Association of p60^{fyn} with Middle Tumor Antigen in Murine Polyomavirus-Transformed Rat Cells

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Rabbit antisera raised against human FYN-specific peptides were used to evaluate the expression of the *fyn* gene product in normal and murine polyomavirus middle tumor antigen (MTAg)-transformed rat cells. The antisera were capable of detecting p60^{fyn} in both normal and MTAg-transformed cells. Two different antisera directed against unique p60^{fyn} sequences were found to detect p60^{fyn}-MTAg complexes in cell lysates from the MTAg-transformed cells. The MTAg molecules immunoprecipitated by FYN antisera were phosphorylated on tyrosine during immune-complex kinase reactions at sites similar to those found on MTAg in complexes with pp60^{c-src}. Whereas the abundance of p60^{fyn} was estimated to be less in the MTAg-transformed cells than in their normal counterparts, the specific activities of p60^{fyn} molecules in the normal and transformed cells were similar.

Oncogenic transformation of rodent cells by polyomavirus is dependent on the expression of middle tumor antigen (MTAg) (reviewed in reference 18). Transformation-competent MTAg molecules possess associated tyrosine-specific protein kinase activity that represents the functions of at least two members of the *src* family of protein kinases: pp60^{c-src} (4, 9-11) and p62^{c-yes} (15). One of the regions of MTAg that is important for stable association with pp60^{c-src} (and presumably p62^{c-yes}) has been localized to the amino-terminal portion of MTAg between residues 78 and 191 (3, 7, 19). At least one of the pp60^{c-src} sequence elements critical for MTAg complex formation has also been identified and corresponds to the carboxy-terminal residues Asp-Tyr-Phe-Thr-Ser-Thr-Glu-Pro, encompassing amino acids 518 to 525 (8). With the exception of an alanine at position 522, this sequence is the same as that of p62^{c-yes} and another member of the *src* family of tyrosine protein kinases, p60^{fyn} (14, 20). Because of the sequence identity between p62^{c-yes} and p60^{fyn} in this region, we conducted experiments to evaluate whether the *fyn* gene product is expressed in rodent cells susceptible to transformation by MTAg and to determine whether the *fyn* gene product is able to associate with MTAg in these cells.

Detection of p60^{fyn} by antisera to FYN peptides. Rabbit polyclonal antibodies to four different peptides predicted from the *fyn* gene sequence were generated (14, 20). The sequences selected (Fig. 1A) and the corresponding antisera were designated for the various FYN peptides used for immunization. Three of the antisera were directed against peptides representing amino-terminal sequences predicted to be unique to the *fyn* gene product (FYN1, FYN2, and FYN3); the fourth antiserum was directed against a peptide representing the last 13 predicted FYN amino acids (FYN4). In addition, rabbit antibodies to a peptide representing the last 25 carboxy-terminal amino acids of pp60^{c-src} (SRC2) were generated. The amino acid sequence variation between

the *fyn* and *src* gene products within this carboxy-terminal region is indicated in Fig. 1A.

All five of the antisera were able to immunoprecipitate the human *fyn* gene product from detergent lysates of FYN^d cells, which are NIH 3T3 cells overexpressing the normal human *fyn* gene (Fig. 1B) (13). Each of the antisera was able to support immune-complex protein kinase reactions (Fig. 1C) and could detect the denatured *fyn* gene product in immunoblot assays (data not shown). Quantitation of these and other, unpublished results revealed that FYN3 represented the most efficient of the three amino-terminal FYN antisera for immunoprecipitating the *fyn* gene product. These evaluations also demonstrated that the human *fyn* gene product comigrated in our sodium dodecyl sulfate-polyacrylamide gels with avian pp60^{c-src}, a result consistent with the predicted molecular mass of 60,856 daltons for the *fyn* primary translation product (including the initiating methionine). For comparison, the primary avian *src* translation product is predicted to have a molecular mass of 60,053 daltons. Although previous studies have referred to the *fyn* gene product as p59^{fyn} (6, 13, 16), in this study we have designated it p60^{fyn}.

Detection of p60^{fyn} in Fisher rat (FR) 3T3 cells. The sequence of the rodent *fyn* gene is not known. However, the amino acid sequence of pp60^{c-src} is highly conserved between species, which suggests that antisera recognizing human p60^{fyn} might also be expected to recognize the rodent *fyn* gene product. We therefore evaluated by immune-complex kinase assays expression of FYN-related kinases in normal rodent cells in culture. Our results revealed that FYN3-reactive 60-kilodalton kinases could be detected in all mouse, rat, and hamster cell lines examined (data not shown).

Figure 2A demonstrates the specificity of the FYN3 antiserum for the *fyn* gene product in FR 3T3 cell lysates. As assessed by immune-complex protein kinase assays, the ability of the FYN3 antiserum to immunoprecipitate rat p60^{fyn} could be prevented by preincubating the antiserum with FYN peptide 3 (lane 3) but not with FYN peptide 1

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FIG. 1. Detection of human $p60^{fyn}$ by antisera to FYN peptides. (A) Predicted sequences of the human *fyn* gene product from amino acids 9 to 48 and 513 to 537 used for peptide synthesis. The peptides were synthesized by either Peninsula Laboratories (San Carlos, Calif.) or MilliGen/Bioscience (San Rafael, Calif.). The sequences selected for synthesis and the nomenclature of the antisera generated to the individual peptides are indicated. The sequence variation of FYN and SRC between amino acids 513 and 537 (FYN numbering) is also indicated. (B) SRC2 immunoblot analysis of peptide antiserum immunoprecipitates of FYN^d cells (13). The cells were lysed in TNNE buffer (100 mM Tris hydrochloride [pH 8.0], 100 mM NaCl, 1% Nonidet P40, 2 mM EDTA, 10 μ g of aprotinin per ml, 10 μ g of leupeptin per ml, 100 μ M sodium orthovanadate), adjusted to a protein concentration of 50 μ g/ml, and immunoprecipitated (4) with FYN1 (lane 3), FYN2 (lane 4), FYN3 (lane 5), FYN4 (lane 6), or SRC2 (lane 7) antiserum or with no antiserum (lane 2). For molecular weight comparison, a 100- μ g/ml concentration of a TNNE lysate from NIH 3T3(pMsrc/cos)A cells (NIH 3T3 cells overexpressing avian $pp60^{c-src}$; 12) was immunoprecipitated with SRC2 antiserum (lane 1). The immunoprecipitates were washed with TNNE, separated by electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose, and reacted with SRC2 antiserum and 125 I-protein A (Amersham Corp., Arlington Heights, Ill.) (22). (C) Immune-complex protein kinase assays (4) of TNNE FYN^d lysates (25 μ g/ml) immunoprecipitated with FYN1 (lane 2), FYN2 (lane 3), FYN3 (lane 4), FYN4 (lane 5), or SRC2 (lane 6) antiserum or with no antiserum (lane 1). Positions of $pp60^{c-src}$ (src), $p60^{fyn}$ (fyn), immunoglobulin heavy chain (IgG), and prestained molecular mass markers (Amersham) are indicated.

(lane 4) or other peptides (data not shown). Immunoblot analysis of the FYN3 immunoprecipitates also showed that the rat *fyn* gene product comigrated with the rat *src* gene product (Fig. 2B).

The results of experiments evaluating the ability of the FYN3 antiserum to support $p60^{fyn}$ kinase activity in immune complexes and to detect $p60^{fyn}$ abundance in different amounts of FR 3T3 cell lysates are shown in Fig. 2C and D. In FR 3T3 lysates adjusted to protein concentrations of 10 to 1,250 μ g/ml, the FYN3 antiserum was capable of resolving $p60^{fyn}$ protein kinase activity (Fig. 2C) and abundance (Fig. 2D) at protein concentrations of approximately 20 to 1,000 μ g/ml. Thus, the FYN3 antiserum was adequate for quantitating the relative levels of $p60^{fyn}$ abundance and protein kinase activity over a 50-fold range in these cell lysates.

Detection of $p60^{fyn}$ -MTAg complexes in MTag-transformed FR 3T3 cells. To evaluate whether $p60^{fyn}$ and MTag are complexed in polyomavirus-transformed cells, FYN3 im-

mune-complex protein kinase assays were performed on detergent lysates from FR 3T3 cells and FR 18-1 cells. FR 18-1 (FR10281/18-1) (1) is a clonal cell line derived from a single focus that arose after transfection of FR 3T3 cells with pPYMT-1, a recombinant plasmid capable of encoding only MTag (21). The results of the FYN3 protein kinase assays along with those of monoclonal antibody (MAb) 327 (a monoclonal antibody specific for $pp60^{c-src}$) (17) and hamster T serum (serum from hamsters bearing polyomavirus-induced tumors) (4) immune-complex protein kinases assays from parallel lysates are shown in Fig. 3. A 56-kilodalton protein that comigrated with MTag was phosphorylated in the FYN3 immune complexes from the FR 18-1 lysates (lane 4). Phosphoamino acid analysis revealed that this protein was phosphorylated primarily on tyrosine residues, although phosphoserine was also detected (data not shown). The same protein was also found to be phosphorylated when immune-complex kinase assays were conducted with the

FIG. 2. Detection of rat $p60^{fyn}$ by antisera to human FYN peptides. (A) FR 3T3 cells were lysed in TNNE buffer, the protein concentration was adjusted to 500 $\mu\text{g/ml}$, and equal portions were immunoprecipitated with FYN3 antiserum (lane 2), FYN3 antiserum plus 5 μg of FYN3 peptide (lane 3), FYN3 antiserum plus 5 μg of FYN1 peptide (lane 4), MAb 327 (17) (lane 5), or no added serum (lane 1); immune-complex kinase assays were then conducted. (B) FR 3T3 cells were lysed and immunoprecipitated as described above with no serum added (lane 1), FYN3 antiserum (lane 2), or MAb 327 (lane 3), and SRC2 immunoblot analysis was conducted. (C and D) FR 3T3 cells were lysed, the protein concentration adjusted to the indicated amounts, equal portions were immunoprecipitated with FYN3 antiserum, and immune-complex protein kinase assays (C) or SRC2 immunoblot analysis (D) were conducted.

FYN1 antiserum (Fig. 3B, lane 3). However, proteins with similar mobilities were found to be phosphorylated intermittently in FYN3 and FYN1 immune complexes when lysates from FR 3T3 cells were used (Fig. 3A, lane 3; Fig. 3B, lane 1).

To establish the possible identities of the 56-kilodalton phosphoproteins detected in the immune-complex kinase assays, V8 peptide analysis was conducted. At least one of the 56-kilodalton proteins phosphorylated on tyrosine in the FYN3 immune-complex kinase assays was MTA γ (Fig. 3C, lane 2). The additional V8 phosphopeptides observed were found to originate from the comigrating protein that could be detected in FYN3 immune complexes from FR 3T3 lysates (lane 1).

Partial V8 proteolysis was also used to evaluate the $p60^{fyn}$ molecules phosphorylated in these assays. Phosphorylated $p60^{fyn}$ derived from the FR 3T3 cells (Fig. 3C, lane 5) yielded the same V8 phosphopeptides as did the protein derived from the FR 18-1 cells (Fig. 3C, lane 6). Interestingly, the autophosphorylated rat $p60^{fyn}$ V8 phosphopeptides were

also indistinguishable from those observed after V8 digestion of autophosphorylated human $p60^{fyn}$ (Fig. 3C, lane 10).

The protein kinase-specific activities of $pp60^{c-src}$ and $pp62^{c-yes}$ molecules associated with MTA γ have been shown to be elevated (2, 4, 5, 9, 15). To determine whether the $p60^{fyn}$ molecules associated with MTA γ might also have increased protein kinase activity, immune-complex kinase assays, using rabbit muscle enolase as an exogenous substrate, and SRC2 immunoblot analysis were conducted on FYN3 immunoprecipitates from equal amounts of FR 3T3 and FR 18-1 cell lysates. The total protein kinase activity of $p60^{fyn}$ from FR 18-1 cells was found to be less than that of $p60^{fyn}$ from FR 3T3 cells (Fig. 4A). Quantitation of the parallel SRC2 immunoblots (Fig. 4B) revealed that the level of $p60^{fyn}$ protein kinase activity was proportional to the amount of $p60^{fyn}$ immunoprecipitated by the FYN3 antiserum in both cell types. Similar results were obtained with use of the FYN1 antiserum (data not shown).

The results presented in this report establish that rabbit antisera raised against human FYN-specific peptide se-

FIG. 3. Detection of $p60^{fyn}$ -MTAg complexes in FR 18-1 cell lysates. (A) FR 3T3 (odd lanes) and FR 18-1 (even lanes) cells were lysed in TNNE buffer, the protein concentration was adjusted to 500 $\mu\text{g}/\text{ml}$, and equal portions were immunoprecipitated with MAb 327 (lanes 1 and 2), FYN3 antiserum (lanes 3 and 4), or hamster T serum (lanes 5 and 6) before immune-complex kinase assays were performed. (B) FR 3T3 (lanes 1 and 2) and FR 18-1 (lane 3) cells were lysed in TNNE buffer, the protein concentration was adjusted to 500 (lanes 1 and 3) or 200 (lane 2) $\mu\text{g}/\text{ml}$, and equal portions were immunoprecipitated with FYN1 antiserum before immune-complex kinase assays were performed. (C) The phosphorylated bands (a through h in panels A and B) were excised, and V8 protease analysis (4) was conducted (lanes 1 through 8). Lanes 9 and 10, V8 analysis of $pp60^{c-src}$ and $p60^{fyn}$ isolated after immune-complex autophosphorylation from pMcsrc/cosA (lane 9) or FYN^d (lane 10) cells.

quences are capable of recognizing $p60^{fyn}$ in normal and transformed rodent cells. These antisera were used to demonstrate specifically that $p60^{fyn}$ is expressed in normal FR 3T3 cells and that $p60^{fyn}$ can be detected in association with polyomavirus MTag in lysates of MTag-transformed FR 3T3 cells.

Using different cell systems, two other independent groups have also found that the product of the *fyn* gene is capable of associating with MTag (6, 16). Of these studies, the murine cell system used by Kypta et al. (16) most closely

resembles the rat cell system presented in this report. Although there are some differences in the results obtained for the mouse and rat cell systems, the general conclusions reached by Kypta et al. concerning the interactions of the rodent *fyn* gene product and MTag (16) are strongly supported by the results described here.

The number of MTag molecules stably associated with $p60^{fyn}$ in FR 18-1 cells, as evaluated by FYN1-FYN3 immune-complex kinase assays and SRC2 immunoblot analysis, appears to be low. This conclusion is supported by experiments in which the amount of MTag immunoprecipitated with $p60^{fyn}$ by either FYN1 or FYN3 antiserum was found to be below the level of detection by PAb 815 immunoblot analysis (data not shown). PAb 815 is a monoclonal antibody that recognizes MTag (2) and has been used in immunoblot assays for determining that 10 to 15% of the total MTag in FR 18-1 cells is associated with $pp60^{c-src}$ (2). Thus, our results with this assay suggest that less than 5% of MTag in FR 18-1 cells can be immunoprecipitated with the available antisera to $p60^{fyn}$. Additional support for this view is provided by experiments in which clearing of all detectable $p60^{fyn}$ from FR 18-1 lysates with use of FYN1 and FYN3 antisera did not significantly diminish the level of MTag-associated protein kinase activity in hamster T serum and MAb 327 immune complexes (data not shown). These results are in contrast to those in which clearing of FR 18-1 lysates with MAb 327 reduced MTag-associated kinase activity by as much as 75% (2). Interestingly, immunological clearing of $pp60^{c-src}$ - and $pp60^{c-src}$ -associated MTag from FR 18-1 lysates with MAb 327 did not diminish the level of detectable $p60^{fyn}$ -MTag complexes (data not shown). These results are consistent with the idea that, as with $p62^{c-yes}$ -MTag complexes, $p60^{fyn}$ and MTag are in complexes separate from the $pp60^{c-src}$ -MTag complexes.

Our experiments do not provide evidence that implicates or eliminates the involvement of $p60^{fyn}$ in MTag-mediated transformation. Evidence suggesting that $p60^{fyn}$ may not be

FIG. 4. Analysis of $p60^{fyn}$ protein kinase activity and abundance in FR 3T3 and FR 18-1 cell lysates. TNNE buffer lysates of FR 3T3 and FR 18-1 cells were adjusted to the same protein concentration (250 $\mu\text{g}/\text{ml}$) and immunoprecipitated with FYN3 antiserum. (A) Immune-complex kinase assays using rabbit muscle enolase as an exogenous substrate; (B) SRC2 immunoblot analysis of parallel FYN3 immune complexes.

involved in MTag transformation events includes the observations that no alterations in the specific activity of $p60^{fyn}$ molecules associated with MTag could be detected and that the abundance of $p60^{fyn}$ in these MTag-transformed FR 3T3 cells was less than that in normal FR 3T3 cells. However, because alterations in the specific activity and abundance of *src*-related tyrosine kinases in immune complexes may not reflect potentially important functions provided by this group of enzymes *in vivo*, the lack of detectable changes in $p60^{fyn}$ kinase in the experiments reported here should be interpreted cautiously. In addition, our evaluation of the ability of $p60^{fyn}$ to associate with transformation-defective MTags (e.g., NG59, SD15, dl23, and dl1015) proved not to be helpful in distinguishing the possible importance of $p60^{fyn}$, since the pattern of $p60^{fyn}$ association with these MTag molecules was the same as that previously established for $pp60^{c-src}$ (18). The same conclusions were reached by Kyta et al. (16).

It is now clear that at least three of the seven known members of the *src* family of tyrosine protein kinases ($pp60^{c-src}$, $p62^{c-yes}$, and $p60^{fyn}$) are capable of associating with transformation-competent MTag molecules. These three proteins are all expressed in the rodent fibroblast-derived cells and cell lines most commonly used for evaluating MTag transformation, thereby making the potential roles of each of these protein kinases more difficult to determine. Generation of additional immunological and molecular reagents will be essential for future progress in understanding these interactions.

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