Development and Characterization of Antisera Specific for Amino- and Carboxy-Terminal Regions of pp60^{src}

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Antisera to the Rous sarcoma virus transforming protein pp60^{src} were produced in rabbits immunized with pp60^{src} purified from recombinant *Escherichia coli*. Immunization with isolated V8 proteolytic fragments of pp60^{src} resulted in antisera specific for the amino- and carboxy-terminal domains. The reactions of these antisera with pp60^{v-src} and the normal cell protein p60^{c-src} were characterized.

Cellular transformation by Rous sarcoma virus (RSV) is mediated by expression of the viral *src* gene product (12), a 60,000-*M_r* (60K) polypeptide designated pp60^{v-src} (1), which exhibits protein kinase activity (3, 5). The sera from animals bearing RSV-induced tumors have been used extensively to study the biochemistry of pp60^{src}. Unfortunately, these sera (TBR) vary widely with respect to antibody titer and specificity and also contain antibodies to viral structural proteins (1). Recently, purified preparations of antigenic p60, prepared from bacterial recombinants containing a lac-*src* plasmid (9, 10), have been used to raise antibodies. Using the bacterially expressed p60^{src}, this laboratory produced high-titer p60-specific antiserum (αp60) (10) in immunized rabbits. This αp60 serum was instrumental in identifying a novel population of pp60^{src} molecules previously unrecognized by TBR sera (20). Immunization with bacterially expressed p60^{src} has also proved to be a successful approach for generating monoclonal antibodies (16, 18, 19). In addition, several groups have obtained antisera from animals immunized with synthetic peptides corresponding to regions of the carboxy terminus of pp60^{v-src} (8, 22, 25, 27). However, these antisera tend to be of relatively low titer toward pp60^{src} (25) and often cross-react with other cellular proteins (8, 22, 27).

Our knowledge of the structure of the pp60^{v-src} polypeptide has been advanced by the identification of several functional domains in the molecule. The region of the polypeptide which interacts with membranes appears to be localized near the amino terminus (6, 15), whereas carboxy-terminal regions are involved in kinase activity (15, 25) and interaction with the cellular proteins pp50 and pp90 (25). Tyrosine residues in the amino- and carboxy-terminal regions of the molecule can be differentially phosphorylated depending on the concentration of ATP (21). Clearly, the availability of high-titer antisera directed against different domains of pp60^{src} would facilitate the study of these reactions. In this study, we utilized the p60 expressed in *Escherichia coli* to produce antisera specific for the amino- and carboxy-terminal regions of pp60^{src}.

Lysates from bacteria containing the lac-*src* plasmid (11) were prepared as described previously (9, 11), except that 0.05% Nonidet P-40 was added after lysozyme treatment to facilitate lysis. The 16,000 × *g* pellet was washed once with 1% Nonidet P-40–0.5% sodium deoxycholate–100 mM NaCl–1 mM EDTA–10 mM Tris (pH 7.2), twice with 150 mM

NaCl–50 mM Tris (pH 7.2), and once with 10 mM Tris (pH 7.2)–5 mM MgCl₂. The washed pellet was suspended in 1.5 ml of buffer containing 10 mM Tris (pH 7.2)–5 mM MgCl₂–1 μM ATP–25 μCi of [γ-³²P]ATP, and catalytic subunit of cyclic AMP-dependent protein kinase was added to label p60^{src} for 30 min at room temperature. The reaction was quenched by the addition of 10 volumes of ice-cold 150 mM NaCl–50 mM Tris (pH 7.2)–1 mM EDTA and centrifuged at 16,000 × *g* for 30 min, and the pellet was solubilized in sample buffer (14) by sonication and boiling. After clarification at 10,000 × *g* for 5 min, the solubilized pellet was electrophoresed through a 10% polyacrylamide gel (14). The radiolabeled band corresponding to p60^{src} was excised from the wet gel, minced with a razor blade, and eluted with three changes in 1 to 2 ml each of 0.05 M NH₄HCO₃ (pH 8.5) and 0.1% sodium dodecyl sulfate over 12 h at 37°C. The eluate was filtered through glass wool, diluted with 2 volumes of 0.025 M NH₄HCO₃, pH 8.5, and dialyzed against 1 liter of 0.025 M NH₄CO₃, pH 8.5, with three to four changes over 2 days. The dialysate was lyophilized to dryness, resuspended in phosphate-buffered saline, and stored at –70°C. The amount of p60 in the preparation was quantitated by comparing Coomassie blue-stained bands with known amounts of stained bovine serum albumin.

To prepare the isolated amino- and carboxy-terminal-derived fragments, p60^{src} was suspended in 125 mM Tris (pH 6.8)–0.1% sodium dodecyl sulfate–1 mM EDTA–30% glycerol–0.005% bromophenol blue, boiled for 1 min, and treated with protease V8 from *Staphylococcus aureus* (Miles Laboratories, Elkhart, Ind.) at an enzyme/protein ratio of 250 ng of V8 to 20 μg of p60^{src} for 5 min. The mixture was electrophoresed through 10% gels. The radiolabeled amino-terminal band and the carboxy-terminal band (identified by comparison with V8 fragments from ³²P-labeled pp60^{v-src}) were excised from wet gels and processed separately as described above.

For the initial immunization, protein in phosphate-buffered saline was mixed with an equal volume of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) and an emulsion was formed in a Sorvall Omni-Mixer (DuPont, Norwalk, Conn.). Immunization was by injection into the popliteal lymph nodes of a rabbit (23), 20 μg of protein in 100 μl per lymph node. The first boost, 2 to 3 weeks later, was with an emulsion of 100 to 200 μg of protein in incomplete Freund adjuvant injected subcutaneously and intramuscularly. A second boost was administered in the same manner approximately 3 to 5 weeks later. The rabbits were

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FIG. 1. Detection of α p60-reactive epitopes by Western immunoblotting. Field vole cells transformed by the Schmidt-Ruppin D strain of RSV (1T cells) labeled with 32 P were immunoprecipitated with α p60 antiserum followed by polyacrylamide gel electrophoresis. Gel slices containing pp60^{V-src} were then subjected to partial proteolysis (2) with 0, 5, and 50 ng of *S. aureus* protease V8 during re-electrophoresis through a 10% polyacrylamide gel. The peptide fragments were then electrophoretically transferred to nitrocellulose (25) and reacted sequentially with α p60 antibody, anti-rabbit IgG-peroxidase conjugate, and finally 4-chloro-1-naphthol (a peroxidase substrate) (13). (A) Autoradiograph of nitrocellulose sheet showing transfer of pp60^{V-src} and V8 proteolytic fragments derived from the amino-terminal (34 kd, 20 kd, 18 kd) and carboxy-terminal (26 kd) halves of the molecule. (B) Colorimetrically developed nitrocellulose sheet (same as in A). Mainly fragments derived from the amino terminus of pp60^{V-src} reacted with α p60 antibody. Lane 1, 50 ng of V8; lane 2, 5 ng of V8; lane 3, untreated.

bled at intervals of 2 to 8 weeks after the second boost, during which time antibody titers peaked and remained maximal.

As reported previously (7, 10), the antisera raised against bacterially expressed p60^{src}, α p60 sera, recognized pp60^{V-src} from a variety of RSV strains. To further characterize the specificity of the α p60 antibody, the antigenic sites recognized on pp60^{V-src} were probed by Western immunoblotting analysis (13, 26). Mild protease V8 treatment cleaves pp60^{V-src} into two fragments, a 34K M_r amino-terminal portion and a 26K M_r fragment derived from the carboxy terminus (4). Electrophoretic transfer of V8-proteolyzed p60 revealed that, although both amino- and carboxy-terminal fragments were transferred to nitrocellulose, only the 34K amino-terminal domain was efficiently recognized by α p60 antibody (Fig. 1). A faint reaction with the carboxy-terminal fragment was sometimes evident (see Fig. 2). More extensive proteolysis further cleaves the 34K fragment into 18K and 20K fragments, both of which retain the amino terminus of pp60^{V-src} (4) and are immunoreactive toward α p60 antibody (Fig. 1B, lane 1). Based on the putative location of the V8 cleavage sites in the pp60^{V-src} sequence (24), the data presented in Fig. 1 imply that the predominant antigenic determinants which react with α p60 are localized to the first 160 amino acids of pp60^{V-src}. The same results were obtained with sera from three different rabbits immunized with p60. It is interesting to note that several monoclonal antibodies also seem to be directed against sequences in the amino-terminal region (19).

We next used purified proteolytic fragments of the bacterially expressed p60 to produce antisera specific for different

regions of the polypeptide. Immunization with the isolated amino-terminal-derived V8 proteolytic fragment generated antiserum (α N) which immunoprecipitated intact p60 and the 34K fragment but not the carboxy-terminal fragment (Fig. 2, lanes 2, 5, 8). Moreover, the α N serum also recognized the 18K and 20K amino-terminal fragments on Western immunoblots (data not shown). Conversely, the antiserum directed against the carboxy-terminal 26K fragment (α C) recognized p60 and the 26K peptide, but not the fragment derived from the amino terminus (Fig. 2, lanes 3, 6, 9).

Since the α N and α C antisera were clearly specific for different domains of the pp60^{V-src} molecule, it was of interest to determine if p60 kinase activity was affected differentially by these antibodies. When immune complexes of pp60^{V-src} immunoprecipitated with α p60 serum were incubated with [γ - 32 P]ATP, a phosphopeptide of molecular weight 60,000 was observed. This 60-kilodalton (kd) band was identified as autophosphorylated pp60^{V-src}, since it was absent in phosphotransfer reactions when nonimmune complexes were used (Fig. 3A). Moreover, in the absence of added carrier ATP, phosphorylation occurred almost exclusively in the carboxy-terminal half of the pp60^{V-src} molecule (Fig. 3B). The apparent autophosphorylation of pp60^{V-src} also occurred in immunoprecipitates formed with α N serum, but not α C serum (Table 1). Furthermore, immune complexes of pp60^{V-src} bound to α p60 and α N, but not α C, antibodies

FIG. 2. Specificity of antibodies for various regions of pp60^{V-src}. 1T cells were labeled with 32 P, lysed, and immunoprecipitated with α p60 serum as described previously (1, 20). After the addition of 10 ng of V8 protease, the samples were electrophoresed through 10% polyacrylamide gels (14). The radiolabeled bands corresponding to intact p60, the 34K and 26K fragments, were excised from the wet gel and eluted as described in the text. Each eluate was adjusted to 150 mM NaCl-10 mM Tris (pH 7.4)-1% sodium deoxycholate-1% Triton X-100-0.1% sodium dodecyl sulfate, and 5 μ l of α p60 (lanes 1, 4, 7), α N (lanes 2, 5, 8), or α C (lanes 3, 6, 9) antibody was added to 1 ml containing intact p60 (lanes 1 to 3), the amino-terminal 34K fragment (lanes 4 to 6), or the carboxy-terminal 26K fragment (lanes 7 to 9). Each sample was immunoprecipitated and analyzed by polyacrylamide gel electrophoresis on a 10% gel. Based on these data, we estimate that 10 to 15% of the total antibody population in the α p60 serum is directed against the carboxy-terminal domain.

could support phosphorylation of exogenous substrates such as casein and TBR immunoglobulin G (IgG) (Table 1). However, neither α p60, α N, nor α C IgGs inhibited the phosphorylation of TBR IgG or casein by purified p60^{src} in a soluble system (data not shown).

We have also examined the ability of these antisera to recognize the normal cell protein pp60^{c-src}. The data presented in Table 1 indicate that only α p60 and α N sera, but not α C serum, immunoprecipitated pp60^{c-src} and its associated kinase activity from normal uninfected chicken embryo fibroblasts. Since the major differences between the v-src and c-src polypeptides reside in the carboxy-terminal 20 amino acids (24), it is likely that the α C serum recognizes determinants unique to pp60^{v-src} in this region. However, an additional antigenic region must be involved in α C interaction with pp60^{v-src}, since antibodies directed against only the carboxy-terminal seven amino acids of pp60^{v-src} do not inhibit kinase activity (8, 22). It is therefore probable that α C antibodies recognize a tertiary structure determinant which contains sequences unique to v-src as well as sequences involved in the function of the kinase domain. The inability to observe measurable immunoreactivity of α C serum with the carboxy-terminal 26K fragment on Western blots supports this notion (data not shown).

The expression of p60^{src} by recombinant bacteria has provided an excellent large-scale source of antigen for producing high-titer antisera which recognize various domains of pp60^{v-src} and pp60^{c-src}. The α N and α C antibodies have already been used to identify membrane-bound and

TABLE 1. Summary of the properties of antibodies to pp60^{src}

Reaction ^a	Antibody ^b		
	α p60	α N	α C
Immunoprecipitation of pp60 ^{v-src}	+	+	+
Immunoprecipitation of N-terminal 34K peptide	+	+	—
Immunoprecipitation of C-terminal 26K peptide	(+)	—	+
Phosphorylation of pp60 ^{v-src} in immune complex	+	+	—
Phosphorylation of casein in immune complex	+	+	—
Phosphorylation of TBR IgG in immune complex	+	+	—
Immunoprecipitation of pp60 ^{c-src}	+	+	—
Phosphorylation of pp60 ^{c-src} in immune complex	+	+	—

^a Immunoprecipitation of pp60^{v-src} from transformed vole cells (1T) was performed as described previously (1, 20). Immunoprecipitation of the amino- and carboxy-terminal fragments was performed as described in the legend to Fig. 2. Immunoprecipitation of pp60^{c-src} was performed as described before (20) from normal chicken embryo fibroblasts. In vitro phosphorylation reactions were as previously described (16).

^b +, Positive reaction; —, no reaction; (+), partial reaction.

cytoplasmic domains of pp60^{v-src} (Resh and Erikson, manuscript in preparation), and we anticipate that they will be of value in understanding the structure and function of normal and viral pp60^{src} proteins.

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FIG. 3. Phosphorylation of pp60^{v-src} bound in an immune complex with α p60 antibody. 1T cells were lysed in 1% Nonidet P-40–0.5% sodium deoxycholate–100 mM NaCl–1 mM EDTA–10 mM Tris (pH 7.2) and clarified at 40,000 $\times$ g for 30 min. Antibody and *S. aureus* were added, and the immune complexes were washed as previously described (5, 20). The kinase reaction was initiated by the addition of 50 μ l of 10 mM Tris (pH 7.2)–5 mM MgCl₂–20 μ Ci of [γ -³²P]ATP for 15 min at room temperature. (A) The reaction products were analyzed by electrophoresis through 10% polyacrylamide gels and autoradiography. Lane 1, Nonimmune rabbit serum; lane 2, α p60 serum. (B) The 60-kd region of the gel (lane 2 in A) was excised and subjected to partial proteolysis during re-electrophoresis with 5 (lane 1) or 50 (lane 2) ng of *S. aureus* protease V8. The positions of proteolytic fragments derived from the amino (N34)- and carboxy (C26)-terminal halves of the molecule are marked in the margin.

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