Identification and Characterization of the Hamster Polyomavirus Middle T Antigen

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Hamster polyomavirus (HaPV) is associated with lymphoid and hair follicle tumors in Syrian hamsters. The early region of HaPV has the potential to encode three polypeptides (which are related to the mouse polyomavirus early proteins) and can transform fibroblasts in vitro. We identified the HaPV middle T antigen (HamT) as a 45-kDa protein. Like its murine counterpart, HamT was associated with serine/threonine phosphatase, phosphatidylinositol-3 kinase, and protein tyrosine kinase activities. However, whereas mouse middle T antigen associates predominantly with pp60^{c-src} and pp62^{c-yes}, HamT was associated with a different tyrosine kinase, p59^{lyn}. The ability of HaPV to cause lymphoid tumors may therefore reside in its ability to associate with p59^{lyn}, a potentially important tyrosine kinase in lymphocytes.

Hamster polyomavirus (HaPV) was first described in association with spontaneously arising skin tumors in a colony of Syrian hamsters in Germany, with as many as 10% of hamsters showing overt multiple hair follicle tumors by the age of 3 months (25, 27). However, inoculation of the virus into newborn hamsters from a separate virus-free colony led predominantly to induction of lymphoma and leukemia. These tumors arose with a short latency (4 to 8 weeks) and high incidence (30 to 80%) (26). Similar tumors have been observed in hamster colonies in the United States, and in this case, both B- and T-cell tumors have been described (14). Furthermore, it has recently been shown that mice transgenic for the HaPV genome also develop hair follicle tumors and lymphoma at high frequency (43a). This tumor profile is different from that observed with murine polyomavirus. While hair follicle tumors are among the many tumor types observed when early-passage murine virus is injected into newborn mice, lymphoma and leukemia have not been reported (21, 23, 47). Furthermore, transgenic and chimeric mouse model systems have also failed to reveal any leukemogenic properties of mouse middle T antigen (MomT), the predominant phenotype in this model system being hemangioma (6, 53), with various other tumor types, such as neuroblastoma and carcinoma, arising infrequently (1, 40).

The genome of mouse polyomavirus has been extensively characterized (for reviews, see references 28 and 47). The early region (which has all of the transforming properties) encodes three proteins in overlapping open reading frames: the large, middle, and small T antigens. The large T antigen is able to immortalize primary cells (41), while MomT carries the transforming potential and is the only protein required to transform established cell lines (although this occurs at a somewhat reduced frequency compared with the intact early region) (48). The role of small T is less clear, but it appears to function in tumorigenesis in vivo (2). Biochemical characterization of middle T antigen has shown that it is a protein devoid of intrinsic enzymatic activity but capable of complex

formation with a number of cellular proteins. These include tyrosine kinases of the *src* family (13, 19, 34, 36), a phosphatidylinositol (PI) kinase (52) which phosphorylates at the 3' position on the inositol ring (PI-3 kinase) (51), and serine/threonine phosphatase 2A (PP2A) (39, 50). Genetic analysis has revealed that the protein kinases and the phosphatase bind to the region of middle T antigen which is common to middle T and small T antigens, i.e., within the amino-terminal 192 amino acids (12, 20, 29, 37, 38). By contrast, a protein called p81 or p85, whose presence has been correlated with PI kinase activity (18, 32), requires sequences around the major phosphotyrosine site of middle T antigen (Tyr-315) for binding (18, 38, 46). Middle T antigens unable to bind to any of these proteins fail to transform, suggesting that these interactions are required for transformation. However, to date no mutants have been described which are able to bind phosphatase but not kinase or vice versa. Thus, we know neither the exact binding sites for the kinase and phosphatase on the middle T antigen nor the individual contributions each of these proteins make to the transformation process.

The genome of HaPV was molecularly cloned from a DNA preparation derived from samples of hair follicle tumors and shown to have great similarity to mouse polyomavirus (22). For example, the early region of HaPV also has the coding potential for at least three T antigens and possesses in vitro immortalization and transforming properties similar, but not identical, to those of mouse polyomavirus (5). Comparison of the HaPV putative middle T antigen sequences with the mouse polyomavirus middle T antigen shows an overall identity of approximately 42%. However, these homologies are clustered within the sequence, as shown in Fig. 1. The amino-terminal domains (shared by all of the T antigens and comprising the first 79 amino acids of MomT) have 48% identity, the next domains (common to middle and small T antigens and comprising amino acids 80 to 192 of MomT) have the highest homology at 59%, while the rest of the proteins (which are unique to the middle T antigens) have only 22% similarity. Within this latter region, the homology is concentrated in just two areas: the sequences surrounding the major tyrosine phosphorylation site (Tyr-315 [43]) in

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When we initiated this work, the T antigens encoded by HaPV had not been identified, and therefore it had previously not been possible to correlate the biochemical properties of the middle T antigen with, for example, tumor profile differences. We reasoned that such an analysis would perhaps also greatly facilitate the mapping of the binding sites for tyrosine kinase and phosphatase on MomT (for example, by comparing the ability or inability of hamster middle T antigen [HamT] to bind these proteins with known regions of similarity and dissimilarity in the sequences of the two middle T antigens) and therefore assist in the generation of mutants defective in binding one or the other of these proteins. For these reasons, therefore, we undertook to identify and characterize the middle T antigen of HaPV. In this communication, we describe this characterization.

MATERIALS AND METHODS

Lysis conditions. For most assays, cells were washed in

For one experiment (see Fig. 6A), kinase assays were performed on TBH serum-derived immune complexes as described in this article. Following the kinase assay, the immune complexes were washed once in TBS and then denatured by heating to 95°C for 2 min in 25 mM Tris (pH 7.5)–100 mM NaCl–2 mM EDTA–0.4% SDS–2 mM 2-mercaptoethanol. *S. aureus* was removed by centrifugation for 2 min in a microcentrifuge, and the supernatants were made to 10 mM in iodoacetamide. Following 30 min of incubation in the dark at 30°C, Triton X-100 was added to a final concentration of 2%. The samples were then immunoprecipitated and analyzed as usual. This is a modification of the procedure described by Cohen et al. (15).

RESULTS

In vitro transcription and translation reactions using a plasmid containing the HaPV middle T antigen sequences produced a protein of approximately 45 kDa (Fig. 2A, lane 2). The size of this protein was in good agreement with the

FIG. 2. Detection of HamT and associated proteins. (A) Lanes 1 and 2 show in vitro translation products. In lane 1, no exogenous RNA was added to the translation mixture, whereas in lane 2, the translation mixture contained RNA transcribed from a vector containing putative HamT sequences. Lanes 3 and 4 show immunoprecipitation of denatured lysates of [35 S]methionine-labeled HamT-transformed cells with either normal hamster serum (lane 3) or TBH serum (lane 4). The position of a 45-kDa protein detected in both analyses is indicated. (B) The 45-kDa bands from gels similar to those shown in panel A were excised and digested with 40 (lanes 1 and 3) or 200 (lanes 2 and 4) ng of *S. aureus* V8 protease. In lanes 1 and 2, the 45-kDa protein derived from the in vitro translation reaction, whereas the 45-kDa band analyzed in lanes 3 and 4 derived from in vivo-labeled material. (C) [35 S]methionine-labeled HamT-transformed cells were lysed under nondenaturing conditions and immunoprecipitated with normal hamster serum (lane 1), TBH serum (lane 2), or an anti-peptide antibody specific for the carboxy terminus of the catalytic subunit of PP2A (lane 3). The positions of HamT (p45) and the catalytic (p36) and regulatory (p63) subunits of PP2A are indicated.

predicted molecular weight of the middle T antigen, and furthermore, the protein was immunoprecipitated with anti-serum reactive against HaPV proteins (see below). We therefore believe that p45 is HamT. To determine whether HamT-transformed cells also contained a protein of this size, the cells were labeled with [35 S]methionine, lysed under denaturing conditions, and then subjected to immunoprecipitation. Antibodies specific for the middle T antigen have not been described, so we used sera from hamsters bearing HaPV-induced hair follicle tumors (TBH sera). These sera have previously been shown by immunofluorescence to react with tumor cells but not normal cells and therefore presumably contain antibodies reactive against HaPV-encoded proteins. Analysis of these labeled extracts revealed only one band, at about 45 kDa, whose immunoprecipitation was specific to TBH serum (Fig. 2A, lane 4). This band was not detected when the extracts were derived from normal cells (data not shown) or when preimmune serum was used to make the immunoprecipitates (lane 3). The 45-kDa protein had the same partial proteolytic map (Fig. 2B) as the in vitro translation product. We therefore conclude that the 45-kDa protein produced in HamT-transformed cells is the HaPV middle T antigen. The in vitro translation product migrated perhaps a little faster on these gels. This could be due to the occurrence of posttranslational modification of the middle T antigen in vivo but not in vitro. Indeed, the protein is phosphorylated in vivo (unpublished data), although the low level of labeling we achieved (presumably due to either low

FIG. 3. Association of phosphatase activity with HamT. Immunoprecipitates were made from either NIH 3T3 cells (lanes 1 and 2) or HamT-transformed NIH 3T3 cells (lanes 3 and 4) with either normal hamster serum (lanes 1 and 3) or TBH serum (lanes 2 and 4), washed, and assayed for associated phosphatase activity. Results are expressed as counts per minute released from the substrate in trichloroacetic acid-soluble form in the 10-min reaction. Assays were carried out in duplicate, and the average of the two samples is shown.

abundance of the protein or low stoichiometry of phosphorylation) precluded phosphoamino acid analysis.

Association of protein phosphatase activity with HamT. Immunoprecipitation of [35 S]methionine-labeled denatured lysates showed predominantly one band at 45 kDa. However, when lysates were not denatured before immunoprecipitation, several other proteins were also detected (Fig. 2C, lane 2). Among these were two which were not immunoprecipitated with normal hamster serum (Fig. 2C, lane 1) or from normal cells by using TBH sera (data not shown); their molecular masses were 36 and 63 kDa. These molecular masses were identical to those previously reported for two MomT-associated proteins which have recently been identified as the catalytic (39) and regulatory (39, 50) subunits of PP2A. Indeed, as shown in Fig. 2C, the 63- and 36-kDa bands comigrated with immunoprecipitated PP2A. We attribute the difference in the ratios of the 36- and 63-kDa bands in each case to the fact that the association between the PP2A subunits is known to be weakened by the relatively harsh solutions used to wash such immunoprecipitates. Thus, in the case of the anti-PP2A immunoprecipitate, the antibody is directed against the 36-kDa subunit and therefore the 63-kDa subunit is underrepresented, whereas in the TBH immunoprecipitate the 63-kDa subunit is strongly associated with the middle T antigen and the 36-kDa protein is underrepresented. We detected no HamT in the PP2A immunoprecipitate in this exposure (lane 3), presumably because PP2A is a very abundant protein and HamT is not, and the assay was not sensitive enough to detect the very minor HamT-associated form of PP2A. We next wanted to confirm that the HamT-associated proteins were indeed PP2A. We have previously described an immune complex phosphatase assay which uses cyclic AMP-dependent protein kinase-phosphorylated casein as a substrate and is a very sensitive way of detecting PP2A activity (48a). By using this assay, we tested immune complexes from normal and HamT-transformed cells for associated phosphatase activity, as shown in Fig. 3. We detected phosphatase activity in HamT immune precipitates (lane 4) but not when the immune complexes were derived from normal cells (lane 2) or when normal hamster serum was used to make the immune complexes

FIG. 4. Association of kinase activity with HamT. (A) Lysates of NIH 3T3 cells (lanes 1 and 2) or HamT-transformed equivalents (lanes 3 to 6) were adjusted to equal protein concentrations and immunoprecipitated with anti-cst.1 preblocked with peptide (lanes 1 and 3), anti-cst.1 (lanes 2 and 4), normal hamster serum (lane 5), or TBH serum (lane 6), and kinase assays were performed. The proteins of 81, 60 and 45 to 48 kDa specifically detected in this assay are indicated. (B) *S. aureus* V8 protease analysis using 40 (lanes 1 and 3) and 200 (lanes 2 and 4) ng of enzyme of the 45- to 48-kDa proteins equivalent to those from lanes 4 (lanes 1 and 2) and 6 (lanes 3 and 4) of panel A.

(lanes 1 and 3). This activity was inhibited by low concentrations of okadaic acid (K_i , ~ 1 nM) but was insensitive to inhibitor 2, hallmarks of PP2A (16). Furthermore, the 36-kDa component in HamT complexes was immunoprecipitable with an antibody against the catalytic subunit of PP2A (data not shown). By all of these criteria, the phosphatase associated with HamT is PP2A.

Association of protein tyrosine kinase activity with HamT. We next determined whether HamT was also able to associate with cellular tyrosine kinases. To do this, we immunoprecipitated HamT with TBH serum and performed immune complex kinase assays, the results of which are shown in Fig. 4A. We detected labeled products, including those with approximate molecular masses of 81, 60, and 45 to 48 kDa (lane 6). All of these proteins were phosphorylated on tyrosine residues (data not shown). Immune complex kinase assays using TBH serum and lysates of normal cells (data not shown) or normal hamster serum (lane 5) showed only weak phosphorylation of proteins from higher-molecular-weight bands. We frequently observed such bands, regardless of the antibody or lysate used, and assumed that they were background bands. However, the presence of other, tyrosine-phosphorylated proteins specific to the immunoprecipitates made from HamT transformed with TBH serum led us to conclude that HamT was associated with a cellular tyrosine kinase(s).

Since MomT has previously been shown to complex with tyrosine kinases of the *src* family, and given the similarities between HamT and MomT, we next determined whether the HamT-associated tyrosine kinase was a member of the *src* family. To do this, we made use of an antibody (anti-cst.1) which recognizes all three *src* family tyrosine kinases which are expressed in fibroblasts, pp60^{c-src}, p59^{lyn}, and pp62^{c-yes} (35). Kinase assays of immune complexes derived from normal cells with this antibody showed a predominant band at approximately 60 kDa which represents autophosphorylated *src* family kinases, as well as smaller amounts of other

FIG. 5. Association of PI kinase activity with HamT. (A) *S. aureus* V8 protease analysis using 40 (lanes 1, 3, and 5) and 200 (lanes 2, 4, and 6) ng of enzyme of the 81-kDa proteins phosphorylated in kinase assays of TBH serum (lanes 1 and 2) and anti-cst.1 (lanes 3 and 4) immunoprecipitates from HamT-transformed cells and the 81-kDa protein detected in immunoprecipitates of MomT (lanes 5 and 6). (B) PI kinase assays of immunoprecipitates made with TBH serum and lysates of NIH 3T3 cells (lane 1) or their HamT (lane 2)- or MomT (lane 3)-transformed counterparts. The positions of authentic PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃ are shown.

proteins which may represent either cross-reactive proteins or contaminants (lane 2). When the immune complexes were derived from HamT-transformed cells, we detected, in addition to these bands, extra bands with molecular masses of 81 and 45 to 48 kDa (lane 4). We note that the intensity of labeling of the 45- to 48-kDa bands was very variable from experiment to experiment and always stronger when TBH serum was used to make the immune complexes. Nevertheless, this ladder of bands at 45 to 48 kDa had a partial proteolytic map very similar to that of the bands labeled in TBH immune complexes (Fig. 4B) and also appeared to represent a subset of those bands derived by V8 proteolysis of [³⁵S]methionine-labeled HamT. (The small differences in the higher-molecular-weight fragments were attributed to contamination with other proteins.) We therefore conclude that the tyrosine kinase associated with HamT is a member of the *src* family and that one of the substrates of this kinase is HamT itself. It is also evident from Fig. 4 that autophosphorylation of the 60-kDa band was greatly increased in HamT-transformed cells compared with their normal counterparts, suggesting that association with HamT resulted in activation of the kinase.

Association of PI kinase activity with HamT. The kinase assays of both TBH sera and anti-cst.1 immune complexes detected a protein with a molecular mass of 81 kDa (Fig. 4A, lanes 4 and 6). This band also comigrated with the 81-kDa protein previously shown to associate with MomT (see Fig. 6). Figure 5 shows that by the criterion of partial proteolytic mapping using V8 protease (panel A), these proteins are indeed identical. Since the presence of p81 in immune complexes has been shown to correlate with PI-3 kinase activity (18, 32), we next tested whether PI-3 kinase was also associated with HamT. The results are presented in Fig. 5B. We did indeed demonstrate PI-3 kinase activity in TBH serum immune complexes derived from HamT-transformed cells (lane 2). The specificity of the reaction was shown by the lack of activity when TBH serum was used to make

FIG. 6. Association of $p59^{fyn}$ with HamT. NIH 3T3 cells (lanes 1, 4, and 7), HamT-transformed NIH 3T3 cells (lanes 2, 5, 8, 10, and 11), and MomT-transformed NIH 3T3 cells (lanes 3, 6, and 9) were immunoprecipitated with antibodies specific for $pp60^{c-src}$ (lanes 1 to 3), $p59^{fyn}$ (lanes 4 to 6, 10, and 11), or $pp62^{c-yes}$ (lanes 7 to 9), and associated proteins were detected by kinase assay. In lane 10, the anti- $p59^{fyn}$ antibody was incubated with a cognate peptide prior to addition of the lysate. The positions of autophosphorylated kinases (p60) and associated proteins p81, MomT, and HamT are indicated. The exposure time for lanes 1 to 3 was fourfold less than that for lanes 4 to 9.

immune complexes from normal (lane 1) or MomT-transformed (lane 3) cells. As the substrate for the lipid kinase reaction, we used a mixture of PI, PI(4)P, and PI(4,5)P₂. The associated PI kinase was able to phosphorylate all three lipids, demonstrating its similarity to the MomT-associated and platelet-derived growth factor receptor-associated enzyme (3, 49). We also detected PI-3 kinase activity in anti-cst.1 immunoprecipitates of HamT-transformed cells (data not shown), and we therefore conclude that the complex of HamT and a *src* family tyrosine kinase also contains PI-3 kinase activity.

Preferential association of HamT with $p59^{fyn}$. It has previously been shown that MomT associates with three members of the *src* family of tyrosine kinases, although not with equal affinities. Of the total kinase-associated form of middle T antigen, approximately 50 to 75% is bound to $pp60^{c-src}$, about 25 to 50% is bound to $pp62^{c-yes}$, and less than 10% is bound to $p59^{fyn}$ (7, 31, 34, 36). We wished to know whether HamT would show the same specificity of binding. To investigate this, we made use of antipeptide antibodies specific for the three kinases, as shown in Fig. 6. Lanes 1, 4, and 7 show kinase assays using these antibodies and lysates from the parental NIH 3T3 cells to demonstrate that in each assay the predominant product was the autophosphorylated kinase itself. Lanes 2, 5, and 8 represent kinase assays using HamT lysates. We first screened for the presence of labeled p81 in the immune complexes and found that most of it was associated with $p59^{fyn}$ (lane 8), with very little detectable in $pp60^{c-src}$ (lane 2) and $pp62^{c-yes}$ (lane 5) immunocomplexes on prolonged exposure. While the associated middle T antigen itself was barely visible in this assay, we did observe its presence in longer exposures of $p59^{fyn}$ immunoprecipitates (lane 11) but not in peptide-blocked controls (lane 10) or $pp60^{c-src}$ and $pp62^{c-yes}$ immunoprecipitates, and we confirmed its identity by V8 digestion (data not shown). HamT may be a poor substrate because the protein is already phosphorylated at this site(s) in vivo; this requires further investigation. We doubt that the results we achieved were due to inability of our antibodies to $pp60^{c-src}$ and $pp62^{c-yes}$ to

FIG. 7. Identification of the 60-kDa protein associated with HamT as predominantly $p59^{fyn}$. (A) Immune complexes were formed by using TBH serum and a lysate of HamT-transformed cells, washed, and labeled in a kinase reaction. The products of the reaction were then denatured and reprecipitated as described in Materials and Methods. The second immunoprecipitation was with normal rabbit serum (lane 1) or anti- $pp60^{c-src}$ (lane 2), anti- $p59^{fyn}$ (lane 3) or anti- $pp62^{c-yes}$ (lane 4) specific antibodies. The arrow marks the position of the 60-kDa HamT-associated protein. We assume that the band detected in all of the lanes migrating faster than p60 was the immunoglobulin heavy chain, which became labeled during the kinase reaction. (B) *S. aureus* V8 protease analysis using 40 (lanes 1, 3, and 5) and 200 (lanes 2, 4, and 6) ng of enzyme of the 60-kDa proteins phosphorylated in kinase assays of anti- $pp60^{c-src}$ (lanes 1 and 2), TBH serum (lanes 3 and 4), and anti- $p59^{fyn}$ (lanes 5 and 6) immunoprecipitates from HamT-transformed cells. (C) Lysates of HamT-transformed cells were immunoprecipitated with normal rabbit serum (lanes 1 and 2) or antibodies specific for $p59^{fyn}$ (lanes 3 and 4) under conditions which we have previously shown to remove at least 80% of $p59^{fyn}$ from the lysate (35). Following removal of these immunocomplexes, the depleted lysates were subjected to a second round of immunoprecipitation using either normal hamster serum (lanes 1 and 3) or TBH serum (lanes 2 and 4), and kinase assays were performed. The positions of p81, p60, and HamT are marked.

recognize their antigens efficiently. (i) With these antibodies, we routinely precipitated more $pp60^{c-src}$ than $p59^{fyn}$ from a lysate and the exposure time for lanes 1 to 3 was fourfold shorter than that for lanes 3 to 9. (ii) The expected binding pattern of MomT and associated p81 with the three kinases was observed, with the most binding to $pp60^{c-src}$ (lane 3), less binding to $pp62^{c-yes}$ (lane 6), and very little binding to $p59^{fyn}$ (lane 9). From these results, we concluded that HamT, unlike its murine counterpart, associates predominantly with $p59^{fyn}$. We estimate from sucrose density gradient centrifugation that approximately 10% of $p59^{fyn}$ is associated with the middle T antigen. This complex also contains p81 and sediments to approximately the same position in the gradient as the middle T antigen- $pp60^{c-src}$ complex (17a).

We wished to confirm these data by using a different approach. The kinase assays of TBH serum immunoprecipitates revealed a band at approximately 60 kDa. Since the tyrosine kinases of the *src* family share this molecular mass and autophosphorylate in such kinase reactions, we next sought to identify this band. In the first analysis, kinase assays were performed on TBH-derived immune complexes, and the resulting products were denatured and then subjected to a second round of immunoprecipitation (Fig. 7A). The 60-kDa band was efficiently reprecipitated with antibodies specific for $p59^{fyn}$ (lane 3), perhaps very weakly with anti- $pp62^{c-yes}$ (lane 4), but not with normal rabbit serum (lane 1) or antibodies against $pp60^{c-src}$ (lane 2). Independent tests

FIG. 8. Activation of $p59^{fyn}$ by HamT. (A) $p59^{fyn}$ was immunoprecipitated from either NIH 3T3 (lane 1)- or HamT (lane 2)-transformed NIH 3T3 cell lysates which had been adjusted to equal protein concentrations, and kinase assays were performed. (B) Immunoprecipitates made with either anti-cst.1 (lanes 1 and 2) or anti- $p59^{fyn}$ (lanes 3 and 4) from normal (columns 1 and 3) or HamT-transformed (columns 2 and 4) cells were assayed for kinase activity by using the exogenous substrate enolase. Depicted is relative kinase activity. The value has been set to 1 for each immunoprecipitate from normal cells. The results are the average of two experiments with anti-cst.1 and three with anti- $p59^{fyn}$.

established that the antibodies used were capable of recognizing their cognate denatured antigen (data not shown). Secondly, V8 proteolytic analysis (Fig. 7B) showed that the 60-kDa band (lanes 3 and 4) had a map which was identical to that of authentic autophosphorylated $p59^{fyn}$ (lanes 5 and 6) but subtly different from those of $pp60^{c-src}$ (lanes 1 and 2) and $pp62^{c-yes}$ (data not shown). From this we conclude that the 60-kDa HamT-associated protein detected by kinase assay was predominantly $p59^{fyn}$.

To assess how much of the total HamT-associated kinase activity could be attributed to $p59^{fyn}$, we performed the following experiment. Lysates of HamT-transformed cells were subjected to immune precipitation with either inappropriate or $p59^{fyn}$ -specific antibodies. Following removal of these immune complexes, a second round of immunoprecipitation was carried out with either normal or tumor-bearing hamster serum. Figure 7C shows that compared with the control (lane 2), predepletion of $p59^{fyn}$ from the lysates resulted in removal of most of the HamT-associated kinase activity (lane 4). We conclude, therefore, that most (>75%) of the associated kinase activity was attributable to $p59^{fyn}$.

It has been demonstrated that MomT activates $pp60^{c-src}$ kinase activity at least 10-fold (8, 10, 17). It is also able to activate $pp62^{c-yes}$, although not to such a great extent (34). We had already observed that the presence of HamT greatly increased the autophosphorylation observed in anti-cst.1 immunoprecipitates (Fig. 4A, compare lanes 3 and 6). We investigated this in more detail. Figure 8A shows that $p59^{fyn}$ is activated in its autophosphorylation capacity in HamT-transformed cells, even though by Western blotting (immunoblotting) we detected no change in the level of the protein (data not shown). Furthermore, by using both anti-cst.1 and anti- $p59^{fyn}$ antibodies, we showed activation of the kinase in HamT-transformed cells by using the exogenous substrate enolase (Fig. 8B). However, the activation was, at best, only on the order of twofold and therefore not as striking as the activation of $pp60^{c-src}$ in MomT-transformed cells.

DISCUSSION

We have identified the product of the HaPV middle T gene as a 45-kDa phosphoprotein. We have further shown that in cells transformed by this protein, the middle T antigen is associated with a number of cellular proteins, including PP2A, tyrosine kinase, and PI-3 kinase. In many respects, therefore, HamT closely resembles MomT. From [³⁵S]methionine labeling experiments (30; this study), it appears that most of HamT and MomT is associated with PP2A. The conservation of this interaction suggests that it is very important in transformation, although the functional consequences of PP2A association with middle T antigen have yet to be elucidated.

In the case of both middle T antigens, kinase assay revealed the presence of a protein of approximately 81 kDa and PI-3 kinase activity. Recent results have shown that the p81 protein itself is unlikely to possess catalytic activity and suggest instead that it is a noncatalytic subunit of PI kinase (37a). It has been shown that the presence of a tyrosine in MomT at position 315 is an important determinant in the binding of PI-3 kinase to the protein (46) and that p81 appears to bind to middle T antigen which is phosphorylated at this site (15). We note that although the carboxy-terminal half of HamT is extremely divergent from that of MomT, a tyrosine and surrounding residues in an equivalent region of the hamster protein have been conserved. It will be interesting to create mutations in these sequences of HamT and test them for the ability to associate with PI-3 kinase activity.

HamT does, however, differ from MomT in a very important regard: it binds preferentially to $p59^{fyn}$ and only very weakly to $pp60^{c-src}$ and $pp62^{c-yes}$. While the low titer and scarcity of the tumor-bearing hamster sera precluded confirmation of these experiments by Western blotting, we have recently carried out the experiment shown in Fig. 7A by using [³⁵S]methionine-labeled HamT immunoprecipitates and obtained the same results (data not shown). In contrast, MomT binds mostly to $pp60^{c-src}$ and $pp62^{c-yes}$. Mutational analysis of $pp60^{c-src}$ has shown that one region critical for binding of MomT is that near tyrosine 527 (13). We noted that this region is almost perfectly conserved among these three members of the *src* family, suggesting that sequences elsewhere in the molecule also influence binding. Construction of chimeric molecules of $pp60^{c-src}$ and $p59^{fyn}$ should be very informative for mapping of the determinants important for binding of HamT and MomT more accurately. It has been shown that MomT is able to activate $pp60^{c-src}$ kinase approximately 10-fold and that this is achieved, at least in part, by prevention of phosphorylation of $pp60^{c-src}$ at the major negative regulatory site, Tyr-527 (11). In contrast, we detected only very weak activation of $p59^{fyn}$ by binding of HamT. While this may reflect the failure of our in vitro kinase assays to measure $p59^{fyn}$ activity accurately (none of the in vitro substrates available to us were as well phosphorylated by $p59^{fyn}$ as they were by $pp60^{c-src}$ [unpublished data]), it is also possible that large changes in overall kinase activity are not important in transformation, but rather the ability to associate with and phosphorylate certain proteins, e.g., p81, is a critical determinant.

Overall, HamT and MomT have 42% identity, the regions of greatest similarity being found in the amino-terminal halves of the proteins. Genetic analyses have shown that both the phosphatase and $pp60^{c-src}$ bind to this amino-terminal half of MomT but have been unable to separate the two binding sites. Indeed, it was possible that tyrosine kinase did not bind to middle T antigen directly but rather

bound to the associated PP2A. While this was unlikely, since no association between PP2A and pp60^{c-src} had been demonstrated in cells lacking middle T antigen (48a), we believe it is ruled out by the results presented here. Both HamT and mouse MomT are able to bind PP2A, yet they bind the tyrosine kinases of the *src* family with differing affinities. This suggests that the binding sites on the middle T antigens for phosphatase and kinase are distinct. Production of chimeric HamT-MomT should allow us to define the regions involved in tyrosine kinase binding. Such experiments are now in progress.

The function of the carboxy-terminal half of MomT is rather obscure. Mutational analysis of this region has identified several stretches of amino acids which are unimportant in transformation (see reference 45 for a review). However, many more such mutants are able to associate with all of the proteins described to date (i.e., phosphatase, tyrosine kinase, and lipid kinase) yet fail to transform (18, 38). It is interesting that MomT and HamT are rather divergent in this region (with the exception of possible tyrosine phosphorylation sites and the transmembrane domain) yet both are able to transform fibroblasts, albeit it with different efficiencies. Production of chimeric middle T antigens and analysis of their transforming properties may help to resolve this issue.

One of the most striking differences between the HaPV and mouse polyomavirus is their different transformation properties in vivo. Firstly, the murine virus is not normally associated with disease in its normal host; only inoculation of immunocompromised or newborn animals reveals its pathogenicity (47). In contrast, HaPV is associated with disease in at least 10% of the colonies in which it is endemic. Furthermore, inoculation of previously virus-free hamsters with the virus results in tumors with very short latency and high penetrance. Perhaps the most striking difference is that whereas mouse polyomavirus causes a number of different tumors but never lymphoma or leukemia, HaPV is associated predominantly with lymphoma-leukemia (4, 14, 26), with high incidences of epithelioma also appearing in one hamster strain (25, 27). It is possible that the differences between the mouse and hamster viruses reside in their different abilities to replicate in the tissues in question. However, in the case of MomT, lymphoma and leukemia have also never been seen in transgenic mice expressing the middle T antigen under the control of a number of different promoters-enhancers, including a construct containing the immunoglobulin E enhancer (40). It is therefore possible that middle T antigen itself dictates the tumor profile, although in the case of high- and low-tumor strains of mouse polyomavirus the middle T antigens are not responsible for the tumor profiles (24). In regard to lymphomas, it is noteworthy that lymphocytes have appreciable levels of p59^{lyn} (33, 44). Interestingly, it has recently been shown that some fraction of p59^{lyn} in T cells is associated with the T-cell receptor and it has been proposed that this enzyme is responsible for tyrosine phosphorylation of the receptor following antigen presentation (42). One could speculate that HamT binds to and activates p59^{lyn} in these T cells, thus circumventing the need for external signals to activate the cells. In any event, it will be interesting to introduce HamT into T cells to study its effects directly.

Use of the embryonal stem cell system has shown that one of the primary targets for MomT in embryo are endothelial cells (53). Middle T antigen appears to disrupt the normal controlled development of these cells, causing formation of hemangiomas. It is not known whether these effects are due to association of middle T antigen with the host cell phos-

phatases or kinases or both. Expression of HamT in the same system will allow us to approach this issue. These experiments are also in progress.

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