Inhibition of the Tyrosine Kinase Activity of *v-src*, *v-fgr*, and *v-yes* Gene Products by a Monoclonal Antibody Which Binds Both Amino and Carboxy Peptide Fragments of pp60^{v-src}

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A monoclonal antibody, R2D2, raised to the *src* gene product of Rous sarcoma virus was found to inhibit the tyrosine protein kinase activity of pp60^{v-src} in autophosphorylation reactions and in reactions involving exogenously added substrates, such as casein and histone. R2D2 also inhibited the enzymatic activity of two related viral transforming proteins, pp70^{gag-fgr} and pp90^{gag-yes}. The inhibitory ability of R2D2 was dependent upon immunoglobulin concentration and could be demonstrated in both immune complexes formed directly with R2D2 and preformed immune complexes to which R2D2 was added. Binding sites in both the amino-terminal 110 amino acid residues and the carboxy-terminal 240 amino acids of pp60^{v-src} were identified for R2D2. These results indicate that at least part of the epitope recognized by R2D2 resides within a region of the *src* protein which is required for protein kinase activity. The localization of the R2D2 epitope to the amino- as well as to the carboxy-terminal portions of pp60^{v-src}, together with results of studies analyzing the relative binding efficiencies of R2D2 to the intact protein and to V-8 proteolytic fragments of pp60^{v-src}, are consistent with the view that the R2D2 epitope is conformational in nature and that it is assembled from residues contained within both N-terminal and C-terminal regions of the molecule.

The ability of Rous sarcoma virus (RSV) to transform cells in culture or to induce tumors in animals is dependent upon the functional expression of its transforming gene, *v-src* (5, 28). The protein encoded by this gene is a phosphoprotein of 60 kilodaltons (pp60^{v-src}) which exhibits a tyrosine-specific phosphotransferase activity (9, 16, 31, 35). The carboxy-terminal 30-kilodalton proteolytic fragment of pp60^{v-src} contains the catalytic activity (8, 34) and shares amino acid sequence homology with the purported catalytic domains of other members of the tyrosine protein kinase family, including the transforming proteins *v-yes* (33), *v-abl* (51), *v-fes/v-fps* (27, 54), *v-fgr* (38), *v-ros* (40), and *v-fms* (26), encoded by several oncogenic retroviruses, certain growth factor receptors (59, 60), and the catalytic subunit of cyclic AMP-dependent kinase (2). In contrast to the homologies between the catalytic regions of these proteins, amino acid sequences outside of the catalytic domains diverge significantly. It has been suggested that these nonhomologous sequences play a role in substrate specificity or in the modulation of the activity of the kinase domain.

Many biochemical data relating to pp60^{v-src} structure and function have accumulated over the past several years. Two major sites of phosphorylation in pp60^{v-src} have been identified, i.e., Ser-17 and Tyr-416 (15, 20, 48, 55, 58). Tyr-416 is the major site of *in vitro* autophosphorylation (49), but the conditions under which Ser-17 is phosphorylated are not well defined. Although the presence of Tyr-416 is not absolutely required for transformation (20, 56), the presence of four other residues, i.e., Ala-430, Pro-431, Glu-432, and Ala-433 (in close proximity to Tyr-416), has been shown to be important for kinase activity and obligatory for transformation (10). Additionally, directed mutagenesis of selected amino-terminal sequences has shown that specific sites in this region are required for myristylation, ATP binding, membrane association, and morphological transformation

(19, 32, 41, 42, 57). Furthermore, Collett et al. (14, 17) and Purchio et al. (50) have provided evidence which suggests that purified pp60^{v-src} specific tyrosine protein kinase activity can be increased by additional amino-terminal phosphorylations. Proteolytic removal of the amino terminus from the catalytic domain of pp60^{v-src} results in an increase in the specific activity of the protein compared with that of the holoenzyme (8). Thus, it appears that regions of the amino-terminal domain of pp60^{v-src} may influence the activity of the catalytic domain, and this influence may be mediated by covalent modifications of sites within the amino terminus.

In this report, we describe a monoclonal antibody (MAb), R2D2, which was found to inhibit the tyrosine kinase activity of pp60^{v-src}, pp70^{v-fgr}, and pp90^{v-yes} both in immune complexes and in solutions containing preformed complexes. Evidence is presented showing that the epitope recognized by R2D2 is contained not only on a carboxy-terminal peptide of pp60^{v-src} corresponding to a portion of the catalytic domain shared by the three proteins but also on a peptide containing the amino-terminal 110 amino acids of pp60^{v-src}. These results, together with those of relative binding efficiency studies, are discussed in the context of the nature of the epitope proposed for R2D2 and the potential regulatory mechanisms for pp60^{v-src} kinase activity.

MATERIALS AND METHODS

Cells, viruses, and plasmids. The cells used in these experiments were chicken embryo cells (CE cells), transformed by the Prague A strain of RSV (PrA-RSV) or by the Yamaguchi-73 strain of avian sarcoma virus (Y73-ASV), and mink fibroblasts, transformed by the Gardner-Rasheed strain of feline sarcoma virus (GR-FeSV). The cells were maintained in culture as previously described (46). The bacterial plasmid pS+M, which encodes the fusion protein *src-myc-β-galactosidase*, containing the first 110 amino acids of pp60^{v-src}, was constructed and propagated as outlined by Morgan et al. (37).

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Immunoglobulin and antisera. The derivation and partial characterization of R2D2 and other pp60^{v-src}-specific MABs have been described previously (43, 44). R2D2 is of the mouse immunoglobulin G3 (IgG3) subclass and was obtained from a fusion of immune BALB/c lymph node cells with the mouse myeloma NS.1. R2D2 IgG3 is characterized by a homogenous heavy chain and two distinct light chains when analyzed by gel electrophoresis and Coomassie blue staining. One light chain is R2D2 specific, and the other comigrates with that secreted by the fusion partner, NS.1. As indicated in the figure legends, either culture supernatants or R2D2 immunoglobulin purified from mouse ascites fluid by ammonium sulfate precipitation (alone or by ammonium sulfate precipitation followed by chromatography on protein A-Sepharose [21]) was used as a source of antibody. Rabbit anti-bp60^{src} and rabbit anti-myc sera were raised against gel-purified proteins expressed in bacteria (25, 37, 44). Goat anti-feline leukemia virus (FeLV) antiserum, containing antibodies specific for the feline retrovirus structural proteins and the FeSV *gag* moiety of pp70^{v-gag-fgr}, was kindly provided by Keith Robbins (National Institutes of Health, Bethesda, Md.). Rabbit anti-RSV serum was raised against the Schmidt-Ruppin-D strain of RSV and contained antibodies with specificities for the avian retrovirus structural proteins, as well as the *gag* portion of pp90^{v-gag-yes}.

Radiolabeling of cultured cells and immunoprecipitation. Cells in culture were labeled for 2 or 3 h in phosphate-free medium 199 (GIBCO Laboratories, Grand Island, N.Y.) containing 5% dialyzed calf serum and 500 μ Ci of carrier-free ³²P_i per ml (ICN Pharmaceuticals Inc., Irvine, Calif.) or for 6 h in methionine-free Dulbecco modified Eagle medium containing 5% dialyzed fetal calf serum and 150 μ Ci of [³⁵S]methionine per ml (800 to 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). Extracts were prepared in RIPA buffer, and aliquots were immunoprecipitated as previously described (44). Immune complexes were collected on formaldehyde-fixed, heat-inactivated *Staphylococcus aureus* Cowan I bacteria; washed; and fractionated on sodium dodecyl sulfate (SDS)-9% polyacrylamide gels. Gels containing [³⁵S]methionine-labeled proteins were fluorographed, dried, and exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.) at -70°C. Gels containing ³²P_i-labeled proteins were dried and autoradiographed at -70°C with X-Omat film by using intensifying screens (Cronex Lightning-Plus, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

Immune complex protein kinase assay. To measure protein kinase activity, cells were lysed in RIPA buffer lacking SDS, and extracts were immunoprecipitated as described above. Immune complexes were collected, washed in RIPA buffer lacking SDS and then in phosphate-buffered saline (PBS) (10 mM sodium phosphate [pH 7.2], 0.15 M NaCl), suspended in 30 μ l of kinase buffer (20 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 7.2], 10 mM MnCl₂, 10 μ Ci of [γ -³²P]ATP [6,000 Ci/mmol; ICN]), and incubated at 22°C for 30 min. Where indicated, a second antibody was added to the washed immune complexes in a total volume of 10 μ l and incubated for 30 min at 4°C before addition of the kinase buffer. Exogenous substrates were added immediately before the addition of the kinase buffer. The reactions were stopped by the addition of 15 μ l of three-times-concentrated sample buffer. Labeled samples were heated for 5 min at 100°C and subjected to electrophoresis on SDS-9% polyacrylamide gels, and the phosphorylated proteins were visualized by autoradiography. The amount of ³²P_i incorporated was determined by densitometric scanning of autoradio-

graphs or by spectrophotofluorometric analysis of eluted gel slices. To test the ability of specific peptides to reverse the effects of R2D2, peptides solubilized in PBS were incubated for 2 h at 4°C with 300 μ l of R2D2 culture supernatant in a total volume of 350 μ l. The peptide-immunoglobulin solution was then added to a preformed, washed immune complex for an additional 30 min. The entire mixture was subsequently pelleted, washed one time in PBS, and assayed for kinase activity. Peptides 7 to 15, 152 to 162, and 498 to 512, corresponding to sequences in Prague strain C RSV-encoded pp60^{v-src}, were generously provided by L. Gentry.

RESULTS

Cross-reactivity of pp60^{v-src} MAB, R2D2, with pp70^{v-fgr} and pp90^{v-yes}. Detergent extracts of ³²P_i-labeled PrA-RSV-transformed CE cells, GR-FeSV-transformed mink fibroblasts, and Y73-ASV-transformed CE cells were prepared as described in Materials and Methods, and aliquots were immunoprecipitated with R2D2 MAB or with control antisera specific for each of the transforming proteins. The MAB R2D2 immunoprecipitated proteins which comigrated with pp70^{v-fgr} (Fig. 1A, lane 7) and pp90^{v-yes} (lane 11), as well as with pp60^{v-src} (lane 3), from extracts of the appropriate transformed cell lines, but not from extracts of uninfected control cells. The efficiency of immunoprecipitation of pp70^{v-fgr} and pp90^{v-yes} was significantly less than that of pp60^{v-src} and could not be improved by increasing the concentration of antibody. R2D2 did not immunoprecipitate pp130^{v-fps} from Fujinami avian sarcoma virus-transformed rat cells, pp120^{v-abl} from Abelson murine leukemia virus-transformed NIH 3T3 cells, pp68^{v-ros} from UR-2 avian sarcoma virus-transformed CE cells, or pp60^{c-src} from cultured avian, rodent, or human fibroblasts (data not shown). R2D2 was unique among our panel of pp60^{v-src}-specific MABs in its ability to reproducibly immunoprecipitate two closely related viral tyrosine protein kinases other than pp60^{v-src}, i.e., pp70^{v-fgr} and pp90^{v-yes}.

V-8 protease peptide analysis of pp70^{v-fgr} and pp90^{v-yes} immunoprecipitated by R2D2. To confirm the identity of the proteins immunoprecipitated by MAB R2D2 as pp70^{v-fgr} and pp90^{v-yes}, the proteins were labeled with [³⁵S]methionine and subjected to *S. aureus* V-8 protease partial proteolytic peptide analysis by the method of Cleveland et al. (12). The peptide profiles of radiolabeled pp70^{v-fgr} and pp90^{v-yes}, as immunoprecipitated by R2D2, were compared with those immunoprecipitated by polyclonal antisera directed towards the *gag* portion of each of the transforming proteins. R2D2 immunoprecipitated proteins with peptide profiles identical to those immunoprecipitated by antisera with specificities for the *gag* moieties of pp70^{v-fgr} and pp90^{v-yes} (Fig. 2). The specificity of R2D2 for pp60^{v-src} had previously been verified (43). In addition, phosphoamino acid analysis of pp60^{v-src}, pp70^{v-fgr}, and pp90^{v-yes}, immunoprecipitated by R2D2 from extracts of ³²P_i-labeled cells, revealed the presence of phosphoserine and phosphotyrosine (data not shown). These data further support the conclusion that the MAB R2D2 cross-reacted with pp70^{v-fgr} and pp90^{v-yes} and not with cellular proteins of identical molecular weight.

Inhibition of pp60^{v-src}, pp70^{v-fgr}, and pp90^{v-yes} autophosphorylations by R2D2 culture supernatant. To determine whether the kinase activity of the transforming proteins could be detected in complex with the MAB, immune precipitates containing v-*src*, v-*fgr*, and v-*yes* proteins were incubated in kinase buffer containing [γ -³²P]ATP as outlined in Materials and Methods. Autokinase activity was not

detected in R2D2 immune complexes formed with pp60^{v-src}, pp70^{v-fgr}, or pp90^{v-yes} (Fig. 1B, lanes 3, 7, and 11, respectively), whereas immune complexes formed with polyclonal antibodies exhibited strong autokinase activity (lanes 1, 5, and 9). (The heavy chain of monoclonal immunoglobulin did not serve as substrate in these reactions.) It appeared, therefore, that binding of MAb R2D2 to pp60^{v-src}, pp70^{v-fgr}, and pp90^{v-yes} inhibited the autophosphorylation activities of these proteins. R2D2 was the only MAb in our panel of pp60^{v-src}-specific MABs which exhibited this inhibitory activity (see below).

Dependence on R2D2 for inhibition of pp60^{v-src} autokinase

FIG. 1. Immunoprecipitation of *v-src*, *v-fgr*, and *v-yes* gene products and inhibition of their tyrosine autophosphorylation activities by the MAb R2D2. (A) PrA-RSV-infected CE cells (lanes 1 to 4), GR-FeSV-infected mink fibroblasts (lanes 5 to 8), and Y73-ASV-transformed CE cells (lanes 9 to 12) were labeled for 2 h with ³²P_i, as described in the text. Extracts were prepared, and aliquots representing approximately 2×10^6 cells were immunoprecipitated with polyclonal antibody or MAb and analyzed by SDS-9% polyacrylamide gel electrophoresis and autoradiography. (B) Extracts of unlabeled PrA-RSV-transformed CE cells (lanes 1 to 4), GR-FeSV-transformed mink fibroblasts (lanes 5 to 8) and Y73-ASV-transformed CE cells (lanes 9 to 12) were prepared as described in Materials and Methods and immunoprecipitated with specific antibody. Immune complex kinase activity was determined as described in Materials and Methods. Lanes: 1, 3 μ l of rabbit anti-pp60^{v-src} serum; 2 and 10, 3 μ l of NRS; 3, 7, and 11, 500 μ l of culture supernatant of MAb R2D2; 4, 8, and 12, 500 μ l of culture supernatant of NS.1; 5, 5 μ l of goat anti-FeLV *gag* serum; 6, 5 μ l of normal goat serum; 9, 1 μ l of rabbit anti-RSV *gag* serum. Molecular weights of labeled proteins were determined relative to the migration of known molecular weight standards (200,000, myosin; 116,000, β -galactosidase; 92,000, phosphorylase *a*; 66,000, bovine serum albumin; 55,000, human immunoglobulin heavy chain; and 45,000, ovalbumin). Degradation products of pp70^{v-fgr} (confirmed by partial protease mapping) were observed in panel B, lane 5.

activity. To test the possibility that the inhibiting agent in the R2D2 antibody preparations was a factor other than immunoglobulin, R2D2 culture supernatant was cleared of immunoglobulin by repeated incubations with *S. aureus*, and the resultant supernatant was tested for its ability to inhibit pp60^{v-src} activity. pp60^{v-src} was immunoprecipitated from PrA-RSV CE cell extracts by a MAb (EB7) which recognized an epitope distinct from that recognized by R2D2 and which supported the kinase activity of pp60^{v-src} in complex (44, 45). The EB7-pp60^{v-src} immune complexes were collected on *S. aureus* bacteria, washed extensively, and incubated with either adsorbed R2D2 supernatant, unadsorbed R2D2 supernatant, R2D2 immunoglobulin purified by chromatography on protein A-Sepharose, or culture supernatant containing a third MAb (EC10). Protein kinase activity was measured as described above. R2D2 culture supernatant, adsorbed with *S. aureus* to remove immunoglobulin, did not inhibit pp60^{v-src} kinase activity in immune complex with MAb EB7 (Fig. 3, lane 3), whereas an equal volume of unadsorbed R2D2 supernatant (lane 4), added to pp60^{v-src} in immune complex, virtually abolished all protein kinase activity. Addition of purified R2D2 immunoglobulin also inhibited kinase activity (Fig. 3, lane 5). In contrast, culture supernatant from MAb EC10 did not inhibit kinase activity either in direct complex with pp60^{v-src} (Fig. 3, lane 6) or added to a preformed complex containing MAb EB7 (lane 7). Similar results were obtained with yet another pp60^{v-src}-specific MAB, FG1, which binds an epitope different from those recognized by either R2D2 or EB7 (44, 45). The inhibition of pp60^{v-src} activity by R2D2 was dependent upon the amount of R2D2 added to a preformed complex (Fig. 3, lanes 8 to 12; Fig. 4B) and was maximal when R2D2 was the primary precipitating antibody (Fig. 4A, sample number 2). Preparations of R2D2 were found to contain no ATPase or protein phosphatase activities when added to prephosphorylated immunoprecipitates (data not shown). Immunoaffinity-purified pp60^{v-src} autophosphorylation activity was also inhibited by R2D2 culture supernatant or R2D2 purified immunoglobulin in the absence of *S. aureus* (data not shown), indicating that immunoglobulin preparations, not *S. aureus* bacteria or some component nonspecifically adsorbed to it, were responsible for the observed inhibition.

Incubation of adsorbed and unadsorbed R2D2 culture supernatants with preformed immune complexes containing either pp70^{v-fgr} plus goat anti-FeLV antibodies or pp90^{v-yes} plus rabbit anti-RSV antibodies yielded a similar pattern of inhibition of kinase activity (data not shown). Five- to ten-fold-greater amounts of immunoglobulin were required to inhibit pp70^{v-fgr} and pp90^{v-yes} activity in a preformed immune complex than the amount required to inhibit pp60^{v-src} under the same conditions. This may be a reflection of the apparently weaker affinity of R2D2 for pp70^{v-fgr} and pp90^{v-yes} (Fig. 1A). Taken together, these data indicate that the R2D2 immunoglobulin recognizes an antigenic determinant present on three different viral tyrosine protein kinases and that it functions to inhibit the autophosphorylation activity of those proteins.

Inhibition of transphosphorylation by R2D2. To test the ability of R2D2 antibody to inhibit phosphorylation of exogenously added substrates, immune complexes were prepared with pp60^{v-src} and with R2D2, EB7, or control MABs and were subsequently incubated with casein or histone in kinase reaction mixtures (Fig. 5A, B, and D, respectively). In one experiment, R2D2 was added to EB7-immune complexes and incubated for 30 min at 4°C before

FIG. 2. V-8 proteolytic digests of pp70^{v-fgr} and pp90^{v-yes} immunoprecipitated by gag- or src-specific antisera. (A) Mink fibroblasts transformed by GR-FeSV were labeled with [³⁵S]methionine, and extracts were prepared and immunoprecipitated with either goat anti-FeLV gag sera or R2D2 MAb. ³⁵S-labeled pp70^{v-fgr} was resolved by electrophoresis on SDS-9% polyacrylamide gels, localized by autoradiography, and excised from the gels for a second cycle of electrophoresis in the presence of *S. aureus* V-8 protease (Miles Laboratory, Naperville, Ill.) as described by Cleveland et al. (12). Lanes: 1 and 2, no V-8 protease; 3 and 4, 25 ng of V-8 protease; 5 and 6, 100 ng of V-8 protease. Lanes 1, 3, and 5, goat anti-FeLV gag sera; lanes 2, 4, and 6, R2D2 MAb. (B) CE cells infected with Y73-ASV were labeled with ³²P_i and processed as described in panel A. Lanes: 1 and 2, no V-8 protease; 3 and 4, 5 ng of V-8 protease; 5 and 6, 25 ng of V-8 protease. Lanes 1, 3, and 5, rabbit anti-RSV gag sera; lanes 2, 4, and 6, R2D2 MAb. Molecular weights were determined as described in the legend to Fig. 1, with the addition of carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,400).

FIG. 3. Dependence on R2D2 immunoglobulin for inhibition of pp60^{v-src} kinase activity. Extracts of PrA-RSV-transformed CE cells were prepared, and aliquots representing approximately 10⁶ cells were immunoprecipitated with pp60^{v-src}-specific MABs as indicated below. The immune complex kinase activity was determined in the presence or absence of R2D2 immunoglobulin. Lanes: 1, 15 μ l of culture supernatant of MAB EB7; 2, 500 μ l of R2D2 culture supernatant; 3, 15 μ l of EB7 plus 500 μ l of R2D2 supernatant adsorbed with *S. aureus* (see text); 4, 15 μ l of EB7 plus 500 μ l of R2D2; 5, 15 μ l of EB7 plus 5 μ g of purified R2D2 immunoglobulin; 6, 20 μ l of MAB EC10; 7, 15 μ l of EB7 plus 500 μ l of EC10; 8, 15 μ l of EB7 plus 25 μ l of R2D2; 9, EB7 plus 50 μ l of R2D2; 10, EB7 plus 100 μ l of R2D2; 11, EB7 plus 200 μ l of R2D2; 12, EB7 plus 400 μ l of R2D2. In each case, the amount of MAB used was previously determined to quantitatively immunoprecipitate pp60^{v-src} from approximately 10⁶ PrA-RSV-transformed CE cells which had been labeled with [³⁵S]methionine.

the addition of casein or histone (Fig. 5C). In each case, R2D2 antibody inhibited both autophosphorylation and phosphorylation of casein or histone compared with immune complexes prepared with EB7 antibody alone.

Mapping of the R2D2 epitope by using bacterial and viral fusion proteins. Preliminary mapping data (43, 44) localized the epitope recognized by MAB R2D2 to the N-terminal one-third of the v-src molecule. To verify this assignment, MAB R2D2 was used to immunoprecipitate a bacterially expressed src-myc- β -galactosidase (src-myc- β -Gal) fusion protein (p145) containing the first 110 amino acids of pp60^{v-src} (37). Six MABs, including R2D2, immunoprecipitated p145^{src-myc- β -Gal} from extracts of [³⁵S]methionine-labeled *Escherichia coli* containing the plasmid pS+M (Fig. 6A).

FIG. 4. Quantitation of inhibition of pp60^{v-src} autokinase activity by MAB R2D2. (A) The level of pp60^{v-src} autophosphorylation in the presence and absence of R2D2 was measured by densitometric analysis of the 60-kilodalton bands depicted in Fig. 3. Relative activities were determined by comparing the amount of ³²P_i incorporation of a given sample (in arbitrary densitometry units) with that found in an EB7-pp60^{v-src}-*S. aureus* complex. Sample numbers 1 to 6 correspond to lanes 1 to 6 in Fig. 3. (B) Various amounts of R2D2 IgG were added to preformed complexes containing either MAB EB7 (●) or FG1 (×) and the relative activities of pp60^{v-src} were determined as described in the legend to panel A.

FIG. 5. Inhibition of casein and histone phosphorylation by MAb R2D2. Aliquots of extracts of PrA-RSV-transformed CE cells were immunoprecipitated with pp60^{v-src}-specific MAb R2D2 (lanes 1 to 3), EB7 (lanes 4 to 9), or NS.1 culture supernatants (lanes 10 to 12). Immune complexes were incubated with [γ -³²P]ATP and either 5 μ g of casein (lanes 2, 5, 8, and 11) or 5 μ g of histone (lanes 3, 6, 9, and 12) or were incubated without added substrates (lanes 1, 4, 7, and 10). Lanes 7 to 9, EB7 immune complexes incubated with R2D2 culture supernatant before addition of casein or histone.

Rabbit anti-bp60^{v-src} and rabbit anti- β -galactosidase also precipitated a protein of identical molecular weight, whereas MAbs GD11 and EB8 and control antibodies (anti-bovine serum albumin MAb and normal rabbit serum [NRS]) failed to do so. p145^{src-myc- β -Gal} was not precipitated from extracts of *E. coli* lacking the plasmid (Fig. 6B). That the specificity of R2D2 for p145^{src-myc- β -Gal} resided in the *src* sequences and not in the *myc* or β -galactosidase sequences was supported by the observations that R2D2, as well as EB7 (a MAb which binds an epitope different from that recognized by R2D2), did not immunoprecipitate β -galactosidase from wild-type *E.*

coli (Fig. 6B) or p110^{gag-myc} from MC29-infected quail cells (data not shown). In addition, MAbs GD11 and EB8, the epitopes of which have been mapped to that region of pp60^{v-src} bordered by residues 92 to 128 (45), did not immunoprecipitate p145^{src-myc- β -Gal}. Thus, we concluded that R2D2 binds amino acids of pp60^{v-src} mapping within the first 110 residues of the molecule. Interestingly, comparison of the sequences of pp70^{v-fgr} and pp90^{v-yes} with that of pp60^{v-src} revealed limited sequence homology with the N-terminal 110 residues of pp60^{v-src}. As noted previously (33, 38), pp70^{v-fgr} and pp90^{v-yes} displayed a high degree of homology with the C-terminal protein kinase domain of pp60^{v-src}.

Since the inhibitory properties of R2D2 suggested an involvement of C-terminal sequences in epitope recognition, we tested the ability of R2D2 to immunoprecipitate a p130^{gag-myc-src} fusion protein encoded by a genetically engineered viral plasmid vector, pMC29 Δ Bal1 (M. Heaney and J. T. Parsons, personal communication). The 130-kilodalton protein contained the C-terminal 286 amino acids of pp60^{v-src} fused to a prematurely truncated form of the MC29-encoded p110^{gag-myc} (Fig. 7A). MAb R2D2 (Fig. 7C, lane 4), but not MAb EC10, FG1, or EB7 (lanes 1 to 3), immunoprecipitated p130^{gag-myc-src}, as did the control sera, rabbit anti-RSV *gag* (lane 7), rabbit anti-*myc* (lane 8), and, to a lesser extent, rabbit anti-bp60^{v-src} (lane 5), from extracts of [³⁵S]methionine-labeled Cos-1 cells expressing pMC29 Δ Bal1. That the immunoprecipitation of p130^{gag-myc-src} by R2D2 was due to its specificity for the pp60^{v-src} sequences in p130^{gag-myc-src} and not to cross-reactivity with the *gag* or *myc* sequence was shown by the inability of R2D2 to immunoprecipitate authentic MC29-encoded p110^{gag-myc} expressed in Cos-1 cells transfected with the plasmid pMC29 (29) (Fig. 7B and D). In addition, neither p130^{gag-myc-src} nor p110^{gag-myc} was immunoprecipitated by R2D2 from extracts of mock-transfected Cos-1 cells (Fig. 7E). These data suggest that MAb R2D2 recognizes sequences which reside not only within the N-terminal 110 amino acids but also within the carboxy-terminal 240 residues of pp60^{v-src}, a region which shares extensive homology with pp70^{v-fgr} and pp90^{v-yes}.

Relative binding of R2D2 to pp60^{v-src} and its N-terminal and

FIG. 6. Mapping of the R2D2 determinant to the amino-terminal 110 amino acids of pp60^{v-src}. pp60^{v-src}-specific MAbs were tested for their ability to immunoprecipitate a *src-myc- β -galactosidase* fusion protein containing the first 110 amino acids of pp60^{v-src}. (A) *E. coli* D2015 cells containing the plasmid pS+M, which encodes the fusion protein, were labeled with 75 μ Ci of [³⁵S]methionine per ml of sulfate-depleted M9 media for 5 min in the presence of 1 mM isopropyl- β -D-thiogalactopyranoside. Detergent extracts were immunoprecipitated with MAbs or control polyclonal antisera. Lanes: 1, 3 μ l of GD11 ascites fluid; 2, 5 μ g of purified EB8 immunoglobulin; 3, 15 μ g of R2D2; 4, 2 μ g of EB7; 5, 10 μ g of GB6; 6, 15 μ g of GE7; 7, 30 μ g of EC10; 8, 5 μ l of rabbit anti-bp60^{v-src}; 9, 5 μ l of NRS; 10, 15 μ g of anti-bovine serum albumin MAb; 11, 3 μ l of rabbit anti- β -galactosidase sera; 12, 15 μ g of FG1. (B) Detergent extracts of [³⁵S]methionine-labeled *E. coli* D2015 cells, lacking the plasmid pS+M, were prepared, and aliquots were immunoprecipitated with representative MAbs and polyclonal antibodies as described in the legend to panel A. Lanes: 1, GD11; 2, R2D2; 3, EB7; 4, NRS; 5, anti-bovine serum albumin MAb; 6, rabbit anti- β -galactosidase serum. p147 was an *E. coli* protein found in cells containing or lacking a plasmid and was immunoprecipitated only by anti- β -galactosidase antiserum.

FIG. 7. Immunoprecipitation of p130^{gag-myc-src} by Mab R2D2. (A and B) Schematic diagrams of retroviral coding sequences contained in pMC29ΔBal1 and pMC29, respectively. (C) Extracts of pMC29ΔBal1-transfected, [³⁵S]methionine-labeled Cos-1 cells (29) immunoprecipitated with (lanes): 1, EC10 MAb; 2, FG1 MAb; 3, EB7 MAb; 4, R2D2 MAb; 5, rabbit anti-bp60^{src} serum; 6, NS.1 mouse myeloma culture fluid; 7, rabbit anti-RSV *gag* serum; 8, rabbit anti-myc serum; 9, NRS. (D) Extracts of pMC29-transfected, [³⁵S]methionine-labeled Cos-1 cells immunoprecipitated with (lanes): 10, rabbit anti-RSV *gag* sera; 11, NRS; 12, R2D2 MAb; 13, NS.1. (E) Extracts of mock-transfected, [³⁵S]methionine-labeled Cos-1 cells immunoprecipitated with (lanes): 14, rabbit anti-RSV *gag* sera; 15, NRS; 16, R2D2 MAb; 17, NS.1.

C-terminal V-8 peptides in an ELISA. To further elucidate the nature of the interactions between R2D2 and the N-terminal and C-terminal structural elements of pp60^{v-src}, two additional experiments were carried out. The first experiment was designed to assess the relative binding efficiencies of R2D2 to the purified, intact molecule and to the amino and carboxy V-8 fragments in an attempt to distinguish between duplicative and conformational (or assembled) types of determinants. For example, if the epitope were assembled from components of both N and C termini, the binding of R2D2 to the native molecule should be greater than the sum of the binding to the fragments. Conversely, if the epitope

were composed of duplicated, independent parts, the binding to the whole molecule should approximate the sum of the binding to the fragments. To test this hypothesis, ³⁵SO₄-labeled bp60^{v-src} was purified from *E. coli* as previously described (25, 44) and was subjected to *S. aureus* V-8 protease digestion. bp60^{src}, as well as the major V-8 digestion products, bp34 and bp26, were recovered from preparative polyacrylamide gels in PBS. At no time were the preparations subjected to heat denaturation. The amounts of each peptide (picomoles) obtained were calculated from the amount of ³⁵S incorporated, assuming uniform labeling of all cysteine and methionine residues and normalizing the specific activity of bp60^{src} to the specific activity of the crude lysate. Equal amounts (picomoles) of bp60, bp34, and bp26

TABLE 1. Binding of R2D2 to bp60^{v-src}, bp34^{v-src}, and bp26^{v-src} as determined by ELISA^a

Peptide	Binding of the following R2D2 culture supernatant dilution ^b :				
	1:10	1:50	1:200	1:600	1:3,600
bp60	>2.000	1.255	0.489	0.135	0.022
bp34	0.376	0.102	0.034	0.001	0.000
bp26	0.529	0.238	0.078	0.005	0.000

^a A total of 0.83 pmol of peptide was applied to each well, and the binding assay was performed as previously described (47) by using horseradish peroxidase-conjugated goat anti-mouse heavy- and light-chain IgG (Hyclone Laboratories, Logan, Utah) and 2,2'-azino-bis-3-ethyl benzthiazolinesulfonic acid (Sigma Chemical Co., St. Louis, Mo.) as substrate.

^b Results are expressed as optical density at 414 nm and are corrected for background binding of a control MAb (anti-collagenase). The level of binding of R2D2 culture supernatants to an unrelated antigen (bovine serum albumin) was nearly identical to that of the control MAb to the pp60^{src} peptides. Initial dilutions of antibody were chosen to yield maximum binding to bp60 of 2 optical density units per well. R2D2 culture supernatant contained ca. 10 μg of immunoglobulin per ml.

TABLE 2. Binding of EC10 to bp60^{v-src}, bp34^{v-src}, and bp26^{v-src} as determined by ELISA^a

Peptide	Binding of the following EC10 culture supernatant dilution ^b :				
	1:300	1:1,000	1:3,000	1:9,000	1:27,000
bp60	>2.000	1.023	0.432	0.261	0.147
bp34	1.835	0.904	0.282	0.209	0.000
bp26	0.331	0.108	0.024	0.000	0.000

^a See footnote a of Table 1 for procedural details.

^b Results are expressed as optical density at 414 nm and are corrected for background binding of a control MAb (anti-collagenase). The level of binding of EC10 culture supernatants to an unrelated antigen (bovine serum albumin) was nearly identical to that of the control MAb to the pp60^{src} peptides. Initial dilutions of antibody were chosen to yield maximum binding to bp60 of 2 optical density units per well. EC10 culture supernatant contained ca. 4 μg of immunoglobulin per ml.

were then applied to wells of a microtiter plate, and the relative binding capacities of serial dilutions of R2D2 and a control MAb, EC10, were monitored in an enzyme-linked immunosorbent assay (ELISA) (47). The results of a representative experiment are shown in Tables 1 and 2. Approximately four to five times more R2D2 bound to the intact molecule, bp60, than bound to bp34 and bp26 combined. The level of binding to bp34 was similar to (slightly less than) the level of binding to bp26. Approximately 20 times more antibody was required to achieve a level of binding to the fragments which was comparable to the level of binding to the intact molecule, suggesting a difference in the affinity of R2D2 for the V-8 peptides as compared with the whole protein. In contrast, the sum of the binding of EC10 to the fragments was approximately equal to that of the binding to the intact molecule. About 90% of that binding was directed toward the amino, p34 peptide, and nearly equal amounts of antibody achieved comparable levels of binding to the N-terminal fragment and the intact molecule, suggesting that proteolytic preparation of the V-8 fragments did not significantly alter the affinity of EC10 for its epitope. MAb EB7 exhibited binding similar to that of EC10 (data not shown). These results support the interpretation that R2D2 binding is enhanced by a conformational assembly of amino acid residues contributed by both amino and carboxy regions of the molecule. However, binding, albeit weaker in nature, can also occur if these respective portions of the molecule are presented to the antibody.

Effect of alteration of pp60^{v-src} C terminus on inhibitory properties of R2D2. A second experiment was performed to assess the role of C-terminal sequences in the kinase-inhibitory activity of R2D2. As stated previously, R2D2 was incapable of immunoprecipitating pp60^{c-src} and was found to be incapable of inhibiting its activity in preformed immune complexes. Therefore, the ability of R2D2 to inhibit the tyrosine kinase activity of a pp60^{v-src}-pp60^{c-src} hybrid molecule was compared with its ability to inhibit that of the wild-type pp60^{v-src}. The hybrid *src* protein, which transformed tissue culture cells and which could be immunoprecipitated by R2D2, was encoded by a PrA-RSV genome constructed to contain the N-terminal *Bgl*I fragment (residues 1 to 436) of pp60^{v-src} and the C-terminal *Bgl*I fragment (residues 437 to 533) of pp60^{c-src} (PBS-C) (61). In the C-terminal *Bgl*I fragment, pp60^{c-src} encodes a unique residue at position 510 and a different, extended, C-terminal peptide beginning at position 515 (36) (CHPrA-RSV encodes an aspartate residue at position 502, as does *c-src*, in contrast to the PrA-RSV sequence reported by Fincham and Wyke [22]). R2D2 culture supernatant was added at various concentrations to immune complexes formed between EB7 and wild-type pp60^{v-src} or to the hybrid pp60^{v-src}-pp60^{c-src}. The double antibody complexes were assayed for their autophosphorylation activities as described in Materials and Methods. Immunoprecipitates were prepared from [³⁵S]methionine-labeled sister cultures to predetermine the volume of each extract required to assure equal amounts of wild-type and hybrid pp60^{v-src} in the immune complexes. The hybrid molecule was more refractory to inhibition by R2D2 than was the wild-type protein (Fig. 8). To achieve 50% inhibition, more than four times as much R2D2 culture supernatant was needed for the hybrid molecule than was needed for the wild type. These results indicate that alteration of the extreme terminus of pp60^{v-src} affects the ability of R2D2 to inhibit kinase activity but does not abolish this ability altogether. Thus, sequences N terminal, as well as sequences C terminal, to residue 436 appear to play a role in

FIG. 8. Inhibition of pp60^{v-src} and pp60^{v-src}-pp60^{c-src} hybrid protein autophosphorylation activities by R2D2. Various amounts of MAb R2D2 IgG were added to preformed complexes containing MAb EB7 and equal amounts of either wild-type pp60^{v-src} or the hybrid protein pp60^{v-src}-pp60^{c-src}. The relative autophosphorylation activities were determined by comparing the amount of ³²P_i incorporation of a given sample (in counts per minute) with that found in an EB7-*src* protein-*S. aureus* complex lacking R2D2. Maximum activity (100%) of EB7-pp60^{v-src} was 48,200 cpm, whereas that of EB7-pp60^{v-src}-pp60^{c-src} was 43,700 cpm, both after correction for negative antibody controls.

the binding of and inhibition mediated by R2D2. Taken together, the experiments described in this report suggest that the determinant recognized by R2D2 is complex and involves both N-terminal and C-terminal residues.

DISCUSSION

The MAb R2D2, which was raised against the transforming protein of PrA-RSV, pp60^{v-src} (43), was shown in this communication to immunoprecipitate two additional members of the tyrosine protein kinase family, pp70^{gag-fgr} and pp90^{gag-yes}. As with pp60^{v-src}, R2D2 efficiently inhibited the tyrosine protein kinase activities of pp70^{gag-fgr} and pp90^{gag-yes} in immune complex autophosphorylation reactions and in reactions containing the added substrates, casein and histone. The inhibitory activity of R2D2 was dependent upon immunoglobulin concentration, not upon the presence of an unknown inhibitory component in the antibody preparations.

The epitope recognized by R2D2 was previously localized to the N-terminal 204 amino acids of pp60^{v-src} (43). By using *src* fusion proteins, we show in this study that R2D2 is capable of recognizing residues not only in the N-terminal 110 amino acids but also in the C-terminal half of pp60^{v-src}. Thus, the inhibitory activity of R2D2 is most likely mediated by its binding to the catalytic domain which is contained in the carboxyl half of the *src* molecule. By using antibodies

TABLE 3. Regions of homology between the N-terminal and C-terminal amino acids of pp60^{v-src}^a

	N-terminal residues	C-terminal residues	Available synthetic peptides
<u>Seq. 1</u>	1 *** * 22 MGSSKSKPKDPSQRRHSLEPPD	493 *** * 514 DLMCQCWRKDPEERPTFKYLQA	7 *** *15 KPKDPSQRR 498 *** * 512 CWRKDPEERPTFKYL
<u>Seq. 2</u>	53* * * * * * *74 TVATEPKLFWGFNTSDTVTSFQ	428* * * * * * *449 WTAPEAALYGRFTIKSDVWSFG	---
<u>Seq. 3</u>	89 * * *** 110 LYDYESWTETDLSFKKGERLQI	336 * * *** 357 IVIEYMSKGSLLDFLKGEMGKY	---

^a *, Homologous residues.

prepared to the C-terminal peptides, residues 498 to 512 and 409 to 421, of the *v-src* protein, a similar inhibition of *src* kinase activity has been reported by Gentry et al. (23, 24) and Casnellie et al. (11). Since a high degree of homology exists between the C-terminal two-thirds of pp60^{v-src} and the catalytic regions of pp70^{v-fgr} and pp90^{v-yes}, it follows that binding of R2D2 to the kinase domains of these molecules might also cause a reduction of their activities. However, the inability of R2D2 to immunoprecipitate either pp70^{v-fgr} or pp90^{v-yes} as efficiently as the appropriate *gag* antisera or as efficiently as it immunoprecipitated pp60^{v-src} suggested that the complete R2D2 epitope was not presented by the *v-fgr* and *v-yes* proteins. Therefore, the significance of the pp60^{v-src} N-terminal sequences in R2D2 binding was brought into focus.

Several models can be proposed to describe the nature of the pp60^{v-src} epitope identified by R2D2. The ability of R2D2 to bind both amino and carboxyl regions could be due to the presence of (i) identical determinants (i.e., exhibiting homologous continuous or discontinuous residues on both halves of the molecule), (ii) a single epitope assembled from both N- and C-terminal residues, or (iii) two independent epitopes, the similarities of which are based on topography, not on primary amino acid sequence.

In an attempt to determine whether R2D2 recognized the same amino acids in the N-terminal 110 residues as it did in the C-terminal 286 residues of pp60^{v-src} (as encoded by PrA-RSV), we carried out a computer-assisted search for small regions of homology between these domains. The 286 residues of the C terminus were searched for homology to each of five sequential 22-amino-acid segments of the N-terminal 110 residues. Three segments, involving a total of 15 residues, were identified which displayed a homology of 18% or greater (Table 3). Of 22 (18%) amino acids, 4 were identical between residues 1 to 22 and 493 to 514, 6 of 22 (27%) were identical between residues 53 to 74 and 428 to 449, and 5 of 22 (23%) were identical between residues 89 to 110 and 336 to 357. Comparably weak homologies were also found when the N-terminal, 22-residue segments of pp60^{v-src} were compared with the complete sequences of pp70^{gag-fgr} and pp90^{gag-yes}. All of these homologies occurred in the catalytic domains of pp70^{gag-fgr} and pp90^{gag-yes}. The one exception was a very high degree of homology (77%) between residues 89 to 110 of pp60^{v-src} and 373 to 394 of pp90^{gag-yes}. (There were also homologies outside of the N-terminal 110 amino acids and outside of what is normally considered to be the protein kinase domain [e.g., residues 144 to 190 of pp60^{v-src}, 428 to 474 of pp90^{gag-yes}, and 281 to

327 of pp70^{gag-fgr}] [33, 38].) The existence of limited homology between the N-terminal residues of pp60^{v-src} and the specific C-terminal regions of pp60^{v-src}, pp70^{gag-fgr}, and pp90^{gag-yes} suggested that the epitope recognized by R2D2 could be duplicated within the polypeptide chain of pp60^{v-src} and that it could be present in the catalytic regions of pp70^{gag-fgr} and pp90^{gag-yes}.

To test this hypothesis, available synthetic peptides corresponding to some of the regions of homology described above (Table 3) were examined for their ability to reverse the inhibition of kinase activity by R2D2. Peptides corresponding to residues 7 to 15, 152 to 162, and 498 to 512 of pp60^{v-src} were incubated with R2D2 before addition of the MAb to an extract containing pp60^{v-src} or to preformed complexes of either MAb GD11 or EB7 plus pp60^{v-src}. The immune complexes were then tested for kinase activity. In all instances, R2D2 retained the capacity to fully inhibit the *in vitro* kinase activity of the *v-src* protein, even at approximate molar ratios of peptide to R2D2 in excess of 1,000 to 1, respectively. The peptides also did not prevent immunoprecipitation of *in vivo*, ³²P-labeled pp60^{v-src} by R2D2, indicating that the absence of kinase activity was not due to the inability of R2D2 to precipitate the protein. At least two explanations for these results can be offered. First, the peptides contained all or part of the residues composing the R2D2 epitope but were unable to reverse the R2D2 inhibition because they did not assume proper conformations for binding. Second, within any single peptide either insufficient or incorrect residues were presented for binding to R2D2. To attain high-avidity binding of the antibody, different or additional residues from other regions of the polypeptide chain may be required.

Furthermore, comparisons between the N-terminal 110 amino acids of pp60^{v-src} and the sequences in pp60^{c-src}, a protein which R2D2 does not immunoprecipitate, revealed that 14 of the 15 residues sharing homology between the N-terminal 110 amino acids and the C-terminal regions of pp60^{v-src} were identical to those in pp60^{c-src} (residue 501 being the exception). This finding also suggested that residues in addition to or in place of those identified by the homology search could participate in the binding of R2D2 to pp70^{gag-fgr} and pp90^{gag-yes} and in the immunoprecipitation of both N-terminal and C-terminal peptides of pp60^{v-src}. As a consequence of the low degree of homologies observed through our computer search and of the negative results of the peptide inhibition experiments, we gained little further insight into the involvement of specific sequences in the composition of the R2D2 epitope.

What, then, is the nature of the epitope recognized by R2D2 in pp60^{v-src}? The results of the ELISA experiment (Tables 1 and 2) demonstrated that, under identical conditions of peptide preparation, binding of R2D2 to the intact pp60^{v-src} greatly exceeded the sum of the binding to the N-terminal and C-terminal fragments. These results appear to be inconsistent with the duplicative epitope models, in which cases the sum of the binding to the V-8 peptides should have equaled the level of binding to the intact molecule (as it did for the MAb EC10). This should have been true for both sequential and conformational duplicative determinants of identical or fortuitously similar composition, except in the case in which V-8 proteolysis may have reduced the conformational stability of the fragments to a greater extent than that of the intact molecule. This possibility cannot be ruled out.

An alternative description for the R2D2 epitope is that of an assembled determinant in which discontinuous residues are brought into juxtaposition with one another on the surface of the v-*src* protein molecule. Such determinants require native conformation for maximum efficiency of antibody binding and do not exist in complete form within any single fragment of a protein. A case in point is myoglobin; specific antibodies that recognize the complete protein also bind peptide fragments, albeit with lower affinities than they bind native myoglobin (53). The reduced affinities for the myoglobin in peptides are most likely caused by the lack of native conformation assumed by the peptides (4). Another example is lysozyme, with which X-ray crystallography techniques were used to determine the three-dimensional structure of a complex between the antigen and the Fab fragment of a MAb. The antigen-contacting residues belonged to two stretches of the lysozyme polypeptide chain: residues 18 to 27 and 116 to 129. These sequences were nonidentical and were distant in the amino acid sequence but adjacent on the protein surface. The interface between antigen and antibody extended over a large area, with maximum dimensions of 3.0 by 2.0 nm (1). In a related but separate study, Barlow et al. (3) have provided evidence to show that if the recognition zone between a globular antigen protein and an antibody has the same dimensions as those described above, none of the antigen surface will be "continuous." These investigators suggest that all determinants are discontinuous to some extent and that cross-reactive peptides mimic only a portion of the interactive site. Such observations, together with the data presented in this report and the paucity of examples describing duplicative epitopes within a single polypeptide chain, lead us to favor the model of an assembled epitope for R2D2. Short of an X-ray crystallography analysis of pp60^{v-src}, however, no definitive conclusion can be reached.

An assembled-epitope model for R2D2 presupposes a juxtaposition of N-terminal and C-terminal peptides on the surface of pp60^{v-src} and leads to the intriguing possibility that the C-terminal catalytic domain of pp60^{v-src} may be regulated by N-terminal regions of the molecule. Regulation of enzymatic activity may be brought about by association of pp60^{v-src} with other membrane-associated proteins (such as pp60^{c-src} with polyomavirus middle T antigen [6, 18]), by posttranslational modifications, or by mechanisms mediated through the amino terminus. It has recently been shown that phosphorylation of a threonine residue of the epidermal growth factor receptor accompanies negative regulation of its tyrosine protein kinase activity (13). The phosphorylated residue is located in a region of the receptor most proximal to the cytoplasmic face of the plasma membrane (30), a position

also thought to be occupied by the amino terminus of pp60^{v-src} (19). Interestingly, the amino-terminal portion of pp60^{v-src} has been shown to acquire unique tyrosine phosphorylations after incubation either in high concentrations of ATP and Mg²⁺ (17) or in the presence of Na₂VO₄ (7, 14, 52). Such incubations result in an apparent activation of pp60^{v-src}. Whether the tyrosine phosphorylations are the cause or the result of the activations is not clear. It is tempting, however, to speculate that the amino-terminal domain of pp60^{v-src} can either positively or negatively regulate the activity of the catalytic domain, depending upon the type of modifications it undergoes. Multiple examples of protein kinases regulated in such a manner exist (for a review, see reference 39).

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