NOTES

Investigation of the Role of P130^{gag-fps} in Transformation: Generation and Use of a Temperature-Sensitive Mutant P130^{gag-fps}

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Changing Glu-1025 to Asp in Fujinami sarcoma virus P130^{gag-fps} made the protein temperature sensitive for transformation and protein-tyrosine kinase activity. Another mutant, Phe-1073 P130^{gag-fps}, lacking the major autophosphorylation site, has an extended latent period for transformation (G. A. Weinmaster, M. J. Zoller, M. Smith, E. Hinze, and T. Pawson, *Cell* 37:559-568, 1984). By introducing the Asp-1025 lesion into Phe-1073 P130^{gag-fps}, we showed that this mutant protein is required for the maintenance of the transformed phenotype of Phe-1073 P130^{gag-fps}-expressing cells.

Fujinami sarcoma virus (FSV) causes tumors in animals and transforms fibroblasts in culture (12). The FSV transforming protein is a protein-tyrosine kinase (6). FSV mutants temperature sensitive (*ts*) for transformation encode *v-fps* proteins with *ts* protein-tyrosine kinase activity, implying that tyrosine phosphorylation is necessary for transformation by FSV (7, 11, 13, 17). Shibuya and Hanafusa have cloned and sequenced a temperature-resistant strain of FSV, FSV-2, which encodes a 130-kilodalton (kDa) protein (P130^{gag-fps}) (19). This cloned FSV-2 genome has been used to introduce a number of mutations into the protein kinase domain of P130^{gag-fps}. Amino acid substitutions (24, 25), insertions (18, 20), or deletions (1) in the protein kinase domain, which are deleterious for enzymatic activity, impair the transforming potential of P130^{gag-fps}. This impairment establishes the importance of tyrosine phosphorylation in FSV transformation. Substitution of Tyr-1073, the major autophosphorylation site in the protein kinase domain of P130^{gag-fps}, with Phe decreases both the enzymatic activity and transforming ability of the protein (25). In addition, the latent period for focus formation is prolonged by this point mutation, being more than 30 days for the F-1073 FSV compared with approximately 8 days for the wild-type (WT) FSV (2, 25). The reason for the slow morphological change observed in the F-1073 mutant-transformed cells is unclear. Peptide mapping of WT and mutant P130^{gag-fps} proteins indicates that aside from the directed mutation (Tyr to Phe), the proteins are structurally indistinguishable (25). This result suggests that second site mutations in the mutant protein are not responsible for the eventual development of the F-1073-transformed phenotype. The ultimate efficiency of transformation by F-1073 DNA is similar to that of WT DNA when it is transfected into rat-2 cells, giving credence to the idea that dominant cellular alterations are not a cause of the protracted transformation seen with the F-1073-transfected rat-2 cells (25). Thus, it seems reasonable to assume that expression of the F-1073 P130^{gag-fps} protein is involved in achieving and maintaining the transformed phenotype. One could test whether this is the case if an F-1073 P130^{gag-fps} protein *ts* for transformation were available.

Recently, Chen et al. (3) have sequenced the *v-fps* region of the *ts* FSV mutant FL-15, which encodes a P140^{gag-fps} transforming protein, to define the *ts* lesions of this conditional mutant. Comparison of this sequence with the known sequence of the temperature-resistant FSV-2 (19) and the chicken *c-fps* gene (8) identified two putative *ts* mutations. Further analysis of recombinants between the *ts* and temperature-resistant FSV sequences indicates that a change of Glu to Asp in the protein kinase domain, at position 1025, is sufficient for a complete *ts* phenotype (3).

Given these results, we wanted to confirm that a single conservative change in the catalytic domain would confer temperature sensitivity upon the temperature-resistant P130^{gag-fps} protein and then introduce this mutation into the F-1073 mutant to extend its characterization. Glu at position 1025 in both the WT P130^{gag-fps} and F-1073 P130^{gag-fps} proteins was converted to Asp by using the oligonucleotide 5'-AGATGATGGACAATGCCG-3' to change the GAG codon (nucleotides 3452 to 3454 in the genomes of FSV-2 and F-1073 FSV cloned in M13 [M13-FSV-2 and M13-FSV-2-F-1073]) to a GAC codon (25). Mutants were initially identified by their differential hybridization to the ³²P-labeled mutagenic primer and confirmed by DNA sequencing (25). The D-1025 or D-1025/F-1073 FSV genomes were transfected into rat-2 cells as previously described, except that cultures were maintained at 34°C (25). Foci of transformed cells formed on monolayers of D-1025 FSV-transfected fibroblasts with morphology, efficiency, and latent period similar to those occurring with WT FSV DNA-transfected fibroblasts.

In contrast, cells transfected with D-1025/F-1073 FSV DNA did not appear altered until at least 30 days posttransfection, a time similar to that of the latent period for transformation by the parental F-1073 FSV. In addition to the lengthened latent period, the morphological changes seen with the D-1025/F-1073-transfected cells were at first very subtle, but with time the transformed phenotype became more obvious. However, the final morphological phenotype of the D-1025/F-1073-transformed cells was not as pronounced as that displayed by the WT-transformed cells. Despite the greater latency and the morphological differences, the efficiency of focus formation with D-1025/F-1073

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DNA was as great as that with WT FSV DNA. These events recapitulate those previously reported for F-1073 FSV-transfected cells (25) and indicate that the additional mutation (Glu to Asp) within F-1073 P130^{gag-fps} did not drastically alter its transforming potential at 34°C.

Clonal cell lines of D-1025- and D-1025/F-1073-transformed cells were established at the permissive temperature (34°C). The D-1025-transformed cells were refractile and showed a growth pattern similar to that of the WT FSV-transformed cells when they were cultured at 34°C (Fig. 1). However, when the D-1025-transformed cells were shifted to the nonpermissive temperature (39°C), they lost their WT-transformed morphology and appeared more like the flat parental rat-2 fibroblasts (Fig. 1), indicating that the conversion of Glu-1025 to Asp does indeed result in a *ts* P130^{gag-fps}. The flattening out of these cells was apparent within 2 h of the temperature shift, and the final normal fibroblast morphology was achieved within 4 h of incubation at 39°C. When these same cells were shifted back to the temperature permissive for transformation, a slight morphological change was apparent after 2 h of incubation, but the complete transformed phenotype was not observed until after at least 10 h of incubation. Similar results were obtained when the D-1025/F-1073-transformed cells were shifted from 34 to 39°C (Fig. 1), indicating that the mutant protein was *ts* and, more important, that the D-1025/F-1073 P130^{gag-fps} protein was contributing to the phenotype of the cells. However, the recovery of the D-1025/F-1073-transformed morphology was not as rapid as that observed for the D-1025 P130^{gag-fps}-expressing cells.

Consistent with the morphological changes observed when D-1025- or D-1025/F-1073-transformed cells were shifted from 34 to 39°C, these same cells formed colonies in soft agar at 34°C but not at 39°C (data not shown). In contrast, the WT- and F-1073-transformed cells formed colonies in soft agar at both 34 and 39°C (data not shown).

To confirm that the observed changes in cellular morphology correlated with the expression of the respective P130^{gag-fps} proteins, the various FSV-transformed cell lines were metabolically labeled with 100 μ Ci of [³⁵S]methionine per ml at 34 or 39°C for 18 h (Fig. 2). P130^{gag-fps} proteins were immunoprecipitated with an anti-p19^{gag} monoclonal antibody from the various transformed cell lines as previously described (9, 22, 25). Anywhere from one to three forms of P130^{gag-fps} were present, depending on the cell line and the incubation temperature used in the labeling studies, resulting in a broadening of the P130^{gag-fps} labeled band (Fig. 2, arrowhead). These multiple forms are probably due to differential tyrosine phosphorylation, with the upper bands being more highly phosphorylated than the lower bands (14).

D-1025- or D-1025/F-1073-transformed cells labeled with [³⁵S]methionine at 39°C had fewer of the slower migrating forms of P130^{gag-fps} than the same cells labeled at 34°C, presumably because of the failure of the *ts* P130^{gag-fps} proteins to autophosphorylate at 39°C (4, 14). To investigate this possibility, cells were labeled with 1 mCi of ³²P_i per ml overnight at 34 or 39°C to determine if the stoichiometries of phosphorylation were different. Equal amounts of ³²P were incorporated into the WT P130^{gag-fps} and F-1073 P130^{gag-fps} proteins at both 34 and 39°C (Fig. 2). In contrast, when the amount of ³²P incorporated into D-1025 P130^{gag-fps} was adjusted to the amount of [³⁵S]methionine incorporated at each temperature, less than 20% of the phosphate incorporated into D-1025 P130^{gag-fps} at 34°C was detected at 39°C (Fig. 2). The difference in the phosphorylated state of D-1025/F-1073 P130^{gag-fps} at the two growth temperatures

was not as large, but it should be noted that this mutant protein lacks the major autophosphorylation site (Tyr-1073), which greatly reduces the amount of phosphotyrosine in the protein even at 34°C (see below).

Phosphoamino acid analysis of WT P130^{gag-fps} isolated from cells labeled with ³²P_i at 34 or 39°C indicated that both serine and tyrosine residues were phosphorylated to approximately equal extents at both temperatures (Fig. 3A). D-1025 P130^{gag-fps} isolated from cells grown at 34°C was also phosphorylated equally on serine and tyrosine residues, but when it was isolated from cells incubated at 39°C, the amount of phosphotyrosine was considerably reduced compared with that of phosphoserine (Fig. 3B). In F-1073 P130^{gag-fps}, the absence of Tyr-1073 clearly decreased the amount of phosphotyrosine detected in P130^{gag-fps} compared with that of phosphoserine (25), but the temperature shift-up did not alter this ratio (Fig. 3C). The ratio of phosphoserine to phosphotyrosine detected in D-1025/F-1073 P130^{gag-fps} at 34°C was similar to that observed for F-1073 P130^{gag-fps} isolated from cells maintained at both temperatures. However, the amount of phosphotyrosine in D-1025/F-1073 P130^{gag-fps}, although low already, was further decreased by the shift to 39°C (Fig. 3D).

The phosphorylation state of P130^{gag-fps} and its ability to transform cells are directly related to its intrinsic protein kinase activity. We therefore quantitated the protein kinase activity inherent in the P130^{gag-fps} proteins immunoprecipitated from cells cultured at 34 or 39°C. The specific activity of P130^{gag-fps} was determined by including enolase as a substrate in an in vitro kinase assay containing immunoprecipitated P130^{gag-fps} and [γ -³²P]ATP. Specific activities were obtained by dividing the ³²P counts per minute incorporated into enolase by the amount of P130^{gag-fps} in the immune complex kinase reaction, as measured by ³⁵S counts per minute in the immunoprecipitate. The values obtained for the different FSV P130^{gag-fps} proteins were compared with the specific activity for WT P130^{gag-fps} isolated from WT-transformed cells maintained at 34°C, which was set at 100 (Fig. 4). The protein kinase activities for the WT and D-1025 P130^{gag-fps} proteins were comparable at 34°C. As expected, the decreased tyrosine phosphorylation of D-1025 P130^{gag-fps} maintained at 39°C (Fig. 3B) correlated with a lower protein kinase specific activity. In contrast, the protein kinase activity of WT P130^{gag-fps} was relatively unchanged, and the F-1073 P130^{gag-fps} protein kinase activity was only slightly decreased under the same conditions (Fig. 4).

The protein kinase activity of D-1025/F-1073 P130^{gag-fps} was lower than that of F-1073 P130^{gag-fps} at both temperatures, possibly because of the combined deleterious effect of the two mutations on the activity of the protein (Fig. 4). The incremental decrease in protein kinase activity between 34 and 39°C for the double mutant was not as great as that observed for D-1025 P130^{gag-fps}, but the percentage of decrease was similar. Curiously, the activity of D-1025 P130^{gag-fps} isolated from cells maintained at 39°C was equivalent to that determined for D-1025/F-1073 P130^{gag-fps} isolated from cells grown at 34°C (Fig. 4). This is intriguing since the D-1025 P130^{gag-fps}-expressing cells exhibited a normal rat-2 cellular morphology and were incapable of anchorage-independent growth at 39°C, in contrast to the D-1025/F-1073-transformed cells maintained at 34°C.

Because of the apparent discrepancy between in vitro protein kinase activity and cellular phenotype, we attempted to determine in vivo protein kinase activity by using antibodies directed against phosphotyrosine to probe immuno-

FIG. 1. Morphological phenotype of rat-2 cells and FSV-transfected rat-2 cells cultured at the permissive (34°C) and nonpermissive (39°C) temperatures for transformation. Rat-2 cells were transfected with 100 ng of WT, D-1025, F-1073, or D-1025/F-1073 FSV DNA as described previously (25) and examined for the appearance of transformed cells, which were then cloned and expanded into stable cell lines. Phase-contrast photomicrographs ($\times 100$) are of monolayers of rat-2 cells and WT, D-1025, F-1073, or D-1025/F-1073 FSV-expressing cells maintained at 34 or 39°C.

FIG. 2. Expression and phosphorylation of FSV P130^{gag-fps} in WT and mutant FSV-transformed cells at the permissive (34°C) and nonpermissive (39°C) temperatures. P130^{gag-fps} was isolated from equivalent numbers of cells labeled for 18 h with [³⁵S]methionine or ³²P_i at 34 and 39°C by immunoprecipitation with anti-p19^{gag} antibody as described previously (9, 22, 25). Immunoprecipitates were analyzed by electrophoresis on 7.5% sodium dodecyl sulfate-polyacrylamide gels followed by autoradiography (9, 22). Immunoprecipitates from WT, D-1025, F-1073, or D-1025/F-1073 FSV-expressing cells labeled at 34°C are in lanes C, E, G, and I, respectively, and those labeled at 39°C are in lanes B, D, F, and H, respectively. Lane A contains immunoprecipitates prepared from rat-2 cells labeled at 34°C. Exposure times were 18 h for the ³⁵S-labeled gel and 8 h for the ³²P-labeled gel. The arrowhead indicates the P130^{gag-fps} labeled band.

blots of total cellular proteins from WT-, D-1025-, F-1073-, and D-1025/F-1073-transformed cells and rat-2 cells. All cells were grown at 34°C and then shifted to 39°C for 24 h or were maintained continuously at 34°C (Fig. 5). As previously reported, there were distinct differences in the numbers and degree of reactive proteins detected in the rat-2 cells and WT- or F-1073-transformed cell lines (23). The rat-2 cells had very few phosphotyrosine-containing proteins, while the WT-transformed cells had several new phosphotyrosine-containing proteins, including P130^{gag-fps} itself (Fig. 5, asterisks). There were some changes in the intensity of reactive proteins when WT-transformed cells were shifted to 39°C, but these differences did not correlate with any of the morphological or biochemical properties reported here. The intensity and number of phosphotyrosine-containing proteins in F-1073-transformed cells were decreased compared with those of WT P130^{gag-fps}-transformed cells, consistent with the lower level of F-1073 P130^{gag-fps} kinase activity. There was no change in the respective patterns of proteins reactive with the antibody when rat-2 cells or F-1073-transformed cells were shifted from 34 to 39°C (Fig. 5; compare lanes A and B and lanes G and H).

The pattern of reactive proteins obtained from D-1025 P130^{gag-fps}-expressing cells incubated at 34°C was comparable with that detected in WT-transformed cells maintained at both temperatures (Fig. 5). However, when these *ts* cells were grown at 39°C, the pattern of reactive proteins was similar to that of normal rat-2 cells. The low levels of phosphotyrosine-containing proteins in these cells maintained at 39°C is consistent with normal levels of total cellular phosphotyrosine reported for *ts* FSV-infected chick cells at 39°C (17). In fact, the overall differences in phosphotyrosine-containing proteins detected by the antibody correlated well with the morphological phenotypes associ-

ated with the cell lines at 34 and 39°C (Fig. 1). Although D-1025/F-1073 P130^{gag-fps}-expressing cells grown at 34°C had far fewer phosphotyrosine-containing proteins than F-1073-transformed cells did, there were bands present that were not in rat-2 cells (Fig. 5). The pattern of proteins in D-1025/F-1073 cells grown at 39°C was essentially like that of rat-2 cells.

Are any of these phosphotyrosine-containing proteins likely to be involved in the transformed phenotype? A strongly reactive 120-kDa protein (Fig. 5, arrow), whose presence was correlated with characteristics of the phenotype of normal rat-2 cells such as anchorage-dependent growth and a flat fibroblast cellular morphology, was greatly enhanced with *ts* cell lines were shifted to 39°C (Fig. 5, lanes F and J). This protein may be similar to a major phosphotyrosine-containing protein detected in quiescent mouse fibroblasts with antiphosphotyrosine antibodies (16). The only protein positively correlated with the transformed phenotype was a 63-kDa protein (Fig. 5, arrowhead) which was more prominent in cells with a transformed phenotype than in normal cells. This is illustrated by the comparison of normal D-1025 cells grown at 39°C, in which the 63-kDa protein is undetectable, with transformed D-1025/F-1073 cells, which have similar levels of *in vitro* P130^{gag-fps} protein kinase activity and in which the 63-kDa protein is readily detectable. One striking conclusion from the results with the D-1025/F-1073 cells is that only a minimal increase in tyrosine phosphorylation was required to transform normal rat fibroblasts into cells capable of focus formation and anchorage-independent growth (Fig. 5, compare lanes A, I, and J).

The *ts* phenotype and protein-tyrosine kinase activity displayed by D-1025 P130^{gag-fps} confirm the previously reported work of Chen et al. (3) that a Glu-to-Asp change at residue 1025 is sufficient to render the FSV transforming

FIG. 3. Phosphoamino acid analysis of WT, D-1025, F-1073, or D-1025/F-1073 P130^{gag-fps} at 34 and 39°C. Cells expressing WT, D-1025, F-1073, or D-1025/F-1073 FSV P130^{gag-fps} were labeled for 18 h with 1 mCi of ³²P_i per ml at 34 and 39°C. P130^{gag-fps} proteins isolated by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were extracted and hydrolyzed as described elsewhere (5, 9, 22). The acid hydrolysates were separated on thin-layer cellulose plates as previously described (5). Cerenkov counts per minute of indicated samples loaded onto plates and their exposure times (at -70°C with an intensifying screen) were as follows. (A) WT; 1,810 cpm at 34°C and 1,795 cpm at 39°C; 1 day. (B) D-1025; 608 cpm at 34°C and 380 cpm at 39°C; 2 days. (C) F-1073; 792 cpm at 34°C and 650 cpm at 39°C; 2 days. (D) D-1025/F-1073; 174 cpm at 34°C and 156 cpm at 39°C; 5 days. Phosphoserine is in the upper left and phosphotyrosine is in the lower right of each panel.

protein sensitive to temperature. It is remarkable that a conservative change which involves the difference of a single methylene group within a 130-kDa protein can have such a

FIG. 4. Specific protein-tyrosine kinase activities of WT, D-1025, F-1073, and D-1025/F-1073 P130^{gag-fps} proteins isolated from cells incubated at 34 and 39°C. The intrinsic protein-tyrosine kinase activities for enolase phosphorylation by the indicated FSV P130^{gag-fps} proteins at 34 and 39°C were determined as described in the text and elsewhere (25).

FIG. 5. Phosphotyrosine-containing proteins identified by immunoblotting cellular lysates with antiphosphotyrosine antibodies. Equivalent amounts of cellular proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitrocellulose filter, and incubated with antibodies directed against phosphotyrosine as described elsewhere (9a, 21). Lysates were prepared from the following cell lines: rat-2, WT, D-1025, F-1073, or D-1025/F-1073 incubated at the permissive temperature (34°C) (lanes A, C, E, G, and I, respectively) and at the nonpermissive temperature (39°C) (lanes B, D, F, H, and J, respectively). The 120-kDa protein is indicated with an arrow; the 63-kDa protein is marked with an arrowhead. Exposure time was 12 h at -70°C with an intensifying screen.

dramatic effect on protein function and activity. The effect of adding this *ts* lesion to F-1073 P130^{gag-fps} allows us to conclude that mutated P130^{gag-fps} is required for the maintenance of the transformed state of the cells, despite the extended latent period associated with mutation of Tyr-1073 within the protein. The properties of the D-1025 mutants of both WT and F-1073 P130^{gag-fps} show that this mutation has a very stringent *ts* phenotype. It will therefore be useful for creating additional conditional P130^{gag-fps} mutants.

Although we can conclude that F-1073 P130^{gag-fps} is required for the maintenance of the transformed phenotype, the mechanism determining the long latent period for transformation associated with amino acid substitutions of Tyr-1073 in P130^{gag-fps} remains an enigma (2, 25). It seems reasonable to propose that P130^{gag-fps} is needed for the initiation of transformation as well, but it is possible that additional changes, either in cellular or *v-fps* sequences, are involved in the establishment of the transformed state. However, WT FSV DNA or mutant F-1073 FSV DNA cotransfected with the herpes simplex virus *tk* gene yielded similar numbers of hypoxanthine-aminopterin-thymidine-resistant colonies, the same percentage of which have a transformed morphology (25). Thus, if there were a second event, it would have to occur at a very high frequency. In addition, this putative second event cannot be sufficient for transformation, since D-1025/F-1073-transformed cells revert to a normal phenotype at 39°C. An alternative explanation for the long latent period is that the adjacent normal cells suppress the transformed phenotype and clonal expansion of cells transfected with F-1073 FSV DNA. This is a well-documented phenomenon for other types of trans-

formed cells (10, 15). This suppression might prevent the appearance of foci in the case of F-1073 P130^{gag-fps}, which is partially debilitated, until a time when these transfected cells can produce a microenvironment conducive to outgrowth.

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