strong perinuclear localization, which appears to be associated with endocytotic vesicles (Redmond et al., 1992; David-Pfeuty et al., 1990). These localization studies reveal a similar subcellular distribution between p50 and two kinases that interact with p50/hsp90 complexes. The tissue distribution of p50 was determined by protein blot analysis using cytosolic preparations from C57BL/6 mice tissues (Figure 9). The presence of p50 was detected in every tissue examined except skeletal muscle the following ranking of the level of expression was observed: thymus > spleen > brain > heart > kidney > liver > lung > skeletal muscle. The significant level of expression seen in most tissues is consistent with the role of this protein in the function of several protein kinases. The tissue distribution of cdc37 in *Drosophila* indicated a ubiquitous distribution in developing embryos; in addition, cdc37 was localized in the cytoplasm (Cutforth & Rubin, 1994).

Potential Role of p50 in Kinase Function. The putative tyrosine kinase inhibitors geldanamycin (GA) and herbimycin A have been reported to revert the morphology of fibroblasts transformed by many oncogenic tyrosine kinases such as src, fyn, lck, and erbB2. The mechanism of this effect is unknown, but apparently is not due to a direct effect on tyrosine kinase activity (Whitesell et al., 1994). Using immobilized GA and cellular extracts, one protein was found to tightly associate with GA; this protein was identified to be hsp90. Further experimentation demonstrated that GA inhibited the association of hsp90 with pp60^{v-src}, both *in vitro* and in cultured cells (Whitesell et al., 1994). An initial decline in kinase activity and pp60^{v-src} protein levels paralleled the rate of kinase turnover, indicating that GA induced turnover of newly synthesized pp60^{v-src}/hsp90 complexes. The importance of hsp90 in pp60^{v-src} kinase

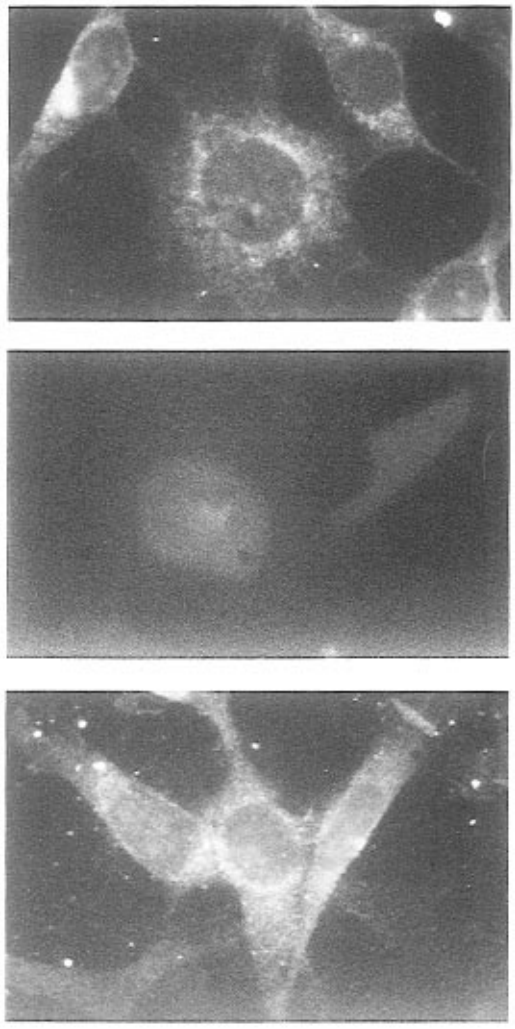

FIGURE 8: Indirect immunofluorescence micrographs of mcdc37 in NIH 3T3 cells. Cells were fixed, permeabilized, blocked with serum, and incubated with primary antibody, followed by incubation with Rhodamine LRSC-conjugated donkey anti-mouse IgG. NIH 3T3 cells were incubated with mAb C1p50 culture supernatant (upper panel), without primary antibody (middle panel), and with anti-p50 polyclonal antibody (lower panel).

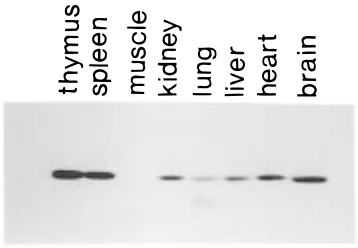

FIGURE 9: Protein blot analysis of the level of expression of mcdc37 in various tissues. Cytosolic extracts (60 μg) from the tissues listed in the figure were subjected to SDS−PAGE and transfered to an Immobilon membrane. The presence of mcdc37 was detected with a 1/400 dilution of mAbC1p50 culture supernatant and the ECL visualization method.

activity was also established in yeast by expressing pp60^{v-src} in yeast that contained a mutation that lowers hsp90 levels, which resulted in a dramatic decrease in pp60^{v-src} activity (Xu & Lindquist, 1993). This effect appears to be specific as it was not observed with pp60^{c-src} and pp160^{v-abl} tyrosine kinases expressed in the same yeast system. This study would support the hypothesis that hsp90 is required for the proper folding and perhaps stabilization of pp60^{v-src} kinase activity. The level of Raf-1 protein is reduced over 90% in cells treated with GA for 16 h in culture, thus underscoring the importance of hsp90/raf-1/p50 complexes in stabilizing raf (Schulte et al., 1995). In these studies, the role of p50 in the stabilization of the complex has not been examined. In addition, whether GA disrupts p50/hsp90 complexes also has not been determined. Whether raf is bound directly to p50, or indirectly through binding to hsp90, is not known. The role of p50 in kinase complexes has not been established, although several hypotheses have been published (Brugge, 1986). These include: (1) p50 plays a role in targeting kinases to the plasma membrane, (2) p50 is required for kinases to bind to hsp90, and (3) p50 may aid in the proper folding of certain kinases after synthesis. Results presented here will facilitate future studies to test these hypotheses and thus precisely define the role of p50 in kinase function.

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