Murine Hepatitis Virus Strain 3 Induces the Macrophage Prothrombinase \( fgl-2 \) through \( p38 \) Mitogen-activated Protein Kinase Activation*

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The clinical syndrome of acute liver failure produced by fulminant viral hepatitis can be reproduced in mice by infection with murine hepatitis virus strain 3 (MHV-3). Although it is clear that MHV-3-induced hepatitis depends upon macrophage activation and the expression of a specific prothrombinase, \( fgl-2 \), the signaling pathways involved in virally stimulated cell activation are unclear. Since we had previously found that MHV-3 induces the tyrosine phosphorylation of cellular proteins, we investigated the roles of the mitogen-activated protein kinase (MAPK) proteins. In a series of Western blots, immunoprecipitation and in \( \text{vitro} \) kinase assay studies, we found that both the extracellular signal-related kinase (ERK) and \( p38 \) MAPK proteins are tyrosine-phosphorylated and activated following exposure of murine peritoneal exudative macrophages (PEM) to MHV-3. Although \( p38 \) phosphorylation and activity are induced soon after MHV-3 exposure, peaking by 1–5 min, ERK phosphorylation and activity increase more gradually, peaking at 20–30 min and gradually fading thereafter. Interestingly, whereas selective \( p38 \) inhibition with SB203580 (1–20 \( \mu \text{M} \)) abolished the virally stimulated induction of \( fgl-2 \) mRNA, protein, and functional activity, selective ERK inhibition with PD98059 (1–50 \( \mu \text{M} \)) limited \( fgl-2 \) functional activity but had little to no effect on \( fgl-2 \) mRNA or protein levels. Moreover, whereas inhibition of ERK had no effect on \( p38 \) activity, \( p38 \) inhibition consistently increased MHV-3-induced ERK activity. To ensure that these pathways were relevant in vivo, MHV-3 was injected intraperitoneally, and peritoneal exudative macrophages were collected. Again, MHV-3 exposure led to increased \( p38 \) and ERK tyrosine phosphorylation. These data argue that MHV-3 induces tightly interconnected ERK and \( p38 \) MAPK cascades in the macrophage both \( \text{in vitro} \) and \( \text{in vivo} \). Although the ERK and \( p38 \) MAPK proteins have discordant effects at the level of \( fgl-2 \) expression, both converge at the level of its activity, suggesting that targeted MAPK inhibition may ultimately be useful in the modulation of viral hepatitis.

The mortality rate associated with fulminant hepatitis remains in excess of 25–45%, despite the use of liver transplantation as an acceptable form of therapy (1). Studies using a model of viral hepatitis induced by infection with murine hepatitis virus strain 3 (MHV-3)\(^1\) have provided significant insights into the mechanisms underlying the pathogenesis of this disease and have suggested novel approaches to therapy (1, 2). Fulminant hepatitis induced by this virus is characterized by the presence of sinusoidal thrombosis and associated hepatocellular necrosis (3–5). These findings occur concomitant with the expression of a virus-induced procoagulant molecule in the sinusoidal lining cells of the liver. This prothrombinase protein, encoded by the \( fgl-2 \) gene, has the ability to activate directly the coagulation cascade, an ability expressed as procoagulant activity (PCA) and measured by standard clotting assays (6, 7). Accumulated evidence suggests that the virus-induced PCA plays a central role in the pathological changes observed in this disease. Following infection with MHV-3, hepatocellular necrosis is seen to occur in regions of sinusoidal fibrin deposition, where concomitant expression of the \( fgl-2 \) gene and its protein product is observed in the sinusoidal lining cells (8). By contrast, other organs simultaneously infected with MHV-3 fail to express \( fgl-2 \) protein and remain uninjured (8). Importantly, pretreatment with a neutralizing monoclonal antibody directed against MHV-3-induced PCA prevents sinusoidal fibrin deposition, hepatocellular necrosis, and mortality in infected mice (5). Thus, there is good reason to conclude that an \( fgl-2 \) up-regulation is essential to the lethal hepatitis induced by MHV-3. These considerations become all the more relevant with the recent discovery of a human \( fgl-2 \) analogue (9), which may contribute to inducible endothelial PCA (10, 11).

MHV-3 infection of murine macrophages represents an excellent model to study the induction of \( fgl-2 \) for several reasons. First, the degree of hepatocellular necrosis following infection correlates well with the induction of macrophage PCA (2, 5, 12). Macrophages from susceptible mouse strains (Balb/cJ) infected with MHV-3 exhibit a marked increase in PCA, whereas those recovered from resistant mice (A/J) fail to do so (13, 14). Moreover, the resistant A/J strains will develop both fulminant hepatitis and macrophage PCA following pretreatment with corticosteroids, which stabilize \( fgl-2 \) mRNA (14). Second, administration of exogenous prostaglandin \( E_2 \) completely abro-

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\(^1\) The abbreviations used are: MHV-3, murine hepatitis virus strain-3; DTT, dithiothreitol; ERK, extracellular signal-related kinase; FCS, fetal calf serum; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MBP, myelin basic protein; m.o.i., multiplicity of infection; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PCA, procoagulant activity; PEM, peritoneal exudative macrophages; HBSS, Hank’s buffered saline solution; PBS, phosphate-buffered saline; PGE\(_2\), prostaglandin E\(_2\).
gates viral induction of macrophage PCA both in vitro and in vivo and prevents the development of fulminant hepatitis (2, 15). Considered together, these findings suggest that macrophage PCA may serve both as a marker of disease and contribute to the pathogenesis of the process by virtue of the ability of the cells to sequester in the infected liver.

The cellular mechanisms underlying the induction of this protein in macrophages as well as in other cells are presently being defined. Detailed studies by Holmes and colleagues (16, 17) have characterized the MHV receptor as a 110–120-kDa glycoprotein in the carcinoembryonic antigen family of glycoproteins. Expression of this receptor in hamster cell lines confers susceptibility to MHV infection (17). Subsequent events in the signaling pathway(s) leading to expression of fgl-2 in MHV-infected macrophages have not been clearly elucidated. Our group previously demonstrated that incubation of macrophages with MHV-3 caused the rapid accumulation of tyrosine phosphoproteins over a range of molecular masses from 33 to 91 kDa. In addition, the nonspecific tyrosine kinase inhibitors genistein, herbimycin, and tyrophostin AG51 inhibited virus-induced PCA, both at the functional level and at the level of gene expression (18). Together, these findings suggest that tyrosine kinase activation in response to MHV-3 stimulation is an important component of the signaling cascade leading to fgl-2 expression. In this regard, a clustering of tyrosine phosphorylation around the 38–44-kDa region was suggestive of activation of members of the MAP kinase family, specifically p38/CSBP/reactivating kinase and extracellular signal-related kinase (ERK)-1 and ERK2. These proteins, which undergo dual phosphorylation on tyrosine and threonine residues during their activation, are known to be involved in the response of cells to a variety of infectious and inflammatory stimuli (19–25). We therefore hypothesized a role for these proteins in MHV-induced macrophage fgl-2 expression. In the present studies, we demonstrate that MHV-3 induces the phosphorylation and activation of both ERK and p38. However, although both of these kinases are activated, the use of specific inhibitors clearly demonstrates that p38, but not ERK, is integral to the induction of fgl-2 mRNA and its protein product.

**EXPERIMENTAL PROCEDURES**

**Animals, Buffers, and Reagents**

Pathogen-free female Swiss-Webster mice aged 6–7 weeks were obtained from Taconic Farms and were chow fed and allowed to acclimate for 1 week prior to experiments. 3% thiglycollate (Life Technologies, Inc.) was prepared as per the manufacturer’s instructions. Endotoxin-free RPMI and HBSS were purchased from Life Technologies, Inc.; fetal calf serum (FCS) was from HyClone. The p38-selective inhibitor SB203580 was the kind gift of Dr. J. C. Lee (SmithKline Beecham) and was prepared in Me2S Ot aa 0m M solution. The selective MAP kinase kinase-1 inhibitor PD98059 (Research Biochemicals International) was prepared in Me2S Ot aa 0m M stock solution.

**Cell and MHV-3 Preparation**

Peritoneal exudative macrophages (PEM) were harvested in ice-cold HBSS 5–6 days after the intraperitoneal injection of 2 ml of sterile thiglycollate. The cells were washed twice in cold HBSS, and resuspended in RPMI 2% FCS, l-Gln at 1–10 × 10⁶ cells/ml. This procedure consistently yields a >96% macrophage cell population by Wright’s stain, with >97% viability by trypan blue exclusion (26). Cells were incubated for 60 min at 37 °C, 5% CO₂ prior to experimentation. MHV-3 was obtained and purified as described previously (18). Virus was titrated in vitro on SW-68 (UVG-11 ultraviolet lamp; Ultra-Violet Products Inc.).

**Cell Activation**

Cells were incubated at 37 °C in 5% CO₂ in the presence or absence of MHV-3 for times ranging from 1 min to 6 h. Unless otherwise indicated, a multiplicity of infection (m.o.i.) of 5:1 was employed. In some studies, PEM were preincubated in the presence or absence of 1–50 µM PD98059 or 1–20 µM SB203580 for 45 min at 37 °C, 5% CO₂. Control cells were exposed to vehicle, 0.1% Me₂SO, during the preincubation period. At the end of the incubation period, reactions were stopped by placing the cells on ice.

**Measurement of PCA**

PEM were pelleted 6 h after exposure to viral particles and resuspended at 1 × 10⁶ cells/ml RPMI. Following a single freeze-thaw cycle at −70 °C, PCA was measured by single stage calcification clotting assay. PCA was expressed as milliunits/10⁶ cells by comparison to rabbit brain thromboplastin as described previously (18, 26). Previous work has established that MHV-3-induced PCA is entirely dependent on the induction of the fgl-2 prothrombinase (5–7, 12); for details, see “Results.”

**Western Blot Analysis**

At various times after virion exposure, PEM were pelleted and lysed in ice-cold cell lysis buffer. Whole cell lysates were prepared with 2× Laemmli, 0.1 mM dithiothreitol (DTT) buffer followed by immediate boiling and loading.

**RNA Extraction and Northern Blot Analysis**

Total RNA from 10 × 10⁶ PEM was obtained using the guanidinium-isothiocyanate method (27). RNA (20 µg) was separated on 1% agarose, blotted to nylon membrane. Hybridization was carried out using a32P-labeled, random-primed murine fgl-2 cDNA probe, after which the blots were exposed to Kodak BIOMAX MR film.

**Immunoprecipitations and Kinase Assays**

**ERK-2 and p38 Immunoprecipitations—**Cells (3 × 10⁶) were lysed as above and the postnuclear supernatants preclariﬁed with protein G-Sepharose (Amersham Pharmacia Biotech). Cellular proteins were immunocomplexed using rabbit polyclonal anti-ERK-2 or anti-p38 antibody (Santa Cruz Biotechnologies) for 1 h at 4 °C. Protein G-Sepharose (Amersham Pharmacia Biotech) was washed twice with 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM sodium orthovanadate, 10 µg/ml leupeptin, 50 µg/ml NaF, 5 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride. Postnuclear supernatants were collected following centrifugation at 10,000 × g for 5 min and diluted with 2× Laemmli buffer, 0.1 mM dithiothreitol (DTT). Lysates prepared from 100,000 cells were separated on 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Blots were then probed with polyclonal rabbit anti-phosphoysotrysin (Transduction Laboratories), anti-phospho-ERK or anti-phospho-p38 (New England Biolabs) antibody, or rabbit anti-fgl-2 antibody (Dr. G. Levy, University of Toronto). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech), blots were developed using an ECL-based system (Amersham Pharmacia Biotech).

**Immunofluorescence**

The sialic acid of the fusocanol receptor was visualized with z32P-labeled, random-primed murine fgl-2 cDNA probe, after which the blots were stripped and probed for 18 S RNA or glyceraldehyde-3-phosphate dehydrogenase mRNA to ensure equal loading.

**ERK-2 and p38 Kinase Assays—**ERK2 and p38 immunocomplexes were washed with 5 changes of cold phosphate-buffered saline, 0.01% Tween 20, and then separated from beads by 2× Laemmli buffer, 0.1 mM DTT and boiling at 100 °C for 5 min. Beads were then sedimented by ultracentrifugation, and the supernatant was collected for Western blot analysis.

**RESULTS.**

**Measurement of PCA**

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**Immunofluorescence**

PEM were allowed to adhere to autocolored glass coverslips for 1 h at 37 °C, 5% CO₂, incubated in the presence or absence of 50 µM PD98059, and then infected with MHV-3 at an m.o.i. of 1. Following a 6-h incubation with the virus, the cells were fixed in fresh 4% paraformaldehyde (Sigma) for 20 min at room temperature, quenched for 10 min with 100 mM glycine, and blocked overnight at 4 °C with 5% normal goat serum (Sigma) in PBS (pH 7.4). Rabbit anti-fgl-2 primary antibody was diluted
Fig. 1. Induction of ERK tyrosine phosphorylation and activity by MHV-3. Following activation with MHV-3, cytosolic PEM proteins were prepared for Western blot analysis or immunoprecipitation of the p42 ERK2 as described under “Experimental Procedures.” A, Western blot (WB) staining PEM lysates with pAb specific to the tyrosine-phosphorylated form of ERK (New England Biolabs). Note the bands of tyrosine-phosphorylated ERK migrating at 42 and 44 kDa and corresponding to ERK2 and ERK1, respectively. B, immunoprecipitated (IP) ERK2 was probed for tyrosine-phosphorylated residues using polyclonal anti-phosphotyrosine pAb. To control for loading, the blot was stripped and probed for ERK2 protein, as shown in the lower panel. C, immunoprecipitated ERK2 was allowed to phosphorylate ultra-pure MBP (Upstate Biotechnology Inc.) for 30 min at 30 °C, using [32P]ATP. A typical autoradiogram of the 20-kDa band is shown.

1:100 in PBS, 1% normal goat serum and incubated with fixed PEM for 2 h at room temperature. Cells were washed with five changes of PBS and then incubated with a 1:500 dilution of Cy3-conjugated goat anti-rabbit pAb (Jackson ImmunoResearch) for 2 h at room temperature. Cells were washed with five changes of PBS, 1% normal goat serum and incubated with fixed PEM for 2 h at room temperature. Cells were washed with five changes of PBS and then incubated with a 1:500 dilution of Cy3-conjugated goat anti-rabbit pAb (Jackson ImmunoResearch) for 2 h at room temperature. After five washes in PBS, the coverslips were mounted using SlowFade antifade reagents (Molecular Probes) and evaluated by confocal microscopy using a Bio-Rad MRC 600 confocal microscope with Comos 7.0 software.

In Vivo PEM Stimulation

At day 5 following intraperitoneal injection of thioglycollate, mice were infected with 50 × 10⁸ virions in 500 μl of RPMI, 2% FCS, L-Gin at 37 °C by intraperitoneal injection. Control animals were injected with 500 μl of medium alone, pre-warmed to 37 °C. At 10, 20, and 30 min after injection of the virus, mice were euthanized by cervical dislocation and PEM collected by peritoneal lavage using 10 ml of ice-cold HBSS. Cells were immediately placed on ice, sedimented, and prepared for Western blot analysis as described above. Protein amounts were standardized by Bradford protein assay (Bio-Rad) prior to gel loading.

Statistical Analysis

Continuous data are represented as the mean ± S.E. of the indicated number of experiments. Where representative studies are shown, these are indicative of at least three equivalent studies performed independently. Statistical comparisons were made using one-way analysis of variance with post hoc Tukey.

RESULTS

Induction of ERK Tyrosine Phosphorylation and Activity by MHV-3—Two approaches were used to determine whether the ERK MAP kinase was targeted by the response to MHV-3. Fig. 1A shows the time course of the appearance of tyrosine-phosphorylated p44 ERK1 and p42 ERK2 in response to MHV-3 stimulation. MHV-3 induced a rise in phospho-ERK as early as 5 min, reaching a peak at 20–30 min, and fading over the ensuing 30 min. As confirmation of this effect, ERK2 was immunoprecipitated and evaluated for tyrosine-phosphorylated residues. As shown in Fig. 1B, tyrosine phosphorylation of ERK2 peaked at 20–30 min after exposure to MHV-3 and persisted through 45 min. By having shown the tyrosine phosphorylation of ERK2, in vitro kinase assays were performed to evaluate its activation (Fig. 1C). MHV induced ERK2 activation with a time course consistent with its pattern of tyrosine phosphorylation.

Induction of p38 Tyrosine Phosphorylation and Activity by MHV-3—MHV-3 also induced rapid tyrosine phosphorylation of p38. Fig. 2, A and B, illustrates the time course of phosphorylation of p38 using the phosphospecific anti-p38 antibody and immunoprecipitation, respectively. It should be noted that in our work and that of others (28), the p38 MAP kinase migrates at 42 kDa. In contrast to the activation of the ERK pathway, MHV-3 induces a much faster and briefer tyrosine phosphorylation of p38. Phosphorylation was seen as early as 1 min, generally peaking by 1–5 min, and fading over the next 10–20 min. The time course of p38 tyrosine phosphorylation was reflected directly in p38 activity, as revealed by in vitro kinase assay (Fig. 2C). Considered together with the results in Fig. 1, these studies demonstrate that MHV-3 induces both ERK and p38 activation, albeit with markedly different time courses.

Although endotoxin (LPS) has been shown to activate both ERK and p38 in cells of the monocyte/macrophage lineage (21, 24), it is very unlikely that LPS contamination contributed to our results for the following reasons. All media and culture materials were endotoxin-free, both by commercial testing and Limulus amebocyte lysate assay, and strict attention was paid to sterile techniques. Moreover, endotoxin induces a very different pattern of ERK and p38 activation in PEm; a 1 μg/ml dose of Escherichia coli O111:B4 LPS leads to a more profound tyrosine phosphorylation of the ERK1 and ERK2 proteins that persists through 60 min and beyond and induces p38 tyrosine phosphorylation that peaks at 20–30 min and persists to 60 min (data not shown). Furthermore, treatment of cells with 50 μg/ml LPS-complexing polymyxin B greatly attenuated LPS-induced PEM.
Inhibition of Virus-induced ERK and p38 Activation Using PD98059 and SB203580—Two compounds have recently been described that act as selective inhibitors of the ERK and p38 pathways. PD98059 selectively inhibits MAP kinase kinase-1, the tyrosine kinase immediately upstream of ERK (29, 30), whereas the bicyclic imidazole SB203580 directly inhibits p38 kinase activity (31, 32). Prior to evaluating the role of these MAP kinases in virus-induced fgl-2 expression, initial studies were performed to determine the effect of these inhibitors on activation of ERK2 and p38 following MHV-3 stimulation. Pre-treatment of cells with PD98059 caused a dose-dependent decrease in ERK2 activity, with complete inhibition of ERK2 following MHV-3 stimulation. Pre-treatment with PEM preincubated in the presence or absence of PD98059 or SB203580 were treated with MHV-3 as before, and in vitro kinase assays were performed on immunoprecipitated p38 MAP kinase. Note that whereas a 10 μM dose of SB203580 greatly decreased p38 activity, a similar dose of PD98059 had no effect. A typical autoradiogram of the 60-kDa 32P-MBP band is presented; all points are taken at 20 min after treatment with MHV-3. B, PEM preincubated in the presence or absence of PD98059 or SB203580 were treated with MHV-3 as before, and in vitro kinase assays were performed on immunoprecipitated p38 MAP kinase. Note that whereas a 10 μM dose of SB203580 greatly decreased p38 activity, a similar dose of PD98059 had no effect. A typical autoradiogram of the 20-kDa 32P-MBP band is presented; all points are taken at 20 min after treatment with MHV-3. B, PEM preincubated in the presence or absence of PD98059 or SB203580 were treated with MHV-3 as before, and in vitro kinase assays were performed on immunoprecipitated p38 MAP kinase. Note that whereas a 10 μM dose of SB203580 greatly decreased p38 activity, a similar dose of PD98059 had no effect. A typical autoradiogram of the 20-kDa 32P-MBP band is presented; all points are taken at 20 min after treatment with MHV-3. B, PEM preincubated in the presence or absence of PD98059 or SB203580 were treated with MHV-3 as before, and in vitro kinase assays were performed on immunoprecipitated p38 MAP kinase. Note that whereas a 10 μM dose of SB203580 greatly decreased p38 activity, a similar dose of PD98059 had no effect.
Fig. 5. *fgl-2* mRNA expression is selectively abrogated by inhibition of p38 MAP kinase. Following pretreatment with PD98059 (20 μM) or SB203580 (20 μM), PEM were exposed to MHV-3 and incubated for 4 h at 37 °C, 5% CO₂. The mRNA from 10 × 10⁶ cells was isolated, separated, and probed for *fgl-2* as described under “Experimental Procedures.” Note that although PD98059 had little to no effect on MHV-3-induced *fgl-2* mRNA expression, the increase was blocked by selective p38 MAP kinase inhibition with SB203580. A typical Northern blot is shown and is representative of results obtained in at least four independent experiments. Note that doses of PD98059 up to 50 μM had no effect on *fgl-2* mRNA expression (data not shown). *g3pdh*, glycéraldehyde-3-phosphate dehydrogenase.

Fig. 6. Effect of ERK and p38 MAP kinase inhibition on Fgl-2 activity. 6 h after exposure to MHV-3, PEM were washed, resuspended at 10⁶ cells/ml RPMI, and freeze-thawed at −70 °C. Fgl-2 activity, manifested as PCA, was determined by single stage recalcification assay. Both PD98059 and SB203580 were able to dose-dependently inhibit PCA. Data mean ± S.E., n = 3/group. Stats: analysis of variance with post hoc Tukey, **p < 0.001, *p < 0.05 versus control.

activity or an effect on an as yet unknown *fgl-2* cofactor.

Effect of ERK Inhibition on Fgl-2 Protein Localization—Recent studies have suggested that the ERK MAP kinase proteins can be associated with cytoskeletal elements such as microtubules and phosphorylate regulatory proteins such as dynamin, suggesting that they may play some role in intracellular transport (33–35). We postulated that one mechanism through which ERK inhibition could be affecting Fgl-2 activity is through inhibition of its transport to its biologically active location in the plasma membrane. To address this issue PEM were infected with MHV-3 and then assessed for Fgl-2 localization using immunofluorescence. As demonstrated in Fig. 7, PEM pretreatment with a 50 mM dose of the ERK-selective PD98059 had no gross effect on either the amount or localization of the Fgl-2 protein. When considered together with the studies outlined above, these results suggest that the ERK MAP kinase is not essential for either the expression or the intracellular transport of Fgl-2.

Effect of UV Irradiation on MHV-3-induced PCA and p38 Activation—By having determined that p38 MAP kinase activation is essential for *fgl-2* synthesis, we performed studies using UV-irradiated virus in an effort to dissect whether this activation is sufficient for macrophage prothrombinase expression. As shown in Fig. 8A, UV irradiation eliminated the ability of MHV-3 to stimulate functional PCA. These data are consistent with the finding that UV-irradiated MHV-3 does not induce *fgl-2* mRNA. However, the induction of phosphorylated p38 MAP kinase was unchanged (Fig. 8B). These data suggest that p38 MAP kinase activation is necessary but not sufficient for *fgl-2* synthesis.

**Vivo p38 and ERK Tyrosine Phosphorylation in Murine PEM by MHV-3**—To determine whether MHV-3 was able to stimulate ERK and p38 phosphorylation in vivo, animals were inoculated intraperitoneally with MHV-3 or medium vehicle at 5 days after PEM elicitation with thioglycollate. At various times following MHV-3 injection, cells were harvested from animals and subjected to Western blot analysis. Compared with cells recovered from mice injected with medium alone, cells from MHV-3-treated animals exhibited a significant increase in the tyrosine-phosphorylated forms of p38 and ERK (Figs. 9, A and B, respectively). The lower panel in each figure confirms equivalent loading of the indicated protein among lanes.

**DISCUSSION**

The present data provide a detailed assessment of the cellular mechanisms by which a pathogenic virus, MHV-3, causes the activation of physiologic intracellular signaling cascades and leads to the production of a biologically active protein responsible for disease. Expression of the prothrombinase encoded by the *fgl-2* gene is central to the pathogenesis of hepatitis caused by MHV-3 (5). A previous report from our group (18) demonstrated that induction of tyrosine phosphorylation by MHV-3 was necessary for macrophage expression of this molecule. In the present studies, components of the signaling cascade leading to *fgl-2* expression are further defined. Specifically, we demonstrate that MHV-3 is able to rapidly induce the

*Dr. G. Levy, unpublished observations.*
tyrosine phosphorylation and activation of two members of the MAP kinase family, p38 and ERK. By using specific inhibitors of both pathways, p38 activation is shown to be required for induction of fgl-2 gene expression and elaboration of its protein product. Despite being activated by MHV-3, ERK does not appear to be essential for fgl-2 gene induction, although it may participate in the post-translational modification of the protein or alternatively in the action of a cofactor required for its biological activity. When considered in conjunction with the in vivo data demonstrating tyrosine phosphorylation of both these kinases in peritoneal macrophages following intraperitoneal injection of MHV-3, these findings strongly support the idea that these pathways are critical in the development of hepatitis following MHV-3 infection.

The signaling pathways upstream of the MHV-3-induced ERK and p38 activation remain to be determined. The rapid activation of ERK and p38 activity is consistent with the conclusion that viral replication per se is not required for the early signaling events that ultimately contribute to fgl-2 expression. This is further supported by the observation that UV-irradiated MHV-3 similarly induces p38 phosphorylation. The receptor for MHV-3 is a 110-kDa glycoprotein, which is a member of the murine carcinoembryonic antigen family. Its short intracellular domain lacking tyrosine residues precludes its ability to function as a receptor tyrosine kinase or act as a binding site for SH2 domains of nonreceptor tyrosine kinases.

However, variants of the receptor with a long cytoplasmic tail containing tyrosine residues, derived by alternative mRNA splicing, have been reported to serve as MHV receptors (16). Phosphorylation of one of these carcino embryonic antigen-related glycoproteins may have participated directly in the signaling pathways (36, 37). In this regard, members of the carcino embryonic antigen-related glycoprotein family have been reported to associate with molecules that could feed into downstream MAP kinases. Tyrosine-phosphorylated biliary glycoprotein can reversibly associate with the protein tyrosine phosphatase SHP-1 (38), whereas CD66a, a human homologue, can be tyrosine-phosphorylated and associate with pp60src, leading to increased c-Src activity in vitro (39, 40). Activated c-Src is a classic inducer of the Ras and Raf proteins, both potentially upstream of ERK (41, 42), and it is clear that tyrosine phosphatase activity can regulate both the ERK and p38 MAP kinases (43–47). An analogous situation may be found in the human immunodeficiency virus tyrosine phosphorylation response, in which gp120 binds to the T-cell CD4 antigen, leading to the release of the CD4-associated, src family tyrosine kinase p56lck and the subsequent phosphorylation of the MAP kinase kinase kinase Raf-1 (48). Consistent with the notion of the role of c-Src in the MHV-3 ERK signal, we have found that PP-1, an src family inhibitor (49), inhibits MHV-3-dependent ERK tyrosine phosphorylation (data not shown). Alternatively, it is possible that the extracellular domains of MHV receptor lacking a cytoplasmic tail might associate with a transmembrane receptor capable of initiating an intracellular signal, as has been reported for the interleukin-6 receptor (50). It is interesting to speculate that the different responses to MHV-3 in susceptible and non-susceptible mice might be partially due to differences in receptor-mediated signaling, since Bgp variants derived from alternative mRNA splicing are expressed differently in susceptible and non-susceptible mouse strains (17).

Inhibition of Fgl-2 protein by selective p38 inhibition with SB203580 appears to be at the level of transcription. Although the decrease in mRNA levels may be due to decreased tran-
sorption rates, other groups have also described a role for p38 MAP kinase in the maintenance of mRNA transcript stability (51). This finding is consistent with the presence of AUUU-rich regions in the 3’-region of the fgl-2 mRNA transcript.2 By contrast, ERK inhibition did not affect fgl-2 mRNA or protein expression but abolished its activity as reflected in the PCA clotting assay. Control studies testing the effect of PD98059 on PCA assay itself indicated that this inhibition was not related to a direct effect of the compound on clotting per se. As demonstrated in Fig. 7, selective ERK inhibition did not appear to affect Fgl-2 localization within macrophages. Rather, the inhibition appears to be either posttranslational or related to the synthesis of a necessary cofactor. In this regard, our findings are consistent with previous observations made during evaluation of the effect of prostaglandin E2 on Fgl-2 function. PGE2 inhibited Fgl-2-dependent PCA and liver necrosis (2) but had no effect on Fgl-2 protein levels (15). A recent study found that PGE2 reduced the induction of ERK activity by platelet-derived growth factor and epidermal growth factor in rat mesangial cells (52), suggesting that the effect of PGE2 on MHV-3-stimulated ERK activity may, in part, be due to inhibition of the ERK pathway. Consistent with increased cyclic AMP leading to decreased ERK activation, adenosine simultaneously increased cyclic AMP and inhibited the tyrosine phosphorylation of ERK-2 in human cultured mast cells following Fe epsion RI receptor cross-linking (53). The role of the ERK MAP kinase in regulating Fgl-2 activity deserves further investigation. The fact that ERK inhibition does not affect Fgl-2 protein migration by PAGE argues against protein cleavage as a post-translational modification; however, Fgl-2 phosphorylation (should it occur) could be under the influence of ERK. It is possible that a cofactor protein is necessary for Fgl-2 function, in a manner analogous to the recently described effector cell protein receptor-1 protein and the classical factor Xa-Va prothrombinase complex (54). The expression or activity of such a protein could be directly influenced by ERK activity. In any case, our data suggest that the ERK and p38 MAP kinase pathways act in a coordinated fashion to regulate MHV-3-induced prothrombinase activity.

Further evidence for the close interaction of the ERK and p38 MAP kinase pathways comes from the fact that selective p38 inhibition with SB203580 consistently increased ERK tyrosine phosphorylation and activity in response to MHV-3. This finding suggests an inhibitory feedback of ERK by p38 MAP kinase activation. These data are consistent with the recent finding of a similar p38-mediated inhibition of ERK in mast cells stimulated by IgE aggregation (55). Although the mechanism for such cross-talk is unclear, feedback modulation of ERK activity has been previously described to proceed via the mSOS adaptor protein (56), ERK-dependent expression or modulation of phosphatases (57, 58), inactivation of the MAP kinase kinase Raf-1 by a GTP-sensitive tyrosine phosphatase (47), and possibly cytosolic phospholipase A2-mediated signaling events (55). The functional significance of this cross-talk between MAP kinase pathways following MHV-3 stimulation is unclear. Further elucidation of the significance of ERK activation in response to MHV-3 may clarify this issue.

Our results have important implications both for viral hepatitis and for virally induced inflammatory responses. Several viruses have been demonstrated to stimulate tyrosine phosphorylation and through this contribute to aspects of the acute inflammatory response as well as virus-induced cellular transformation. For example, both the increased tumor necrosis factor-α expression by astrocytomas exposed to Newcastle disease virus and the increased c-myel and c-jun expression which follows simian virus 40 binding to growth-arrested cells occur in a tyrosine kinase-dependent fashion (58, 59). Hepatitis B has been shown to activate MAP kinase cascades via the virus-specific HBx protein (60–62), and human immunodeficiency virus infection of lymphocytic cells leads to the prompt activation of both the p38 MAP kinase and the MAP kinase kinase Raf-1 (48, 63). Although MAP kinase cascades have been suggested to be important for virally induced cell cycle events (64), their role in virally induced inflammatory events remains unclear. The finding that the p38 MAP kinase in particular is essential for the viral induction of the fgl-2 prothrombinase, an important inflammatory mediator in viral hepatitis, adds to a growing body of evidence suggesting that this kinase can play an integral role in the inflammatory response elicited by a number of stimuli (24, 25, 65–68).

The relative roles of the ERK and p38 MAP kinase pathways in the pathogenesis of viral hepatitis in vivo remain to be determined. We have demonstrated that both ERK and p38 are tyrosine-phosphorylated in vivo in PEM exposed to MHV-3 by intraperitoneal injection. Since it has been previously demonstrated that inhibition of fgl-2 PCA by specific monoclonal antibody greatly attenuates MHV-3-induced liver necrosis and mortality (5), the data suggest that selective inhibition of p38 MAP kinase activation, and possibly of ERK, might be of benefit in vivo. In favor of this notion is recent work using the Tyrphostin family of nonspecific tyrosine kinase inhibitors, resulting in improved survival and organ function in a lethal endotoxemia model in mice and an intra-abdominal sepsis model in dogs (69–71). Preliminary work in our laboratory has suggested that pretreatment of mice with Tyrphostin AG126 markedly inhibits the increase in hepatic fgl-2 mRNA following infection with MHV-3. Future studies to define the effect of nonspecific and selective inhibition of tyrosine phosphorylation signaling routes in this murine model of fulminant viral hepatis may ultimately suggest novel treatment strategies for the clinical disease.

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