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Protein–tyrosine kinase activity of alternate protein products of the *Drosophila Dsrc28C* locus

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The *Dsrc28C* gene is a unique member of the extensive tyrosine kinase family. Two proteins, $p66^{Dsrc28C}$ and $p55^{Dsrc28C}$, are encoded by the gene. Each contains a highly conserved tyrosine kinase domain and each lacks the usual amino-terminal myristylation signal. The protein–tyrosine kinase activity of the two proteins was investigated through a recombinant baculovirus expression system. $p66^{Dsrc28C}$ expressed from a recombinant baculovirus phosphorylated a large number of Sf9 cell proteins on tyrosine. A group of proteins of approximately 100 kDa were the preferred substrates. No evidence of $p66^{Dsrc28C}$ autophosphorylation was found. In contrast to $p66^{Dsrc28C}$, $p55^{Dsrc28C}$ did not exhibit protein–tyrosine kinase activity when expressed from a recombinant baculovirus. A deletion derivative of $p66^{Dsrc28C}$ lacking the SH3 and SH2 domains also failed to phosphorylate Sf9 cell proteins. These results suggest that the protein–tyrosine kinase activity of Dsrc28C proteins is tightly regulated.

src enzyme activity; Signal transduction

1. INTRODUCTION

The *src* gene super-family is a diverse group of protein kinases sharing a highly conserved catalytic domain. The *src* gene itself represents the major sub-family of cytoplasmic tyrosine kinases. Because src and related proteins are positioned at the inner surface of the cell membrane an involvement in normal cellular signal transduction processes is suggested. Despite extensive studies, the precise physiological roles of the normal cellular or transforming variants of the cytoplasmic src family of oncoproteins are not completely understood. Additional members of this extensive gene family have been revealed through classical and molecular genetic studies (reviewed in [1]). Protein–tyrosine kinase or protein–serine/threonine kinase activity has been directly demonstrated for proteins encoded by numerous *src*-related genes. It is widely assumed that proteins encoded by other *src*-family members also possess protein kinase activity because specific amino acid positions within the kinase domain are conserved.

We have been studying a *Drosophila src*-related gene, referred to as *Dsrc28C*. Analysis of key residues within the kinase domain of this gene indicates that it is most closely related to *v-src* and other tyrosine kinases. The SH3 and SH2 domains of *v-src* tyrosine kinases are also well conserved in *Dsrc28C*. These domains are present in the amino-terminal region of cytoplasmic src family of proteins and are known to contain elements that affect kinase activity [2].

Although *Dsrc28C* represents a mainstream *src* family member when highly conserved amino acids are considered, it is by other criteria a unique member of this gene family. At least two proteins, $p66^{Dsrc28C}$ and $p55^{Dsrc28C}$, are encoded by the gene. The similar proteins are distinguished by a lack of the first 125 amino acids in $p55^{Dsrc28C}$ [3,4]. Cytoplasmic src proteins commonly contain a sequence motif at the amino-terminus, including a glycine residue in the second position that is required for post-translational myristylation and membrane association [5,6]. Neither Dsrc28C protein has the Gly-2 required for myristylation [3]. Each protein encoded by *Dsrc28C* has a unique temporal and spatial pattern of expression. $p66^{Dsrc28C}$ is expressed predominantly in epidermal cells, while $p55^{Dsrc28C}$ is expressed predominantly in the nervous system.

Due to the unusual properties of the *Dsrc28C* gene, it was important to test directly the postulated kinase activity of the encoded proteins. We report that $p66^{Dsrc28C}$ expressed in Sf9 cells via a recombinant baculovirus has protein–tyrosine kinase activity. However, this protein does not autophosphorylate, nor does it utilize classical src substrates. In contrast, similarly expressed $p55^{Dsrc28C}$ does not exhibit detectable protein–tyrosine kinase activity.

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2. MATERIALS AND METHODS

2.1. *Cells and virus infection*

Spodoptera frugiperda cells (Sf9) adapted for growth in serum-free medium were obtained from Gibco BRL Life Technologies Inc. Cells were cultured as recommended by Gibco. *Autographia californica* nuclear polyhedrosis virus (AcNPV) was a gift from Dr. Max Summers. Recombinant virus was titered by limiting dilution and dot blot hybridization [7]. Infection of Sf9 cells was carried out at a multiplicity of infection (MOI) of 5. Virus propagation was performed as suggested by Summers and Smith [8].

2.2. *Construction of recombinant baculoviruses*

All constructs in this study employed the pVL1393 transfer vector [9] in which the initiator codon is provided by the cDNA insert. Thus, the Dsrc28C proteins expressed in Sf9 cells do not contain exogenous amino acids. To generate the transfer vector encoding $p66^{Dsrc28C}$, pCapser-hs28C [4] was restricted with *Hin*dIII, repaired with Klenow polymerase, and digested with *Xba*I. The resulting cDNA fragment was ligated to pVL1393 that had been cleaved with *Sma*I and *Xba*I. To produce the transfer vector encoding $p55^{Dsrc28C}$, a 2.2 kb *Xba*I cDNA fragment was removed from pCasper-hs28C-S [4] and ligated to *Xba*I-restricted pBluescript-KS$^+$ (Stratagene Inc.). The cDNA segment from this plasmid was inserted into pVL1393 as described above. To generate the transfer vector encoding the $p66^{Dsrc28C}$ deletion derivative lacking the SH3 and SH2 src homology domains ($p66^{dSH3SH2}$), *Nco*I sites were introduced at nucleotide positions 550 and 1,130 within the pHS28C cDNA by oligonucleotide-directed mutagenesis. The mutagenesis was carried out as previously described [4]. The resulting plasmid was digested with *Nco*I and ligated to form a cDNA segment with the original translational reading frame but lacking the SH3 and SH2 domains. The deleted cDNA was inserted into pVL1393 as described above for the $p66^{Dsrc28C}$ cDNA. To generate recombinant baculoviruses, 2 μg of a recombinant baculovirus transfer vector was mixed with 1 μg of wild-type viral DNA and used to transfect Sf9 cells as described by Summers and Smith [8]. After 8 days, the virus in the medium was purified by limiting dilution and dot blot hybridization [7]. Viruses encoding $p55^{Dsrc28C}$ and $p66^{dSH3SH2}$ were also identified and partially purified by limiting dilution and dot blot hybridization. The titer of the recombinant baculovirus was determined by dot blot hybridization.

2.3. *Expression of recombinant baculovirus-encoded proteins*

To monitor production of proteins produced by recombinant baculovirus, 9×10^4 cells were infected with a given recombinant baculovirus at a MOI of 5. For each time point, cells were collected, washed in PBS, lysed in 10 μl of Laemmli buffer [10], fractionated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Staining of the immunoblots was carried out with MabI19 or MabB3 to monitor production of Dsrc28C proteins as previously described [4]. The recombinant baculovirus stock encoding $p60^{c\text{-}src}$ was provided by Dr. Helen Piwnica-Worms. Production of $p60^{c\text{-}src}$ was monitored using Mab327 provided by Dr. Joan Brugge. Protein–tyrosine kinase activity was analyzed using MabPY20 (obtained from ICN Biochemicals).

3. RESULTS

3.1. *In vivo protein–tyrosine kinase activity of the p66 form of Dsrc28C protein*

Both major forms of protein encoded by the *Dsrc28C* gene, p66 and p55, contain the highly conserved tyrosine kinase domain [3,11] and would thus be expected to function as tyrosine kinases. When expressed in *E. coli*, tyrosine kinase activity could not be detected from either form of protein, nor could activity be detected in the standard immune-complex reaction using *Drosophila* embryonic protein and monoclonal antibodies (R.J. Gregory, W.S. Vincent, and S.C. Wadsworth, unpublished data). Inherent difficulties with these approaches that could explain the inability to detect enzyme activity are the improper folding of foreign proteins in *E. coli* and the low abundance of Dsrc28C proteins in embryos.

We next attempted to make use of heat-shock promoters to drive expression of *Dsrc28C* cDNAs. We have described previously a P element-transformed fly line expressing $p66^{Dsrc28C}$ protein from a heat-shock promoter [4]. Following heat shock, $p66^{Dsrc28C}$ itself was not significantly phosphorylated on tyrosine. Other proteins containing phosphotyrosine residues were also not detected. This result had not been anticipated because the autophosphorylation site in Dsrc28C is well conserved with respect to other src family members that readily undergo autophosphorylation [3]. These results indicated that an alternate system for the production of the Dsrc28C proteins would have to be utilized in order to study the protein–tyrosine kinase activity of these proteins.

As an alternate system for high-level production of recombinant proteins, we chose to express $p66^{Dsrc28C}$ in Sf9 cells via a recombinant baculoviral vector [8]. One advantage of this system is that it is known to be permissive for the expression of foreign proteins with tyrosine kinase activity [12]. The cDNA sequences encoding the $p66^{Dsrc28C}$ protein were introduced into the baculovirus genome downstream from the polyhedrin promoter as described in section 2. The structure of the protein encoded by this construct is diagrammed in Fig. 1A. Expression of $p66^{Dsrc28C}$ in cells infected with recombinant virus was monitored by staining immunoblots with the monoclonal antibody MabI19, which recognizes a $p66^{Dsrc28C}$ epitope somewhere within the SH3 or SH2 domains [4]. Within 24 h of infection, a MabI19-staining protein of the expected electrophoretic mobility is detected (Fig. 1B). Characteristically, a strong doublet of MabI19-staining material is seen by 32 h post-infection. This doublet represents the major MabI19-staining material, although in longer infection times, breakdown and aggregation products are evident.

To determine whether the expressed $p66^{Dsrc28C}$ has tyrosine kinase activity, a parallel immunoblot was stained with PY20, which is a monoclonal antibody that is specific for phosphorylated tyrosine residues. As the level of $p66^{Dsrc28C}$ increases in infected cells, PY20-staining of numerous Sf9 cell proteins could be detected (Fig. 1C). Several proteins in the range of 100–110 kDa were routinely the most heavily phosphorylated species (Fig. 1C). However, $p66^{Dsrc28C}$ proteins were not major phosphotyrosine-containing proteins even though the region corresponding to Tyr-416 in $p60^{c\text{-}src}$ is well conserved [3]. One PY20-staining protein exhibited an electrophoretic mobility similar to the upper band of the $p66^{Dsrc28C}$ doublet, but this protein could be resolved

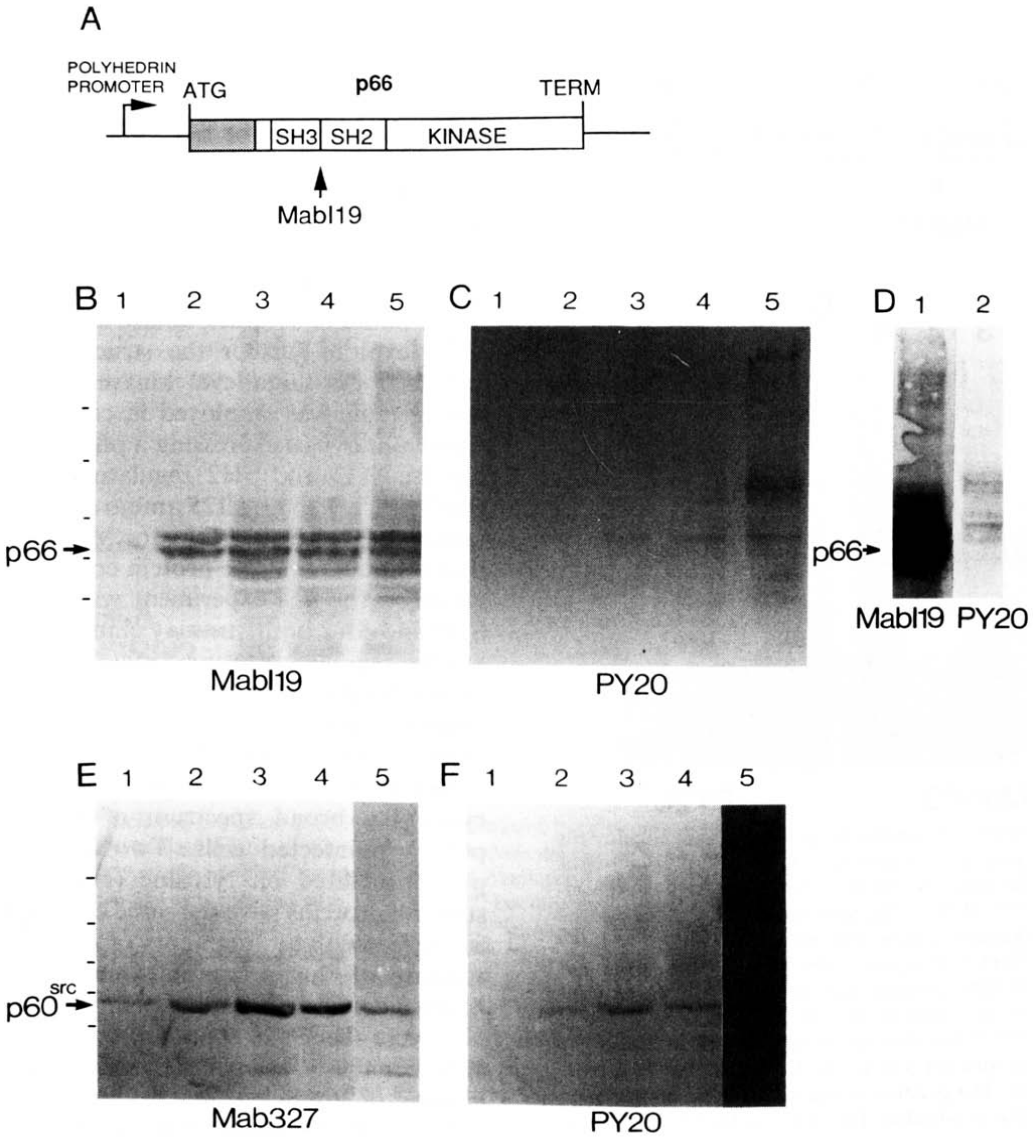

Fig. 1. Expression of $p66^{Dsrc28C}$ protein from recombinant baculovirus and analysis of tyrosine kinase activity. (A) $p66^{Dsrc28C}$ baculovirus expression vector. The indicated initiator and terminator codons are derived from the $p66^{Dsrc28C}$ cDNA. The recognition site of Mabl19 is not precisely known but is present within the SH3–SH2 region of the protein. The filled block represents the 125 amino acid amino-terminal p66-specific domain. (B) Immunoblot of proteins from Sf9 cells infected with the $p66^{Dsrc28C}$ baculovirus reacted with Mabl19. (C) Immunoblot of proteins from Sf9 cells infected with the $p66^{Dsrc28C}$ baculovirus reacted with PY20. (D) Immunoblot of $p66^{Dsrc28C}$ recombinant baculovirus-infected Sf9 cell proteins reacted with Mabl19 (lane 1) or PY20 (lane 2). These protein samples were electrophoresed for a longer time to resolve the $p66^{Dsrc28C}$ proteins from the PY20-staining Sf9 cell proteins. (E) Immunoblot of proteins from Sf9 cells infected with the $p60^{c\text{-}src}$ baculovirus reacted with Mab327. (F) Immunoblot of proteins from Sf9 cells infected with the $p60^{c\text{-}src}$ baculovirus reacted with PY20. In panels B, C, E, and F, lanes 1–5 contain proteins from infected cells at 24, 32, 40, 48, and 72 h post-infection, respectively. The positions of the recombinant baculovirus-encoded proteins are indicated with labeled arrows. The dashes at the left indicate the position of pre-stained protein markers of 180 kDa, 116 kDa, 84 kDa, 58 kDa, and 48 kDa.

from $p66^{Dsrc28C}$ when electrophoresis was carried out for longer periods of time (Fig. 1D). These results indicate that although $p66^{Dsrc28C}$ is able to utilize a variety of Sf9 cell proteins as substrates, it does not itself serve as an efficient substrate. These results do not exclude the possibility that a small fraction of $p66^{Dsrc28C}$ proteins are phosphorylated. Consistent with these findings, we were unable to demonstrate autophosphorylation of $p66^{Dsrc28C}$ in an immune-complex kinase assay that employed monoclonal antibodies raised against $p66^{Dsrc28C}$ (i.e. Mabl19 or MabB3; data not shown). Enolase was also not phosphorylated in this assay (data not shown).

For comparison, Sf9 cells were infected with a recombinant baculovirus expressing the avian $p60^{c\text{-}src}$ protein [12]. Production of $p60^{c\text{-}src}$ was monitored by staining with the src-specific antibody, Mab327 (Fig. 1E). $p60^{c\text{-}src}$ can be detected within 24 h post-infection and accumulates to high levels thereafter. A broad spectrum of cell proteins react with PY20 after 40 h of infection but preferential phosphorylation of individual Sf9 cell

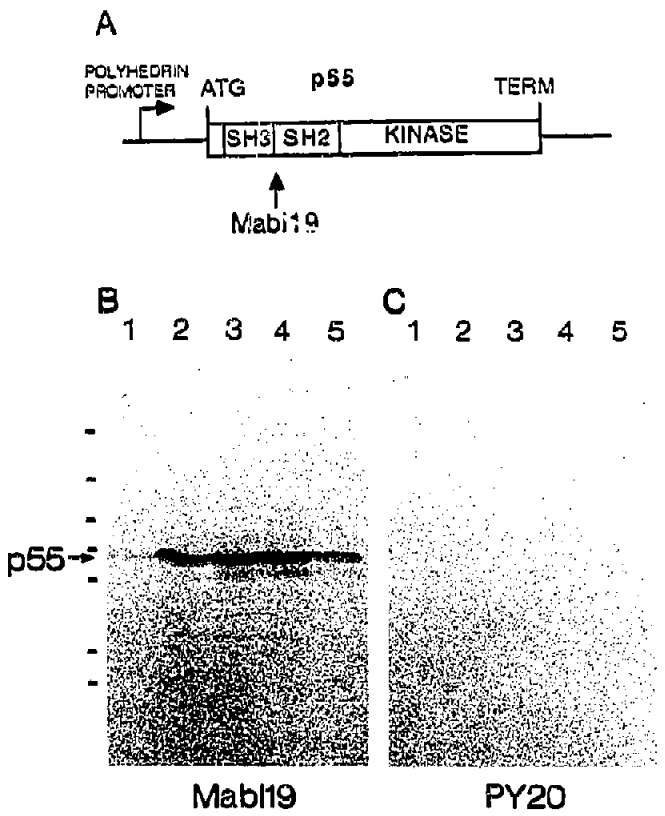

Fig. 2. Expression of $p55^{Dsrc28C}$ protein from recombinant baculovirus and analysis of tyrosine kinase activity. (A) $p55^{Dsrc28C}$ baculovirus expression vector. The indicated initiator codon corresponds to Met-126 within the $p66^{Dsrc28C}$ cDNA. The terminator codon is the normal $p66^{Dsrc28C}$ cDNA terminator codon. The recognition site of MabI19 is present within the SH3–SH2 region of the protein. (B) Immunoblot of proteins from Sf9 cells infected with the $p55^{Dsrc28C}$ baculovirus reacted with MabI19. (C) Immunoblot of proteins from Sf9 cells infected with the $p55^{Dsrc28C}$ baculovirus reacted with PY20. Lanes 1–5 contain proteins from infected cells at 24, 32, 40, 48, and 72 h post-infection, respectively. The position of recombinant baculovirus-encoded $p55^{Dsrc28C}$ protein is indicated. The dashes at the left indicate the positions of pre-stained protein markers of 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48 kDa, 37 kDa, and 27 kDa.

proteins was not evident (Fig. 1F). At all times, $p60^{c\text{-}src}$ was the most abundant phosphotyrosine-containing protein. Under the conditions used, cells infected with wild-type baculovirus did not exhibit detectable binding to any of the antibodies used in this study including PY20 (data not shown).

3.2. *Full-length $p66^{Dsrc28}$ is required for maximal tyrosine kinase activity*

The *Dsrc28C* gene encodes two major protein forms, $p66^{Dsrc28C}$ and $p55^{Dsrc28C}$. The mechanism for the production of these alternate protein forms has not been established. However, the two proteins have unique temporal and spatial patterns of expression, suggesting that each might have a specialized function. Therefore, to examine the protein-tyrosine kinase activity of $p55^{Dsrc28C}$, a recombinant baculovirus was constructed to express $p55^{Dsrc28C}$ in Sf9 cells (Fig. 2A). Since the kinase domain is present within $p55^{Dsrc28C}$, this protein would be expected to function as a tyrosine kinase. Sf9 cells infected with the recombinant $p55^{Dsrc28C}$ virus expressed a protein of the expected size and immunoreactivity (Fig. 2B). However, no detectable phosphorylation of cell proteins nor of $p55^{Dsrc28}$ was observed (Fig. 2C). Furthermore, $p55^{Dsrc28C}$ produced in Sf9 cells did not phosphorylate enolase nor did it autophosphorylate in the immune-complex kinase assay (data not shown).

To explore further the structural requirements of $p66^{Dsrc28C}$ for high-level kinase activity, site-directed mutagenesis was employed in constructing a recombinant baculovirus expressing a $p66^{Dsrc28C}$ derivative lacking the SH3 and SH2 regulatory domains (Fig. 3A; $p66^{\Delta SH3SH2}$). The first 125 amino acids of $p66^{Dsrc28C}$ containing the recognition site for MabB3 were retained so that production of the protein could be monitored. The rationale for this experiment was that removal of the SH3 and SH2 domains may eliminate regions that negatively regulate tyrosine kinase activity, as has been shown for $p60^{c\text{-}src}$ [2]. A protein of the expected size and immunoreactivity was expressed from this recombinant virus, as well as some breakdown products (Fig. 3B). However, it is clear that $p66^{\Delta SH3SH2}$ did not phosphorylate the broad spectrum of cell proteins seen in $p66^{Dsrc28C}$ infected cells. Two smaller proteins were phosphorylated on tyrosine (Fig. 3C). These PY20-staining proteins have the same electrophoretic mobility as the proteolytic fragments of $p66^{\Delta SH3SH2}$. Subsequent staining of the immunoblot shown in Fig. 3C with MabB3 confirmed that the $p66^{\Delta SH3SH2}$ breakdown products were the PY20-stained proteins (data not shown). Whether these fragments possess autophosphorylation activity or are substrates for $p66^{\Delta SH3SH2}$ has not been determined. Assuming that the electrophoretic mobilities of the proteolytic fragments are proportional to their molecular weights, only the larger fragment could contain the kinase domain in addition to the MabB3 epitope. None of the proteins in cells infected with the $p66^{\Delta SH3SH2}$ recombinant virus exhibited kinase activity in the immune-complex kinase reaction (data not shown).

4. DISCUSSION

The proteins encoded by the *Dsrc28C* gene are different in many respects from other src-family proteins. Two major distinctions are that: (i) two proteins of significantly different size and domain structure are encoded by the same gene; and (ii) each protein lacks the usual structural motif at the amino-terminus for post-translational myristylation. Nonetheless, both the $p66^{Dsrc28C}$ and $p55^{Dsrc28C}$ forms of Dsrc28C protein contain the highly conserved tyrosine kinase domain, including the ATP-binding site and the autophosphoryla-

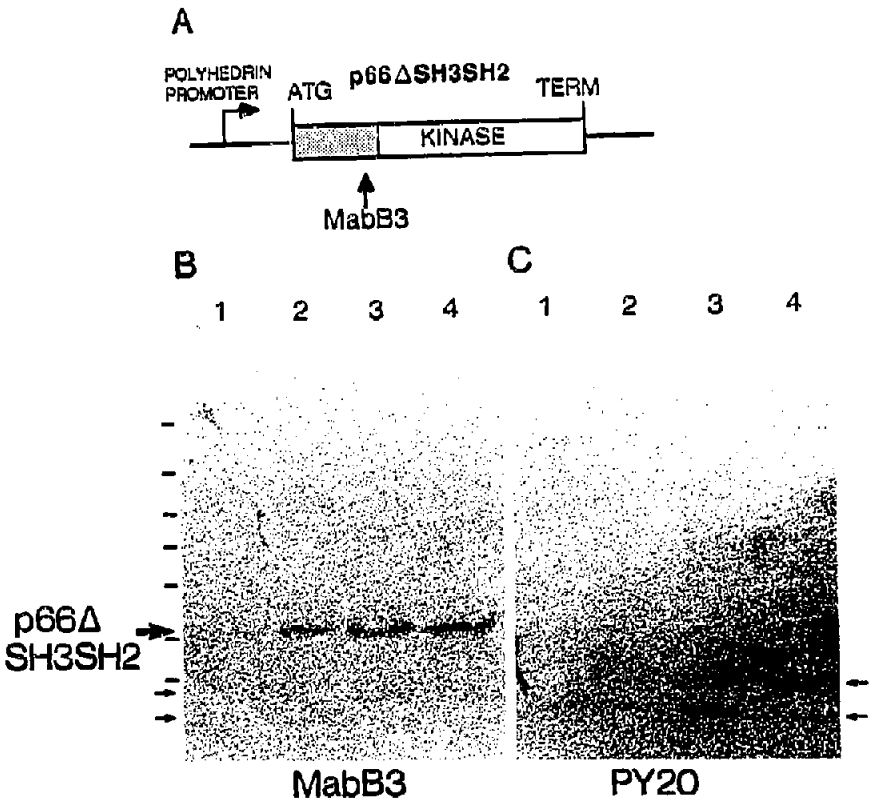

Fig. 3. Expression of $p66^{\Delta SH3SH2}$ deletion mutation protein from recombinant baculovirus and analysis of tyrosine kinase activity. (A) $p66^{\Delta SH3SH2}$ baculovirus expression vector. The indicated initiator and terminator codons are derived from the $p66^{Dsrc28C}$ cDNA. The recognition site of MabB3 is within amino acids 111 and 125 of the $p66^{Dsrc28C}$ protein. The filled block represents the 125 amino acid amino-terminal p66-specific domain. (B) Immunoblot of proteins from Sf9 cells infected with the $p66^{\Delta SH3SH2}$ baculovirus reacted with MabB19. (C) Immunoblot of proteins from Sf9 cells infected with the $p66^{\Delta SH3SH2}$ baculovirus reacted with PY20. Lanes 1–5 contain proteins from infected cells at 24, 32, 40, and 48 h post-infection, respectively. The position of recombinant baculovirus-encoded $p66^{\Delta SH3SH2}$ protein is indicated by the large labeled arrow. Small arrows indicate the positions of either the MabB3 or PY20-stained proteolytic products of $p66^{\Delta SH3SH2}$. The dashes at the left indicate the positions of pre-stained protein markers of 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48 kDa, 37 kDa, and 27 kDa.

tion region corresponding to Tyr-416 in c-src [3]. Based on the conservation of the kinase domain, we have proposed previously that the Dsrc28C proteins function as tyrosine kinases. The results obtained in this study demonstrate that $p66^{Dsrc28C}$ has protein–tyrosine kinase activity. These results have also shown that $p66^{Dsrc28C}$ has properties that differ from conventional src tyrosine kinases. A hallmark of src-family tyrosine kinases is their ability to phosphorylate the tyrosine residue corresponding to Tyr-416 within the c-src protein, in a process generally referred to as autophosphorylation. Tyr-416 is the principal site of phosphorylation in vitro and in vivo in Sf9 cells infected with recombinant $p60^{c\text{-}src}$ baculovirus [12]. Kinase-inactive src is not phosphorylated, thus ruling out Sf9 cell enzymes as the source of tyrosine kinase activity [12]. Interestingly, $p66^{Dsrc28C}$ itself was not a major substrate in vitro or in vivo even though the analog of c-src Tyr-416 is conserved in the protein. The success of the baculovirus expression system in the analysis of $p60^{c\text{-}src}$ eliminates the possibility that high levels of protein–tyrosine phosphatases exist in Sf9 cells that might preclude the detection of phosphotyrosine on $p66^{Dsrc28C}$. Moreover, experiments carried out in parallel demonstrate that the level of $p66^{Dsrc28C}$ protein expression and tyrosine kinase activity surpasses the expression and kinase activity of $p60^{c\text{-}src}$ (data not shown). The lack of in vivo autophosphorylation activity is not an artifact of the baculovirus expression system since $p66^{Dsrc28C}$ expressed in bacteria or yeast and $p66^{Dsrc28C}$ expressed from a heat-shock promoter in adult flies did not exhibit autophosphorylation activity (R.J. Gregory and S.C. Wadsworth, unpublished data). We have also shown that $p66^{Dsrc28C}$ does not exhibit in vitro kinase activity under standard assay conditions. Therefore, all of the available data indicate that the Dsrc28C proteins do not have high-levels of autophosphorylation activity.

The alternate, naturally occurring form of Dsrc28C protein, $p55^{Dsrc28C}$, is not enzymatically active under the assay conditions employed, even though the protein contains a kinase domain identical to that found in $p66^{Dsrc28C}$. The existing data do not allow an adequate explanation of this phenomenon. However, the low kinase activity of the $p66^{\Delta SH3SH2}$ protein offers some in-

sight into this question. On the basis of similar deletion mutations in avian *c-src* [2], the expectation in making this deletion mutant was that regulatory domains would be removed leading to increased kinase activity on cellular proteins. However, in contrast to this expectation, the $p66^{\Delta SH3SH2}$ protein has minimal tyrosine kinase activity as compared to the parent $p66^{Dsrc28C}$ protein. The $p55^{Dsrc28C}$ protein and the $p66^{\Delta SH3SH2}$ protein are essentially reciprocal deletion products of the $p66^{Dsrc28C}$ protein. Thus, it seems that both the amino-terminal 125 amino acids of $p66^{Dsrc28C}$ and the SH3 and SH2 domains are necessary for full kinase activity in the assay systems employed.

Despite the inability to find assay conditions under which $p55^{Dsrc28C}$ is active, it would be premature to conclude that the protein does not function as a tyrosine kinase. The kinase domain is present and it seems likely that the enzyme is active in particular developmental environments. We can conclude, however, that there are significant differences between the activities of $p66^{Dsrc28C}$ and $p55^{Dsrc28C}$. An important question is whether these differences in enzyme activity have important biological consequences. As mentioned above, the two proteins are expressed in unique temporal and spatial patterns [4,13]. The most striking difference in their expression pattern is that $p55^{Dsrc28C}$ is the only form of *Dsrc28C* gene product that is present in the central nervous system. It is possible that the function of $p55^{Dsrc28C}$ is independent of kinase activity as has been shown for the pupal function of the *Drosophila abl* homolog [14]. Alternatively, in light of the restricted activity of $p66^{Dsrc28C}$, it is plausible that the conditions appropriate for $p55^{Dsrc28C}$ kinase activity exist specifically in the cells of the central nervous system.

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