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Monoclonal antibody against the carboxy terminal peptide of pp60^{src} of Rous sarcoma virus reacts with native pp60^{src}

Teruko Tamura and Heinz Bauer*

Institut für Virologie, Fachbereich Humanmedizin der Justus-Liebig-Universität Giessen, Frankfurter Str. 107, D-6300 Giessen, FRG

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A monoclonal mouse antibody has been prepared against a synthetic peptide corresponding to the six carboxy-terminal amino acids (C′ peptide) of the *src* gene product pp60$^{v\text{-}src}$ of Rous sarcoma virus (RSV). The antibody was able to precipitate pp60$^{v\text{-}src}$ and to bind pp60$^{v\text{-}src}$ kinase activity in a competition test, indicating that this peptide can serve as an antibody-binding site (epitope). Furthermore, the finding that three out of 28 pp60^{src}-specific tumor-bearing rabbit (TBR) sera contained antibody against the C′ peptide argues for an *in vivo* role for the carboxy terminus of pp60^{src}. C′ peptide-specific IgG was purified from one TBR serum using affinity chromatography, and was shown to precipitate significant amounts of pp60^{src}, and bind most of the pp60^{src} kinase activity from SRA, PrA, and B77-C strains of avian sarcoma virus (ASV), but not endogenous pp60$^{c\text{-}src}$, a cellular homologue to the viral pp60$^{v\text{-}src}$. Similar results were obtained with IgG isolated from a C′ peptide immune rabbit serum. None of the three C′ peptide-specific IgGs could serve as a phosphate acceptor in an immune complex protein kinase reaction.

Key words: monoclonal antibody/pp60^{src}/ synthetic oligopeptide

Introduction

The *in vitro* transformation of cells and the *in vivo* induction of tumors by Rous sarcoma virus (RSV) is mediated by the viral *src* gene which codes for a phosphoprotein of mol. wt. ~60 000 (pp60$^{v\text{-}src}$) that has tyrosine-specific, cAMP-independent phosphokinase activity (Collett and Erikson, 1978; Collett *et al.*, 1980; Erikson *et al.*, 1979; Hunter and Sefton, 1980). It is generally assumed that this kinase activity plays a key role in the transformation mechanism, and several candidate target proteins for this enzyme have been described (Radke and Martin, 1979; Burr *et al.*, 1980; Erikson and Erikson, 1980; Hunter and Sefton, 1980; Radke *et al.*, 1980; Presek *et al.*, 1980; Boscheck *et al.*, 1981; Cooper and Hunter, 1981; Sefton *et al.*, 1981; Rübsamen *et al.*, 1982; Bauer *et al.*, 1982).

There are, on the other hand, a multitude of transformation-related phenotypic alterations to be observed, in cellular morphology, metabolism, and physiology. The relationship of these changes to the action of pp60^{src} is by no means understood. A pleiotropic or multifunctional effect of the *src* gene product has been postulated because of different cellular phenotypes as induced by transformation-defective, temperature-sensitive (td-ts) mutants of RSV (Calothy and Pessac, 1976; Becker *et al.*, 1977; Friis *et al.*, 1977; Weber and Friis, 1979). This allows speculation that pp60^{src} in different configurations or in different cellular compartments exerts different biological effects.

That pp60$^{v\text{-}src}$ has more than one antigenic site is evident from the various degrees of cross-reactivity by individual pp60^{src} rabbit immune sera when tested against different strains of RSV (Rübsamen *et al.*, 1979). Therefore, monospecific antisera to pp60^{src} epitopes may potentially allow the study of structural and functional relationships of this protein. One approach in this direction would be the preparation of monoclonal antibodies (Köhler and Milstein, 1975). To our knowledge, no such pp60^{src}-specific monoclonal antibodies have been described, and we ourselves have failed to prepare such antibodies when pp60^{src} itself was used as antigen, probably because the assay for pp60^{src}- specific antibodies is not sensitive enough for that method.

An alternative approach for obtaining monospecific antibody is the immunization against synthetic peptides which correspond to certain regions of a protein. This method has been successfully used in several cases. Thus, antibodies prepared against a peptide, the sequence of which had been predicted from the nucleotide sequence of a retrovirus genome, have allowed the detection of a previously unknown protein (Sutcliffe *et al.*, 1980). Walter and his colleagues have succeeded in preparing peptide antibodies which are specific for the large T-antigen of SV40 and the middle-size tumor antigen of polyomavirus (Walter *et al.*, 1980, 1981).

Wang and Goldberg (1981) reported the preparation of antibodies against a synthetic peptide representing an amino acid sequence of pp60^{src} containing the phosphate-accepting tyrosine residue as predicted from the nucleotide sequence of the *src* gene of RSV as published by Czernilofsky *et al.* (1980). These antibodies precipitated pp60^{src} from RSV-transformed cells.

We report here the preparation of monoclonal antibodies against a synthetic peptide containing the six amino acid residues at the carboxy terminus of pp60^{src} plus an additional N-tyrosine residue (C′ peptide). This antibody reacts with native pp60$^{v\text{-}src}$ and binds pp60^{src} kinase activity in a competition test. In addition, C′ peptide-reactive antibodies having similar specificity to the monoclonal mouse antibody have been detected in three out of 28 tumour-bearing rabbit (TBR) sera. Our analysis of a rabbit antiserum obtained after immunization with the same C′ peptide and supplied by G.Walter (Freiburg) revealed similar specificity as compared to C′ peptide-specific IgG from TBR serum. None of the various C′ peptide-reactive rabbit antibodies serve as phosphate acceptors in an immune complex protein kinase reaction.

Results

Establishment of monoclonal antibody against the C′ peptide

For the analysis of the biological functions of pp60^{src}, TBR sera have the disadvantage of containing antibodies against a variety of other antigens such as viral structural proteins. For instance, immune localization studies with TBR sera are always hampered by the possibility that antigens other than pp60^{src} might be detected. Also, the pp60^{src} specificity of

*To whom reprint requests should be sent.

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TBR sera varies from serum to serum (Rübsamen *et al.*, 1979). Monoclonal antibodies would provide an unlimited supply of antibodies of a stable specificity and could be expected to lead to the detection of different pp60^{src} epitopes responsible for the different functions of pp60^{src}.

Unfortunately, our attempts at preparing monoclonal antibodies by immunization of mice with whole pp60^{src} or by Rous sarcoma induction were unsuccessful for unknown reasons. With the aim of developing antibodies against different epitopes of pp60^{src}, we have, therefore, designed a protocol for preparing antibodies against oligopeptides corresponding to certain amino acid sequences of pp60^{src}. We have started with the heptapeptide Tyr-Val-Leu-Glu-Val-Ala-Glu (C′ peptide) which consists of the six carboxy-terminal amino acids of pp60^{src} and an additional amino-terminal tyrosine to facilitate coupling of the peptide to carrier proteins.

The mouse which was immunized with a bovine serum albumin (BSA)-C′ peptide conjugate developed an antibody titer of 1:5000 against the peptide, as tested by immunoprecipitation of [^{125}I]ovalbumin (OA)-C′ peptide. Spleen cells from this mouse were hybridized to X63-Sp8-653 myeloma cells and 32 out of 100 such hybridoma cultures were found to produce IgG. The supernatants from these 32 cultures were tested with [^{125}I]OA-C′ peptide, and one hybridoma supernatant was found to precipitate 7- to 10-fold more C′ peptide OA-conjugate than the others. This hybridoma clone was subcloned three times before the cells were injected i.p. into BALB/c mice. Ascites fluid was obtained from mice 2–4 weeks later and IgG was purified immediately using DEAE-cellulose chromatography. The specificity of the IgG was then tested in a competition assay by preincubating 1 μg each of purified IgG with either 5 μg of cold OA, OA-C′ peptide, BSA-C′ peptide, keyhole limpet hemocyanin (KLH) C′ peptide, or peptide alone for 1 h on ice before incubation with 25 ng of [^{125}I]OA-C′ peptide. After precipitation with protein A-containing *Staphylococcus aureus* Cowan-I strain, the precipitates were washed three times with lysis buffer, and the radioactivity measured. The result is shown in Table I indicating that any preincubation of the IgG with C′ peptide, i.e., with OA-C′ peptide, BSA-C′ peptide, KLH-C′ peptide, or peptide alone, inhibited the precipitation of [^{125}I]C′ peptide-OA by 60–90%, while cold OA had no competitive effect at all.

Table I. Demonstration by immuno-competition assay of C′ peptide-specific monoclonal mouse IgG

Preincubation of IgG with[a]	Following immunoprecipitation of [^{125}I]OA-C′ peptide (in % inhibition)
–[b]	0[b]
OA	0
OA-C′ peptide	81.8
BSA-C′ peptide	62.6
KLH-C′ peptide	90.8
C′ peptide	88.4

[a]Preincubation was done by mixing 1 μg of monoclonal ascites IgG with equal amounts of the respective probes.
[b]Untreated IgG was taken as control and its precipitating capacity set as 0% inhibited.

Characterization of monoclonal antibody

When the mouse C′ peptide IgGs were reacted with extracts from [^{32}P]orthophosphate-labeled, SR-RSV-A-transformed chick embryo cells (CEC), a 60-K and a 52-K phosphoprotein (pp60 and pp52) were precipitated (Figure 1, track 1). This precipitation was inhibited by preincubation of IgG with C′ peptide (track 2). (The faint remaining band appears to have a mol. wt. lower than 50 K.) In contrast, the immunoprecipitation of the 60-K and 52-K phosphoproteins by TBR-IgG (track 3) is barely competed by preincubation of the IgG with C′ peptide (track 4). In a control experiment, 10 μg of purified IgG from myeloma ascites did not precipitate these phosphoproteins (track 5). In addition, no

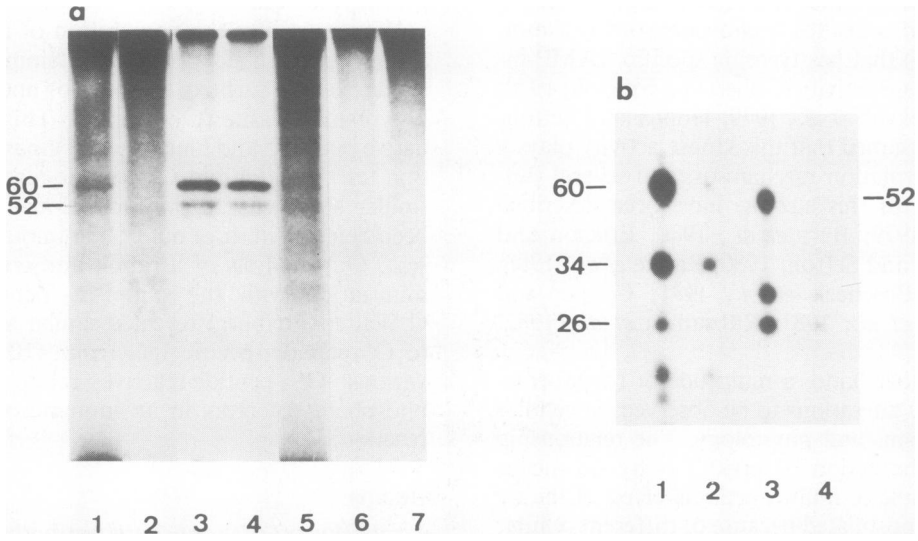

Fig. 1. a: Autoradiogram of SDS-polyacrylamide gel analysis of [^{32}P]orthophosphate-labeled proteins immunoprecipitated with various IgGs. Extracts were prepared from cultures of ^{32}P-labeled SR-RSV-A-transformed CEC (**tracks 1–5**) and from non-infected CEC (**tracks 6, 7**). The following IgG preparations were used: **track 1**: monoclonal C′ peptide-IgG (10 μg); **track 2**: monoclonal C′ peptide-IgG after absorption with C′ peptide; **track 3**: total TBR-IgG (50 μg); **track 4**: TBR-IgG after preabsorption with C′ peptide; **track 5**: myeloma ascites IgG (10 μg); **track 6**: monoclonal C′ peptide-IgG (10 μg); **track 7**: myeloma ascites IgG (10 μg); **b**: Partial proteolysis of pp60 and pp52 proteins as shown in **a**. The protein bands were excised from polyacrylamide gels and subjected to limited proteolysis during re-electrophoresis as described by Cleaveland *et al.* (1977). Digestion was with 0.08 μg of *S. aureus* protease V8. **Track 1**: pp60 from **track 3** in Figure 1; **track 2**: pp60 from **track 1**; **track 3**: pp52 from **track 3**; **track 4**: pp52 from **track 1**.

phosphoproteins were precipitated from [^{32}P]orthophosphate-labeled, non-infected CEC either by monoclonal C′ peptide-IgG (track 6) or myeloma ascites IgG (track 7). A partial hydrolysis with the V8 protease shows that the pp60 and the pp52, as immunoprecipitated by total TBR-IgG (tracks 1 and 3) and by mouse-C′ peptide-IgG (tracks 2 and 4), are identical. Thus, the pp60 and the pp52 immunoprecipitated by mouse-C′-peptide-IgG appear to be pp60^{src} and its cleavage product pp52 which has been described previously (Courtneidge *et al.*, 1980).

No phosphorylation of the mouse-C′ peptide-IgG could be observed upon reaction with pp60^{src} and [^{32}P]ATP in a protein kinase assay. Therefore, as a further test for the specificity of the mouse IgG, the ability of these antibodies to bind and remove pp60^{src}-associated kinase activity was tested in a competition assay. For this purpose, extracts from SR-RSV-A-transformed cells were pretreated as indicated in the legend to Figure 2. After 1 h incubation at 0°C, the mixtures were centrifuged and the supernatants incubated with total TBR serum for 1 h at 0°C. Immunoprecipitation was then performed by addition of *S. aureus* and the precipitates tested for IgG phosphorylation. As shown in Figure 2, the mouse C′ peptide-IgG removes ~70% of kinase activity (track 3), and this effect was completely inhibited by preincubation with C′ peptide (track 4). As controls, preincubation with 50 μg of total TBR-IgG absorbed almost 100% of kinase activity from the extract (track 2), while IgG from control ascites did not absorb kinase activity at all (track 5). Thus, these data provide additional evidence for the pp60^{src}-specific reaction of the monoclonal C′ peptide antibody.

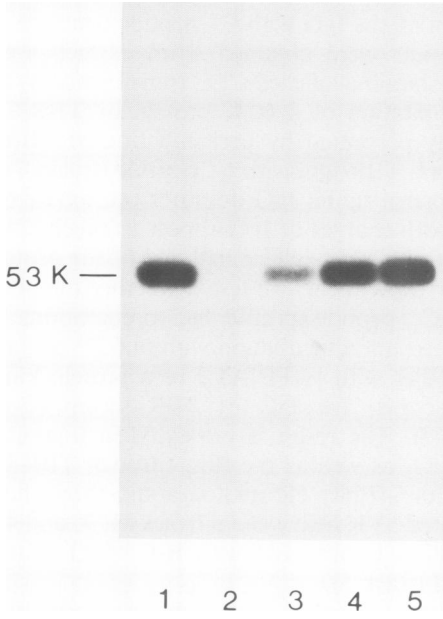

Fig. 2. Kinase absorption test with lysates from SR-RSV-A-transformed CEC. Extracts were incubated for 1 h at 0°C with **1**: *S. aureus* only; **2**: *S. aureus* loaded with total TBR-IgG (50 μg); **3**: *S. aureus* loaded with mouse-C′ peptide-IgG (5 μg); **4**: as in **3** but after preabsorption of IgG with C′ peptide; **5**: *S. aureus* loaded with myeloma ascites-IgG (10 μg); then centrifuged and the supernatants tested with total TBR-IgG for kinase reaction.

Isolation of C′ peptide-reactive antibodies from TBR-serum

Since C′ peptide-specific antibody reacted with native pp60^{src} it appeared that the C′-terminal portion of pp60^{src} is immunogenic in tumor-bearing animals. A total of 28 TBR sera were therefore tested for antibodies against the C′ peptide.

In one experiment, 10 different TBR sera were checked for

Table II. Demonstration by immuno-competition assay of C′ peptide-specific antibody in a TBR-serum

Preincubation of TBR-serum with[a]	Following immunoprecipitation of [^{125}I]OA-C′ peptide (in % inhibition)
–[b]	0[b]
OA	0
OA-C′ peptide	100
BSA-C′ peptide	99.8
KLH-C′ peptide	99.8
C′ peptide	82.6

[a]Preincubation was done by mixing 10 μl TBR serum with equal amounts of the respective probes.
[b]Untreated serum was taken as control and its precipitating capacity set as 0% inhibited.

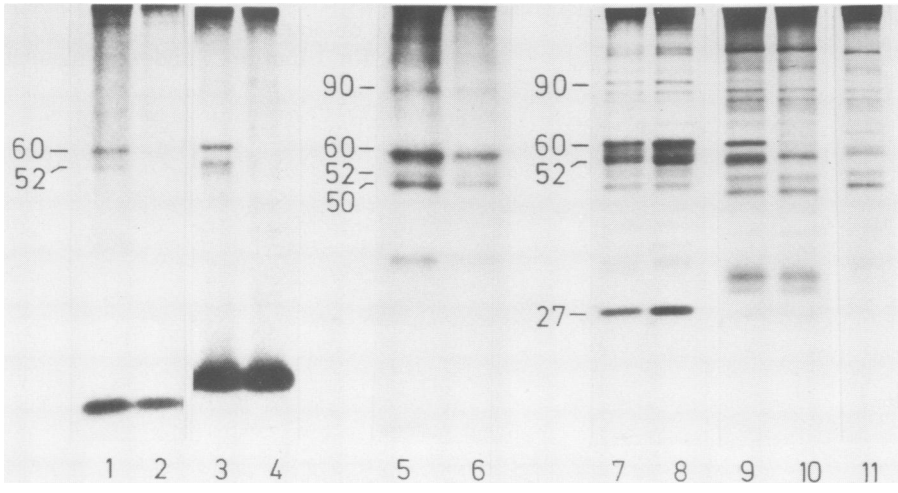

Fig. 3. Immunoprecipitation with extracts from [^{32}P]orthophosphate **(tracks 1–6)** and [^{35}S]methionine **(tracks 7–11)** labeled SR-RSV-A-transformed CEC. **1**: R-C′ IgG (10 μg); **2**: R-C′ IgG after pretreatment with C′ peptide; **3**: TBR-C′ IgG (3 μg); **4**: TBR-C′ IgG after pretreatment with C′ peptide; **5**: total TBR-IgG (10 μg); **6**: total TBR-IgG after treatment with C′ peptide; **7**: total TBR-IgG (20 μg), **8**: total TBR-IgG after pretreatment with C′: peptide; **9**: TBR-C′ IgG (5 μg); **10**: TBR-C′ IgG after pretreatment with C′ peptide, **11**: pre-immune rabbit IgG (10 μg).

immunoprecipitation of ^{125}I-labeled C′ peptide which was conjugated to ovalbumin (OA-C′ peptide). One of these sera showed a specific precipitation of OA-C′ peptide as compared to the background reaction with a normal rabbit serum. Additionally, 18 different TBR sera were tested by the enzyme-linked immunosorbent assay (ELISA) and two of these were found to be reactive with the C′ peptide. The positive serum of the first series was further analyzed. When it was preincubated with either 5 μg of cold OA, OA-C′ peptide, KLH-C′ peptide, BSA-C′ peptide, or C′ peptide alone for 1 h at 0°C and then incubated with 25 ng of [^{125}I]OA-C′ peptide under the same conditions, all cold peptide-carrying protein conjugates as well as the C′ peptide alone, but not cold OA, inhibited the precipitation of [^{125}I]OA-C′ peptide to 80 – 100% (Table II).

30 mg of IgG as isolated from this TBR serum by DEAE-cellulose column chromatography was added to a CH-Sepharose 4B column coupled with C′ peptide. About 50 μg of IgG was bound to this column and after elution could be shown to be specifically C′ peptide reactive.

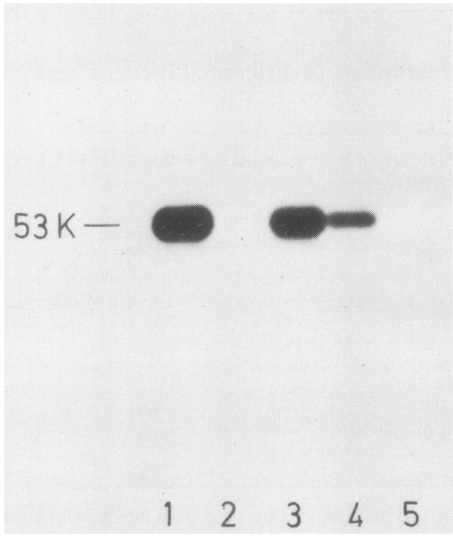

Fig. 4. Phosphorylation of IgG heavy chain after incubation of lysate from SR-RSV-A-transformed cells with **1**: total TBR-IgG (50 μg); **2**: R-C′ IgG (10 μg); **3** and **4**: non-binding fraction of TBR-IgG after C′ peptide affinity chromatography (30 μg; 3 μg), **5**: TBR-C′ IgG (3 μg).

Properties of C′ peptide-specific antibody derived from TBR serum and of antibody prepared by immunization of a rabbit with C′ peptide

C′ peptide-specfic antibodies isolated from TBR serum (TBR-C′ IgG) were compared with the antibodies of a rabbit serum which was prepared by immunizing a rabbit with the C′ peptide (R-C′ IgG), given to us by G.Walter.

As shown in Figure 3, both IgG preparations precipitate a 60-K and a 52-K phosphoprotein from extracts of CEC transformed by SR-RSV-A and labelled for 4 h with [^{32}P]orthophosphate. This precipitation was completely inhibited by preincubation of either IgG with C′ peptide (tracks 1 – 4). In contrast, the immune precipitation of the 60-K and 52-K phosphoproteins by total TBR-IgG is barely competed by preincubation of the IgG with C′ peptide (tracks 5,6).

Similar results were obtained when extracts were taken from [^{35}S]methionine-labelled CEC transformed by SR-RSV-A. The precipitation of a 60-K protein by TBR-C′ IgG is almost totally prevented by preincubation of TBR-C′ IgG but not of total TBR-IgG with C′ peptide (tracks 7 – 10). As a control, normal rabbit IgG does not precipitate 60-K protein. Thus, both charges of IgG appear to precipitate pp60^{src} by binding to the carboxy-terminal end of the protein.

In another experiment, we tested whether the heavy chain of the two C′ peptide-specific IgG preparations could be phosphorylated upon incubation with pp60^{src}-containing cell extracts together with [γ-^{32}P]ATP in a protein kinase reaction, as described for IgG of TBR serum (Collett and Erikson, 1978). The result is presented in Figure 4. While total TBR-IgG, as well as the flow-through TBR-IgG from the C′ peptide affinity chromatography, show the classical phosphorylated 53-K heavy chain (tracks 1, 3, and 4) no such phosphorylation is observed with R-C′ IgG (track 2) and TBR-C′ IgG (track 5).

To ascertain whether the immunoprecipitated 60-K phosphoprotein is indeed the phosphokinase-associated pp60^{src}, we checked in a competition assay whether the C′ peptide-specific IgG could remove pp60^{src} kinase activity from cell extracts. For this purpose, lysates from CEC transformed either by SR-RSV-A, Pr-RSV-A, or B77-ASV-C, and lysates of normal primary CEC were incubated with total TBR-IgG for kinase reaction after having been pretreated as indicated in the legend to Figure 2. The lysate supernatants were obtained by centrifugation after incubation of the mixture for 1 h at 0°C, and were incubated with total TBR-IgG

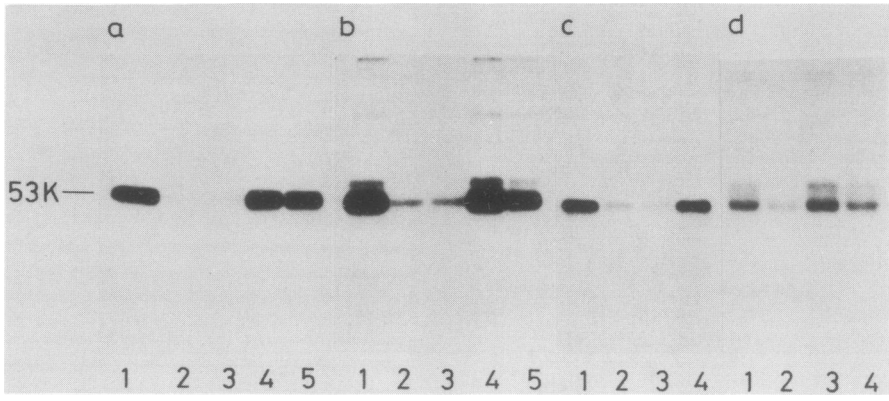

Fig. 5. Kinase absorption assay. Lysates from SR-RSV-A **(a)**, Pr-RSV-A **(b)**, B77-ASV-C **(c)** transformed CEC and from normal primary CEC **(d)** were pretreated with **1**: *S. aureus*; **2**: total TBR-IgG (50 μg) adsorbed to *S. aureus*; **3**: R-C′ IgG (5 μg) adsorbed to *S. aureus*; **4**: IgG as in **3** but after preabsorption with C′ peptide; **5**: preimmune rabbit IgG (50 μg); centrifuged and the supernatants tested with total TBR-IgG for kinase reaction.

for 1 h at 0°C before kinase reaction. As shown in Figure 5, R-C′ IgG removed kinase activity from virus-transformed cells or extracts as well as did whole TBR-IgG. Preincubation of the R-C′ IgG with the C′ peptide blocked this absorption capacity, so it can be concluded that C′ peptide-specific IgGs indeed react with pp60^{src}. This experiment further demonstrates that the carboxy-terminal end of pp60^{src} from three different sarcoma virus strains SR-RSV-A, Pr-RSV-A, and B77-ASV-C are antigenically related or identical. In contrast, endogenous pp60$^{c\text{-}src}$ does not appear to react with C′ peptide-specific antibody (Figure 5, panel d).

Discussion

To begin preparing monoclonal antibodies against different epitopes of pp60^{src}, we have investigated whether the carboxy-terminal peptide of pp60^{src} could be employed. The *in vitro* synthesized C′ peptide containing the six C′-terminal amino acids of pp60^{src}, with a tyrosine in place of a cysteine at the amino terminus (Tyr-Val-Leu-Glu-Val-Ala-Glu), has been used for this purpose.

After immunization of a mouse with the C′ peptide, it was possible to prepare monoclonal C′ peptide-specific antibodies which likewise reacted with native pp60^{src} as extracted from virus-transformed cells. About 10% of the TBR sera tested were found to contain pp60^{src}-specific antibodies which could be isolated by C′ peptide-specific affinity column chromatography. C′ peptide itself is immunogenic in rabbits as was shown with a rabbit C′ peptide antiserum (obtained from G.Walter, Freiburg) which was also reactive against native pp60^{src}. Thus, all three types of C′ peptide-specific antibodies react with native pp60^{src}, suggesting that the carboxy-terminal portion of pp60^{src} functions antigenically *in vivo*.

That most of the TBR sera failed to show significant amounts of C′ peptide-specific antibody is not surprising, since individual TBR sera, even those derived from the same litter of rabbits, reveal a great variation in titer and in specificity of pp60^{src} antibody. For instance, only few TBR sera are useful for the detection of endogenous pp60$^{c\text{-}src}$.

R-C′ peptide-IgG as well as TBR-C′ peptide-IgG, even in excess, was not capable of precipitating all of the pp60^{src} detectable with total TBR-IgG. In particular, the result with TBR-C′ peptide-IgG which had been induced by native pp60^{src}, suggests that a certain fraction of pp60^{src} may exist in a form which leaves the carboxy terminus inaccessible to antibody. Interestingly, both these IgG preparations were able to bind the total kinase activity in a kinase absorption test. In contrast, the monoclonal mouse-C′ peptide-IgG removes only ~70% of kinase activity, possibly as a consequence of low avidity often seen with monoclonal antibodies. As an alternative explanation, the carboxy-terminal six amino acids might be involved in two different epitopes of which one is only detected by the monoclonal antibody, and the other may not be accessible in all pp60^{src} molecules expressing kinase activity. In spite of the inability of the monoclonal C′ peptide-IgG to precipitate and remove quantitatively pp60^{src} and a kinase activity, respectively, this antibody has been successfully used for the purification of pp60^{src}. Further experiments, including study of other monospecific antibodies, are in progress to further define the possible existence of different configurations of pp60^{src}.

A 50-K as well as a 90-K phosphoprotein, are co-immunoprecipitated by TBR serum with a small fraction of wild-type RSV pp60^{src} (Hunter and Sefton, 1980; Brugge *et al.*, 1981). The complexed pp60^{src} molecules contain greatly reduced amounts of phosphotyrosine and their IgG phosphorylating activity has been found to be lower by at least a factor of 10 than that of the monomeric form of pp60^{src} (Brugge *et al.*, 1981). It was therefore determined whether the C′ peptide-specific antigenic site is altered in complexed pp60^{src}, i.e., whether C′ peptide-specific antibody could co-precipitate pp90 and pp50. This was examined with cells infected by the ts-NY68 mutant of RSV which have been shown to contain higher proportions of complexed pp60^{src} as compared to wild-type virus-infected cells (Brugge *et al.*, 1981). R-C′ peptide-IgG, as well as one TBR-C′ peptide-IgG, co-precipitate pp50 and pp90 with pp60^{src}. However, C′ peptide-IgG from a second TBR-serum did not precipitate the complex at all (data not shown). Whether this reflects more than one epitope expressed in the C′ peptide sequence is not clear and awaits further clarification.

None of the three kinds of C′ peptide-IgG is phosphorylated upon reaction with pp60$^{v\text{-}src}$ in the presence of [γ-^{32}P]-ATP suggesting that the antibody must bind to a specific site of pp60^{src} in order to serve as a phosphate acceptor in the immune complex protein kinase reaction. This has also been suggested by other workers who have prepared antibody against a synthetic peptide from a different region of pp60^{src} (Wang and Goldberg, 1981). Nevertheless, all C′ peptide-IgG preparations were able to bind and remove pp60^{src} kinase activity in a competition test.

In light of the failure, thus far, to produce monoclonal antibodies to pp60^{src} using the native protein as immunogen, it was interesting to see that monoclonal pp60^{src}-specific antibody could be prepared against an oligopeptide homologous to a portion of pp60$^{v\text{-}src}$. This suggests that the preparation of monoclonal antibody made against pp60^{src} may be possible as soon as a sensitive assay becomes available, for instance with radiolabelled purified pp60^{src}. As one can imagine, the existence of epitopes of native pp60^{src} which are not defined by the primary structure, but rather by the tertiary structure of the protein (Sela *et al.*, 1967), and which could be specific for a certain function of pp60^{src}, it would be desirable to prepare antibodies against such epitopes by the hybridoma-derived monoclonal antibody technique.

According to the nucleotide sequence of the *src* genes from the two RSV strains SR-RSV and Pr-RSV which have been determined (Czernilofsky *et al.*, 1980; Schwartz *et al.*, 1982), both *src* genes encode identical amino acid sequences at the 3′ end. Accordingly, the antibody prepared against C′ peptide reacts with the pp60^{src} encoded by both viruses. The B77 avian sarcoma virus is not a direct derivative of the original RSV but a separate field isolate (Thurzo *et al.*, 1963). While the *src*-gene of B77-ASV and its gene product are highly related in structure and function to those of the RSV strains, the nucleotide sequence of B77 *src* gene is not yet known. Our finding that the C′ peptide-IgGs react with pp60^{src} from B77-transformed cells points to a homologous or highly related amino acid sequence at the C′ end of the pp60^{src} of the three sarcoma viruses. This is of special interest in the light of the failure of these antibodies to precipitate cellular pp60^{src}.

Very little is known about possible differences in the biology and structure between cellular and viral pp60^{src}. The question arises, for example, why is the viral, but not the cellular pp60^{src}, inhibited by the nucleotide diadenosine

5′,5″-P¹P⁴-tetraphosphate (Schartl and Barnekow, 1982). The inability of our C′ peptide-IgG to react with the cellular pp60^{src} suggests a structural difference. Either the carboxy end of pp60$^{c\text{-}src}$ has an amino acid structure which differs from that of pp60$^{v\text{-}src}$, or it has a conformation with makes it inaccessible to antibody. It would be interesting to discover if these differences between cellular and viral pp60^{src} result in differences in function. The quantitative analysis of pp60$^{c\text{-}src}$ activity in normal tissue has led to the speculation that the function of pp60$^{c\text{-}src}$ is modulated under specific physiological conditions and in specific tissues (Bauer, 1982).

It is hoped that antibodies prepared against oligopeptides from other sequence regions of pp60^{src} will be useful in answering this and other questions with respect to the function of both cellular and viral pp60^{src}.

Materials and methods

Viruses and cells

The Schmidt-Ruppin strain and the Prague strain of RSV, both of subgroup A (SR-RSV-A; Pr-RSV-A), and the Bratislava avian sarcoma virus B77 of subgroup C (B77-C) were used for the transformation of chick embryo cells *in vitro* according to published procedures (Friis *et al.*, 1975).

Antigen preparation and immunization

The synthetic heptapeptide Tyr-Val-Leu-Glu-Val-Ala-Glu, corresponding to the six amino acid residues at the carboxy terminus of pp60^{src} with a N-terminal tyrosine instead of a cysteine as predicted from the nucleotide sequence of the RSV *src* gene (Czernilofsky *et al.*, 1980), was made by Bachem Company, Bubendorf, Switzerland and obtained through the courtesy of G.Walter, Freiburg. This peptide was coupled to BSA using bis-diazotized benzidine (Bassiri *et al.*, 1979) resulting in 6–8 peptide molecules bound per molecule of BSA. 100 μg of BSA-C′ peptide conjugate in Freund's complete adjuvant were injected s.c. into a BALB/c mouse. Five weeks later the mouse was boostered with the conjugate in the absence of adjuvant. After 1 week the mouse was sacrificed for hybridoma production.

The rabbit C′ peptide antiserum was prepared by injection of BSA-C′ peptide conjugate (G.Walter, personal communication), and the TBR sera as described earlier (Ziemiecki and Friis, 1980).

Hybridoma formation

A total of 1 x 10^8 mouse spleen cells were fused with 4 x 10^9 cells of the X63-Sp8-653 myeloma cell (Köhler and Milstein, 1976) according to the procedure described by St.Groth and Scheidegger (1980) using polyethylene glycol 4000. The cell suspension was distributed into 96 microwells of tissue culture plates and cultured in HAT-medium for the selection of hybrid cells. Mouse IgG from hybridoma supernatant was detected and quantitated by ELISA (Engvall and Perlman, 1972). For this purpose, alkaline phosphatase (Sigma type VII) was conjugated with glutaraldehyde to IgG purified from goat-anti-mouse IgG serum. The wells of microplates were coated with goat-anti-mouse IgG overnight, and then each incubated with the supernatant of an individual hybridoma culture. Positive cultures were identified by the addition of phosphatase-conjugated goat-anti-mouse IgG.

Assay for antibody against the C′ peptide

To test for specific antibody against the C′ peptide, the peptide was coupled to ovalbumin (OA) and then labelled with [^{125}I]Bolton-Hunter reagent ([^{125}I]OA-C′ peptide). 100 μl of supernatant from IgG positive cultures were incubated with 10 000 c.p.m. of [^{125}I]OA-C′ peptide in PBS substituted with 1% OA, 1% BSA and incubated for 1 h on ice and then precipitated by formaldehyde-fixed *S. aureus* for 30 min on ice. The precipitate was washed three times with lysis buffer (pH 7.3) (0.01 M Tris, 0.1 M NaCl, 1% Triton X-100, 1% DOC, 1% Trasylol, 0.001 M EDTA) and its radioactivity measured.

The hybridoma cells which produced specific antibody against C′ peptide were twice cloned by limiting dilution method and then cloned in 0.5% soft agar. After the third cloning, 2 x 10^6 hybridoma cells were injected i.p. into BALB/c mice which had been treated with pristane. Two to 4 weeks later, ascites was obtained from these mice. IgG was precipitated from ascites fluid by 40% ammonium sulphate and immediately afterwards purified by DEAE-cellulose chromatography.

Purification of C′ peptide-specific antibody by affinity chromatography

5 mg of the C′ peptide was coupled to 1 g of CH-Sepharose 4B by the carbodiimide coupling procedure of Pharmacia. TBR serum was rinsed through the Sepharose column and the bound IgG was eluted with 0.1 M glycine (pH 2.5). The eluted fractions were neutralized immediately by addition of Tris-base.

Immunoprecipitation of pp60^{src}

Chicken embryo cells were transformed by avian sarcoma virus and labelled with [^{32}P]orthophosphate for 3–5 h. Cells were washed with PBS and lysed in 1 ml of extraction buffer (10 mM PO_4, 10 mM EDTA, 40 mM NaF, 0.5% Triton-X-100, 1% Trasylol). 50 μl aliquots of cell lysate were incubated with antibody for 1 h on ice. Immunoprecipitates were adsorbed on fixed *S. aureus* (Kessler, 1975) and washed three times with washing buffer (10 mM PO_4, 10 mM EDTA, 40 mM NaF, 0.2% Triton X-100, 1 M NaCl, 1% Trasylol). The precipitates were then analyzed by electrophoresis on 11% SDS-polyacrylamide and by subsequent autoradiography.

Protein phosphokinase assay

The assay for protein kinase activity in immune complexes was basically as described by Collett and Erikson (1978). Antibody and transformed CEC or mouse cell (D17) extracts were incubated for 1 h on ice and the immune complexes precipitated with fixed *S. aureus*. Immunoprecipitates were washed three times with washing buffer, once with distilled water and then incubated for 3 min on ice in 10 μl of a buffer containing 30 mM bis-Tris-propane (BTP), 50 mM $MgCl_2$ and 2 μCi of [γ-^{32}P]ATP. The samples were analyzed by 11% SDS-polyacrylamide gel electrophoresis and subsequent autoradiography.

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