H-Ras and Phosphoinositide 3-Kinase Cooperate to Induce α(1,3)-Fucosyltransferase VII Expression in Jurkat T Cells*

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The α(1,3)-fucosyltransferase FucT-VII is essential for the biosynthesis of selectin ligands, but the signaling pathways mediating FucT-VII induction in T cells and other lymphocytes are poorly understood. We have shown previously that sustained activation of Ras in Jurkat T cells induces FucT-VII transcription, which requires the Raf-MEK-ERK pathway. In this study we report that FucT-VII induction is specific to the H-Ras isoform. Jurkat T cells retrovirally transduced with constitutively active H-Ras but not N- or K-Ras up-regulated expression of FucT-VII. Pharmacological inhibition studies also revealed that phosphoinositide 3-kinase (PI3K) activity is required for H-Ras-mediated FucT-VII induction. However, the ability of H-Ras to selectively induce FucT-VII is not a function of the inability of the N- or K-Ras isoforms to activate Raf or PI3K pathways. The use of effector-loop domain mutants of H-Ras, which are impaired for their ability to interact selectively with individual effectors alone or in combination with active Raf, indicated that induction of FucT-VII requires the concomitant activation of at least three signaling pathways. These studies show that H-Ras mediates FucT-VII induction in Jurkat T cells via the activation of the Raf, PI3K, and a distinct, H-Ras-specific effector signaling pathway.

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One such enzyme, the α(1,3)-fucosyltransferase FucT-VII, is essential for the biosynthesis of all selectin ligands in all cells in which it has been examined, including monocytes, neutrophils, and other myeloid cells as well as the biosynthesis of E- and P-selectin ligands on activated T cells (7, 8). Neutrophils from mice deficient in FucT-VII exhibit sharply reduced E- and P-selectin ligands, whereas activated T cells from these mice do not express detectable levels of selectin ligands (9, 10). Although myeloid cells constitutively express L-selectin and functional ligands for E- and P-selectins, expression of selectin ligands in T cells is inducible and highly regulated (3). Naïve CD4+ T cells do not display selectin ligands due to the lack of expression of the α(1,3)-fucosyltransferase FucT-VII (11, 12). Induction of FucT-VII in CD4+ T cells requires T cell activation, and expression levels are high in Th1 cells and substantially lower in Th2 cells generated in vitro (11–14). Moreover, studies from our laboratory reveal that engagement of the T cell receptor (TCR) leads to the induction of FucT-VII, which is further enhanced by interleukin 12 and inhibited by interleukin 4 (12, 15). However, TCR engagement results in the activation of a number of signaling molecules (16), and it is not well understood which are relevant for FucT-VII induction.

We have recently shown that enforced expression of constitutively active Ras in Jurkat T cells leads to the expression of FucT-VII, implicating Ras as a FucT-VII regulator (17). Ras proteins are small guanine nucleotide-binding proteins that function as molecular switches in signal transduction cascades, regulating cell proliferation, survival, and differentiation (18, 19). Mammalian cells express four isoforms of Ras: H-Ras, N-Ras, K-Ras4A, and K-Ras4B, with K-Ras4A and -4B resulting from alternative splicing of the fourth exon of the K-Ras gene (18). K-Ras4B accounts for more than 90% of the total K-Ras and will be referred to as K-Ras. These Ras proteins are 85% homologous (18) and are identical up to the last 24 carboxyl-terminal amino acids (20). Despite their great structural and biochemical similarity, mounting evidence suggests that the Ras isoforms are not merely redundant. Ras isoforms exhibit cell-specific differences in their intrinsic transforming potential (21), their ability to be activated by guanine nucleotide exchange factors or deactivated by GTPase activating factors (22–24), to activate certain specific signal transduction pathways, such as the NF-κB pathway (25), and to determine certain TCR signaling outcomes (26). Further evidence from knock-out mice attest to the distinct functions of the Ras iso-
forms; N-Ras- and H-Ras-deficient mice develop normally (27, 28), and even double knock-outs are normal (29), but K-Ras-deficient mice die during embryonic development (30).

Gaining insight into the mechanism of Ras-induced FucT-VII expression is complicated, given the plethora and diverse functions of Ras effectors. Ras can stimulate the Raf serine/threonine kinases, subsequently activating the ERK mitogen-activated protein kinases (18), which have been shown to be essential for FucT-VII induction (17). Apart from Raf, other known effectors include the phosphoinositide 3-kinase (PI3K), protein kinase C, Raf-1, MEK1, and Nore1 (18, 31). Ras also activates a family of guanine nucleotide exchange factors for the Ral small GTPases, the RalGDS dissociation stimulators (RalGDS) (32–34), which along with the PI3K and Raf, are the best defined Ras effectors (18).

In this study we show that induction of FucT-VII in Jurkat T cells exhibits isoform specificity, with only H-Ras, but not N- or K-Ras, able to induce FucT-VII expression. We demonstrate that PI3K, an important Ras downstream effector, is required for H-Ras-induced FucT-VII expression and that H-Ras mediates FucT-VII induction through the concomitant activation of at least three signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Pharmacological Inhibitors—**Jurkat cells were grown in RPMI 1640 with 10% fetal calf serum plus antibiotics. The PI3K inhibitor Ly294002 was added at the time of retroviral infection and was replenished every 24 h. Ly294002 was dissolved in dimethyl sulfoxide (Me2SO) and used at a range of final concentrations, from 10 μM up to 50 μM, as indicated. No effect in viability was observed at these concentrations, although the efficiency of retroviral infections, as assessed by GFP levels, was progressively decreased.

**Retroviral Infections—**Production of recombinant retrovirus pseudotype virus with the vesicular stomatitis virus glycoprotein G was performed as described previously (17). Briefly, cDNA encoding the different proteins was subcloned into murine stem cell virus (MSCV)-IRESGFP (35) upstream of the internal ribosome entry site (IRES) and enhanced green fluorescent protein (GFP). This plasmid was cotransfected with the plasmid encoding vesicular stomatitis virus glycoprotein G under the control of the cytomegalovirus late promoter into GP293 cells (Clontech) using the combination of LipofectAMINE and Plus Reagent (Invitrogen). Supernatants containing recombinant retroviruses were recovered 48 h later and were either used immediately for retroviral infections or frozen at −80 °C for later use. In some experiments, cells were coinfected with two retroviruses, the MSCV-IRES-GFP and an empty plasmid, to allow for visualization of the cells containing a distinct cDNA and in which the GFP sequence was replaced by the cDNA encoding murine H-2Kk*. The major histocompatibility class I antigen (36). Jurkat cells were spin-infected in the presence of 10 mM Tris, pH 8.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitors (1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, 0.5 mM sodium orthovanadate, 1 mM sodium fluoride), and phosphatase inhibitors (0.5 mM Na3VO4, 50 mM NaF, 50 mM β-glycerophosphate). Jurkat cell lysates were incubated on ice for 15 min and submitted to ultracentrifugation at 390,000 × g for 20 min at 4 °C. Supernatants were boiled for 5 min in SDS-PAGE sample buffer with 6.5% 2-mercaptoethanol, electrophoresed on a 10 or 15% polyacrylamide gel, and transferred to nitrocellulose. The H-Ras effector mutants T35S, E37G, and Y40C were provided by the laboratory of Dr. S. Shima (National Institute of Biomedical Innovation, Tokyo, Japan) and were confirmed by sequencing.

**RESULTS**

**H-Ras Specifically Induces FucT-VII—**We have previously shown that retroviral transduction of the Jurkat T cell line with a constitutively active form of H-Ras leads to FucT-VII induction (17). To determine whether there is isoform specificity in Ras-induced expression of FucT-VII, we infected Jurkat T cells with retroviral constructs expressing the cDNA of activated forms of H-, N-, or K-Ras (K-Ras4B). Because the retroviral vector produces a bicistronic message encoding both GFP and the cloned cDNA, the amount of GFP fluorescence serves as a quantitative marker of the Ras protein levels on a per-cell basis. The expression of FucT-VII in Jurkat T cells is quantitatively associated with cell surface expression of epitopes defined by the monoclonal antibody HECA-452 (8, 37), allowing the use of this antibody as a reporter for FucT-VII expression on a per-cell basis. We analyzed by cell flow cytometry the percent of retrovirally infected Jurkat cells that stain brightly with the HECA-452 antibody (Fig. 1A). As expected, H-Ras induced FucT-VII in a significant portion of the cells expressing the highest levels of GFP. In contrast, cells infected with K-Ras expressed FucT-VII at comparatively very low levels, although higher than the empty retrovirus. Furthermore, N-Ras-transduced cells stained with HECA-452 close to the empty vector levels (Fig. 1B). These data show that FucT-VII induction in Jurkat T cells is H-Ras-specific.

**Ras Isoform Specificity in FucT-VII Induction Is Not Due to Inability to Activate ERK1/2—**Previous studies from our laboratory showed that H-Ras induces FucT-VII expression in part via activation of the Raf-MEK-ERK pathway (17). To determine whether the ability of H-Ras to specifically induce FucT-VII is due to differential activation of the Raf-MEK-ERK pathway, we retrovirally transduced Jurkat T cells with retrovirus containing no cDNA, H-Ras, N-Ras, or K-Ras cDNA. The cells were lysed 2 days after the infection, and the lysates were subjected to SDS-PAGE and Western analysis using antibodies against ERK1/2 proteins as well as the phosphorylated ERK1/2 forms (phospho-ERK1/2) (Fig. 1C). Each of the Ras isoforms strongly activated the ERK1/2 proteins, whereas the empty vector control exhibited almost no ERK1/2 activation. These
data show that the H-Ras isoform specificity in FucT-VII induction is not due to the inability of the N- or K-Ras isoforms to activate ERK1/2 in these cells.

The Ability of H-Ras to Induce FucT-VII Requires Activation but Is Not a Function of the Activating Mutation—The activated form of H-Ras (v-Ha-Ras) contains two activating point mutations, the Gly-12 to Arg and Ala-59 to Thr (G12R/A59T). Both mutations activate H-Ras and other Ras family proteins by blocking their intrinsic GTPase activity (38, 39). However, the N- and K-Ras isoforms contain only the activating mutation Gly-12 to Val (G12V), which also blocks GTPase activity (39). We, therefore, investigated whether the ability of H-Ras to induce FucT-VII is due to the specific activating mutation.

We generated the H-Ras single-point activating mutants 12R (T59A) and 12V (T59A) as well as the wild-type form of H-Ras (12G, 59A) via site-directed mutagenesis. Jurkat cells were retrovirally transduced with empty vector constructs or constructs expressing H-Ras G12R/A59T, H-Ras G12R, H-Ras G12V, or wild-type H-Ras. The ability of the different Ras mutants to induce FucT-VII expression was determined by staining with the monoclonal antibody HECA-452. All the activation mutants were able to induce FucT-VII at the same, if not higher levels as H-Ras 12R/59T (Fig 2, A and B). Hence, the Ras isoform specificity observed is not due to the different mutations that activate the protein. Importantly, the wild-type H-Ras failed to induce FucT-VII, indicating that overexpression of the protein per se is not sufficient for FucT-VII expression but, rather, that H-Ras activation is required.

PI3K Activity Is Required for Induction of FucT-VII by H-Ras—Previous studies from our laboratory show that although the Ras effector Raf is required for the H-Ras induced FucT-VII expression in Jurkat cells, it is not, however, sufficient (17). To investigate the possible involvement of PI3K, another prominent downstream effector of Ras, we determined whether inhibition of PI3K activity abolished H-Ras induction of FucT-VII. Jurkat cells were retrovirally transduced with H-Ras or empty vector and were grown in the presence or absence of Ly294002, a highly specific pharmacological inhibitor of PI3K (40). At the concentration of 50 μM Ly294002, induction of FucT-VII in H-Ras-transduced cells was abolished, with HECA-452-staining levels equivalent to empty vector (Fig. 3, A and B). Before, a significant portion of the Jurkat cells infected with H-Ras and grown in the absence of Ly294002 stained with HECA-452 at high levels (Fig. 3B). Furthermore, Ly294002 inhibited FucT-VII induction by H-Ras in a dose-dependent manner ranging from 10 to 50 μM (Fig. 3C). Similar results were obtained when wortmannin, a chemically distinct PI3K inhibitor, was used (data not shown). PI3K activity is, therefore, required for the H-Ras induced FucT-VII expression in Jurkat cells.

The Ras Isoform Specificity Is Not a Function of Differential PI3K Activation—We have shown that although the Ras effector Raf is required for FucT-VII induction (17), the observed Ras isoform specificity is not due to the ability or inability of the Ras isoforms to activate ERK1/2 (Fig. 1C). Because PI3K activity is also involved in the regulation of FucT-VII expression, we investigated whether the observed Ras isoform specificity is a function of the differential ability of the Ras isoforms to activate PI3K. As a measure of PI3K activity, we used the phosphorylation levels of Akt, a major downstream effector of
PI3K. Jurkat cells retrovirally transduced with empty vector or active H-Ras, expressing one of several different activating mutations, and stained with HECA-452. The double point mutant G12R/A59T is the form of active H-Ras used in the experiments throughout this study; the G12V-activating mutation is the point mutation present in N- and K-Ras. Wild-type (WT) H-Ras refers to the H-Ras 12G/59A. Results from one representative experiment. B, quantitative analysis of the percent of Jurkat cells infected with the different Ras-activating mutants that exhibit high levels of HECA-452 staining. The bar graphs represent the mean ± S.D. of high HECA-452 staining on infected cells from three experiments. *, \( p < 0.05 \) versus the empty vector.

Fig. 2. The ability of H-Ras to induce FucT-VII requires activation but is not a function of the activating mutation. A, Jurkat cells were retrovirally transduced with empty vector or active H-Ras, expressing one of several different activating mutations, and stained with HECA-452. The double point mutant G12R/A59T is the form of active H-Ras used in the experiments throughout this study; the G12V-activating mutation is the point mutation present in N- and K-Ras. Wild-type (WT) H-Ras refers to the H-Ras 12G/59A. Results from one representative experiment. B, quantitative analysis of the percent of Jurkat cells infected with the different Ras-activating mutants that exhibit high levels of HECA-452 staining. The bar graphs represent the mean ± S.D. of high HECA-452 staining on infected cells from three experiments. *, \( p < 0.05 \) versus the empty vector.
Induction of FucT-VII by H-Ras Requires the Activation of at Least Three Signaling Pathways—Ras proteins activate a plethora of downstream effectors, of which the best characterized are Raf, PI3K, and the RalGDS (18, 31–33). In an attempt to identify Ras effectors that participate in the regulation of FucT-VII, we employed mutated H-Ras proteins that are impaired for their ability to interact with specific subsets of effectors. The H-Ras mutants were generated via site-directed mutagenesis and carry, in addition to the activating double point mutations G12R/A59T, different point mutations in the effector-loop domain (residues 32–40), which is essential for interactions with most, if not all, of the known effector proteins. Replacement of the Thr-35 with Ser (T35S) enables the selective activation of Raf (42) but abolishes interaction with RalGDS and PI3K (43). The mutant H-RasE37G can no longer bind Raf nor activate PI3K but retains binding to RalGDS (43). Last, H-RasY40C (Tyr-40 with Cys) selectively binds PI3K and fails to interact with RalGDS or Raf (43). Jurkat cells were retrovirally transduced with empty vector, H-Ras, or the H-Ras effector-loop domain point mutants and were stained with HECA-452 (Fig. 4). Although H-Ras-infected cells stained at high levels with HECA-452, cells infected with each of the different effector mutants did not (Fig. 4, A and B). In particular, H-RasT35S, which activates the Raf-signaling pathway, which we have shown to be essential for FucT-VII induction (17), failed to induce FucT-VII, consistent with previous results showing that expression of active Raf is not sufficient to up-regulate FucT-VII expression. As mentioned above, due to the PTEN deficiency in Jurkat cells, the PI3K signaling pathway is constitutively activated. Thus, cells expressing H-RasT35S did not up-regulate FucT-VII expression. We further investigated whether different combinations of H-Ras effector-loop mutants and Ras effectors could induce FucT-VII. To achieve this goal, two retroviral vectors were employed, one encoding a bicistronic message for the cloned cDNA and GFP, and the other encoding the catalytic domain of PI3K. PI3K activity is required for the induction of FucT-VII by H-Ras. Jurkat cells were retrovirally transduced with H-Ras or empty vector and were grown in the presence or absence of the PI3K pharmacological inhibitor Ly294002. A, representative experiment of Jurkat cells infected with empty and H-Ras retroviral vectors grown in the absence (top panel) or presence of 50 μM Ly294002 (lower panel), stained with HECA-452. B, quantitative analysis of Jurkat cells infected with empty and H-Ras retroviral vectors grown in the absence and presence of 50 μM Ly294002 that stained with HECA-452 at high levels. Results represent the mean ± S.D. of at least three experiments. *, p < 0.01 versus other groups. C, dose-dependent inhibition of H-Ras FucT-VII induction by Ly294002. H-Ras retrovirally transduced Jurkat cells were grown in different concentrations of Ly294002 ranging from 10 to 50 μM and stained with HECA-452. D, Jurkat cells were retrovirally transduced with H-, N-, K-Ras, and empty vector control, and equal numbers of cells were lysed 2 days after the onset of infection. The lysates were subjected to SDS-PAGE and Western blot analysis using antibodies against the phosphorylated form of Akt (phospho-AKT) and total Akt (AKT).
c-Raf-1, RafBxB (17), and the mouse major histocompatibility I H2-Kk gene (36). Jurkat cells were coinfected with the retroviruses and stained with the anti-H2-Kk monoclonal antibody conjugated to phycoerythrin and HECA-452. Expression of the GFP and H2-Kk genes served as markers of the cells infected with the two different retroviruses as analyzed via 3-color flow cytometry. Cells expressing both GFP and the H2-Kk antigen were analyzed for staining with HECA-452 as before (Fig. 5, A and B). Jurkat cells were infected with various combinations of retroviral vectors (Fig. 5B, Table I). Cells coinfected with the empty retroviruses did not stain with HECA-452, whereas the H-Ras-infected cells exhibited high levels of HECA-452 staining, as expected. Surprisingly, all combinations of H-Ras effector mutants plus Raf failed to induce FucT-VII in Jurkat cells.

**Fig. 4.** Point mutations in the H-Ras effector loop abolish FucT-VII induction. A, Jurkat cells were retrovirally transduced with empty vector, H-Ras, or the effector-loop domain point mutants H-RasT35S, H-RasE37G, and H-RasY40C. Two days after the infection the cells were stained with the monoclonal antibody HECA-452. Representative results from one such experiment are shown. B, quantitative analysis of the Jurkat cells infected with the corresponding retroviruses. The results are depicted as the mean ± S.D. of the percent of infected cells that stained with HECA-452 at high levels from three experiments. *, $p < 0.01$ H-Ras versus the mutants.
Even cells coinfected with H-RasE37G and Raf, which activates the Raf signaling pathway, respectively, failed to induce FucT-VII. In these coinfected cells, due to the combination of PTEN deficiency and the retroviral infections, all three major Ras signaling pathways, Raf, PI3K, and RalGDS, were activated, but FucT-VII induction was still not observed. Furthermore, coexpression of Raf and H-RasY40C, which activates the PI3K and the Raf pathway, also failed to induce FucT-VII, further confirming that although Raf and PI3K signaling pathways are essential, their activation is not sufficient to induce FucT-VII expression. Finally, expression of the constitutively active forms of the Rho GTPases Rac1 and Rac2, which can interact with some Ras-induced pathways but also trigger a distinct set of effectors, did not lead to FucT-VII induction either alone or in combination with active Raf.

(Fig. 5B, Table I). Even cells coinfected with H-RasE37G and Raf, which activates the Raf signaling pathway, respectively, failed to induce FucT-VII. In these coinfected cells, due to the combination of PTEN deficiency and the retroviral infections, all three major Ras signaling pathways, Raf, PI3K, and RalGDS, were activated, but FucT-VII induction was still not observed. Furthermore, coexpression of Raf and H-RasY40C, which activates the PI3K and the Raf pathway, also failed to induce FucT-VII, further confirming that although Raf and PI3K signaling pathways are essential, their activation is not sufficient to induce FucT-VII expression. Finally, expression of the constitutively active forms of the Rho GTPases Rac1 and Rac2, which can interact with some Ras-induced pathways but also trigger a distinct set of effectors, did not lead to FucT-VII induction either alone or in combination with active Raf.

**Fig. 5.** H-Ras-induced FucT-VII expression requires the concomitant activation of at least three signaling pathways. Jurkat cells were coinfected with two different retroviruses, each expressing the corresponding cDNA or no cDNA at all (vector), along with GFP or the mouse major histocompatibility I H2-Kk gene. Cells expressing both GFP and the H2-Kk antigen were analyzed for staining with HECA-452 at high levels. B, the bar graph depicts the collective quantitative analysis of the effect of the different retroviral vectors or their combinations on FucT-VII induction. Results are represented as the mean ± S.D. of the percent of doubly infected cells that stained with HECA-452 at high levels from at least three experiments. *, p < 0.05 H-Ras versus the other groups.
**H-Ras and PI3K Cooperate to Induce FucT-VII**

Retroviral transduction with signaling molecules and their effect on FucT-VII expression

Jurkat cells were transduced with retroviral vectors encoding the activated forms of Rac1, Rac2, Raf, H-Ras, and H-Ras effector-loop domain point mutants or coinfected with combinations of the above retroviral constructs as described under “Experimental Procedures.” Results represent the mean ± S.D. of the percent of infected cells that stained with HECA-452.

| Retroviral transduction with % of HECA-452−infected cells |
|----------------------------------------------------------|
| **RafBxB**                                               |
| H-RasT35S (activates Raf)                                |
| H-RasT35S + RafBxB                                       |
| H-RasE37G (activates RalGDS)                             |
| H-RasE37G + RafBxB                                       |
| H-RasY40C (activates PI3K)                               |
| H-RasY40C + RafBxB                                       |
| Rac1                                                    |
| Rac1 + RafBxB                                            |
| Rac2                                                    |
| Rac2 + RafBxB                                            |
| H-Ras                                                  |
| Vector                                                  |

Taken together, our results suggest that activation of at least three signaling pathways, the Raf, PI3K, and a third unknown pathway, is essential for FucT-VII induction in Jurkat T cells. Our results further indicate that the third pathway must be H-Ras-specific, since both the Raf and the PI3K signaling cascades are activated in Jurkat cells expressing H-, N-, or K-Ras, whereas only H-Ras is able to induce FucT-VII expression.

**DISCUSSION**

Ras proteins are small guanine nucleotide-binding proteins that function as molecular switches in signal transduction cascades, regulating cell proliferation, survival, and differentiation (18, 19). Four Ras isoforms are expressed in mammalian cells, H-Ras, N-Ras, K-Ras4A, and K-Ras4B, with K-Ras4A and -4B resulting from alternative splicing of the K-Ras gene (18). Mounting evidence suggests that, despite their structural and biochemical similarities, the different isoforms have distinct functions. Knock-out mouse systems revealed that although the Ras isoforms tested (G12R/A59T in H-Ras and G12V in N- and K-Ras), we also determined that the inability of N- and K-Ras to induce FucT-VII was not due to the different activating mutations. H-Ras proteins bearing only the G12R or G12V point mutation up-regulated the expression of FucT-VII as well as H-Ras G12R/A59T (v-H-Ras), indicating that the activating mutation could not account for the isoform specificity. Furthermore, induction of FucT-VII required active mutants of H-Ras and was not due to mere overexpression because wild-type H-Ras (12R/59A) had no effect on FucT-VII expression. Collectively, these results show that the ability of H-Ras to induce FucT-VII in Jurkat cells requires activation but is not a function of the activating mutation.

Results from a previous study in our laboratory, where Raf is required but not sufficient to induce FucT-VII expression (17) prompted the hypothesis that concomitant activation of two or more signaling pathways may be required for FucT-VII induction. Apart from Raf, another well characterized Ras effector is PI3K (17). Our data show that Lt294002, a highly specific pharmacological inhibitor of PI3K (40), inhibited expression of FucT-VII in Jurkat cells transduced with the active form of H-Ras in a dose-dependent manner and completely blocked expression at the highest concentrations. The structurally unrelated PI3K inhibitor wortmannin (46) also inhibited FucT-VII expression (results not shown). We, therefore, examined whether the Ras isoform specificity of FucT-VII induction is a function of differential activation of PI3K by determining the levels of activated Akt, a major downstream effector of PI3K. However, cells transduced with the empty virus exhibited Akt phosphorylation levels equivalent to cells transduced with each Ras isoform, a phenomenon that can be attributed to the PTEN deficiency in Jurkat cells, leading to strong constitutive activation of PI3K (41). Studies have shown that all three Ras isoforms are equally potent in activating the p110α and p110γ isoforms of the PI3K catalytic subunits (44). It is possible that in primary T cells Ras-induced activation of PI3K is required for FucT-VII induction, but in Jurkat cells PI3K activity levels are already high so that further activation by the Ras isoforms cannot be achieved. Regardless, our results indicate that PI3K activity is required for FucT-VII induction by H-Ras. Whether Akt or members of the Tec family of non-receptor tyrosine kinases, such as Itk (47), which are recruited to the plasma membrane as a result of PI3K activity or other pathways, may actually mediate FucT-VII induction remains to be determined.

To gain insight into other Ras effectors that may mediate FucT-VII expression, we employed H-Ras effector-loop-domain mutants that have been characterized as impaired in their ability to recruit specific subsets of effectors. Our results show that activation of only Raf (by the H-Ras T35S mutant), RalGDS (E37G mutant), or PI3K (Y40C mutant) was not sufficient to induce FucT-VII expression, consistent with results above showing a requirement for both Raf and PI3K. However, although both Raf and PI3K signaling pathways are essential for FucT-VII induction, concomitant activation of these two pathways is not sufficient to induce FucT-VII. Activation of the Raf pathway either by expressing active RafBxB or the H-Ras mutant T35S failed to induce FucT-VII expression in Jurkat T cells, in which the PI3K pathway is constitutively active. Moreover, coexpression of Raf and the H-Ras mutant Y40C, which activates the PI3K pathway, also failed, further confirming...
that activation of more than 2 pathways is necessary for FucT-VII induction. In addition, activation of at least Raf, PI3K, and RalGDS by coexpressing Raf and the H-Ras E37G mutant also did not induce FucT-VII expression, suggesting that the third essential pathway is not mediated by RalGDS but, rather, by a distinct downstream effector. However, Ras proteins interact with a plethora of downstream effectors, and although the ability of Ras partial-loss-of-function mutants to activate Raf, PI3K, or RalGDS has been well characterized and has contributed to the analysis of Ras signaling pathways in a variety of systems (43, 44, 48), little is known about other effector interactions that are retained or abolished in these mutants. Our data indicate that this third Ras effector-signaling pathway is activated by H-Ras but not by at least some of the H-Ras effector-loop domain mutants. Furthermore, the inability of the N- and K-Ras isoforms to induce FucT-VII indicates that this third unknown effector is selectively activated only by H-Ras and, therefore, likely accounts for the observed H-Ras specificity of FucT-VII induction.

Recent studies focus on differences between Ras isoforms in localization to distinct plasma membrane microdomains to explain their distinct biological functions. The Ras isoforms differ in the last 25 carboxyl-terminal amino acids, the hypervariable region (49), which contains a CAAX motif necessary for posttranslational modifications that control localization to the inner surface of the plasma membrane (50). Further studies show H-Ras localizing in caveolae, lipid rafts, and disordered membrane, N-Ras in caveolin-positive and caveolin-negative domains, and K-Ras in disordered, non-raft plasma membrane (51, 52). Therefore, differential localization of the Ras isoforms may account for their ability or inability to induce FucT-VII expression, a possibility that will have to be investigated. Endomembrane signaling, initiating from the Golgi apparatus or the endoplasmic reticulum, has also been considered as an explanation for the isoform differences (26, 53). Ectopically expressed H-Ras in Jurkat cells localizes, apart from the plasma membrane, to the Golgi apparatus, where H-Ras-mediated TCR-signaling requires phospholipase Cγ and RasGRP1 (54). FucT-VII regulation could be mediated by H-Ras activation on the Golgi apparatus, with the involvement of phospholipase Cγ and RasGRP1. Differences in posttranslational modifications in Ras isoforms, such as palmitoylation (18), affect not only subcellular localization, as mentioned before, but also interactions with certain regulators and/or effectors (55, 56).

Thus, the specific ability of H-Ras to induce FucT-VII expression may be due to the distinct localization of H-Ras in microdomains in the plasma membrane or Golgi apparatus, where interactions take place with specific effectors that colocalize in those domains. Alternatively or in addition, the posttranslational modifications per se may be responsible for the H-Ras specificity by hindering or favoring interactions with specific regulators and effectors.

Based on our results, FucT-VII induction in Jurkat cells is specifically mediated by H-Ras via the activation of at least three signaling pathways (Fig. 6). Although all three Ras isoforms can activate the Raf-MEK1/2-ERK1/2 signaling cascade, which is required for FucT-VII expression, only H-Ras exhibits the ability to activate an additional unknown downstream effector essential for FucT-VII induction. Furthermore, PI3K activity is required for H-Ras induction of FucT-VII, but differential PI3K activation cannot account for the isoform specificity, at least in Jurkat T cells. Last, effector-loop domain point mutants alone or in combination with active Raf failed to induce FucT-VII, pointing to a requirement for three or more signaling pathways in FucT-VII regulation. Taken together, these results indicate that H-Ras induces FucT-VII expression via concomitant activation of Raf, PI3K, and a third, H-Ras-specific, signal transduction cascade.

**REFERENCES**

1. Ley, K., and Kansas, G. S. (2004) Nat. Rev. Immunol. 4, 325–335
2. Lowe, J. B. (2002) Immunol. Rev. 186, 19–36
3. Kansas, G. S. (1996) Blood 88, 3259–3287
4. Vestweber, D., and Blanks, J. E. (1999) Physiol. Rev. 79, 181–213
5. Collins, T., Read, M. A., Neish, A. S., Whithey, M. Z., Thanos, D., and Maniatis, T. (1985) FASEB J. 9, 899–905
6. Tu, L., Delahuntly, M. D., Ding, H., Luscinskas, F. W., and Toddler, T. F. (1999) J. Exp. Med. 189, 241–252
7. Maly, P., Thall, A., Petryniak, B., Rogers, C. E., Smith, P. L., Marks, R. M., Kelly, R. J., Gersten, K. M., Cheng, G., Saunders, T. L., Camper, S. A., Camphausen, R. T., Sullivan, F. X., Ieong, Y., Hindsgaul, O., von Andrian, U. H., and Lowe, J. B. (1996) Cell 86, 643–653
8. Knibbs, R. N., Craig, R. A., Natsuka, S., Chang, A., Cameron, M., Lowe, J. B., and Stoolman, L. M. (1996) J. Cell Biol. 133, 911–920
9. Knibbs, R. N., Craig, R. A., Maly, P., Smith, P. L., Wolber, F. M., Faulkner, N. E., Lowe, J. B., and Stoolman, L. M. (1998) J. Immunol. 161, 6305–6315
10. Smithson, G., Rogers, C. E., Smith, P. L., Scheidegger, E. P., Petryniak, B., Myers, J. T., Kim, D. S., Homeister, J. W., and Lowe, J. B. (2001) J. Exp. Med. 194, 611–614
11. Lim, Y. C., Henault, L., Wagers, A. J., Kansas, G. S., Luscinskas, F. W., and Lichtman, A. H. (1999) J. Immunol. 162, 3193–3201
12. Wagers, A. J., Waters, C. M., Stoolman, L. M., and Kansas, G. S. (1998) J. Exp. Med. 188, 2225–2231
13. Austrup, F., Vestweber, D., Borges, E., Lohning, M., Fraurer, R., Her, U., Renz, H., Hallmann, R., Scheffold, A., Radvaksh, A., and Hamann, A. (1997) Nature 385, 81–83
14. Borges, E., Tietz, W., Steegmaier, M., Moll, T., Hallmann, R., Hamann, A., and Vestweber, D. (1997) J. Exp. Med. 185, 573–578
15. Wagers, A. J., and Kansas, G. S. (2000) J. Exp. Med. 191, 5011–5016
16. Kane, L. P., Lin, J., and Weiss, A. (2000) Curr. Opin. Immunol. 12, 242–249
17. Barry, S. M., Zsoulis, D. G., Neal, J. H., Clipstone, N. A., and Kansas, G. S. (2000) Blood 102, 1771–1778
18. Shields, J. M., Pratt, K., McFall, A., Shaub, A., and Der, C. J. (2000) Trends Cell Biol. 10, 147–154
19. Crespo, P., and Leon, J. (2000) Cell. Mol. Life Sci. 57, 1613–1636
H-Ras and PI3K Cooperate to Induce FucT-VII

20. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) _Cell_ 57, 1167–1177

21. Maher, J., Baker, D. A., Manning, M., Dibb, N. J., and Roberts, I. A. (1995) _Oncogene_ 11, 1639–1647

22. Jones, M. K., and Jackson, J. H. (1998) _J. Biol. Chem._ 273, 1782–1787

23. Clyde-Smith, J., Silins, G., Gartside, M., Grimmmond, S., Etheridge, M., Apolloni, A., Hayward, N., and Hancock, J. F. (2000) _J. Biol. Chem._ 275, 32880–32887

24. Bollag, G., and McCormick, F. (1991) _Nature_ 351, 576–579

25. Millan, O., Ballester, A., Castrillo, A., Oliva, J. L., Traves, P. G., Rojas, J. M., and Bosca, L. (2003) _Oncogene_ 22, 477–483

26. Perez, d. C., I, Bivona, T. G., Philips, M. R., and Pellicer, A. (2004) _Oncogene_ 22, 3485–3496

27. Umanoff, H., Edelmann, W., Pellicer, A., and Kucherlapati, R. (1995) _Proc. Natl. Acad. Sci. U. S. A._ 92, 1709–1713

28. Ise, K., Nakamura, K., Nakas, K., Shimizu, S., Harada, H., Ichise, T., Miyoshi, J., Gendo, Y., Ishikawa, T., Alba, A., and Katsuki, M. (2000) _Oncogene_ 19, 2951–2956

29. Esteban, L. M., Vicario-Abejon, C., Fernandez-Salguero, P., Fernandez-Medarde, A., Swaminathan, N., Tyngier, K., Lopez, E., Malumbres, M., McKay, R., Ward, J. M., Pellicer, A., and Santos, E. (2001) _ Mol. Cell. Biol._ 21, 1444–1452

30. Johnson, L., Greenbaum, D., Cichowski, K., Mercer, K., Murphy, E., Schmitt, E., Bronson, R. T., Umanoff, H., Edelmann, W., Kucherlapati, R., and Jacks, T. (1997) _Genes Dev._ 11, 2468–2481

31. Malumbres, M., and Pellicer, A. (1998) _Front. Biosci._ 3, 887–912

32. Albright, C. F., Giddings, B. W., Liu, J., Vito, M., and Weinberg, R. A. (1993) _EMBO J._ 12, 339–347

33. Kikuchi, A., Demo, S. D., Ye, Z. H., Chen, Y. W., and Williams, L. T. (1994) _ Mol. Cell. Biol._ 14, 7483–7491

34. Wolthusius, R. M., and Ros, J. L. (1999) _Curr. Opin. Genet. Dev._ 9, 112–117

35. Van Parijs, L., Ise, K., Alba, A. K., and Baltimore, D. (1999) _Immunity._ 11, 763–770

36. Porter, C. M., and Clipstone, N. A. (2002) _J. Immunol._ 168, 4936–4945

37. Wagers, A. J., Stoolman, L. M., Kannagi, R., Craig, R., and Kansas, G. S. (1997) _J. Immunol._ 159, 1917–1929

38. Gibbs, J. B., Sigal, I. S., Poe, M., and Scolnick, E. M. (1984) _Proc. Natl. Acad. Sci. U. S. A._ 81, 5704–5708

39. Bos, J. L. (1989) _Cancer Res._ 49, 4682–4689

40. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) _J. Biol. Chem._ 269, 5241–5248

41. Abraham, R. T., and Weiss, A. (2004) _Nat. Rev. Immunol._ 4, 301–308

42. White, M. A., Nicolette, C., Minden, A., Pulverino, A., Van Aelst, L., Karin, M., and Wigler, M. H. (1995) _Cell_ 80, 533–541

43. Rodriguez-Viciana, P., Warne, P. H., Kiwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) _Cell_ 89, 457–467

44. Rodriguez-Viciana, P., Sabatier, C., and McCormick, F. (2004) _Mol. Cell. Biol._ 24, 4943–4954

45. Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vangheebroek, R., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994) _Nature_ 370, 527–532

46. Arcaro, A., and Wynnham, M. P. (1995) _Biochem. J._ 296, 297–301

47. Lucas, J. A., Miller, A. T., Atherly, L. O., and Berg, L. J. (2003) _Immunol. Rev._ 191, 119–138

48. Czyzyk, J., Brodgon, J. L., Badou, A., Henegariu, O., Preston, H. P., Flavell, R., and Bottomly, K. (2003) _Proc. Natl. Acad. Sci. U. S. A._ 100, 6683–6688

49. Lowy, D. R., and Williamson, B. M. (1993) _Annu. Rev. Biochem._ 62, 851–891

50. Williamsen, B. M., Christensen, A., Hubbert, N. L., Paageeruge, A. G., and Lowy, D. R. (1984) _Nature_ 310, 583–586

51. Prior, I. A., Harding, A., Yan, J., Sluimer, J., Parton, R. G., and Hancock, J. F. (2001) _Nat. Cell Biol._ 3, 368–375

52. Kransenburg, O., Verlaan, I., and Mulleenaar, W. H. (2001) _Curr. Biol._ 11, 1880–1884

53. Chia, Y. K., Bivona, T., Hach, A., Sajous, J. B., Silette, J., Wiener, H., Johnson, R. L., Cox, A. D., and Philips, M. R. (2002) _Nat. Cell Biol._ 4, 343–350

54. Bivona, T. G., Perez, d. C., I, Ahearn, J. M., Grana, T. M., Chiu, V. K., Lockyer, P. J., Cullen, P. J., Pellicer, A., Cox, A. D., and Philips, M. R. (2003) _Nature_ 424, 694–698

55. Thissen, J. A., Gross, J. M., Subramanian, K., Meyer, T., and Casey, P. J. (1997) _J. Biol. Chem._ 272, 30362–30370

56. Rubis, I., Wittig, U., Meyer, C., Heinze, R., Kaderweit, D., Waldmann, H., Downward, J., and Wetzker, R. (1999) _Eur. J. Biochem._ 266, 70–82
