An Identity Crisis for fps/fes: Oncogene or Tumor Suppressor?

Waheed Sangrar,1,2 Ralph A. Zirnibl,1,2 Yan Gao,1 William J. Muller,1 Zongchao Jia,3 and Peter A. Greer1,2,3

1Division of Cancer Biology and Genetics, Queen's University Cancer Research Institute and Departments of Pathology and Molecular Medicine and 2Biochemistry, Queen's University, Kingston, Ontario, Canada and 3Molecular Oncology Group, McGill University Health Center, Montreal, Quebec, Canada

Abstract

Fps/Fes proteins were among the first members of the protein tyrosine kinase family to be characterized as dominant-acting oncogenes. Addition of retroviral GAG sequences or other experimentally induced mutations activated the latent transforming potential of Fps/Fes. However, activating mutations in fps/fes had not been found in human tumors until recently, when mutational analysis of a panel of colorectal cancers identified four somatic mutations in sequences encoding the Fps/Fes kinase domain. Here, we report biochemical and theoretical structural analysis demonstrating that three of these mutations result in inactivation, not activation, of Fps/ Fes, whereas the fourth mutation compromised in vivo activity. These results did not concur with a classic dominant-acting oncogenic role for fps/fes involving activating somatic mutations but instead raised the possibility that inactivating fps/fes mutations might promote tumor progression in vivo. Consistent with this, we observed that tumor onset in a mouse model of breast epithelial cancer occurred earlier in mice targeted with either null or kinase-inactivating fps/fes mutations. Furthermore, a fps/fes transgene restored normal tumor onset kinetics in targeted fps/fes null mice. These data suggest a novel and unexpected tumor suppressor role for Fps/Fes in epithelial cells. (Cancer Res 2005; 65(9): 3518-22)

Introduction

Fps/Fes (hereafter called Fps) and Fer are the only two members of a unique family of cytoplasmic protein tyrosine kinases (for recent reviews, see refs. 1, 2). Fps and Fer contain a central Src homology-2 (SH2) domain and a COOH-terminal tyrosine kinase catalytic domain. They are structurally distinguished from other members of cytoplasmic protein tyrosine kinase subfamilies by the presence of NH2-terminal Fer/CIP4 homology and coiled-coil domains. fps/fes was originally identified as an oncogene from avian (fps) and feline (fes) retroviruses. The viral Fps (v-Fps) proteins encoded by these oncogenes were among the first members of the protein tyrosine kinase family to be characterized as dominant-acting oncogenes. v-Fps proteins consist of NH2-terminal GAG sequences fused to either the full-length cellular Fps protein or variants lacking portions of the coiled-coil domains. The viral GAG sequences confer unregulated tyrosine kinase activity that promotes cytokine-independent differentiation of hematopoietic progenitor cells and reduced growth factor requirements in transformed fibroblasts. When transgenically expressed under the control of a heterologous promoter, v-fps induced tumors in lymphoid and mesenchymal tissues in mice (3). Deletions and point mutations have also been shown to activate the latent transforming potential of cellular Fps (4). These observations were consistent with well-established correlations between activating mutations in tyrosine kinases and oncogenesis and suggested that activating somatic mutations in fps might someday be detected in human cancers. Recently, a mutational analysis of sequences encoding the catalytic domain of 89 tyrosine kinases in a panel of 182 human colorectal cancers revealed four somatic mutations in fps, and the authors speculated that these mutations might have activated the Fps kinase and thereby contributed to cancer (5). Here, we provide biochemical evidence that three of these four reported mutants are actually catalytically inactivated, whereas the fourth retained in vitro but lacked in vivo activity. At first, this biochemical analysis would seem to undermine the pathologic significance of these mutations; however, we also provide genetic evidence that targeted null or kinase-inactivating missense mutations in fps correlated with earlier tumor onset in a transgenic mouse model of breast cancer. Together, these observations suggest that activating mutations in fps could contribute to cancer and that Fps might have an unexpected tumor suppressor role.

Materials and Methods

Transgenic mice. Generation and genotyping of in-bred SVJ/129-CD1 hybrid lines harboring targeted null (fps−/−), targeted catalytically inactive (fpsKR/KR), or transgenic (fpsTg) fps alleles in a targeted fps-null genetic background (fps−/−/fpsTg) have been reported previously (6–8). Generation of in-bred transgenic mice with mouse mammary tumor virus-long terminal repeat–directed, mammary epithelial–specific expression of polyoma virus middle T antigen (PyVmT) has also been described (9). This PyVmT line develops multifocal mammary tumors with 100% penetration in females. All mice were housed at the Animal Care Facility at Queen's University (Kingston, Ontario, Canada) and procedures were approved by the Queen's University Animal Care Committee in accordance with the regulations set forth by the Canadian Council on Animal Care.

Tumor measurements. Initial tumor onset in abdominal mammary gland was assessed daily by physical palpitation of hemizygous PyVmT progeny in the context of fps wild-type (wt), fps−/−, and fps−/−/fpsTg genetic backgrounds. In a second independent series of experiments performed by a different investigator, initial tumor onset was assessed by physical palpitation in hemizygous PyVmT progeny in the context of wt, fpsKR/KR, and fps−/−/fpsTg genetic backgrounds. In this second series of experiments, tumor diameter was continually monitored after the initial onset to generate tumor growth profiles. These profiles were characterized by a dormant phase, which occurred postonset and which preceded emergence of a rapid phase of tumor growth. Estimates of the onset time of this rapid phase of growth were obtained from these profiles.

Biochemical analysis of mutant Fps proteins. PCR mutagenesis was used to generate EcoRI cDNA fragments encoding the human Fps SH2 and kinase domains corresponding to K588R (6), M704V, R706Q, V743M, and S759F mutants. Wild-type and mutant fps cDNA sequences were

Requests for reprints: Peter A. Greer, Division of Cancer Biology and Genetics, Queen's University Cancer Research Institute, Botterell Hall, Room A309, Kingston, Ontario, Canada K7L 3N6. Phone: 613-533-2813; Fax: 613-533-6830; E-mail: greerp@post.queensu.ca. ©2005 American Association for Cancer Research.
cloned into the bacterial expression plasmid pGEX-2T (Promega, Madison, WI). For expression in 293T cells, these sequences were substituted into the mammalian expression plasmids encoding full-length Fps with a COOH-terminal myc epitope tag (10). All constructs were confirmed by sequencing. Kinase reactions were done in kinase reaction buffer containing 375 μmol/L ATP and 125 μg/mL tubulin (Sigma-Aldrich, Oakville, Ontario, Canada) at 30 °C for 20 minutes. Kinase autophosphorylation and phosphorylation of the exogenous substrate tubulin was assessed by Western blotting using a monoclonal anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA). No observable Fps anti-phosphotyrosine signal was apparent in the absence of ATP at the exposure times used to quantify kinase activities. Levels of Fps proteins were determined by immunoblotting with rabbit polyclonal antibody to Fps (for bacterial expression) or with a monoclonal anti-myc antibody (for mammalian expression), and tubulin levels were assessed by immunoblotting with a sheep polyclonal anti-tubulin antibody (Cytoskeleton, Inc., Denver, CO).

Structural modeling. A model of the Fps catalytic domain was generated by threading the human Fps sequence onto the solved crystal structure of the inactivated form of chicken Src determined at 2.35 Å (2 protein tyrosine kinases; ref. 11). The resulting models were energy minimized using GROMOS96 (12) and ribbon diagrams were generated using MOLSCRIPT (13).

Results and Discussion

Activating mutations in several tyrosine kinase-encoding genes have been detected in different human malignancies. In vitro cell transformation and in vivo retroviral infection or transgenic mouse experiments argue strongly that dominant-acting mutations in tyrosine kinases can contribute to human cancer. Although data of this type support the hypothesis that activating mutations in the fps proto-oncogene might also contribute to human cancer, until recently, there have been no reports of fps mutations in any human tumors. At first, the exciting report by Bardelli et al. (5) seemed to provide the long awaited biological evidence that fps could indeed behave like a dominant-acting oncogene in human cancer. Their mutational analysis examined the tyrosine kinome of a panel of 182 colorectal cancers and detected four missense mutations in sequences encoding the kinase domain of Fps (5). Two of these mutations were predicted to cause V743M and S759F substitutions in the COOH-terminal lobe (C-lobe) of the kinase domain (Fig. 1A-C), whereas two other mutations predicted M704V and R706Q substitutions in the activation loop (Fig. 1A and D).

We first assessed these amino acid substitutions by theoretical structural modeling. Energy minimization experiments suggested that three of these mutations had the potential to induce structural perturbations, which might compromise kinase activity. M704 is positioned in the C-lobe of the kinase domain at the beginning of the activation loop near the active site. The M704 side chain points toward the opening of the active site between the NH2-terminal lobe (N-lobe) and the C-lobe of the kinase domain, neighbor amino acids are also shown in yellow. N-lobe (green), C-lobe (blue), activation loop (red/transparent), and catalytic loop (orange).
where few interactions are found (Fig. 1A). Structural modeling suggested that M704 might play an important role in stabilizing interactions between the ATP-binding cleft and the active site, because it makes several van der Waals interactions with residues in the N-lobe, for example, with E607 (Fig. 1D). The shorter side chain of V at this position is predicted to mediate weaker interactions and might therefore destabilize interactions between the ATP-binding cleft and the active site.

R706 is in a region of the activation loop, which was disordered and therefore not determined in the crystal structure template (Fig. 1A). We speculate that the apparent flexibility of this loop region might make the R706 position relatively tolerant of the R706Q substitution and it might therefore not have a substantial effect on kinase activity.

V743 is located within a tight hydrophobic pocket in the central core of the C-lobe of the catalytic domain (Fig. 1A). Replacement by the larger M residue would not be easily accommodated because it would clash with F807 and R804 (Fig. 1B). Consequent crowding would cause repulsion in this hydrophobic pocket that would destabilize the hydrophobic core. This might push apart the two α-helices located immediately below the catalytic loop, and this could in turn disrupt the conformation of the catalytic loop and the active site D683 residue.

S759 is on the surface of the C-lobe of the kinase and is therefore solvent exposed (Fig. 1A and C). When substituted by the hydrophobic residue F, the aromatic side chain is predicted to fold back toward the protein core to reduce solvent exposure. In so doing, it would interact with W726. Although W726 is ~6 Å away from the catalytic base D683, there is no residue or atom located in between (Fig. 1C). Thus, with some conformational flexibility during activation, W726 could influence D683.

To test these predictions, we compared the activity of bacterially expressed glutathione S-transferase fusion proteins corresponding to the SH2 and kinase domains of these four mutants with wild-type or a known kinase-inactivating K588R mutant. The position of K588 in the N-lobe is indicated in Fig. 1A. This kinase-inactivating K588R substitution has previously been characterized biochemically and introduced into the endogenous fps locus in the targeted fpsKR/KR mice (6). As predicted by the theoretical modeling, three of the four mutations reported in colon cancer (M704V, V743M, and S759F) greatly compromised the in vitro kinase activity of bacterially expressed Fps proteins, both with respect to autophosphorylation (Fig. 2A and C) or tubulin phosphorylation (Fig. 2B and D) activities were quantified as the ratio of pTyr to kinase expression levels and normalized to wild-type Fps. These values are given numerically under the corresponding lanes. *0*, no pTyr signal above background was detected. E, anti-pTyr and anti-myc blots of whole cell lysates from mammalian cells expressing the different full-length Fps proteins. Longer exposures of the top anti-pTyr blot (data not shown) did not reveal any differences in pTyr-containing proteins other than the signal generated by in vivo autophosphorylation of wild-type Fps.
Fps are shown below the corresponding lanes for autophosphorylation (Fig. 2C) and phosphorylation of tubulin (Fig. 2D), respectively.

Full-length versions of wild-type and Fps mutants were next expressed in mammalian cells and their in vitro activities were assessed in immune complex kinase assays. The M704V, V743M, and S759F mutants were inactive with respect to in vitro tubulin phosphorylation (Fig. 2B); however, unlike the reduction in activity seen in bacteria, the R706Q mutant displayed a slight (1.5-fold) increase in tubulin phosphorylation relative to wild-type Fps when expressed in mammalian cells. The R706Q mutant was comparably active with respect to in vitro autophosphorylation, and the M704V and S759F mutants also displayed some autophosphorylation when expressed in mammalian cells (Fig. 2A). Interestingly, anti-phosphotyrosine immunoblotting of whole cell lysates clearly indicated that only the wild-type Fps protein was able to autophosphorylate in vivo (Fig. 2E). Thus, although some of these mutants retain in vitro activity, their in vivo activity may be substantially more compromised.

Interestingly, the positions of the M704V and R706Q mutations in Fps correspond closely to the L858R and L861Q mutations in the epidermal growth factor receptor that were shown recently to activate that kinase and render it more sensitive to gefitinib in vitro. Although we could not model the R706Q mutant displayed a slight (1.5-fold) increase in tubulin phosphorylation relative to wild-type Fps when expressed in mammalian cells. The R706Q mutant was comparably active with respect to in vitro autophosphorylation, and the M704V and S759F mutants also displayed some autophosphorylation when expressed in mammalian cells (Fig. 2A). Interestingly, anti-phosphotyrosine immunoblotting of whole cell lysates clearly indicated that only the wild-type Fps protein was able to autophosphorylate in vivo (Fig. 2E). Thus, although some of these mutants retain in vitro activity, their in vivo activity may be substantially more compromised.

These biochemical analyses established that three of four Fps mutations recently identified in colon cancer were inactivating rather than activating, whereas the fourth had slightly increased in vitro activity but abolished in vivo activity. Although they did not support a hypothesis that activating mutations in fps/fes contributed to colon cancer, these observations did raise the interesting new possibility that fps/fes might function as a tumor suppressor in epithelial cells. To test this novel hypothesis, we first measured tumor onset in a PyVmT transgenic mouse model of breast cancer in the context of a fps-null (fps-/-) genetic background. Tumor onset occurred earlier in fps-/- mice relative to the wild-type fps genetic background, and this effect could be rescued by a fps transgene (fpsK/K; Fig. 3, left; P = 0.017). A second independent study recapitulated the early onset time observed in fps-/- genetic backgrounds (Fig. 3, right; P ≤ 10^-6). This second study also showed that mean tumor onset time in a targeted kinase-dead (fpsK/K/R) fps genetic background occurred earlier, although not as early as the onset time observed in fps-/- backgrounds (Fig. 3, right; P ≤ 10^-5).

Immediately postonset, tumors remained dormant before entering a rapid growth phase. We observed that the emergence of this rapid growth phase exhibited the same relative pattern of onset as observed for the initial tumor onsets in fpsK/K/R and fps-/- backgrounds (wt: 79 ± 18 days, n = 21; fps-/-: 57 ± 6 days, n = 11; P = 0.0004; fpsK/K/R: 65 ± 9 days, n = 24; P = 0.002). However, no differences in the rate of this growth phase were observed (data not shown).

The genetic data presented here strongly support the idea that loss-of-function fps genetic backgrounds (null or kinase-inactivating) promote epithelial tumorigenesis. Earlier tumor onset in fpsK/K/R and fps-/- backgrounds is consistent with a tumor suppressor function for Fps in epithelial cells and suggests both a phosphorylation-dependent and a protein association–dependent aspect of this Fps function. Our finding that three of the four fps mutations detected in human colorectal cancers were kinase-inactivating suggests that the kinase-dependent function of Fps might be a minimal critical requirement for this suppressor
function. Although we cannot conclude that these kinase-inactivating mutations played a causal or contributing role in these human colon cancers, the mouse model work described here certainly provides strong independent evidence to support that hypothesis. It will be important to determine if tumorigenesis in other oncogene- or carcinogen-based mouse models of epithelial cancers is similarly affected by loss of Fps function.

PyVmT has been proposed to function as a viral analogue of activated growth factor receptors based on its ability to recruit and activate signaling pathways downstream of Shc, phosphatidylinositol 3-kinase, Src, phospholipase Cγ, and protein phosphatase 2A (15). The observed early tumor onset in the PyVmT model of breast cancer therefore implicates Fps in negatively regulating PyVmT signaling pathways. In this respect, increased tyrosine phosphorylation in v-fps-transformed fibroblasts has been observed in several growth factor–inducible signaling proteins, including RasGAP, Shc, and phosphatidylinositol 3-kinase (reviewed in ref. 2). Hence, diminished pathway activity due to hypophosphorylated states of the latter signaling proteins may affect PyVmT-mediated tumorigenesis in loss-of-function fps genetic backgrounds. Potential tumor suppressor roles for the tyrosine kinases Syk (16), Csk (17), and EphB2 (18) have been described. However, to our knowledge, this is the first example of a tyrosine kinase with known oncogenic properties that might also act as a tumor suppressor. In fact, the opposite has been shown for Src, which was required for breast tumorigenesis in the same PyVmT-based breast tumor model used here (19).

In preliminary experiments, we failed to observe an effect of wild-type Fps or kinase-dead Fps on the transforming ability of PyVmT in NIH3T3 cells. However, because Fps is expressed in epithelial cells but not in fibroblasts, it will be important to repeat these experiments on epithelial cells from the fps−/− mice. If transduction with wild-type Fps reduces PyVmT-induced transformation of these cells, it would further support the hypothesis that Fps might act as a tumor suppressor in breast epithelial cells.

The loss of adhesion junctions as a result of E-cadherin dysfunction in epithelial cells is thought to contribute to tumorigenesis (20). It has been shown recently that the closely related Fer kinase plays a role in maintaining adherens junctions by regulating the tyrosine phosphorylation status of β-catenin (21). It will be interesting to see if tumorigenesis is similarly affected in mice lacking Fer and if Fps plays a role in regulating adherens junctions in epithelial cells.

Acknowledgments

Received 9/24/2004; revised 2/16/2005; accepted 3/8/2005.

Grant support: National Cancer Institute of Canada with funds from the Canadian Cancer Society and postdoctoral traineeship award (W. Sangrar) from the U.S. Army Breast Cancer Research Program (BC001029). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

An Identity Crisis for \textit{fps/fes}: Oncogene or Tumor Suppressor?

Waheed Sangrar, Ralph A. Zirgnibl, Yan Gao, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/9/3518

Cited articles
This article cites 21 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/9/3518.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/9/3518.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.