The EMBO Journal vol.7 no.12 pp.3845-3855, 1988

Peptide antibodies to the human c-*fyn* gene product demonstrate pp59$^{c\text{-}fyn}$ is capable of complex formation with the middle-T antigen of polyomavirus

Seng H.Cheng, Robert Harvey, Pearl C.Espino, Kentaro Semba[1], Tadashi Yamamoto[1], Kumao Toyoshima[1] and Alan E.Smith

Laboratory of Cellular Regulation, Integrated Genetics Inc., One Mountain Road, Framingham, MA 01701, USA and [1]The Institute of Medical Science, The University of Tokyo 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan

Communicated by A.E.Smith

The c-*fyn* proto-oncogene is a member of a family of closely related genes of which c-*src* is the prototype. Using peptide antibodies which had been raised against sequences predicted to be specific for the human c-*fyn* gene product, the c-*fyn* protein was identified. It is a tyrosine kinase with apparent mol. wt of 59 kd that is also phosphorylated and myristylated. Like pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$, pp59$^{c\text{-}fyn}$ is able to form a stable complex with middle-T antigen, the transforming protein of polyomavirus. The transformation-defective middle-T mutant NG59, which is unable to associate stably with pp60$^{c\text{-}src}$ does not associate with pp59$^{c\text{-}fyn}$. In contrast to pp60$^{c\text{-}src}$, complex formation with middle-T antigen does not lead to a significant increase in the tyrosine kinase activity of pp59$^{c\text{-}fyn}$. These findings lead us to suggest that middle-T mediated transformation may be a consequence of the deregulation of several members of the *src*-family of protein tyrosine kinases.

Key words: middle-T antigen/peptide antibodies/polyomavirus/pp59$^{c\text{-}fyn}$/*src*-family of protein tyrosine kinases

Introduction

Middle-T antigen, the transforming protein of polyomavirus, is both necessary and sufficient for transformation of established rodent cell lines in culture (for reviews, see Smith and Ely, 1983; Fried and Prives, 1986). Middle-T itself does not possess any known intrinsic catalytic activities. However, it is capable of interacting with and modulating the activities of several proteins (for review, see Cheng *et al.*, 1986b). In polyomavirus infected cells, middle-T is able to associate with cellular proteins to form a complex with ~mol. wt of 200 kd when measured on sucrose density gradients (Walter *et al.*, 1982; Courtneidge and Smith, 1983). Cellular proteins which may constitute part of this complex include the tyrosine kinases pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1983) and pp62$^{c\text{-}yes}$ (Kornbluth *et al.*, 1987), and the p81/85 phosphatidylinositol kinase (Whitman *et al.*, 1985; Kaplan *et al.*, 1986, 1987; Courtneidge and Heber, 1987).

Both pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$ belong to the *src*-family of protein tyrosine kinases which also includes c-*fyn*, c-*fgr*, c-*lck*, c-*hck*, c-*lyn* and c-*tkl* (for review, see Cooper, 1988). Complex formation with middle-T antigen results in activation of the kinase activities of both pp60$^{c\text{-}src}$ (Bolen *et al.*, 1984; Courtneidge, 1985) and pp62$^{c\text{-}yes}$ (Kornbluth *et al.*, 1987). With pp60$^{c\text{-}src}$, this activation may be due in part to the ability of middle-T to stabilize a population of pp60$^{c\text{-}src}$ which is not phosphorylated at Tyr-527 (Cartwright *et al.*, 1986), a site which has been implicated in the regulation of the kinase and transforming activities of the enzyme (Courtneidge, 1985; Cooper and King, 1986; Piwnica-Worms *et al.*, 1987; Kmiecik and Shalloway, 1987; Cartwright *et al.*, 1987; Reynolds *et al.*, 1987; Cheng *et al.*, 1988). Dephosphorylation of Tyr-527, the major site of phosphorylation *in vivo* (Cooper *et al.*, 1986) results in activation of both the tyrosine kinase and transforming activities of pp60$^{c\text{-}src}$.

Analysis of mutants of middle-T antigen indicates that the ability to associate with pp60$^{c\text{-}src}$ is necessary for cellular transformation (Bolen *et al.*, 1984; Courtneidge, 1985; Cheng *et al.*, 1986a; Markland *et al.*, 1986). Recently, it was demonstrated that the region bordered by amino acids 518 and 525 at the carboxy terminus of pp60$^{c\text{-}src}$ is necessary for complex formation (Cheng *et al.*, 1988). The close proximity of this putative middle-T binding domain to Tyr-527 led to the suggestion that a mechanism by which middle-T may activate pp60$^{c\text{-}src}$ is by direct masking of Tyr-527.

The region in pp60$^{c\text{-}src}$ suggested to be involved in complex formation with middle-T is also highly conserved amongst the other *src*-family of tyrosine kinases (Cooper, 1988; see Table I). It is therefore conceivable that other members of the family may be equally capable of associating

Table I. Predicted sequences of the carboxy termini of the *src*-family of protein tyrosine kinases

c-*src*	A F L E D Y F T S T E P Q Y Q P G E N L	(533)
c-*yes*	S F L E D Y F T A T E P Q Y Q P G E N L	(543)
c-*fyn*	S F L E D Y F T A T E P Q Y Q P G E N L	(537)
c-*fgr*	S F L E D Y F T S A E P Q Y Q P G D Q T	(529)
c-*lck*	S V L D D F F T A T E G Q Y Q P Q P	(509)
c-*hck*	S V L D D F Y T A T E S Q Y Q Q Q P	(505)
c-*lyn*	S V L D D F Y T A T E G Q Y Q Q Q P	(512)
c-*tkl*	S V L E D F F T A T E G Q Y Q Q Q P	(457)
Consensus	S F E YF AT P P G E N L L D T E QYQ A V D FY S A G QQP – – * * *	

* End of kinase domain

** Phosphorylated tyrosine

The amino acids of pp60$^{c\text{-}src}$ within the boxed region have been shown to be necessary for complex formation with middle-T antigen (Cheng *et al.*, 1988). This table has been adapted from the review of Cooper (1988).

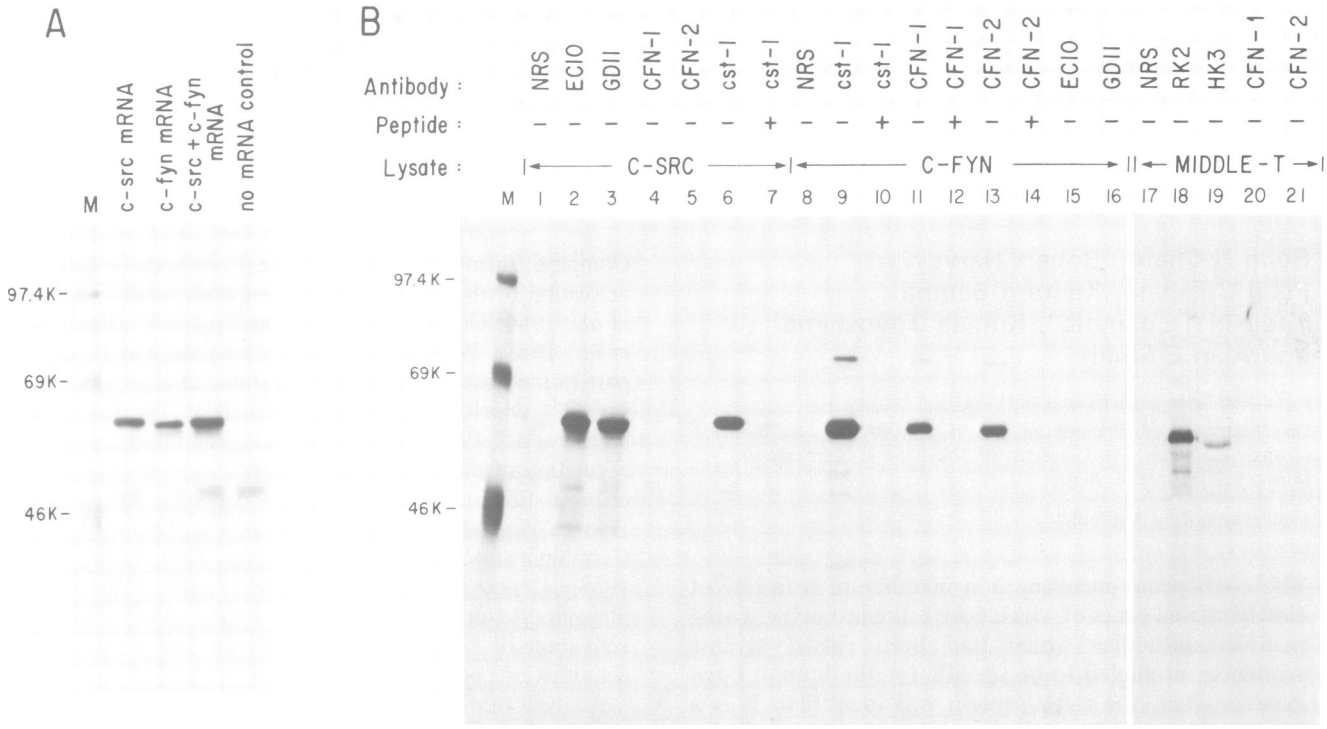

Fig. 1. Specificity of the c-*fyn* peptide antibodies. pSP65 c-src, pSP65 c-fyn and pSP64 MT containing the cDNAs for the avian c-*src*, human c-*fyn* and middle-T respectively, were transcribed with SP6 RNA polymerase. Following purification, the RNA transcripts were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Samples were then either analyzed directly by SDS−PAGE (**A**) or immunoprecipitated with antibodies raised against pp60$^{c\text{-}src}$, the c-*fyn* gene product or middle-T antigen (**B**). **Lanes 1−7** were immunoprecipitates from lysates containing *in vitro* synthesized c-*src*, **lanes 8−16** were from lysates containing *in vitro* translated c-*fyn* and **lanes 17−21** were from middle-T containing lysates. NRS, non-immune rabbit serum; EC10 and GD11 are monoclonal antibodies specific for pp60$^{c\text{-}src}$ (Parsons *et al.*, 1984); CFN-1 and CFN-2 are peptide antibodies raised against sequences unique to human c-*fyn*; cst-1 is a peptide antibody raised against the carboxy-terminal seven amino acids of pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1984); RK2 and HK3 are antisera from a rat and hamster respectively, bearing tumors induced by polyomavirus (Cheng *et al.*, 1986a). When peptide antisera were used, (+) indicates that the immunoprecipitation reactions were performed in the presence of the cognate peptides and (−) in the absence of added peptides. A minor band of ~75 kd was also translated by the c-*fyn* RNA transcripts. The origin of this band is unclear but the protein was shown to be *fyn*-related by peptide mapping (data not shown). **Lanes M** contain molecular size markers; sizes are on the left in kilodaltons (K). The [^{35}S]methionine gels were fluorographed and exposed for 24 h.

with and of being activated by middle-T. The report that pp62$^{c\text{-}yes}$ is also able to bind middle-T supports this suggestion (Kornbluth *et al.*, 1987). To examine this hypothesis in greater detail and also ultimately to ascertain the relative contribution of each of these proto-oncogenes towards middle-T mediated transformation, we generated peptide antibodies specific for the recently described c-*fyn* gene product (Semba *et al.*, 1986; Kawakami *et al.*, 1986).

The human c-*fyn* was initially detected in a normal placental library using a 1.5 kb v-*yes* DNA fragment, as a probe (Semba *et al.*, 1985; 1986). Unlike c-*src* and c-*yes*, no naturally occurring mutated and oncogenic version of c-*fyn* has been described. The predicted carboxy terminus of c-*fyn* shares extensive amino acid sequence homology with c-*src* but is very different within the amino terminal 81 amino acids. The corresponding sequences surrounding Tyr-527 of pp60$^{c\text{-}src}$ are conserved in c-*fyn* (Table I) and thus may be similarly regulated by phosphorylation.

To investigate the biochemical activities associated with the c-*fyn* gene product in greater detail and to probe whether it too is able to interact with and be activated by middle-T, we have generated antibodies against synthetic peptides corresponding to sequences predicted to be unique to the human c-*fyn* gene product.

Results

Specificity of the anti-c-fyn peptide sera CFN-1 and CFN-2

Except for the amino terminal 81 amino acids, the predicted sequences of the *src*-family of tyrosine kinases are highly homologous throughout (Cooper, 1988). By using peptide sequences corresponding to residues 7−19 (CFN-1) and 16−30 (CFN-2) of human c-*fyn* (Semba *et al.*, 1986) as antigens, peptide antibodies specific for the c-*fyn* gene product were generated. The specificity of both CFN-1 and CFN-2 sera were initially analyzed by monitoring their ability to immunoprecipitate avian c-*src* and c-*fyn* proteins which had been produced by transcription *in vitro* followed by translation in rabbit reticulocyte lysates. The c-*fyn* *in vitro* product exhibited a slightly smaller mol. wt than that for c-*src* (Figure 1A).

EC10 and GD11 are monoclonal antibodies against avian pp60$^{c\text{-}src}$ (Parsons *et al.*, 1984) and as expected were able to immunoprecipitate the translation product from the reticulocyte lysates containing c-*src* transcripts (Figure 1B, lanes 2 and 3) but not from those containing c-*fyn* transcripts (Figure 1B, lanes 15 and 16). In contrast, neither CFN-1 nor CFN-2 were able to immunoprecipitate *in vitro* translated

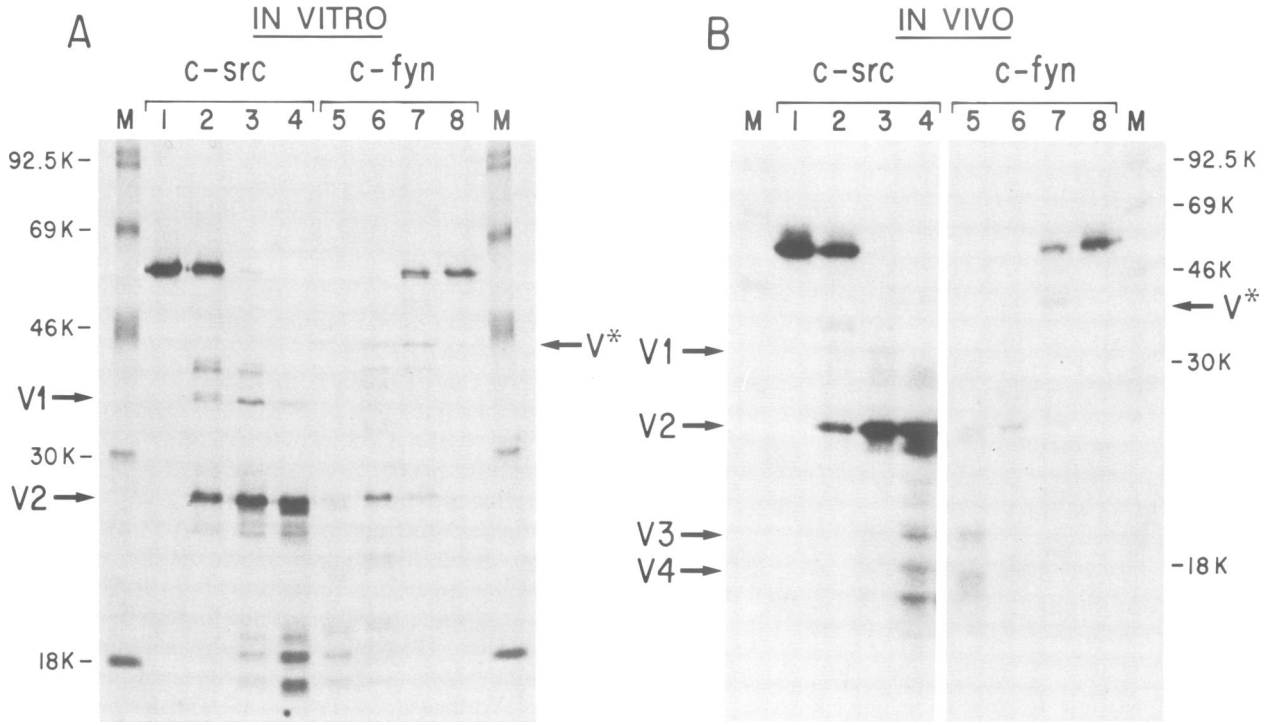

Fig. 2. One-dimensional peptide analysis of pp59$^{c\text{-}fyn}$ and pp60$^{c\text{-}src}$ (**A**) c-*src* and c-*fyn* proteins which had been synthesized *in vitro* in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine were immunoprecipitated with EC10 and CFN-2 antisera respectively, extracted from the polyacrylamide gels, digested with *S.aureus* V8 protease and then electrophoresed on a 15% polyacrylamide gel. (**B**) The products of *in vitro* kinase reactions (see Materials and methods) of immunoprecipitates obtained using either EC10 or CFN-2 antisera from a pp60$^{c\text{-}src}$ or pp59$^{c\text{-}fyn}$ (TR # 2) NIH 3T3 overproducer cell line respectively, were subjected to digestion with increasing amounts of *S.aureus* V8 protease. Proteins in **lanes 2** and **7** were digested with 50 ng of *S.aureus* V8 protease, proteins in **lanes 3** and **6** were digested with 500 ng of enzyme and those in **lanes 4** and **5** were digested with 5 μg of enzyme. **Lanes 1** and **8** were untreated samples. **Lanes M** contain molecular size markers; sizes are in kilodaltons (K). Exposure time was 3 days for **panel A** and 24 h for **panel B**.

pp60$^{c\text{-}src}$ (Figure 1B, lanes 4 and 5) but were capable of recognizing the c-*fyn* product (Figure 1B, lanes 11 and 13). Furthermore, the ability to immunoprecipitate the c-*fyn* protein could be specifically inhibited when the corresponding peptides were included in the reaction (Figure 1B, lanes 12 and 14). The previously reported peptide antiserum, cst-1, was directed against a synthetic peptide corresponding to residues 527–533 of pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1984). These residues are homologous in the corresponding region in c-*fyn*. As expected cst-1 was found to be capable of immunoprecipitating both the c-*src* and c-*fyn* *in vitro* translated products (Figure 1B, lanes 6 and 9). We have also extended this analysis and showed that neither CFN-1 nor CFN-2 were capable of recognizing middle-T antigen (Figure 1B, lanes 20 and 21). Therefore by raising antibodies against peptides with sequences unique to human c-*fyn*, we were successful in generating peptide antisera specific for c-*fyn* and which did not cross-react with pp60$^{c\text{-}src}$ or middle-T antigen.

Characterization of the in vitro synthesized c-src and c-fyn gene products

To characterize further the proteins synthesized *in vitro*, partial proteolytic digestion with *Staphylococcus aureus* V8 protease was performed. The *in vitro* translated c-*fyn* protein displayed only a slightly different *S.aureus* V8 digestion pattern from that for *in vitro* synthesized pp60$^{c\text{-}src}$ (Figure 2A). This was expected since the predicted amino acid sequences for both the c-*src* and c-*fyn* gene products exhibit extensive homology. The amino acid sequences of the two proteins are predicted to differ most significantly at the amino terminus. In accord with this, the equivalent in c-*fyn* of the V1 fragment of pp60$^{c\text{-}src}$ (which corresponds to the amino-terminal portion of the protein—Collett *et al.*, 1979) migrated with a different mobility when analyzed on polyacrylamide gels (Figure 2A). The amino terminal equivalent V1 fragment of the c-*fyn* protein has not been unequivocally identified. However, *S.aureus* V8 protease digestion of the c-*fyn* protein generated a 44-kd peptide (V*) which was absent in pp60$^{c\text{-}src}$. This V* fragment was phosphorylated (Figure 2B, see below) and is a candidate for the V1 fragment equivalent of the c-*fyn* protein. As expected, since the carboxy termini of both proteins are highly conserved, the V2 bands (corresponding to the carboxy terminal portion of the proteins) were indistinguishable.

To ascertain whether the c-*fyn* protein exhibited intrinsic kinase activity, immune-complex kinase assays were performed on immunoprecipitates obtained from lysates containing *in vitro* synthesized c-*fyn* using either cst-1, CFN-1 or CFN-2 antisera (Figure 3). As in the case of pp60$^{c\text{-}src}$, the *in vitro* translated c-*fyn* protein was capable of being autophosphorylated suggesting that the kinase activity is intrinsic to the c-*fyn* gene product.

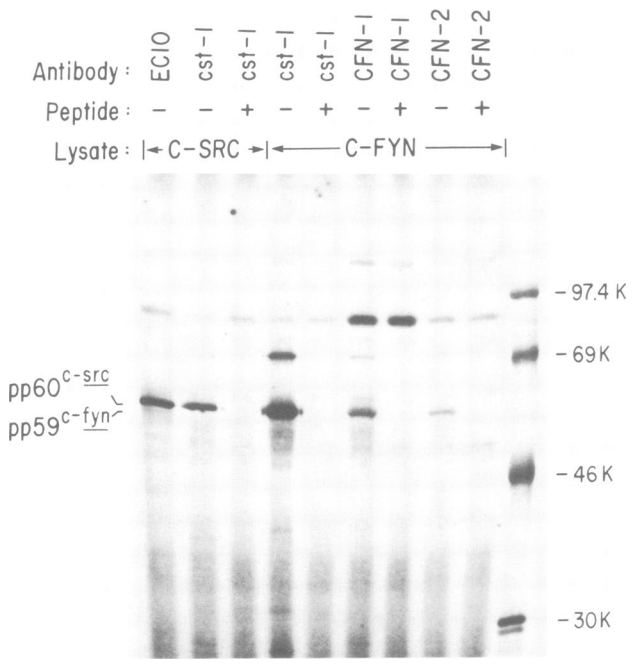

Fig. 3. Kinase assays of c-*fyn* and c-*src* synthesized *in vitro*. The c-*src* and c-*fyn* RNA transcripts were translated *in vitro* in the rabbit reticulocyte lysate in the absence of [^{35}S]methionine. Immunoprecipitates obtained from either the c-*src* (using EC10 or cst-1 antisera) or c-*fyn* (using CFN-1, CFN-2 or cst-1 antisera) containing lysates were assayed for kinase activity. (+) indicates that the kinase reactions were performed on immunoprecipitates obtained using antisera which had been preblocked with the cognate peptides and (−) in the absence of peptides. Autoradiography was for 24 h.

Biological and biochemical characterization of the human c-fyn gene product in NIH 3T3 cells

To investigate the biological and biochemical properties of the human c-*fyn* protein, stable cell lines expressing the c-*fyn* gene product were generated. This was achieved by cloning the human c-*fyn* cDNA (Semba *et al.*, 1986) into pLJ, a murine retroviral expression vector (Piwnica-Worms *et al.*, 1987). Recombinant retroviruses were generated by transfection of the pLJ c-*fyn* into ψ-2 packaging cells which were then used to infect NIH 3T3 cells. Following infection, G418 selection was imposed and the drug-resistant colonies clonally expanded.

Identification of a 59-kd protein as the human c-fyn gene product

G418-resistant NIH 3T3 cells were screened for the presence of the c-*fyn* gene product using the CFN-1 and CFN-2 antibodies. Cell lysates were reacted with the anti-c-*fyn* peptide sera and the immunoprecipitates subjected to *in vitro* kinase reactions. The analysis of one such cell line TR # 2, the highest overproducer, is shown in Figure 4. Immune complex kinase assays of TR # 2 cells using both the c-*fyn*-specific antibodies revealed a 59-kd protein in the lysates (Figure 4A, lanes 5 and 7). The 59-kd band like the *in vitro* translated material migrated slightly faster than pp60$^{c\text{-}src}$ (Figure 4A, compare lanes 5 and 7 with 10). The fact that CFN-1 and CFN-2 sera which were raised against two adjacent regions were both capable of immunoprecipitating the same protein, argues that the 59-kd protein is the c-*fyn* gene product. Further corroborative evidence was provided by partial proteolysis. Analysis of the 59-kd band using V8 protease (Figure 2B) demonstrated it had a digestion profile similar to that observed for *in vitro* translated c-*fyn* but different from that for pp60$^{c\text{-}src}$ (Figure 2). Like the *in vitro* synthesized c-*fyn* protein, the 59-kd band displayed a 44-kd V* fragment which is absent in pp60$^{c\text{-}src}$.

Phosphoamino acid analysis of the 59-kd band revealed that it was phosphorylated primarily on tyrosine residues (Figure 4B). Together these data strongly suggest that the human c-*fyn* gene product is a tyrosine kinase with apparent mol. wt of 59 kd.

Overexpression of the human pp59$^{c\text{-}fyn}$ does not transform NIH 3T3 cells

Overexpression of pp60$^{c\text{-}src}$ to 15-fold above the normal level does not transform NIH 3T3 cells (Shalloway *et al.*, 1984; Iba *et al.*, 1985; Piwnica-Worms *et al.*, 1986). However, like pp60$^{c\text{-}src}$, variant forms of pp59$^{c\text{-}fyn}$ exhibit transforming activity (Kawakami *et al.*, 1986) and a recent report suggests that normal overexpression of pp59$^{c\text{-}fyn}$ is sufficient for induction of morphologic transformation, albeit at a very low frequency (Kawakami *et al.*, 1988). To assay the focus forming activity of the human pp59$^{c\text{-}fyn}$, we infected NIH 3T3 cells with recombinant retroviruses encoding the human c-*fyn* gene essentially as described by Piwnica-Worms *et al.* (1987) and Cheng *et al.* (1988). Multiple infections using different clones of recombinant retroviruses encoding the c-*fyn* gene (titers ranging between 4×10^3 and 3×10^4 G418-resistant colonies/ml) however showed no focus forming activity.

It was not possible to quantitate the level of expression of human pp59$^{c\text{-}fyn}$ over the endogenous murine counterpart since neither CFN-1 nor CFN-2 were capable of detecting pp59$^{c\text{-}fyn}$ kinase in the parent NIH 3T3 cells (Figure 4A, lane 2). This could either be due to the low level of expression of the endogenous pp59$^{c\text{-}fyn}$ in NIH 3T3 cells or to the inability of our antibodies to recognize the murine equivalent. Irrespective, overexpression of the human pp59$^{c\text{-}fyn}$ in NIH 3T3 cells, as in the TR # 2 cell line, did not induce either any observable morphological changes or focus formation.

pp59$^{c\text{-}fyn}$ is a phosphoprotein and is myristylated

Immunoprecipitation of ^{32}P-labeled TR # 2 cells using CFN-2 serum also identified a 59-kd band indicating that pp59$^{c\text{-}fyn}$, like pp60$^{c\text{-}src}$ is a phosphoprotein (Figure 5A). Furthermore, pp59$^{c\text{-}fyn}$ could also be labeled *in vivo* with [^{3}H]myristic acid, presumably at Gly-2 (Figure 5B). These results are consistent with the notion that pp59$^{c\text{-}fyn}$, like pp60$^{c\text{-}src}$ (Buss and Sefton, 1985; Cooper *et al.*, 1986), may be regulated by phosphorylation and is probably associated with the cellular membranes.

pp59$^{c\text{-}fyn}$ is capable of complex formation with the middle-T antigen of polyomavirus

Biochemical analysis of mutants of middle-T antigen indicates that its ability to associate with pp60$^{c\text{-}src}$ is necessary for cellular transformation (Bolen *et al.*, 1984; Courtneidge and Smith, 1984; Cheng *et al.*, 1986a; Markland and Smith, 1987). Recently, we demonstrated that a region at the carboxy terminus of pp60$^{c\text{-}src}$ is necessary for stable interaction with middle-T antigen (Cheng *et al.*, 1988). Since this region is highly conserved amongst the *src*-family of tyrosine kinases, it was possible that pp59$^{c\text{-}fyn}$ may

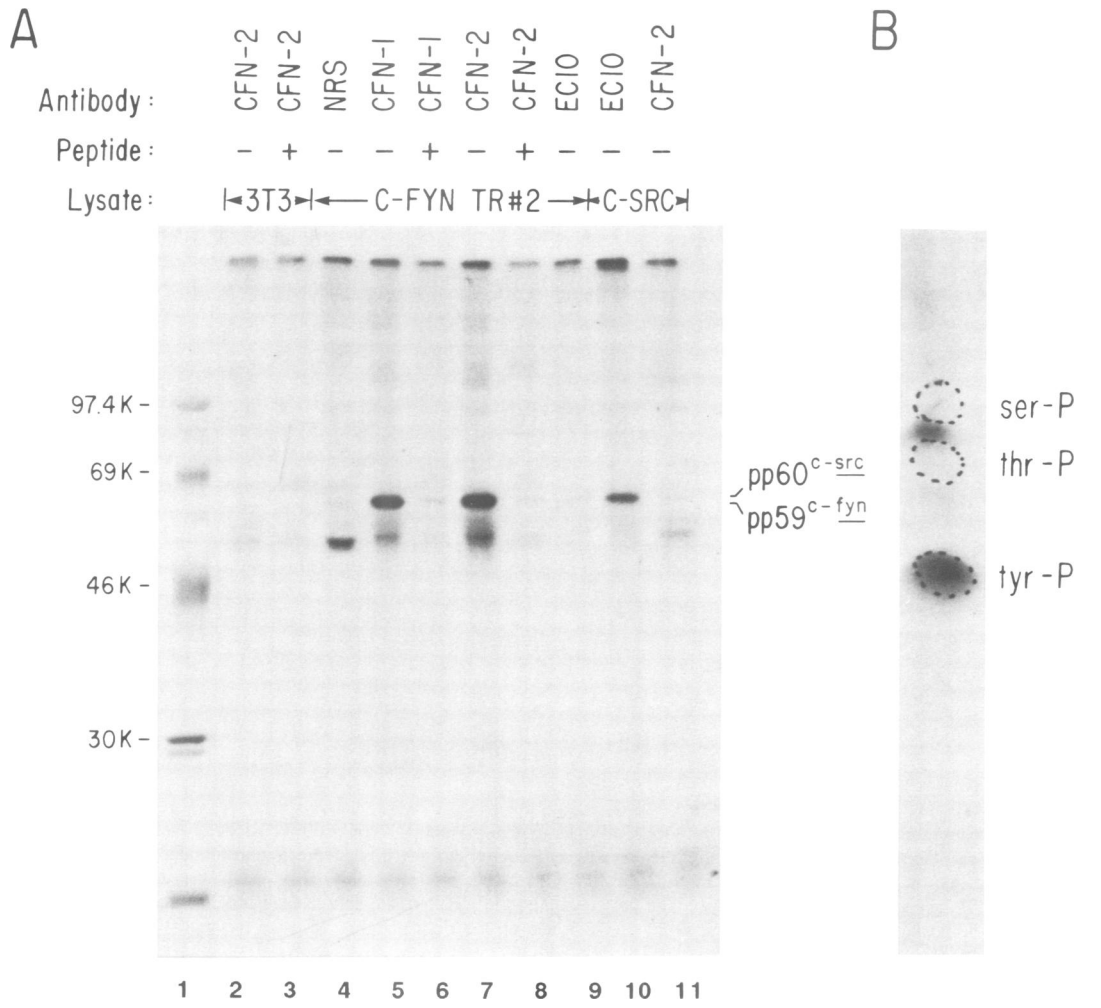

Fig. 4. Immunoprecipitation and phosphoamino acid analysis of the 59-kd protein from TR # 2 cell lysates using CFN-1 and CFN-2 peptide antibodies. (**A**) Cell lysates from normal NIH 3T3 cells and NIH 3T3 cells expressing the human c-*fyn* (TR # 2) and avian c-*src* gene products were prepared and immunoprecipitated with either NRS, CFN-1, CFN-2 or EC10 antisera. Kinase assays were performed and the reaction products then resolved on a 9% SDS−polyacrylamide gel. Antisera were either preblocked (+) or not (−) with the corresponding peptides. The position of pp59$^{c\text{-}fyn}$ and pp60$^{c\text{-}src}$ is indicated on the margin. Autoradiography was for 5 h. (**B**) Phosphoamino acid analysis was performed on the 59-kd protein phosphorylated *in vitro* in anti-CFN-2 immunoprecipitates as described in Materials and methods. Positions of ninhydrin stained phosphoamino acid standards are indicated by discontinuous lines. Ser-P, phosphoserine; Thr-P, phosphothreonine; Tyr-P, phosphotyrosine. Exposure time was 2 days.

similarly be capable of associating with middle-T antigen.

To ascertain whether pp59$^{c\text{-}fyn}$ was able to form a complex with middle-T, the cell line TR # 2 was either mock infected or infected with polyomavirus (Figure 6). At 24 h post infection, cell lysates were prepared and normalized for protein content. Kinase assays were performed on immunoprecipitates obtained by using either HK3 serum, an antiserum specific for the early region of polyomavirus or by CFN-2 serum, which recognizes the human pp59$^{c\text{-}fyn}$. Complex formation was monitored by the ability of immunoprecipitates obtained by using CFN-2 to phosphorylate middle-T in the kinase assay. As a control, an avian pp60$^{c\text{-}src}$ overproducer cell line was also mock infected and infected with polyomavirus. Lysates derived from these cell lines were analyzed using the monoclonal antibody EC10 which is able to recognize avian pp60$^{c\text{-}src}$ (Parsons *et al.*, 1984; Cheng *et al.*, 1988).

Figure 6A shows *in vitro* kinase assays of immunoprecipitates obtained using HK3. The signal observed represents the total kinase active fraction of middle-T present in these lines following infection. *In vitro* kinase assays of immunoprecipitates obtained using the CFN-2 serum, like that for pp60$^{c\text{-}src}$ revealed an additional phosphorylated band (~58 kd) which was absent in the mock infected sample and which comigrated with middle-T (Figure 6B, lane 5). However, on longer exposures, a trace signal could be observed in the mock infected c-*fyn* sample (Figure 6B, lane 4). This band most likely represents phosphorylated immunoglobulin heavy chain. As a consequence, similar samples were also analyzed under non-reducing conditions (Figure 6C). When treated under these conditions, no signal was observed in the mock infected sample and it was very clear that middle-T was co-immunoprecipitated with pp59$^{c\text{-}fyn}$ (Figure 6C, lane 5). That the 58-kd phosphorylated band was indeed middle-T antigen was confirmed by comparing its *S.aureus* V8 protease digestion profile with that of authentic middle-T (Figure 7) and with published maps (Harvey *et al.*, 1984). These findings indicate that pp59$^{c\text{-}fyn}$ is capable of associating with middle-T antigen.

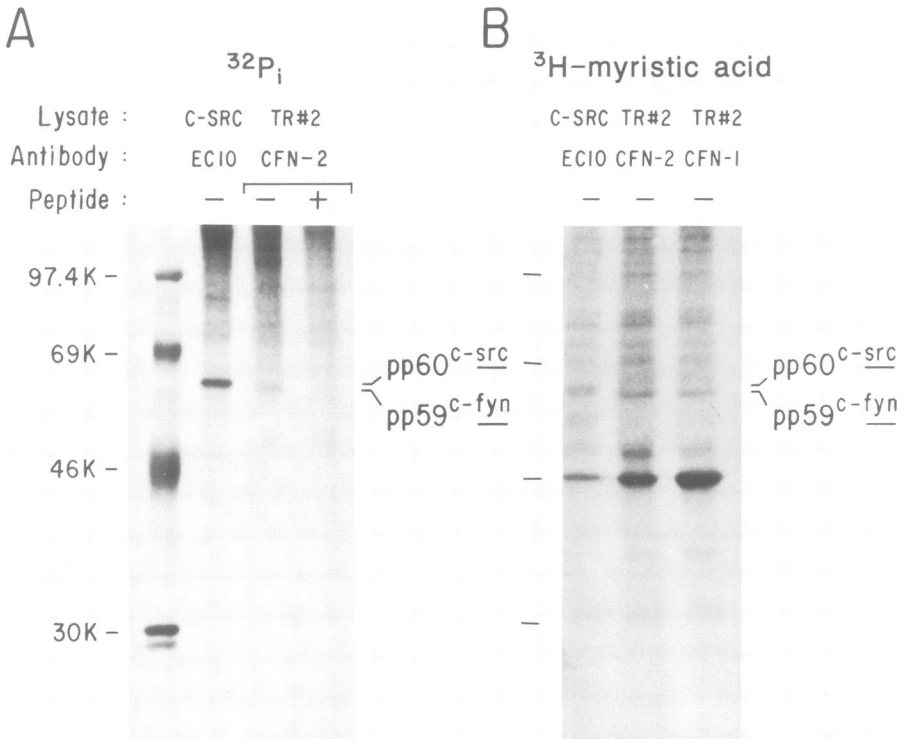

Fig. 5. pp59$^{c\text{-}fyn}$ is a phosphoprotein and is myristylated *in vivo*. Cell lines expressing pp60$^{c\text{-}src}$ or pp59$^{c\text{-}fyn}$ (TR # 2) were labeled with $^{32}P_i$ (**A**) or [^{3}H]myristic acid (**B**) as described in Materials and methods. Lysates were prepared and the immunoprecipitates obtained by using EC10 from the pp60$^{c\text{-}src}$ overproducer cell line or by using CFN-1 and CFN-2 from the TR # 2 cell line were analyzed on 9% SDS–polyacrylamide gels. Autoradiography for the $^{32}P_i$-labeled proteins was for 24 h. The gel containing the [^{3}H]myristic acid-labeled proteins was fluorographed and exposed for 4 weeks at −70°C. The positions for pp59$^{c\text{-}fyn}$ and pp60$^{c\text{-}src}$ are indicated on the right margins. Sizes of the mol. wt markers are indicated on the left margin in kilodaltons (K).

Although both the infected c-*src* and c-*fyn* overproducer cell lines displayed approximately equivalent amounts of kinase activity associated with middle-T (Figure 6A, lanes 3 and 5), much more middle-T appeared to be phosphorylated by pp60$^{c\text{-}src}$ than by pp59$^{c\text{-}fyn}$ (Figure 6B and C, compare lanes 3 and 5). This suggested that perhaps more pp60$^{c\text{-}src}$ than pp59$^{c\text{-}fyn}$ was capable of complex formation with middle-T antigen. However, we cannot discount the possibility that middle-T may be a less efficient substrate for pp59$^{c\text{-}fyn}$ than for pp60$^{c\text{-}src}$.

The middle-T:pp59$^{c\text{-}fyn}$ complex migrates with mol. wt of ~200 kd on sucrose density gradients

That the pp59$^{c\text{-}fyn}$ was capable of forming a stable complex with middle-T was confirmed by sucrose density gradient analysis of the cell lysates. When the fractionated cell extract of the TR # 2 cell line was assayed with CFN-2 serum, the kinase activity was detected in fractions (3–7) corresponding to a mol. wt of ~60 kd consistent with the active form of the pp59$^{c\text{-}fyn}$ kinase being monomeric (Figure 8C). This is similar to that observed for the pp60$^{c\text{-}src}$ kinase (Figure 8B). By contrast when fractions form the polyomavirus infected TR # 2 cells were assayed, the pp59$^{c\text{-}fyn}$ kinase sedimented over a broad range of the gradient (fractions 3–18) with two discernible peaks of activity (Figure 8D). One peak sedimented at the same position as pp59$^{c\text{-}fyn}$ from the uninfected cells, whereas the second peak sedimented at ~200 kd, similar to that observed for the middle-T associated kinase (Figure 8A). This sedimentation profile was very similar to that observed for the middle-T:pp60$^{c\text{-}src}$ complex (data not shown). Hence infection of TR # 2 with polyomavirus caused a fraction of pp59$^{c\text{-}fyn}$ to sediment more rapidly in a position comigrating with the kinase active fraction of middle-T. We interpret this to mean that pp59$^{c\text{-}fyn}$ forms a stable complex with middle-T.

pp59$^{c\text{-}fyn}$ is unable to associate with the middle-T encoded by NG59

To evaluate the role of pp59$^{c\text{-}fyn}$ in middle-T transformation, the ability of pp59$^{c\text{-}fyn}$ to associate with NG59, a transformation-defective middle-T mutant, was examined. The middle-T encoded by the hr-t mutant, NG59, also lacks *in vitro* kinase activity and stably associated middle-T:pp60$^{c\text{-}src}$ (Carmichael and Benjamin, 1980; Courtneidge and Smith, 1984; Cheng *et al.*, 1986a). Extracts from TR # 2 cells which had been infected with NG59 virus were fractionated on a sucrose density gradient and the fractions immunoprecipitated with CFN-2 antibody. As shown in Figure 8E, pp59$^{c\text{-}fyn}$ from NG59 infected cells sedimented as a single peak at ~60 kd, consistent with it being a monomer in these cells.

Complex formation with middle-T antigen does not significantly activate the tyrosine kinase activity of pp59$^{c\text{-}fyn}$

The tyrosine kinase activity of pp60$^{c\text{-}src}$ in complex with middle-T is elevated several-fold over that of the unassociated counterpart (Figure 9A). To investigate whether the kinase

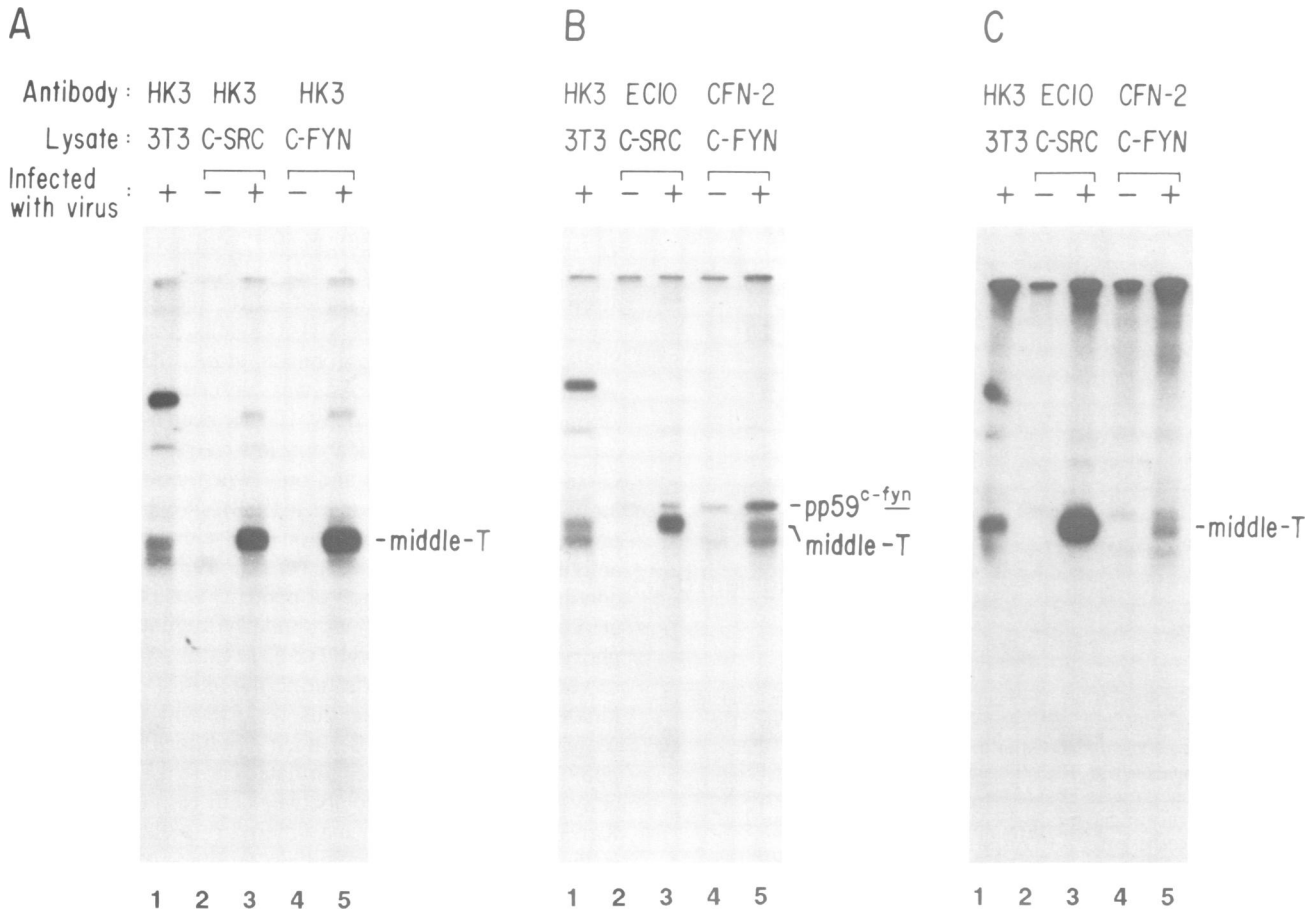

Fig. 6. Association of pp59$^{c\text{-}fyn}$ with middle-T antigen. Normal NIH 3T3 cells and cells expressing pp59$^{c\text{-}fyn}$ or pp60$^{c\text{-}src}$ were either mock infected (−) or infected with polyomavirus (+). At 24 h post-infection, lysates were prepared, normalized for protein content, immunoprecipitated with either HK3, EC10 or CFN-2 antisera and phosphorylated *in vitro* by using [γ-^{32}P]ATP. **Panel C** is similar to **panel B** except that the samples in **panel C** were treated under non-reducing conditions prior to electrophoresis. The positions of pp59$^{c\text{-}fyn}$ and middle-T antigen are indicated on the right margins. Autoradiography was for 4 h.

activity of pp59$^{c\text{-}fyn}$ was enhanced by middle-T binding, the abilities of pp59$^{c\text{-}fyn}$ from mock infected and from polyomavirus infected TR#2 cells to phosphorylate an exogenous substrate were compared (Figure 9A). Examination of the activity by enolase phosphorylation demonstrated that unlike pp60$^{c\text{-}src}$, middle-T binding only induced a very small increase in the kinase activity of complexed pp59$^{c\text{-}fyn}$ when compared to the free form. Quantitation from an average of three experiments revealed a 1.4-fold enhancement in the pp59$^{c\text{-}fyn}$ kinase activity in samples containing complexed pp59$^{c\text{-}fyn}$ over that containing only free pp59$^{c\text{-}fyn}$ (Figure 9B). It is, however, undetermined whether this was because a smaller amount of pp59$^{c\text{-}fyn}$ than pp60$^{c\text{-}src}$ was in complex with middle-T or because pp 59$^{c\text{-}fyn}$ kinase is not activated to the same degree as pp60$^{c\text{-}src}$ by middle-T. Furthermore, it could be that enolase may be a better substrate for pp60$^{c\text{-}src}$ than for pp59$^{c\text{-}fyn}$.

Discussion

Members of the *src*-family encode highly related protein tyrosine kinases which may participate in the control of cell proliferation. Recently, we and others have demonstrated that the tyrosine kinase and transforming activities of pp60$^{c\text{-}src}$ are suppressed by phosphorylation of Tyr-527 (Courtneidge, 1985; Cooper and King, 1986; Piwnica-Worms *et al.*, 1987; Kmiecik and Shalloway, 1987; Cartwright *et al.*, 1987; Reynolds *et al.*, 1987; Cheng *et al.*, 1988). Since all the *src*-like protein kinases contain a tyrosine residue at the site equivalent to that of Tyr-527 of pp60$^{c\text{-}src}$, it is possible that they may be subjected to similar regulation. The observation that the mutant *lck* proto-oncogene product, pp56$^{lck\text{-}F505}$ which harbors an altered Tyr-505 (Tyr−Phe) is also transforming in NIH 3T3 cells lends credence to this supposition (Marth *et al.*, 1988; Amrein and Sefton, 1988).

pp60$^{c\text{-}src}$ which is in complex with polyomavirus middle-T antigen differs from the unassociated counterpart in two ways: (i) the kinase activity of complexed pp60$^{c\text{-}src}$ is enhanced several-fold (Bolen *et al.*, 1984; Courtneidge, 1985) and (ii) complexed pp60$^{c\text{-}src}$ lacks phosphorylation at Tyr-527 but is phosphorylated at Tyr-416 (Cartwright *et al.*, 1986). Hence middle-T binding abrogates phosphorylation of Tyr-527 and this, it is supposed, leads to activation of the tyrosine kinase and transforming activities of the complexed pp60$^{c\text{-}src}$. This, coupled with the recent demonstration that the putative middle-T binding domain may reside immediately upstream of Tyr-527, suggests to us that a mechanism by which middle-T may activate pp60$^{c\text{-}src}$ is by physically blocking the phosphorylation of this residue (Cheng *et al.*, 1988). Prompted by the observation that this domain is also highly conserved in the tyrosine kinases of the *src*-family, we asked here whether the c-*fyn* gene product,

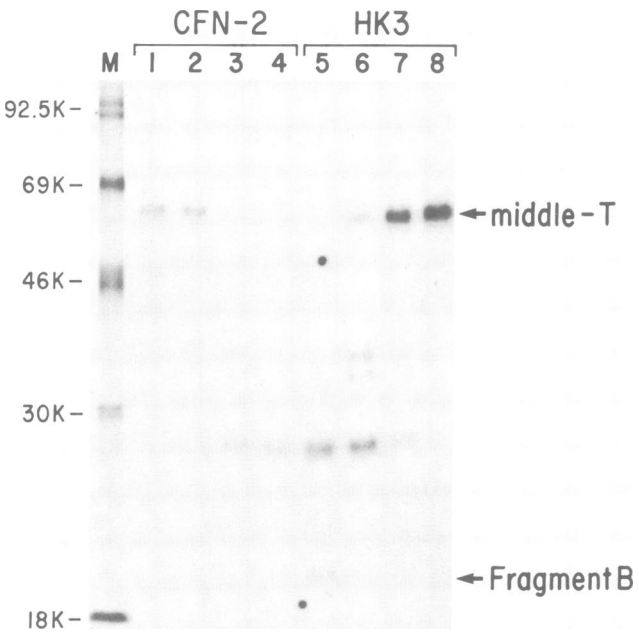

Fig. 7. One dimensional peptide analysis of the 58-kd protein (middle-T) co-immunoprecipitated with pp59$^{c\text{-}fyn}$ using CFN-2 antibody. The 58-kd protein (middle-T) labeled in the *in vitro* kinase assay after immunoprecipitation with HK3 (Figure 6B, lane 1) or with CFN-2 (Figure 6B, lane 5) were excised and then subjected to *S.aureus* V8 protease digestion exactly as described in the legend to Figure 2. Fragment B indicates the ~19-kd partial cleavage product characteristic of wild type middle-T (Harvey *et al.*, 1984). **Lane M** contains molecular size markers; sizes are in kilodaltons (K). Exposure time was 3 days.

a member of this family, is equally capable of complexing with and being activated by middle-T binding.

Identification of the human c-fyn gene product as a tyrosine kinase of 59 kd

We first identified and characterized the human c-*fyn* gene product using peptide antibodies specific for the c-*fyn* protein. Specificity was demonstrated by the observation that the antibodies were only able to recognize c-*fyn* but not c-*src* or middle-T translated *in vitro* in a rabbit reticulocyte lysate. Using these antibodies, we identified the human c-*fyn* product expressed in NIH 3T3 cells as a 59-kd phosphoprotein with intrinsic tyrosine kinase activity. The protein is also myristylated, presumably at Gly-2.

The close structural conservation between pp60$^{c\text{-}src}$ and pp59$^{c\text{-}fyn}$ argues that they share similar functional properties. It is, however, difficult to rationalize the need for both these proteins and potentially for at least six other *src*-like tyrosine kinases in the mammalian genome if all have identical functions. One suggestion is that they may differ in substrate specificity or regulation and perhaps have specialized functions in different tissues. In accord with this suggestion, members of this family have been shown to be present at different levels in different tissues (for review, see Cooper, 1988). Hence it is possible that several members of the family may be present in a particular tissue, but because they are subject to different regulation, only certain members are activated and of these, only those for which appropriate substrates are present, are physiologically relevant. Furthermore, as was recently demonstrated by Sudol *et al.* (1988), c-*yes* and c-*src* display a differential pattern of expression in neural tissues leading them to speculate that perhaps the two proteins may perform similar functions but at different times. These various possibilities warrant further study.

Expression of the human pp59$^{c\text{-}fyn}$ in NIH 3T3 cells

In this study, the effect of overexpression of the human c-*fyn* cDNA in NIH 3T3 cells was investigated. Unlike the report by Kawakami *et al.* (1988), pp59$^{c\text{-}fyn}$ did not display focus-forming activity using the assay described here. Similar discrepancies have been reported for the effect of over-expression of pp60$^{c\text{-}src}$ in NIH 3T3 cells (Iba *et al.*, 1984; Shalloway *et al.*, 1984; Johnson *et al.*, 1985; Piwnica-Worms *et al.*, 1986). As was described for pp60$^{c\text{-}src}$ (Johnson *et al.*, 1985), pp59$^{c\text{-}fyn}$ has been reported to be capable of inducing foci at a very low frequency (1% of total G418-resistant colonies) and only when expressed at very high levels (Kawakami *et al.*, 1988). We have observed previously that overexpression can enhance the kinase activity of pp60$^{c\text{-}src}$ (Cheng *et al.*, 1988). Under these conditions, a small fraction of pp60$^{c\text{-}src}$ was phosphorylated at Tyr-416. If pp59$^{c\text{-}fyn}$ is similarly regulated by phosphorylation at the carboxyl Tyr-531, expression of pp59$^{c\text{-}fyn}$ to very high levels may saturate the ability of the cellular kinases to phosphorylate this residue and thereby down regulate its activity. Under such conditions, a fraction of pp59$^{c\text{-}fyn}$ may be unphosphorylated at Tyr-531 and hence activated. The discrepancy between our results and those of Kawakami *et al.* (1988) may therefore reflect differences in the level of expression and also perhaps of the recipient cell type used.

The middle-T:pp59$^{c\text{-}fyn}$ complex

Examination of lysates of NIH 3T3 cells co-expressing both the human pp59$^{c\text{-}fyn}$ and middle-T antigen revealed that pp59$^{c\text{-}fyn}$ was capable of associating with middle-T antigen. Using the c-*fyn*-specific peptide antibodies, pp59$^{c\text{-}fyn}$ was observed to co-immunoprecipitate with another protein of ~58 kd shown to be middle-T antigen. That they were stably complexed was confirmed by the demonstration that a fraction of pp59$^{c\text{-}fyn}$ from polyomavirus infected cells sediments at ~200 kd on sucrose density gradients. Presently, it is not understood how middle-T regulates qualitatively and quantitatively, the amount of the tyrosine kinases (pp60$^{c\text{-}src}$, pp62$^{c\text{-}yes}$ and pp59$^{c\text{-}fyn}$) with which it associates.

In an attempt to assess the contribution of the middle-T:pp59$^{c\text{-}fyn}$ complex towards transformation by polyomavirus, we showed that pp59$^{c\text{-}fyn}$ like pp60$^{c\text{-}src}$, was not able to interact with the transformation-defective mutant middle-T, NG59. This suggests that as in the case of pp60$^{c\text{-}src}$, the ability of pp59$^{c\text{-}fyn}$ to associate with middle-T may be necessary for polyomavirus middle-T mediated transformation. The mutation of NG59 maps to a region at the amino terminus of middle-T shown previously to be necessary for complex formation with pp60$^{c\text{-}src}$ (Carmichael and Benjamin, 1980; Templeton and Eckhart, 1984; Courtneidge and Smith, 1984; Cheng *et al.* 1986a; Markland and Smith, 1987). It is therefore possible that this same domain may be responsible for the interaction with pp59$^{c\text{-}fyn}$. Indeed, in retrospect, since some of the previous studies on mutants of middle-T were assayed using cst-1 antiserum, raised against the carboxyl terminal seven amino acids of pp60$^{c\text{-}src}$,

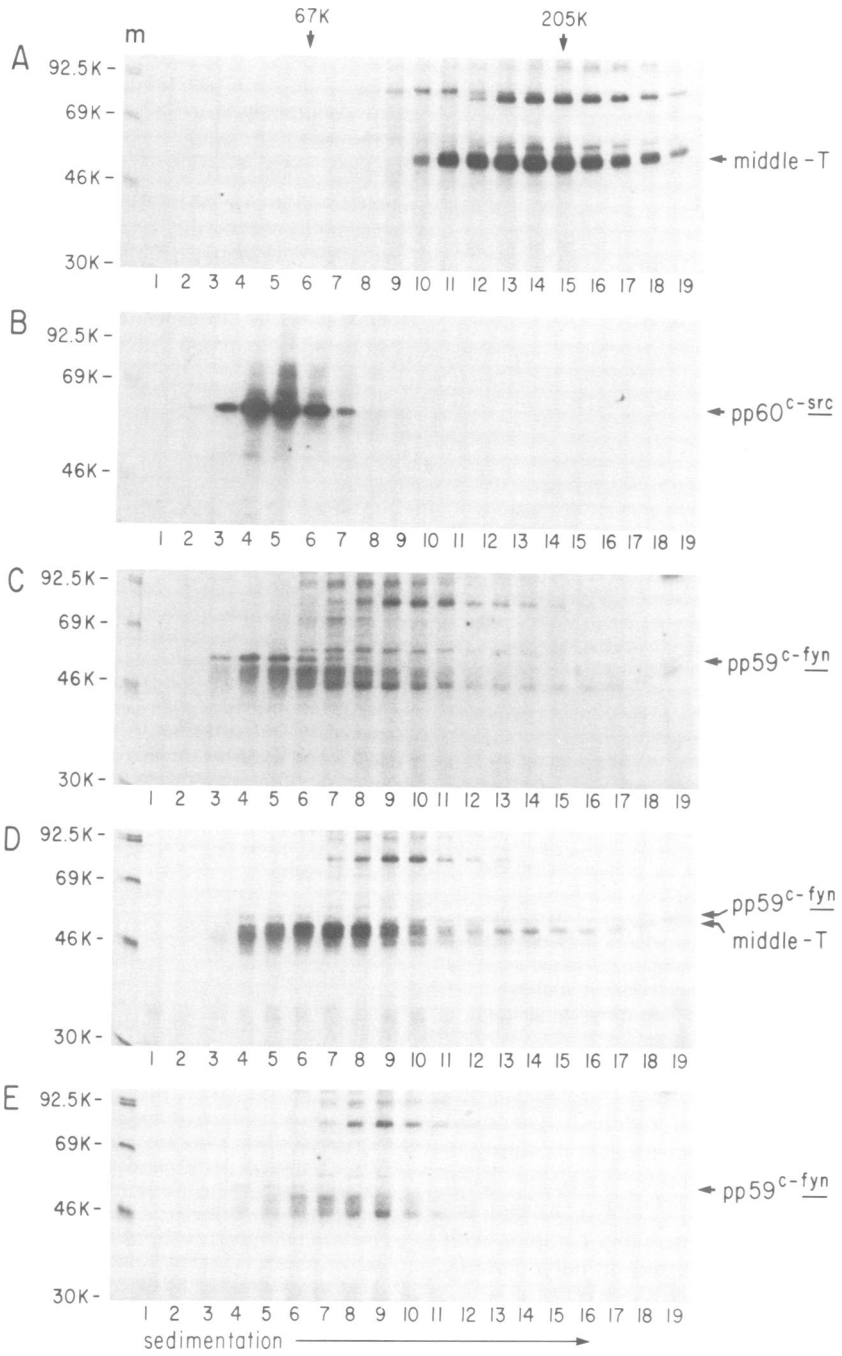

Fig. 8. Sucrose density gradient analysis of lysates of uninfected TR#2 cells and TR#2 cells infected with NG59 and wild type polyomavirus. Cells were lysed, fractionated on sucrose gradients, harvested and assayed for kinase activities as described in Materials and methods. (**A**) Fractionated cell lysate of normal NIH 3T3 cells infected with wild-type polyomavirus and assayed using HK3 antiserum. (**B**) Fractionated cell lysate of NIH 3T3 cells expressing avian pp60$^{c\text{-}src}$ and assayed using the EC10 monoclonal antibody. (**C**) Fractionated cell lysates of TR#2, (**D**) TR#2 infected with wild-type polyomavirus and (**E**) TR#2 infected with NG59 virus, assayed using the CFN-2 antibody. Middle-T, pp60$^{c\text{-}src}$ and pp59$^{c\text{-}fyn}$ are indicated on the right margin. **Lane M** contains mol. wt markers; sizes are in kilodaltons (K). Autoradiography was for 1−2 days.

which we now know is conserved amongst at least two other known members (c-*yes* and c-*fyn*) of the *src*-family of tyrosine kinases, those middle-T mutants which failed to be phosphorylated in the immune-complex kinase assays were presumably also unable to associate with either of these tyrosine kinases (Courtneidge and Smith, 1984; Cheng *et al.*, 1986a; Markland *et al.*, 1986). These included middle-T mutants which contained lesions around the NG59 site and those lacking the putative membrane localization domain at the carboxy terminus.

Unlike pp60$^{c\text{-}src}$, complex formation with middle-T antigen did not induce a significant increase of the pp59$^{c\text{-}fyn}$ kinase over that of the unassociated counterpart at least under the conditions tested. However, this may reflect the possibility that a much smaller fraction of the pp59$^{c\text{-}fyn}$ than pp60$^{c\text{-}src}$ in the lysates was present in the middle-T:tyrosine kinase super complex. Therefore, examination of the entire population of pp59$^{c\text{-}fyn}$ may obscure the true degree of activation. Hence, complex formation with middle-T antigen may have activated the kinase activities of pp60$^{c\text{-}src}$ and pp59$^{c\text{-}fyn}$ to the same degree but because the latter was present in a much smaller proportion, the extent

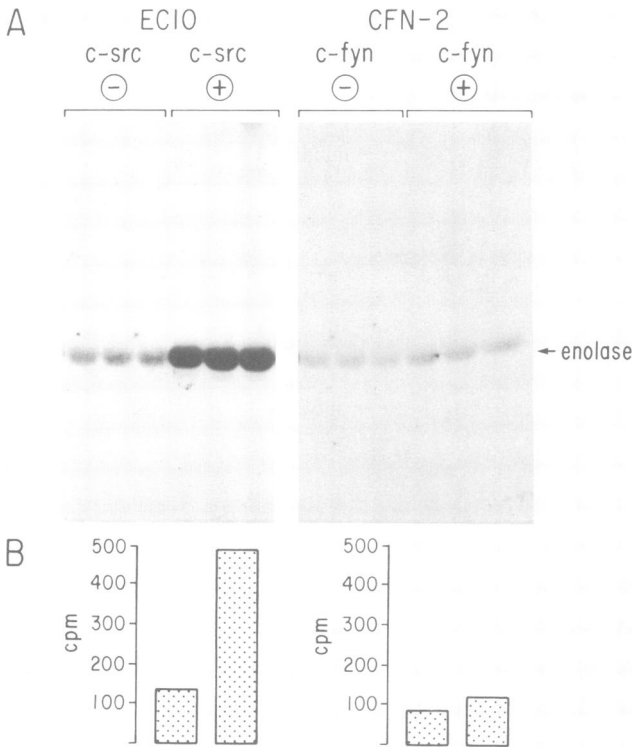

Fig. 9. Quantitative kinase assay of pp59$^{c\text{-}fyn}$ in complex with middle-T antigen. (**A**) Cell lines expressing the avian pp60$^{c\text{-}src}$ and pp59$^{c\text{-}fyn}$ (TR # 2) were either mock infected (−) or infected with polyomavirus (+). Cell lysates were prepared, normalized for protein content and then immunoprecipitated with EC10 (for pp60$^{c\text{-}src}$) or CFN-2 (for pp59$^{c\text{-}fyn}$). Kinase assays were performed as in Figure 3 except that enolase was added to the reactions. Exposure was for 2 h. (**B**) Gel pieces containing enolase were excised and counted. Depicted are results from an average of three separate experiments in triplicate. Shown is the amount of radioactivity (in c.p.m.) in enolase after a 3-min. reaction.

to which its activity was enhanced relative to the total appears to be less. Alternatively, the action of middle-T may not be to activate the kinase of pp59$^{c\text{-}fyn}$ but to alter its substrate specificity or some other activity yet to be defined. Even though the kinase activity was not appreciably enhanced, the observation that pp59$^{c\text{-}fyn}$ was able to stably complex with middle-T argues it may well have a role in polyomavirus mediated transformation.

The middle-T:tyrosine kinase super complex

It would appear that the middle-T antigen is capable of associating with the gene products of c-*src*, c-*yes* and c-*fyn*. However, it is unlikely that this represents the full complement of tyrosine kinases which are capable of complex formation with middle-T. There are at least five other known tyrosine kinases of this family and they all contain that domain identified initially in pp60$^{c\text{-}src}$ as being necessary for association with the viral protein. These findings prompt us to suggest that polyomavirus mediated transformation may be a consequence of the ability of middle-T to interact with and deregulate several members of the *src*-family of proteins. Deregulation may involve either activation or alteration of the substrate specificity of these enzymes. This would mean that transformation results as a consequence of untimely phosphorylation of key cellular regulatory proteins or of phosphorylation of inappropriate substrates.

Presently, it is unclear whether all the tyrosine kinases have equal affinity for middle-T antigen. Neither is it established whether the middle-T complex which is ~200 kd (Walter *et al.*, 1982; Courtneidge and Smith, 1983) is composed of more than one tyrosine kinase and if so, whether the enzymes are homogeneous or heterogeneous in each complex. Furthermore, it is not known whether the interaction of middle-T with more than one proto-oncogene product contributes to cellular transformation in a qualitative manner mediated by the effects of the different proto-oncogenes on different regulatory pathways, or merely in a quantitative manner mediated by some common pathway.

Materials and methods

Antibody production

Antisera specific for residues 7–19 (KDKEATKLTEERD(Y)) and 16–30 (EERDGSLNQSSGYRY) of the human c-*fyn* gene product were obtained through immunization of New Zealand white rabbits with peptide coupled to keyhole limpet hemocyanin (KLH) using benzidine (Bassiri *et al.*, 1979) at a mass ratio of 3:10 (peptide:carrier). The tyrosine residue at the carboxy terminus of the first peptide is not part of the c-*fyn* sequence and was added to facilitate coupling. These peptide sequences are not shared by any other known members of the *src*-family of tyrosine kinases. The degree of coupling was established using ^{125}I-labeled peptide as tracer followed by dialysis. The immunization and serum collection regimes were essentially as outlined by Harvey *et al.* (1982; 1984). The peptide antisera collected were affinity purified by sequential chromatography on a KLH–Sepharose column and a peptide–Sepharose column as described by Paucha *et al.* (1984).

Construction of c-fyn retroviral expression vector

The plasmid pSN-Mlu I which contains the human c-*fyn* cDNA is composed of the 1666-bp *Sau*3AI–*Stu*I fragment of the c-*fyn* cDNA cloned into pUC9 (Semba *et al.*, 1986). This construct was cleaved with *Mlu*I and the 1666-bp c-*fyn* containing fragment was isolated, blunt-ended using Klenow and then ligated to pSP65 (Promega Biotec.) which had been linearized with *Xba*I. Transformed DH5α bacterial cells containing the human c-*fyn* coding sequence in the sense orientation (pSP65 c-fyn) were isolated. The *Bam*HI–*Sal*I c-*fyn* containing fragment of pSP65 c-fyn was then inserted into the retroviral expression vector pLJ (Piwnica-Worms *et al.*, 1986) between the *Bam*HI and *Sal*I sites to create pLJ c-fyn.

Retrovirus production and infection of NIH 3T3 cells

To generate helper-free recombinant retroviruses in ψ-2 packaging cells, $2-3 \times 10^5$ cells per 50-mm dish were transfected with 10 μg of pLJ c-fyn (Mann *et al.*, 1983). At 24 h post-transfection, supernatants (transient supernatants) were harvested and used to infect NIH 3T3 cells. In addition, medium supernatants were also collected from stable ψ-2 producer lines as described previously (Piwnica-Worms *et al.*, 1986; 1987). Stable ψ-2 lines were established by adding the neomycin analog G418 (400 μg/ml) to the medium. G418-resistant clones were then either clonally expanded or selected in bulk.

Viral stocks from the transient supernatants and from three different ψ-2 clones were used to infect NIH 3T3 cells. At 24 h post-infection, cells were trypsinized and replated onto two 100-mm dishes. Following an additional 24 h of growth, one set of the culture dishes was placed under selection with G418 while the other was scored for focus-forming ability (Piwnica-Worms *et al.*, 1987; Cheng *et al.*, 1988). Transforming activity was scored by dividing the number of foci observed by the number of G418-resistant colonies. Stable NIH 3T3 cell lines expressing the human c-*fyn* gene product were also isolated by clonal expansion of the G418-resistant clones.

Transcription and translation of c-src, c-fyn and middle-T in vitro

The cDNAs for avian c-*src* (Piwnica-Worms *et al.*, 1986), human c-*fyn* (Semba *et al.*, 1986) and middle-T (Zhu *et al.*, 1984) were cloned into the SP64 and SP65 transcription vectors (Promega). pSP65 c-src was constructed essentially as outlined by Piwnica-Worms *et al.* (1986). The construction of pSP65 c-fyn was described above. pSP64 MT was constructed by inserting the *Nar*I–*Eco*RI fragment containing the middle-T cDNA generated from pPyMT1 (Zhu *et al.*, 1984) between the *Acc*I and *Eco*RI sites of pSP64. Following linearization with *Sal*I (for pSP65 c-src and pSP65 c-fyn) or *Eco*RI (for pSP64 MT), the vectors were transcribed with SP6 RNA polymerase essentially as recommended by the manufacturers. The RNA transcripts

were treated with 1 unit of DNase/μg RNA, purified and then translated *in vitro* in a micrococcal nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Radioactive methionine was omitted from the translation reactions when *in vitro* synthesized proteins were required for kinase assays.

Immunoprecipitation and in vitro kinase assays

The procedures for isotopic labeling cells with [^{35}S]methionine or $^{32}P_i$, preparation of lysates, immunoprecipitation of proteins and SDS–PAGE have all been described before (Courtneidge and Smith, 1983; 1984; Cheng *et al.*, 1986a; 1988). Conditions for immunoprecipitation of cellular extracts and of the proteins synthesized *in vitro* using the various antisera have been described in detail (Smith *et al.*, 1978; Cheng *et al.*, 1986a; 1988). Labeling of NIH 3T3 cells with [^{3}H]myristic acid was performed as described by Buss and Sefton (1985). Immune-complex kinase assays and quantitative kinase assays using enolase were conducted as described previously (Courtneidge, 1985; Cheng *et al.*, 1986a; 1988; Piwnica-Worms *et al.*, 1987).

Staphylococcus aureus V8 protease mapping and phosphoamino acid analysis

Extraction and recovery of radioactive proteins from gels and peptide mapping using *Staphylococcus aureus* V8 protease were performed as described previously (Cheng *et al.*, 1988). Phosphoamino acid analysis of recovered proteins was performed after acid hydrolysis at 110°C for 3 h in 4 M HCl under vacuum after the oxygen had been removed with nitrogen (Hunter and Sefton, 1980). Traces of HCl were removed by washing twice with water and lyophilization. The hydrolysates were dissolved in a marker mixture containing 2.5 mM phosphoserine, phosphothreonine and phosphotyrosine. The acid hydrolysates were then spotted onto cellulose TLC plates and analyzed by electrophoresis at pH 3.5 for 90 min at 750 V in glacial acetic acid/pyridine/H_2O, 50:5:945 (vol/vol). The markers were visualized with ninhydrin.

Sucrose density gradient centrifugation

Cells expressing pp60$^{c\text{-}src}$, pp59$^{c\text{-}fyn}$ and pp59$^{c\text{-}fyn}$ which had been infected with wild type polyomavirus or NG59 virus (obtained from Dr T.Benjamin) were harvested as described previously (Cheng *et al.*, 1988). Cell extracts were prepared and a 0.5-ml aliquot loaded onto a 4.5-ml continuous gradient of 5–20% sucrose (w/v) in lysis buffer (20 mM Hepes, pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.1 mM sodium orthovanadate, 1% NP40, 1% aprotinin). Centrifugation was in a Beckman SW50.1 rotor at 48 500 r.p.m. for 12 h at 4°C. Each 200 μl fraction collected from the top of the gradient was diluted with lysis buffer, immunoprecipitated and assayed for kinase activity as described above. Molecular weight markers were analyzed in a parallel gradient.

Acknowledgements

We thank T.Benjamin, B.Schaffhausen and E.Hendrikson for stocks of wild type and NG59 polyomavirus, H.Piwnica-Worms and T.Roberts for the avian c-*src* cDNA and pLJ vector, S.Parsons for EC10 and GD11 monoclonal antibodies, W.Markland for pSP64 MT and J.Reid, R.Schweickhardt, K.Mock, S.Pagan and K.Hehir for technical assistance. We also thank E.Cohen for critically reviewing the manuscript and S.Courtneidge for communicating unpublished results. This work was supported in part by a fellowship from Integrated Genetics; by Public Health Service grant RO1 CA 43186-03 from the National Cancer Institute to S.H.C., R.H., P.C.E. and A.E.S; and by a Grant in-Aid for Special Project Research Cancer-Bioscience from the Ministry of Education, Science and Culture of Japan to K.S., T.Y. and K.T..

References

Amrein,K.E. and Sefton,B.M. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 4247–4251.

Bassiri,R.M., Dvorak,J. and Utiger,R.D. (1979) In Campbell,R.A. (ed.), *Methods of Hormone Radioimmunoassay*. Academic Press, New York, pp. 45–46.

Bolen,J.B., Thiele,C.J., Israel,M., Yonemoto,W., Lipsich,L.A. and Brugge,J.S. (1984) *Cell*, **38**, 767–777.

Buss,J.E. and Sefton,B.M. (1985) *J. Virol.*, **53**, 7–12.

Carmichael,G.G. and Benjamin,T.M. (1980) *J. Biol. Chem.*, **255**, 230–235.

Cartwright,C.A., Kaplan,P.L., Cooper,J.A., Hunter,T. and Eckhart,W. (1986) *Mol. Cell. Biol.*, **6**, 1562–1570.

Cartwright,C.A., Eckhart,W., Simon,S. and Kaplan,P.L. (1987) *Cell*, **49**, 83–91.

Cheng,S.H., Markland,W., Markham,A.F. and Smith,A.E. (1986a) *EMBO J.*, **5**, 325–334.

Cheng,S.H., Markland,W. and Smith,A.E. (1986b) In Kahn,P. and Graf,T. (eds), *Oncogenes and Growth Control*. Springer-Verlag, Berlin, Heidelberg, pp. 298–303.

Cheng,S.H., Piwnica-Worms,H., Harvey,R.W., Roberts,T.M. and Smith,A.E. (1988) *Mol. Cell. Biol.*, **8**, 1736–1747.

Collett,M.S., Erikson,E. and Erikson,R.L. (1979) *J. Virol.*, **29**, 770–781.

Cooper,J.A. (1988) In Kemp,B. and Alewood,P.F. (eds), *Peptides and Protein Phosphorylation*. CRC Press Inc., in press.

Cooper,J.A. and King,C.S. (1986) *Mol. Cell. Biol.*, **6**, 4467–4477.

Cooper,J.A., Gould,K.L., Cartwright,C.A. and Hunter,T. (1986) *Science*, **231**, 1431–1433.

Courtneidge,S.A. (1985) *EMBO J.*, **4**, 1471–1477.

Courtneidge,S.A. and Heber,A. (1987) *Cell*, **50**, 1031–1037.

Courtneidge,S.A. and Smith,A.E. (1983) *Nature*, **303**, 435–439.

Courtneidge,S.A. and Smith,A.E. (1984) *EMBO J.*, **3**, 585–591.

Fried,M. and Prives,C. (1986) *Cancer Cells*, **4**, 1–16.

Harvey,R., Faulkes,R., Gillett,P., Lindsay,N., Paucha,E., Bradbury,A. and Smith,A.E. (1982) *EMBO J.*, **1**, 473–477.

Harvey,R., Oostra,B.A., Belsham,G.J., Gillett,P. and Smith,A.E. (1984) *Mol. Cell. Biol.*, **4**, 1334–1342.

Hunter,T. and Sefton,B.M. (1980) *Proc. Natl. Acad. Sci. USA.*, **77**, 1311–1315.

Iba,H., Takeya,T., Cross,F.R., Hanafusa,T. and Hanafusa,H. (1984) *Proc. Natl. Acad. Sci. USA.*, **81**, 4424–4428.

Iba,H., Cross,F.R., Garber,E.A. and Hanafusa,H. (1985) *Mol. Cell. Biol.*, **5**, 1058–1066.

Johnson,P.J., Coussens,P.M., Danko,A.V. and Shalloway,D. (1985) *Mol. Cell. Biol.*, **5**, 1073–1083.

Kaplan,D.R., Whitman,M., Schaffhausen,B., Raptis,L., Garcea,R.L., Pallas,D., Roberts,T.M. and Cantley,L. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 3624–3628.

Kaplan,D.R., Whitman,M., Schaffhausen,B., Pallas,D., White,M., Cantley,L. and Roberts,T.M. (1987) *Cell*, **50**, 1021–1029.

Kawakami,T., Pennington,C.Y. and Robbins,K.C. (1986) *Mol. Cell. Biol.*, **6**, 4195–4201.

Kawakami,T., Kawakami,Y., Aaronson,S.A. and Robbins,K.C. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 3870–3874.

Kmiecik,T.E. and Shalloway,D. (1987) *Cell*, **49**, 65–73.

Kornbluth,S., Sudol,M. and Hanafusa,H. (1987) *Nature*, **325**, 171–173.

Mann,R., Mulligan,R.C. and Baltimore,D. (1983) *Cell*, **33**, 153–159.

Markland,W. and Smith,A.E. (1987) *J. Virol.*, **61**, 285–292.

Markland,W., Cheng,S.H., Oostra,B.A. and Smith,A.E. (1986) *J. Virol.*, **59**, 82–89.

Marth,J.D., Cooper,J.A., King,C.S., Ziegler,S.F., Tinker,D.A., Overell,R.W., Krebs,E.G. and Perlmutter,R.M. (1988) *Mol. Cell. Biol.*, **8**, 540–550.

Parsons,S.J., McCarley,D.J., Ely,C.M., Benjamin,D.C. and Parsons,J.T. (1984) *J. Virol.*, **51**, 272–282.

Paucha,E., Harvey,R. and Smith,A.E. (1984) *J. Virol.*, **51**, 670–681.

Piwnica-Worms,H., Kaplan,D.R., Whitman,M. and Roberts,T.M. (1986) *Mol. Cell. Biol.*, **6**, 2033–2040.

Piwnica-Worms,H., Saunders,K.B., Roberts,T.M., Smith,A.E. and Cheng,S.H. (1987) *Cell*, **49**, 75–82.

Reynolds,A.B., Vila,J., Lansing,T.J., Potts,W.M., Weber,M.J. and Parsons,J.T. (1987) *EMBO J.*, **6**, 2359–2364.

Semba,K., Yamanashi,Y., Nishizama,M., Sukegawa,J., Yoshida,M.E., Sasaki,M., Yamamoto,T. and Toyoshima,K. (1985) *Science*, **227**, 1038–1040.

Semba,K., Nishizawa,M., Miyajima,N., Yoshida,M.C., Sukegawa,J., Yamanashi,Y., Sasaki,M., Yamamoto,T. and Toyoshima,K. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 5459–5463.

Shalloway,D., Coussens,P.M. and Yacuik,P. (1984) *Proc. Natl. Acad. Sci. USA.*, **81**, 7071–7075.

Smith,A.E. and Ely,B.K. (1983) *Adv. Viral Oncol.*, **3**, 3–30.

Smith,A.E., Smith,R. and Paucha,E. (1978) *J. Virol.*, **28**, 140–153.

Sudol,M., Alvarez-Buylia,A. and Hanafusa,H. (1988) *Oncogene Res.*, **2**, 345–355.

Templeton,D. and Eckhart,W. (1984) *Mol. Cell. Biol.*, **4**, 817–821.

Walter,G., Hutchinson,M.A., Hunter,R. and Eckhart,W. (1982) *Proc. Natl. Acad. Sci. USA.*, **79**, 4025–4029.

Whitman,M., Kaplan,D.R., Schaffhausen,B.S., Cantley,L. and Roberts,T.M. (1985) *Nature*, **315**, 239–242.

Zhu,Z., Veldman,G.M., Cowie,A., Carr,A., Schaffhausen,B. and Kamen,R. (1984) *J. Virol.*, **51**, 170–180.

Received on July 26, 1988; revised on August 31, 1988