Proteomic Screen for Cellular Targets of the Vaccinia Virus F10 Protein Kinase Reveals that Phosphorylation of mDia Regulates Stress Fiber Formation*§

Matthew D. Greseth‡, Dominique C. Carter§, Scott S. Terhune§, and Paula Traktman‡¶

Vaccinia virus, a complex dsDNA virus, is unusual in replicating exclusively within the cytoplasm of infected cells. Although this prototypic poxvirus encodes >200 proteins utilized during infection, a significant role for host proteins and cellular architecture is increasingly evident. The viral B1 kinase and H1 phosphatase are known to target cellular proteins as well as viral substrates, but little is known about the cellular substrates of the F10 kinase. F10 is essential for virion morphogenesis, beginning with the poorly understood process of diversion of membranes from the ER for the purpose of virion membrane biogenesis. To better understand the function of F10, we generated a cell line that carries a single, inducible F10 transgene. Using uninduced and induced cells, we performed stable isotope labeling of amino acids in cell culture (SILAC) coupled with phosphopeptide analysis to identify cellular targets of F10-mediated phosphorylation. We identified 27 proteins that showed statistically significant changes in phosphorylation upon the expression of the F10 kinase: 18 proteins showed an increase in phosphorylation whereas 9 proteins showed a decrease in phosphorylation. These proteins participate in several distinct cellular processes including cytoskeleton dynamics, membrane trafficking and cellular metabolism. One of the proteins with the greatest change in phosphorylation was mDia, a member of the formin family of cytoskeletal proteins with the greatest change in phosphorylation. Induction of F10 induced a statistically significant decrease in the percentage of cells with actin stress fibers; however, this change was abrogated when an mDia Ser22Ala variant was expressed. Moreover, expression of a Ser22Asp variant leads to a reduction of stress fibers even in cells not expressing F10. In sum, we present the first unbiased screen for cellular targets of F10-mediated phosphorylation, and in so doing describe a heretofore unknown mechanism for regulating stress fiber formation through phosphorylation of mDia. Data are available via ProteomeXchange with identifier PXD005246. 

Molecular & Cellular Proteomics 16: 10.1074/mcp.M116.065003, S124–S143, 2017.

The poxvirus family comprises large, complex DNA viruses, the most notorious of which is variola virus, the etiologic agent of smallpox (1, 2). One of the most significant plagues to have affected mankind, smallpox was successfully eradicated through worldwide vaccination with vaccinia virus, a natural attenuated virus that is closely related to variola and now serves as the prototypic poxvirus for experimental research. Today, in the post-vaccination era, there is increasing risk from monkeypox virus, which is endemic to Africa (1). In contrast to their role as pathogens, poxviruses are currently being used for the development of recombinant vaccines (3) and for oncolytic therapy (4). Hence, there remains a strong biomedical rationale for in-depth study of these unusual viruses.

Poxviruses, as illustrated by vaccinia virus, are the only DNA viruses which replicate solely within the cytoplasm of infected mammalian cells. This physical autonomy from the host nucleus is enabled by the 195-kb genome, which encodes >200 genes (2). The vaccinia virus genome encodes a full repertoire of proteins required for entry and egress, transcription, replication and morphogenesis. In addition, numerous proteins that target the innate, intrinsic and adaptive immune response, the antiviral response, and the apoptotic response, facilitate the robust replication of the virus. There is also an increasing appreciation for the intricate relationship between viral replication and the host cell’s cytoskeleton, endomembrane systems, signaling cascades, and bioenergetic status.
Fig. 1. Schematic illustration of the vaccinia virus life cycle. Vaccinia virus binds and enters cells through macropinocytosis or direct fusion with the plasma membrane. Following entry, the lateral bodies (yellow ovals) are released and mitigate host signaling events. In addition, the core (blue rounded rectangle), which can move through the cell on microtubules (green bars) becomes activated and the encapsidated transcriptional machinery initiates early gene expression. The early mRNAs, many of which encode proteins involved in replication of the viral genome, are extruded from the core. After a few hours, the core undergoes a process known as uncoating. Release of the genome allows DNA replication to begin within cytoplasmic factories that are delimited by membranes derived from the ER. Two additional phases of post-replicative gene expression (intermediate and late) then ensue, using transcription factors made in the preceding phase. The next stage of assembly is morphogenesis, which occurs in cytoplasmic zones that have been freed of cellular organelles; mitochondria are often enriched near the assembly zone. Assembly of nascent virions begins with the diversion of membranes from the ER to form crescent membranes (C). These membranes enlarge and pinch off a portion of the viroplasm (gray depot), which contains viral proteins destined for encapsidation. Prior to sealing of the immature virion (IV), the viral DNA is packaged (blue oval). A subset of virions acquire two additional membranes from the trans-Golgi network to become wrapped virions (WV). Microtubules are known to participate in the transport of virions to the periphery, and viral proteins mediate the dissolution of the cortical actin network (networks of red lines) to allow access to the plasma membrane. The WV are released by exocytosis; the actin tails that often form underneath the resultant extracellular virus (EV) are involved in mediating cell/cell spread. During infection, characteristic changes in the actin stress-fibers (pink lines) and the microtubule network are associated with phases of cell rounding and cell flattening. Note that the entire life cycle takes place within the cytoplasm.

A schematic view of the vaccinia virus life cycle (2) is shown in Fig. 1. The virion itself is ~250 × 350 nm, and is delimited by a single membrane. Within the virion are two lateral bodies (yellow ovals) which are adjacent to the concavities of the virion core, which is described as having a dumbbell shape. The core is delimited by a proteinaceous wall (maroon) and encloses the viral genome as well as the full complement of viral proteins needed to mediate the transcription and maturation of early viral mRNAs. The structure and function of the lateral bodies remains poorly understood, but recent data suggests that lateral body proteins are released into the cytoplasm upon viral entry, where they can immediately target cellular signaling pathways (5).

The core remains intact for several hours and is the site of early transcription; capped and polyadenylated mRNAs are then extruded and translated on cellular ribosomes. Many of the early mRNAs encode proteins involved in either DNA replication or intermediate transcription. The encapsidated genome is released from the core via a process known as “uncoating”; after this point, early transcription ceases and genome replication occurs in cytoplasmic “paddocks” that are delimited by two membranes derived from the endoplasmic reticulum (ER) (6, 7). Replicated genomes are utilized for the transcription of intermediate genes; several intermediate mRNAs encode late transcription factors. Many of the late genes that are subsequently expressed encode proteins involved in the complex process of virion morphogenesis.

Assembly of the vaccinia virus mature virion (MV) is quite complex (2, 8). Morphogenesis occurs in cytoplasmic regions that are cleared of cellular organelles. Crescent membranes are the first definitive sign of assembly. These are planar lipid bilayers that are thought to be derived from the endoplasmic reticulum; whether they have hairpin ends or are capped by amphipathic proteins is not known. These crescent membranes grow and enclose material present in “virosomes,” which are highly concentrated deposits of proteins destined for inclusion in the virion interior. Shortly before the growing crescent membrane closes to form an intact immature virion (IV), the genome is incorporated and forms a nucleoid. The convex face of the growing lipid bilayer is supported by a lattice comprised of the D13 protein; this lattice is removed once the IV are complete. Virions then undergo a morphological transition in which the internal core becomes distinct and the lateral bodies appear. Maturation is accompanied by several proteolytic cleavage events mediated by viral proteases. The resultant MVs are fully infectious and represent the vast majority of the virions that form. A subset of MVs become wrapped in two additional membranes derived from the Golgi apparatus; one of these additional membranes is inserted into the plasma membrane during exocytic exit from the cell. The resultant extracellular virions (EV) represent MV wrapped in one additional membrane. Additionally, actin tails often polymerize under the site of virion exocytosis and serve to propel the EV to adjacent cells.

1 The abbreviations used are: ER, endoplasmic reticulum; c.o. codon optimized; CAT, chloramphenicol acetyl transferase; CMV, cytomegalovirus; DAPI, 4',6-diamidino-2-phenylindole; DIAPH1, diaphanous; DMEM, Dulbecco modified eagle medium; ERAD, ER associated degradation; EV, extracellular virion; IV, immature virion; mDia, mammalian homolog of diaphanous; MOI, multiplicity of infection; MV, mature virion; SILAC, stable isotope labeling of amino acids in cell culture; TET, tetracycline; ts temperature-sensitive.
It is now accepted that cellular organelles and cytoskeletal elements play key roles during this complex life cycle. The ER is the source of the membranes surrounding the genome replication factories (6, 7, 9), and membrane biogenesis appears to begin with the diversion of ER membranes. Infection is accompanied by a significant increase in mitochondrial activity and ATP generation (10, 11). Infected cells round up and then re-flatten during infection with the manipulation of both microtubules and the actin cytoskeleton (12). Microtubules play roles in the intracellular movement of virions and cores during entry and egress (13). As wrapped virions move to the cell surface during egress, they encounter and must penetrate the cortical actin network (13). Indeed, we are just at the beginning of understanding the network of complex interactions between the virus and the cell.

Dynamic phosphorylation plays a key role in regulation of the viral life cycle, as it does in nearly all facets of cell biology. Indeed, the virus encodes a dual-specificity phosphatase (H1) (14, 15) and two kinases (B1 and F10) (16–21). The H1 phosphatase, which is expressed as a post-replicative gene, is encapsidated in virions and has been shown to dephosphorylate activated STAT1 in the cytosol shortly after the onset of infection (22–25). H1 also modulates viral proteins, and the loss of H1 leads to defects in early transcription (14) as well as hyperphosphorylation of several viral proteins leading to a loss of virion integrity (26–28). The viral B1 kinase, which is expressed as an early gene and is also encapsidated in virions, plays an essential role in viral DNA replication (16); it’s primary role is to phosphorylate the cellular barrier to auto-integration factor (BAF) protein, which otherwise binds to and sequesters viral DNA in a manner that prevents genome replication (29–31).

The viral F10-kinase, which is the focus of this work, is a unique, dual-specificity kinase (21, 27) that is expressed as a late gene and is also encapsidated in virions. F10 shows little sequence similarity to other kinases except for other poxviral orthologs (32). Although F10 does not show any canonical lipid binding motifs or transmembrane domains, F10 was shown to associate with membranes in vivo and directly binds phosphatidylinositol phosphates in vitro (32). It also undergoes robust auto (or trans) phosphorylation on ser residues (21, 32). Several temperature-sensitive (ts) mutants with lesions in F10 have been characterized, as well as an inducible recombinant in which F10 expression is regulated by the TET operator/repressor (19, 20, 32). The first lethal phenotype seen in infections with the ts mutant, Cts28, which has a single point mutation that renders the F10 kinase inactive at nonpermissive temperatures (39.7 °C), is a dramatic block in morphogenesis (19, 20). Under these conditions, the full repertoire of biochemical events occur unperturbed (DNA replication, three stages of gene transcription and protein synthesis). However, no evidence of membrane biogenesis or virion assembly can be found within the cytoplasmic areas that have been cleared of organelles in preparation for morphogenesis (20). This block is reversible; within 15 min after cells are shifted to the permissive temperature crescent membranes are seen and by 90 min, mature virions are readily observed (32). To date, how F10 initiates viral membrane biogenesis remains unknown. Although proteins that participate in this process have been shown to undergo F10-mediated phosphorylation, mutation of the sites of phosphorylation in no way phenocopies the Cts28 phenotype (33). We therefore propose that F10 targets cellular proteins involved in ER membrane dynamics in order to drive the early events of membrane diversion and crescent biogenesis.

Here, we present for the first time an unbiased screen to identify cellular targets of F10-mediated phosphorylation. Two cell lines that allowed for controlled exogenous expression of the F10 kinase were generated, one of which (293-F10) was used for proteomic analysis and the other (CV1-F10) for comparative biological assays. Interestingly, these cells showed dramatic changes in cellular morphology that mimic changes seen during an infection in which F10 is over-expressed. Using SILAC followed by phosphopeptide enrichment, our LC/MS/MS study identified proteins involved in various cell processes including modification of the cytoskeleton, regulation of membrane trafficking and cellular metabolism. One target, mDia, was investigated further and was confirmed to be phosphorylated in cell culture in an F10-dependent manner. mDia is involved in regulating the actin cytoskeleton, and the consequence of mDia phosphorylation by F10 is a significant loss of stress fiber formation that has consequences for cell structure and function.

## EXPERIMENTAL PROCEDURES

**Experimental Design and Statistical Rationale**—The objective of this experiment was to identify cellular targets of F10-mediated phosphorylation through stable isotope labeling of amino acids in cell culture (SILAC). 293-F10 cells were propagated in heavy (H) or light (L) media for 10 cellular doublings. Mass spectrometry was used to confirm that the majority of tryptic peptides showed >90% incorporation of the heavy isotope. H-labeled cells were treated with doxycycline to induce expression of F10 for 10 h, and then these cells were mixed with uninduced, L-labeled cells in a 1:1 ratio. Three biological replicates were obtained from separately passaged cultures and subjected to trypsin digestion. Tryptic peptides were desalted, enriched for phosphopeptides, and analyzed in triplicate by LC-MS/MS. The resulting nine data files were pooled and phosphoprotein identification and quantification was completed using MaxQuant software (version 1.2.2.5) (34, 35). Differential phosphorylation in response to F10 induction was determined using an outlier score for log protein ratios. The Benjamini-Hochberg test was then used to correct for the biased statistical spread of highly abundant proteins. For subsequent biological experiments, 2–4 biological replicates were performed and all data were analyzed using the appropriate statistical test. Average values and standard errors of the mean are reported for all studies.

**Reagents**—Restriction endonucleases, T4 DNA ligase, calf intestinal phosphatase (CIP), pancreatic RNase, PCR grade deoxynucleoside triphosphates (dNTP’s), TaqDNA polymerase, and DNA molecular weight standards were purchased from Roche Diagnostics.
(Indianapolis, IN) and were used per manufacturer’s specifications. 

32PPi was purchased from Perkin Elmer Life Sciences (Boston, MA). Doxycycline, 3x-FLAG peptide and EZView Red anti-FLAG M2 Affinity gel beads were obtained from Sigma (St. Louis, MO). Hygromycin, Blasticidin, Lipofectamine 2000, and LTX transfection reagents were purchased from Invitrogen (Carlsbad, CA). Oligonucleotide primers were purchased from Integrated DNA Technologies (IDT: Coralville, IA). Protran nitrocellulose was obtained from GE Healthcare Life Sciences (Buckinghamshire, UK).

Cell Culture—Monolayer cultures of African green monkey BSC40 cells were maintained at 37 °C in Dulbecco modified eagle medium (DMEM; Corning, Corning, NY) containing 5% fetal calf serum (FCS) (Thermo Fischer Scientific, Carlsbad, CA). Monolayer cultures of Flp-In T-REx 293 and Flp-in-CV1-TetR cells were maintained at 37 °C in 10% DMEM supplemented with blasticidin and hygromycin as described below.

Generation of Codon Optimized 3xFLAG-F10 CV1 and 293 Stable Cell Lines—3xFLAG-F10 was codon optimized (c.o.) for expression in mammalian cells by GeneArt (Regensburg, Germany) as well as a kinase-dead F10 allele (F10-KD) (32) and cloned into pcDNA5/FRT/TO (Invitrogen) using the HindIII and BamHI restriction sites. Flp-In T-REx 293 cells (Invitrogen) and Flp-In-CV-1-TetR cells (9) were cotransfected with pOG44 and either pcDNA5/FRT/TO-CAT (chloramphenicol acetyl transferase; control) or pcDNA5/FRT/TO-c.o.-3xFLAG-F10, per the manufacturer’s instructions using Lipofectamine2000. Properly targeted cells were selected in media containing 75 μg/ml hygromycin and 15 μg/ml blasticidin for Flp-In T-REx 293 cells; 75 μg/ml hygromycin and 30 μg/ml blasticidin for Flp-In-CV-1-TetR cells to obtain pure populations of cells.

Quantification of c.o.-3xFLAG-F10 Expression—Confluent 35 mm dishes of Flp-In T-REx 293 CAT cells and Flp-In T-REx 293 c.o.-3xFLAG-F10 cells were left untreated or induced with 50 ng/ml doxycycline for 24 h. Additionally, Flp-In T-REx 293 CAT cells were infected with a virus that encodes a 3xFLAG-F10 allele in lieu of the endogenous locus (MOI 5) for 24 h. Cells were harvested on ice. Protein quantification was performed using the Pierce BCA Protein Assay Kit (Thermo Scientific). Fifty micrograms of total protein was resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Analysis and quantitation of immunoreactive bands are described below.

Complementation of Cts28 Infection in c.o.-3xFLAG-F10 Expressing Cells—Confluent 35 mm dishes of Flp-In T-REx 293 c.o.-3xFLAG-F10 cells, Flp-In T-REx 293 c.o.-3xFLAG-F10-KD cells, Flp-In-CV1-TetR c.o.-3xFLAG-F10 cells, Flp-In-CV-1-TetR c.o.-3xFLAG-F10-KD, or control cells were infected with Cts28 (19, 20) (MOI 5) in the absence or presence of 50 ng/ml doxycycline for 24 h under nonpermissive (39.7 °C) or permissive (31.5 °C) conditions, followed by analysis of viral yield and protein accumulation. Viral yield was determined by performing plaque assays on BSC40 cells under permissive or control cells were infected with Cts28 (19, 20) (MOI 5) in the absence or presence of 50 ng/ml doxycycline. Cells were monitored over time for morphological changes by light microscopy and images taken at indicated time points. Following the time course, cells were harvested and analyzed for protein accumulation by immunoblot analysis.

Examination of Cell Morphology—

(A) Induction of c.o.-3xFLAG-F10 in the Absence of Infection—Confluent four-well chamber slides of Flp-In T-REx 293 c.o.-3xFLAG-F10 cells or 50% confluent four-well chamber slides of Flp-In-CV1-TetR c.o.-3xFLAG-F10 cells, as well as control cells, were incubated in the absence or presence of 50 ng/ml doxycycline. Cells were monitored over time for morphological changes by light microscopy and images taken at indicated time points. Following the time course, cells were harvested and analyzed for protein accumulation by immunoblot analysis.

(B) Overexpression of F10—Confluent 35 mm dishes of BSC40 cells were either mock infected, infected with WT vaccinia virus (MOI 4) or vTF7.3 (36) (MOI 4), or co-infected with vTF7.3 (MOI 2) and vTM-fUDG (37, 38) (MOI 2) or with vTF7.3 (MOI 2) and vTM-3xFLAG-F10 (32) (MOI 2). Cells were monitored over time for morphological changes by light microscopy at indicated time points. Following the time course, cells were harvested and analyzed for protein accumulation by immunoblot analysis.

Analysis of cell viability—Cell viability was measured using the CellTiter-Glo Luminescent assay (Promega; Madison, WI) as per the manufacturer’s instructions. Briefly, 20,000 CV1-CAT, CV1-F10 or CV1-F10-KD cells were seeded in a black-walled 96-well plate. Cells were either left untreated or treated with 50 ng/ml doxycycline for 48 h. Similarly, 40,000 293-CAT, 293-F10 or 293-F10-KD cells were seeded followed by treatment with 50 ng/ml doxycycline for 15 h. Cell viability was measured on a Synergy HTX multimode reader (BioTek, Winooski, VT).

Identification of Cellular Substrates of 3xFLAG-F10—

(A) Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC) Labeling—Flp-In T-REx 293 c.o.-3xFLAG-F10 cells were incubated for 10 doublings in SILAC media (Sigma) supplemented with 10% FCS and either 0.398 mM l-arginine and 0.798 mM l-lysine (light media) (Sigma) or 0.398 mM 13C6, 15N4 L-arginine and 0.798 mM 13C6, 15N4 L-lysine (heavy media) (Sigma). Cells grown in the presence of heavy media were then induced with 50 ng/ml doxycycline for 10 h. Cells were harvested and mixed 1:1 (heavy/light). Cells were sedimented at 673 × g for 7 min and washed with 1 ml cold PBS. Cell pellets were swollen in 200 μl Hypotonic Buffer (10 mM Tris pH 8.0, 10 mM KCl with protease and phosphatase cocktails (Sigma)) for 20 min on ice, and then disrupted by bounce homogenization. Nuclei were removed by sedimentation at 931 × g for 10 min.

(B) Trypsin Digestion and Phosphopeptide Enrichment—Proteins from post-nuclear supernatants were precipitated by chlorform-methanol extraction, resuspended in 50 mM ammonium bicarbonate, treated with 10 mM DTT for 30 min at 37 °C followed by 50 mM iodoacetamide for 30 min at room temperature to reduce disulfide bonds and alkylate cysteine residues, respectively. Proteins were digested with trypsin gold, MS grade (Promega) overnight at 37 °C at an enzyme-to-substrate ratio of 1:50. Reactions were stopped through addition of TFA (final concentration 0.1%) and desalted with Oligo R3 columns (Applied Biosystems, Foster City, CA). Desalted peptides were then processed for enrichment of phosphopeptides using a TiO2-based enrichment (39). Briefly, desalted peptides were dried and resuspended in 5 μl of 1% SDS by sonication for 10 min followed by the addition of 100 μl TiO2 loading buffer (1 mM glycric acid in 5% TFA; 80% acetonitrile). TiO2 beads (Tispheres, 5 μm; GL Sciences, Tokyo, Japan) were washed in acetonitrile. Samples were added and incubated at 21 °C for 15 min in a thermomixer prior to the beads being sedimented for 1 min. The unbound supernatant was incubated with new TiO2 beads as above. The two batches of beads were combined and washed using 100 μl of loading buffer at 21 °C for 15 s, then 100 μl washing buffer 1 (80% acetonitrile; 1% TFA) at 21 °C for 15 s and finally 100 μl washing buffer 2 (20% acetonitrile; 0.1% TFA) at 21 °C for 15 s. The TiO2 beads were dried in a vacuum centrifuge for 5 min. Bound peptides were eluted from the dried TiO2 beads with 100 μl of 1% ammonium hydroxide by vortexing at room temperature with a Tommy Shaker for 15 min. After centrifugation, the supernatant was collected, acidified with 50% TFA in water to a pH <2 and desalted using an Oligo R3 column (Applied Biosystems).

(C) LC MS/MS Analysis of Enriched Phosphopeptides—Each sample was analyzed in triplicate by LC-MS/MS on an LTQ Orbitrap Velos interfaced with an AB Sciex Eksigent NanoLC 2D HPLC-Thermo system. Elution occurred over a 240 min 2–98% Buffer B gradient at a flow rate of ~300 nL/min delivered by a NanoLC 2D HPLC pump (AB Sciex Eksigent, Framingham, MA). Buffer A contained 0.1% formic acid in H2O and Buffer B contained 0.1% formic acid in 100 mM ammonium bicarbonate.
were then washed with phosphate free DMEM (Invitrogen) and incubated in the indicated, cells were induced with 50 ng/ml doxycycline for 2 h. Cells were treated with the data set identifier http://proteomecentral.proteomexchange.org/dataset/PXD005246; the uploaded version was generated by the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (41) partner repository with the free online software WebLogo (43, 44) to identify a common F10 sequence, were added. Maximum missed cleavages considered for full trypsin digestion were set to 2. The search criteria were set to: which common, ubiquitous laboratory contaminants, as well the viral peptides and proteins; and minimum number of peptides for identification = 1. FDR calculations were based on matches to a decoy database of reversed protein sequences. Amino acid fixed modifications included carbamidomethyl (C) and variable modifications included oxidation (M), Acetyl (Protein N terminus) and phosphorylation (STY). Quantifications were performed as previously described (40). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (41) partner repository with the data set identifier http://proteomecentral.proteomexchange.org/dataset/PXD005246; the uploaded version was generated by MaxQuant v1.3.0.5, which has been included with the uploaded files.

(D) STRING Analysis—The 27 proteins identified as having statistically significant changes in their H/L ratios were investigated using STRING (string-db.org) (42), which draws on a database of known and predicted protein/protein interactions deduced using a variety of different experimental approaches and computer algorithms.

(E) Identification of the F10 Consensus Sequence—The sequences of the phosphopeptides identified in the SILAC screen were analyzed using the free online software WebLogo (43, 44) to identify a common consensus sequence.

Metabolic Labeling of mDia in Cell Culture—

(A) mDia Phosphorylation in 293 and CV1 Cell Lines—Confluent 60 mm dishes of Flp-In T-REX 293 CAT or Flp-In T-REX 293 c.o.-3xFLAG-F10 cells were transfected with 3 μg FLAG-mDia (45) or FLAG-mDia mutants (described below) using LTx per the manufacturer’s instructions for 24 h. Similar assays were performed with Flp-In-CV-1-TetR CAT and Flp-In-CV-1-TetR c.o.-3xFLAG-F10 cells. As indicated, cells were induced with 50 ng/ml doxycycline for 2 h. Cells were then washed with phosphate free DMEM (Invitrogen) and incubated with phosphate free DMEM supplemented with 10% FBS dialyzed against TBS and 100 μg/ml [32P]orthophosphoric acid/ml for 10 h in the presence (or absence) of 50 ng/ml doxycycline as indicated. Cells were harvested on ice. Washed cell pellets were lysed by the addition of 500 μL FLAG Lysis Buffer (50 mM Tris pH [7.4]; 150 mM NaCl; 1% Triton X-100; 1 mM EDTA; 1 μg/ml leupeptin; 1 μg/ml pepstatin; 1 mM sodium orthovanadate; 1 mM sodium fluoride) and incubated for 30 min at 4 °C with end-over-end mixing. Lysates were clarified by sedimentation for 7 min at 673 × g. FLAG-mDia was retrieved from the clarified lysates by incubation with 30 μL of a washed EZView Red anti-FLAG M2 Affinity bead slurry for 4 h at 4 °C with end-over-end mixing. The beads were then washed 3 times with FLAG TBS (50 mM Tris pH [7.4]; 150 mM NaCl). FLAG-mDia was eluted from the beads by incubation with 50 μL FLAG TBS containing 15 μg 3×-FLAG peptide. The beads were collected by sedimentation and the eluates were collected for analysis. Half of the eluate was subjected to SDS-PAGE and visualized by autoradiography and the remaining half was resolved by SDS-PAGE for immunoblot analysis.

(B) mDia Phosphorylation During WT Vaccinia Virus Infection—Confluent 60 mm dishes of Flp-In T-REX 293 CAT cells were transfected with 3 μg of empty vector or a plasmid encoding FLAG-mDia using LTX per the manufacturer’s instructions for 24 h. As indicated cells were either mock infected or infected with WT vaccinia virus (MOI 5). At 2hpi, cells were washed with phosphate free DMEM (Invitrogen) and incubated with phosphate free DMEM supplemented with 10% FBS dialyzed against TBS and 100μCi [32P]orthophosphoric acid/ml for 10 h. Cells were harvested and processed as above.

Targeted Mutagenesis and Cloning of the mDia Gene—An overlap PCR strategy was utilized to introduce the nucleotide changes needed to alter ser22 of mDia to alanine or glutamate (Ser22Ala or Ser22Asp) into an internal 1177nt EcoRV-BgIII fragment of the mDia cDNA. The template was a plasmid encoding FLAG-mDia under the regulation of a CMV promoter (a kind gift from Toshimasa Ishizaki); the primers utilized are listed in supplemental Table S1 (7). Briefly, in the first round of PCR, two separate reactions using primer U(x) plus XB and primers XC plus D(x) were performed. One microliter of each of the two reaction products was mixed and utilized as the template for the second round of PCR using primers U(x) plus D(x). The resultant PCR (1,177bp in length) was purified and digested with EcoRV and BglII. The FLAG-mDia vector was similarly digested, CIP treated, gel purified and ligated to the inserts. All constructs were subjected to DNA sequencing to verify the presence of the desired mutation and the absence of any spurious mutations.

Immunofluorescence Assay—Flp-In-CV-1-TetR c.o.-3xFLAG-F10 cells grown in four-well chamber slides were transfected with either empty vector, WT mDia, mDia (Ser22Ala), or mDia (Ser22Asp) using Lipofectamine 2000. After 24 h, cells were either induced with 50 ng/ml doxycycline or left untreated for 24h prior to fixation with 4% (v/v) paraformaldehyde in PBS for 15 min on ice. Cells were then washed twice with cold PBS and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. Samples were incubated for 1 h at room temperature in PBS containing rhodamine phalloidin (ThermoFischer Scientific). DAPI was added for 15 min at room temperature. Samples were mounted with Vectashield (Vector Laboratories, Inc. Burlingham CA) and pictures were captured using an Nikon Eclipse Ti microscope and NIS Elements AR4.4 software (Tokyo, Japan). Percent of cells with stress fibers was plotted as an average of two experiments with error bars representing standard error of the mean. One hundred twenty-five cells were counted for each sample in each of the two biological replicates. A student one-tailed t test was employed to determine significance.

Immunoblot Analysis—Whole cell extracts were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Protein concentration was determined using the BCA protein assay kit (Thermo-Scientific). The membranes were analyzed by incubation with a polyclonal antibody directed against calnexin (Enzo, Farmingdale, NY) or monoclonal antibody directed against FLAG (Sigma), followed by a horseradish peroxidase-conjugated secondary antiserum (BioRad, Hercules, CA). After development with chemiluminescent Super Signal WestPico reagents (Pierce, Rockford, Ill.), immunoreactive proteins were visualized on Kodak MR film or captured by exposure on an FluorChem E documentation system (ProteinSimple, Santa Clara, CA) and quantified using AlphaView software (ProteinSimple).

Preparation of Digital Figures—Original data were scanned on a Microtek ScanMaker 9800XL scanner (Hsinchu, Taiwan) and were adjusted with Adobe Photoshop software (Adobe Systems Inc., San Jose, CA). Data from in vivo phosphorylation assays were imaged on a Typhoon FLA 9500 (GE Healthcare Bio-Sciences) and quantified using Image Quant TL imaging software (GE Healthcare Bio-Sciences). Images from immunoblot analyses were acquired using the FluorChem E documentation system (ProteinSimple, Santa Clara, CA). Statistical analysis and graph preparation were performed using Sigma-Plot software (Systat Software, Chicago IL). Final figures were assembled and labeled with Canvas software (Deneba Systems, Miami, FL).
RESULTS

Generation and Characterization of Cell Lines Exogenously Expressing the Vaccinia Virus F10 Kinase—The first lethal phenotype seen upon repression or inactivation of the viral F10 kinase is a dramatic block in the initiation of morphogenesis (19, 20). In the absence of F10’s activity, no signs of membrane biogenesis are seen. The mechanism of F10’s role in this process is poorly understood. The known viral substrates of F10 play roles in later stages of morphogenesis (27, 33, 46–48), and in fact, the loss of these proteins or mutation of their phosphorylation sites does not phenocopy the arrest in morphogenesis observed upon loss of F10’s activity. Because membrane biogenesis is thought to involve the diversion of membranes from the ER, we hypothesized that F10-mediated phosphorylation of cellular substrates would be required for membrane diversion and crescent formation. Moreover, by analogy to the other viral kinases and phosphatases, we would predict that F10’s role goes beyond its known importance in morphogenesis and extends to the alteration of other facets of cellular structure and function.

To address this hypothesis, we generated two different cell lines (293 and CV-1) that can be induced to express the vaccinia virus F10 kinase in the absence of infection. Because vaccinia genes are transcribed in the cytoplasm and never encounter the nucleus, a codon optimized, 3xFLAG epitope tagged F10 allele was purchased from GeneArt to remove cryptic splice sites and optimize GC content and codon usage for expression in mammalian cells. This allele was utilized to generate cell lines in which expression of F10 was regulated in a doxycycline-inducible manner. We utilized the Flp-In system, in which the transgene of interest undergoes Flp-mediated recombination into a single FRT site that has been inserted into the cellular genome. Drug selection allows the isolation of a cell line in which every cell contains a single transgene inserted into the same genomic locus. The transgene (in our case, codon-optimized 3xFLAG-F10), is under the regulation of the CMV promoter and the TET operator/repressor. As a control, comparable cell lines encoding a chloramphenicol acetyltransferase (CAT) transgene were generated in parallel. The resulting cell lines will be referred to as 293-CAT, 293-F10, CV1-CAT, and CV1-F10.

Inducible expression of F10 was validated by immunoblot analysis, and the levels of F10 expression were compared with that observed after infection with a virus encoding a 3xFLAG-F10 allele in lieu of, and in the same genomic locus as, endogenous F10. 293-CAT, and 293-F10 cells were incubated in the presence or absence of 50 ng/ml doxycycline for 24h. In addition, 293-CAT cells were infected with the 3xFLAG-F10 virus in the presence or absence of doxycycline. Cell lysates were collected and 50 μg of total protein were resolved by SDS-PAGE. Immunoblot analysis showed that expression of F10 in the 293-F10 cell line is observed only in the presence of doxycycline, and that it then accumulates to levels that are on average 2.76-fold higher than what is seen during viral infection (supplemental Fig. S1A). In parallel experiments using the CV1 cell lines, similar results were obtained (2.82-fold greater than during infection, supplemental Fig. S1B).

Thus, the Flp-in system offers the advantage of allowing us to observe the impact of F10 when it is expressed at levels close to those seen during vaccinia infection.

We then tested whether expression of the 3xFLAG-F10 in 293 and CV1 cells was competent to rescue virus production during a non-permissive infection with Cts28, which encodes a catalytically inactive form of F10 (32). Factors that could contribute to complementation included the levels of expression, which was not a concern, the timing of expression and the ability to relocate to the sites of morphogenesis after infection. Cells were infected with Cts28 (MOI 5) under permissive (31.5 °C) or non-permissive (39.7 °C) conditions in the absence or presence of 50 ng/ml doxycycline for 24 h. In 293 cells (Fig. 2A) infected under permissive conditions (green bars), viral yield was equivalent in both control cells (CAT) and F10 cells, irrespective of the presence of doxycycline (solid and hatched bars). When 293-CAT cells were infected under nonpermissive conditions (red bars, left) there was a ~400-fold reduction in viral yield that was not mitigated upon addition of doxycycline (solid and hatched bars, respectively). In contrast, when 293-F10 cells were infected under nonpermissive conditions (red bars, middle) in the absence of doxycycline there was a ~500-fold reduction in viral yield (solid bar) that was rescued 27-fold upon addition of doxycycline (hatched bar). Immunoblot analysis confirmed that F10 accumulated at both temperatures only in the presence of doxycycline (Fig. 2A).

Similarly, in CV1 cells (Fig. 2B), viral yield was equivalent in both CV1-CAT and CV1-F10 cells infected + or - doxycycline at the permissive temperature (green bars). When infections were performed under non-permissive conditions in CV1-CAT cells (red bars, left), there was a 114-fold reduction in viral yield that was not affected by the addition of doxycycline (solid and hatched bars, respectively). In CV1-F10 cells, viral yield was reduced 192-fold under non-permissive conditions (red bars, middle) in the absence of doxycycline (solid bar); however, viral yield was rescued 10-fold upon addition of doxycycline (hatched bar). Again, accumulation of F10 was dependent upon addition of doxycycline and its stability was unaffected by temperature (Fig. 2B).

Taken together, these data show that when induced in 293 and CV1 cells, F10 accumulates at levels similar to those seen during a WT infection and is able to support viral replication. Parallel assays were conducted with similar cell lines that express a kinase-dead F10 allele (Lys110A/a and Asp307Gly) (Fig. 2, red bars, right) (32). Expression of this allele was unable to significantly rescue Cts28 infection, in keeping with the prior demonstration that the catalytic activity of F10 is essential for its role during infection (32, 49). A caveat to this experiment is that the kinase-dead protein accumulates to
lower levels than does the WT protein (see immunoblots below graph), although we have previously reported that a 4-fold reduction in the levels of WT F10 does not diminish viral yield (32).

**Expression of F10 in the Absence of Vaccinia Infection Induces Changes in Cell Morphology**—During the course of our characterization of the F10-expressing cell lines, we noted that the induction of F10 was followed by evident changes in cellular morphology. Images documenting these changes are shown in Figs. 3 and Fig. 4. 293-CAT and 293-F10 cells were maintained in the absence or presence of inducer (50 ng/ml doxycycline) and monitored by light microscopy over a 24 h period. Throughout the time course, 293-CAT cells remained adherent with no changes seen upon the addition of doxycycline (Fig. 3 - left two columns). In the absence of induction, 293-F10 cells had a similar morphology as the 293-CAT cells: cells remained adherent with visible protrusions (Fig. 3 - third column). Interestingly, when 293-F10 cells were induced with doxycycline, obvious changes in cell morphology were visible (Fig. 3 - fourth column). Starting at 12h post-induction, the protrusions began to retract and by 24h a majority of the cells were rounded up and refractile.

293 cells are not optimal for microscopic evaluation, and so the same experiment was repeated in our control and experimental CV1 cell lines, which adopt a flatter and more epithelial morphology. Here too we observed a clear impact of F10
expression on cell morphology, but the effect was slower and our time course was carried out for 72 h. During the entirety of the time course, CV1-CAT cells remained adherent; the addition of doxycycline had no impact on cell morphology (Fig. 4 - left two columns). Furthermore, the cells on the sub-confluent plate formed organized colonies with a clear boundary, and tight cell:cell contacts were evident until the gap was filled in (see 24 and 48 h). The same phenotype was observed in the CV1-F10 cells in the absence of doxycycline (Fig. 4 - third column). CV1-F10 cells showed a striking change in cell morphology beginning at 24 h post-induction with doxycycline: there were fewer tight cell:cell contacts at the leading edge and cells began to round up and become refractile (Fig. 4 - right column). This phenotype was exacerbated as the time course progressed with more cells becoming refractile and gaps in the monolayer failing to close.

Fig. 3. Expression of an F10 transgene induces changes in cellular morphology in 293 cells. Sub-confluent monolayers of 293-CAT (left two columns) and 293-F10 (right two columns) cells were either left untreated (-), first and third columns) or treated with 50 ng/ml doxycycline (+), second and fourth columns). Cells were then monitored over time by light microscopy and images were taken from 10h post treatment to 24h post treatment. Scale bars represent 100 μm.

Fig. 4. Expression of an F10 transgene induces changes in cellular morphology in CV1 cells. Sub-confluent monolayers of CV1-CAT (left two columns) and CV1-F10 (right two columns) cells were either left untreated (-), first and third columns) or treated with 50 ng/ml doxycycline (+), second and fourth columns). Cells were then monitored over time by light microscopy and images were taken from 24 h post treatment to 72 h post treatment. Representative phase images are shown. Scale bars represent 100 μm.
F10 Modulates Actin Stress Fibers by Phosphorylation of mDia

One possible explanation for the observed changes in cell morphology is an induction of apoptosis resulting in cell death. Examination of cell viability showed no significant change in the number of living cells 15h after induction of F10 in 293 and 48 h after induction in CV1 cells (supplemental Fig. S2). Taken together, these data show that exogenous expression of the F10 kinase induces changes in cell morphology in the absence of infection, consistent with our hypothesis that F10 can target cellular substrates, including those that modulate cellular architecture.

Identification of Cellular Substrates for the Viral F10 Kinase—The hypothesis underlying this work was the proposal that the role(s) played by F10 during infection involved its phosphorylation of cellular proteins implicated in the regulation and organization of cellular membranes and cytoskeletal elements. We therefore performed a SILAC phosphoproteomic screen of the post-nuclear supernatant of 293-F10 cells with and without F10 induction, as diagrammed in Fig. 5A. 293-F10 cells were maintained in light or heavy medium (heavy labeled L-arginine and L-lysine) for 10 passages to ensure complete saturation of protein labeling (supplemental Fig. S3). Cells maintained in heavy medium were treated with 50 ng/ml doxycycline for 10 h to induce expression of F10, at which point the uninduced and induced cells were collected and mixed in a 1:1 ratio. Proteins within the post-nuclear supernatants were fragmented by tryptic digestion; phosphopeptides were enriched and then analyzed in triplicate by high-accuracy LC-MS/MS.

After combining the three biological replicates, the total number of peptides matched to database entries was 2366, of which 1811 were phosphorylated yielding a 76.5% phosphorylation enrichment. When the data obtained from the 9 MS runs were analyzed together in MaxQuant, 27 proteins were found whose phosphorylation status was statistically significant in induced 293-F10 cells compared with uninduced 293-F10 cells (Table I) (A more complete profile of phosphopeptides and phosphoproteins identified in the SILAC screen can be found in supplemental Tables S2, S3, S4, S5, S6, and S7). Of these 27 proteins, 18 showed increases in heavy/light ratio (H/L, column 2), indicative of increased phosphorylation. The fold increase in the H/L ratio of the phosphopeptide(s) ranged from 20.5-fold to 4.7-fold. Interestingly, nine proteins showed a decrease in their heavy/light ratio. In most-cases, the decrease ranged from <2-fold to 3.3-fold, but one phosphopeptide (NACA) showed an ∼12.8-fold decrease in the H/L ratio. These proteins play roles in diverse cellular processes (last column) including, organization of the cytoskeleton, regulation of membrane trafficking, modulation of cellular metabolism and several other processes. Fig. 5B shows a STRING analysis of all the phosphoproteins identified (left panel), as well as analyses of those whose phosphorylation was increased (right top) or decreased (right bottom). The STRING analysis indicates that whereas there are some known interactions between the target proteins that suggest F10-mediated targeting of several members of a pathway, the majority of the proteins do not show any known interactions.

In addition to determining proteins that undergo F10-mediated phosphorylation, the mass spectrometry data allowed us to identify a consensus phosphorylation sequence. Using the web-based program WebLogo (43, 44), we analyzed the 30 sequences surrounding the identified target sites of F10-mediated phosphorylation (Fig. 5C). We observed a strong preference for a central pS/T - P motif. Together, the SILAC screen has provided, for the first time, an unbiased list of cellular proteins that undergo F10-mediated phosphorylation within 10h of F10 expression, and identify an F10 consensus motif that can be utilized for identification of other F10 substrates.

Validation of DIAPH1 (mDia) as a Target of F10-mediated Phosphorylation—One of the targets that experienced the greatest increase in H/L ratio upon F10-mediated phosphorylation was DIAPH1, also known as mDia, the mammalian homologue of Drosophila Diaphanous. mDia is a member of the formin family of proteins and plays important roles in regulating the actin cytoskeleton. mDia adopts an autoinhibitory structure that is relieved upon the binding of activated RhoA (50, 51). Once active, mDia facilitates the polymerization of actin, thereby inducing the formation of actin stress fibers (45, 52–56). Given our observation that induction of F10 expression induced changes in cellular morphology, mDia was chosen as a target for further examination.

Our first step was to validate the results of the SILAC screen by assessing mDia phosphorylation in cell culture using metabolic labeling with 32PPI. Dishes of confluent 293-CAT or 293-F10 cells were transfected with a plasmid expressing FLAG-mDia (or a control vector); the following day, the cells were left untreated or treated with doxycycline and metabolically labeled. FLAG-mDia (and FLAG-F10 where expressed) was retrieved and analyzed by immunoblot (using an anti-FLAG antibody) and autoradiography. Expression and retrieval of FLAG-mDia was observed in all cells transfected with the plasmid encoding FLAG-mDia (Fig. 6A immunoblot, lanes 3, 4, 7, 8), but not in those transfected with the control plasmid (1, 2, 5, 6). A basal level of phosphorylation was observed in samples from 293-CAT and uninduced 293-F10 cells (autoradiograph, lanes 3, 4, 7), but greatly increased phosphorylation was evident in the sample from F10-expressing cells (lane 8). As expected, radiolabeled 3xFLAG-F10 was also retrieved from doxycycline-induced 293-F10 cells (lanes 6, 8; autoradiograph). Using data obtained from two biological replicates, the level of mDia phosphorylation (normalized to the total mDia retrieved) was increased 8.7-fold ± 1.66 in cells induced to express the vaccinia virus F10 kinase. Similar results were obtained when the CV1 cell lines were analyzed (data not shown).

Given that both mDia and F10 are tagged with the FLAG epitope and are retrieved simultaneously on FLAG beads, we wanted to confirm that phosphorylation of mDia was occur-
ring intracellularly, and not after cell lysis and during the
immunoprecipitation procedure. To address this concern, a
mixing experiment was performed as diagrammed in Fig. 6B.
When lysates from induced 293-CAT cells transfected with
FLAG-mDia were mixed with either lysates from uninduced
293-F10 cells transfected with empty vector (Fig. 6C, lane 1)
or with lysates from induced 293-F10 cells transfected with
empty vector (Fig. 6C, lane 2), only basal levels of phosphor-
ylation were seen, showing that F10 does not phosphorylate
mDia in vitro during the immunoprecipitation protocol. Only
when lysates from uninduced 293-CAT cells transfected with
empty vector were mixed with lysates from induced 293-F10

Fig. 5. Identification of cellular targets of F10-mediated phosphorylation through SILAC phosphoproteomic analysis of 293-F10 cells.
A, Schematic representation of SILAC workflow. 293-F10 cells were maintained in normal (light) or heavy medium for 10 passages. Cells
maintained in heavy medium were then treated with 50 ng/ml doxycycline for 10 h. Following treatment, cell pellets were mixed in a 1:1 ratio.
Post nuclear supernatants from three biological replicate experiments were subjected to trypsin digestion followed by phosphopeptide
enrichment, and then each was analyzed in triplicate by LC/MS/MS. The nine data sets were analyzed together using MaxQuant software. B,
STRING analysis (http://string-db.org) of all 27 proteins identified in the SILAC phosphoproteomic screen (left). Individual STRING networks of
proteins exhibiting an increase (upper panel, right) or decrease in H/L ratio (lower panel, right) are also shown. Lines between proteins indicate
prior evidence of physical or functional interaction. C, Utilizing the online software WebLogo, a consensus sequence was identified for
F10-mediated phosphorylation events. The site of phosphorylation is shown at position 0, as well as the adjacent five amino acids upstream
and downstream. (Basic residues = blue; acidic residues = red; non-polar residues = black).
Identification of cellular targets of F10-mediated phosphorylation

The 27 proteins identified as having phosphopeptides with H/L ratios that were statistically different with significantly low probability of obtaining an outlier significance score on the basis of random chance alone for Log2 transformation of the H/L ratios (Benjamini-Hochberg FDR < 5%) (Sig B, third column) are shown. Proteins are identified in column 1 along with their respective H/L ratios (second column). Proteins shown above the solid line exhibited an increase in their H/L ratio, while proteins below the line showed a decrease in their H/L ratio. Posterior error probability (PEP) (sixth column) indicates the likelihood of a false identification. The phosphorylation site(s) and molecular weight in kDa are shown as determined by the LC-MS/MS analysis (fourth and fifth column, respectively). The last column indicates cellular pathways with which each protein has been associated. The data were obtained from 3 biological replicates, each of which were subjected to LC/MS/MS in triplicate.

| Protein name | BR1–3 Avg | BR1–3 significance | Phospho (STY) site positions | MW [kDa] | PEP | Pathway association |
|--------------|-----------|---------------------|-----------------------------|----------|-----|---------------------|
| ADRM1        | 20.550    | 4.79E-13            | (S)405                      | 42.153   | 3.79E-37 | Ubiquitination      |
| DIAPH1       | 18.842    | 2.31E-12            | (S)22                       | 141.57   | 3.27E-27 | Cytoskeleton        |
| C16orf34/HN1L| 9.883     | 6.60E-08            | (S)68                       | 23.025   | 0.00061023 | Unknown             |
| SEC23IP      | 9.473     | 1.18E-07            | (S)826                      | 111.08   | 3.50E-12 | Membrane trafficking|
| NUDC         | 8.162     | 8.49E-07            | (S)139;(T)145;(S)277        | 38.242   | 8.41E-85 | Cytoskeleton        |
| PNPO         | 7.796     | 1.55E-05            | (S)241                      | 29.988   | 5.77E-13 | Metabolism          |
| HASPP28/PDAP1| 7.374     | 3.01E-06            | (Y)18;(S)19;(S)60;(S)176   | 20.63    | 1.71E-18 | Cell proliferation  |
| RAD23B       | 7.091     | 4.82E-06            | (T)155                      | 43.171   | 9.08E-14 | Ubiquitination      |
| C2orf44      | 6.840     | 5.50E-05            | (S)468                      | 79.135   | 0.017358 | Unknown             |
| HGRG8/YTHDF2 | 6.655     | 7.09E-05            | (S)39                       | 62.333   | 9.19E-40 | Unknown             |
| HOOK1        | 6.600     | 7.66E-05            | (S)167                      | 84.647   | 2.18E-33 | Membrane trafficking|
| PUS4/TRUB1   | 6.347     | 1.74E-05            | (S)11;(T)18                 | 37.252   | 1.05E-13 | Nucleoside synthesis|
| CT1A         | 6.265     | 2.02E-05            | (S)124                      | 17.687   | 8.47E-55 | Membrane trafficking|
| C7orf50      | 6.001     | 3.25E-05            | (S)99;(S)175                | 22.083   | 1.74E-34 | Unknown             |
| ARHGAP1      | 5.881     | 4.05E-05            | (S)51;(S)223                | 50.435   | 1.06E-18 | Cytoskeleton        |
| STAU1        | 5.676     | 5.92E-05            | (S)278                      | 63.182   | 5.87E-11 | RNA binding         |
| HCF1         | 5.236     | 0.000136519         | (S)6;(S)1507                | 208.73   | 1.86E-40 | Unknown             |
| C13orf2/KCTD12| 4.746     | 0.000358816         | (S)176;(S)185               | 35.7     | 4.10E-66 | G-protein signaling |
| EAF1         | 0.617     | 0.000475336         | (T)157;(S)158;(S)165        | 29.042   | 0.034976 | Transcription       |
| GBF1         | 0.531     | 1.36E-05            | (S)1298;(S)1318;(S)1773     | 206.44   | 2.39E-19 | Membrane trafficking|
| PIK3C2A      | 0.523     | 0.000671752         | (S)259                      | 190.68   | 5.01E-05 | Membrane trafficking|
| C11orf96     | 0.513     | 0.00048365          | (T)688;(S)399;(T)407        | 46.113   | 9.67E-38 | Unknown             |
| MORC2        | 0.479     | 0.000136115         | (S)615;(S)725              | 117.82   | 4.11E-06 | Metabolism          |
| ARHGAP17     | 0.462     | 2.72E-07            | (S)162;(S)575;(S)674        | 95.436   | 2.87E-28 | Cytoskeleton        |
| INPP5F       | 0.380     | 1.10E-06            | (S)942                      | 128.41   | 0.00016374 | Membrane trafficking|
| MCM3         | 0.301     | 2.67E-09            | (S)672;(T)722              | 90.98    | 5.20E-64 | Cell cycle          |
| NACA         | 0.078     | 6.58E-53            | (S)2029                     | 205.42   | 6.82E-18 | Protein targeting   |
cells transfected with FLAG-mDia was there an increase in mDia phosphorylation of 8.5-fold (Fig. 6C, lane 3), confirming that the F10-mediated phosphorylation of mDia occurred in cell culture.

The SILAC data indicated that the mDia phosphopeptide that was responsive to F10 was modified on Ser22. To confirm that this residue was an F10 target, and to assess whether Ser22 was the major site of phosphorylation, we generated two additional alleles of FLAG-mDia: a phosphonull allele (Ser22Ala, Ala) and a phosphomimetic allele (Ser22Asp, Asp). Plasmids encoding these mutants, as well as WT mDia and an empty vector control, were transfected into confluent monolayers of 293-F10 cells. Induction, radiolabeling, immunoprecipitation and analysis were performed as described above for Fig. 6. As shown in Fig. 7, all three of the FLAG-mDia variants were expressed well and retrieved during the immunoprecipitation (blot, lanes 3–8). Low levels of basal phosphorylation were seen in all cases (autoradiograph, lanes 3, 5, 7). Phosphorylation of FLAG-WT mDia was increased to performing the immunoprecipitation and immunoblot analysis. An mDia+, F10- lysate was mixed with an mDia-, F10- lysate (lane 1); an mDia+, F10- lysate was mixed with an mDia-, F10+ lysate (lane 2); an mDia-, F10- lysate was mixed with an mDia+, F10+ lysate (lane 3). Only when mDia and F10 had been co-expressed in the same cells (lane 3) was a boost in mDia phosphorylation seen. (Representative experiment; n = 4).

**FIG. 7.** Ser22 of mDia is the major site of F10-mediated phosphorylation. 293-F10 cells were transfected with either empty vector (V) or plasmids encoding FLAG-mDia-WT (WT), FLAG-mDia-Ser22Ala (Ala) or FLAG-mDia-Ser22Asp (Asp) for 24 h. Cells were then left untreated or treated with 50 ng/ml doxycycline for 2 h followed by the addition of 100μCi of 32PPi for 10h. Cells were collected and radiolabeled proteins were retrieved by FLAG immunoprecipitation followed by elution with FLAG peptide. Eluates were resolved by SDS-PAGE and subjected to analysis by autoradiography and immunoblot. (Representative experiment; n = 4).

**FIG. 6.** mDia undergoes F10-mediated phosphorylation in cell culture. A, Phosphorylation of mDia was monitored in cell culture by transfecting 293-CAT (lanes 1–4) or 293-F10 (lanes 4–8) cells with either empty vector (lanes 1, 2, 5, 6) or a plasmid encoding FLAG-mDia (lanes 3, 4, 7, 8) for 24 h. Cells were then left untreated (lanes 1, 3, 5, 7) or treated with 50 ng/ml doxycycline for 2 h followed by the addition of 100μCi of 32PPi for 10h. Cells were collected and radiolabeled proteins were retrieved by FLAG immunoprecipitation followed by elution with FLAG peptide. Eluates were resolved by SDS-PAGE and subjected to analysis by autoradiography and immunoblot. (Representative experiment; n = 11005). B, and C, The F10-mediated phosphorylation of mDia occurs in cell culture and not ex vivo during the immunoprecipitation experiment. After performing an additional experiment similar to that shown in A, lysates were mixed pairwise as illustrated in the schematic shown in panel B prior to performing the immunoprecipitation and immunoblot analysis. An mDia+, F10- lysate was mixed with an mDia-, F10- lysate (lane 1); an mDia+, F10- lysate was mixed with an mDia-, F10+ lysate (lane 2); an mDia-, F10- lysate was mixed with an mDia+, F10+ lysate (lane 3). Only when mDia and F10 had been co-expressed in the same cells (lane 3) was a boost in mDia phosphorylation seen. (Representative experiment; n = 3).
12-fold ± 2.22 (normalized to total mDia retrieved, average of four biological replicates) upon the expression of the F10 kinase (autoradiograph, lane 4). In contrast, the phosphorylation of the Ala and Asp alleles was only increased 2.4-fold ± 0.35 and 2.1-fold ± 0.34, respectively; again, these data were normalized to the total mDia retrieved and represent the average of four biological replicates. Taken together, these data show Ser22 is the major, but not only, site of F10-mediated phosphorylation. Moreover, the basal level of mDia phosphorylation seen in 293-CAT cells or uninduced 293-F10 cells phosphorylation. Moreover, the basal level of mDia phosphorylation seen in 293-CAT cells or uninduced 293-F10 cells indicates that mDia is also a target of cellular kinases.

Phosphorylation of mDia at Ser22 Impacts Stress Fiber Formation—Given that active mDia stimulates the formation of actin stress fibers and that F10 phosphorylates mDia, we wanted to query whether the impact of F10 on cell morphology (see Figs. 3 and 4) was mediated by its phosphorylation of mDia. Actin stress fiber formation was therefore monitored by inducing confluent monolayers of CV1-CAT and CV1-F10 cells with 50 ng/ml doxycycline for 24h. Fixed cells were stained with rhodamine phalloidin to visualize the actin cytoskeleton. A majority of CV1-CAT cells contained actin stress fibers irrespective of the presence of doxycycline (Fig. 8A, ††), as did CV1-F10 cells in the absence of doxycycline. In contrast, we observed a notable decrease in the number of cells with actin stress fibers in CV1-F10 cells that had been induced with doxycycline (Fig. 8A, †). These data imply that the impact of F10 on cellular morphology involves disruption of actin stress fibers.

To determine if stress fiber formation might be modulated by the phosphorylation of mDia, we assessed the impact of WT mDia and the Ala and Asp alleles on stress fiber formation. As shown in Fig. 8B, CV1-F10 cells were transfected with empty vector or the three alleles of mDia and stained with rhodamine phalloidin in the absence (top row) or presence (bottom row) of 50 ng/ml doxycycline. We quantified the percent of cells showing evident stress fibers in Fig. 8C (125 cells per condition scored blindly; n = 2). In uninduced cells (black bars), which do not express F10, 65.2% of cells receiving empty vector and 60% of cells receiving the plasmid encoding WT mDia had evident stress fibers. Upon transfection of the plasmid expressing mDia-Ala, the percentage of cells exhibiting stress fibers increased to 80.4%. Expression of the phosphomimetic variant, mDia-Asp, reduced the frequency of cells with stress fibers to 34.4%. These data strongly suggest that an excess of unphosphorylated mDia augments stress fiber formation, whereas an excess of phosphorymimetic mDia impairs stress fiber formation.

The same analysis was performed in parallel in cells expressing F10 as a consequence of induction with doxycycline (gray bars). The percentage of F10-expressing cells with stress fibers that had been transfected with empty vector or plasmids expressing WT mDia or mDia-Asp was 28.4%, 22.8%, and 28%, respectively. Cells transfected with mDia-Ala showed an increase in the number of cells containing stress fibers to 54.8%. Several conclusions can be drawn from these data. First, expression of F10 within cells receiving empty vector or a plasmid encoding WT mDia lowered the frequency of cells with stress fibers from the ~60% (seen in uninduced cells) to ~24%. Second, expression of the non-phosphorylatable variant of mDia (Ala) nullified the impact of F10, raising the percentage of cells with stress fibers to 54%, comparable to what is seen in uninduced cells expressing WT mDia. Combined, these data confirm that phosphorylation of Ser22 of mDia has a dramatic effect on stress fiber formation.

**mDia is Phosphorylated During WT Vaccinia Virus Infection**—The data presented so far show that expression of F10 results in increased phosphorylation of mDia. We next wanted to take this one step further and determine if mDia phosphorylation was augmented during infection with WT vaccinia virus. 293-CAT cells were transfected with a plasmid encoding FLAG-mDia (or a control vector). Cells were then either mock infected or infected with WT vaccinia virus and mDia phosphorylation was assessed after performing metabolic labeling with 32PPi. FLAG-mDia was retrieved and analyzed by immunoblot (using an anti-FLAG antibody) and autoradiography. As shown in Fig. 9A, expression and retrieval of FLAG-mDia was equivalent in both uninfected and infected cells transfected with FLAG-mDia (lanes 2 and 3, immunoblot bottom). Low levels of basal phosphorylation of mDia was seen in mock infected cells (autoradiograph, lane 2). Phosphorylation of FLAG-mDia was significantly increased 6.2-fold ± 0.9 (autoradiograph, lane 3 (normalized to total mDia retrieved, average of six biological replicates) upon infection with vaccinia virus. In sum, these data show that mDia phosphorylation is increased significantly upon expression of F10 in uninfected cells, and is also increased significantly during vaccinia virus infection.

**DISCUSSION**

The F10 protein kinase is a dual specificity protein kinase that plays an essential role during the vaccinia virus life cycle (19–21, 27, 32). Based on the study of ts mutants and inducible recombinants, key roles for F10 in the initiation of viral membrane biogenesis and in the filling of nascent virions with viroplasm has been observed by electron microscopy. Because F10 is present in mature virions, it is delivered into the cytosol upon virion entry, as well as being expressed as a member of the late family of genes. Although some viral proteins have been shown to be dependent upon F10 for their phosphorylation during infection, it has been difficult to distinguish between direct targets and proteins whose phosphorylation only occurs within the context of ongoing virion assembly. In addition, the presence of the large number of cellular kinases has made clarification of F10-mediated events during infection difficult. Furthermore, the encapsidation of the viral B1 protein kinase and the viral H1 phosphatase, as well as F10, has also made the analysis of phosphorylation within virions challenging (57, 58).
Therefore, we undertook an unbiased phosphoproteomic screen using a cell line in which 3xFLAG-F10 could be induced by the addition of doxycycline to the culture medium. Using SILAC coupled with phosphopeptide enrichment, we identified 27 proteins that showed a significant change in their phosphorylation status upon induction of the F10 kinase (in uninfected cells).

**Fig. 8. The presence of stress fibers is modulated by the phosphorylation of Ser22 of mDia.**

A, Confluent monolayers of CV1-CAT (top row) or CV1-F10 (bottom row) were either left untreated (first column) or treated with 50 ng/ml doxycycline (second column) for 24 h. Cells were then fixed, permeabilized and stained with rhodamine phalloidin. Representative pictures of actin stress fibers are shown. △ represents cells containing stress fibers; △ represents cells that have lost their stress fibers. B, Images showing CV1-F10 cells transfected with empty vector (V) or plasmids expressing FLAG-mDia-WT (WT), FLAG-mDia-Ser22Ala (Ala) or FLAG-mDia-Ser22Asp (Asp) for 24 h in the absence (top row) or presence (bottom row) of 50 ng/ml doxycycline. C, Quantification of actin stress fibers of B. Cells were left untreated (black bars (-)) or treated with 50 ng/ml doxycycline to induce F10 expression (gray bars (+)) for an additional 24 h. Cells were then fixed, permeabilized and stained with rhodamine phalloidin. 125 cells were scored for the presence of actin stress fibers (n = 2; the mean and standard error are shown) (**, p < 0.02; ***, p < 0.001).
Transgene-encoded Expression of F10 Models the Function of F10 During Infection—The cell lines established here provide an excellent model for examining the function of the vaccinia virus F10 kinase. Because vaccinia-encoded genes are not normally expressed from the nucleus, we utilized a codon-optimized allele; upon induction of the transgene with doxycycline, F10 was expressed and accumulated to levels that were comparable (~2–3-fold higher) to what is seen during a typical infection with wild-type (WT) vaccinia infection (supplemental Fig. S1). Induction of F10 was able to mediate a partial but significant (~10–30-fold) rescue of viral production upon non-permissive infection with Cts28 (Fig. 2). Cts28 encodes a mutated F10 protein that is only modestly thermolabile, but a recombinant form of the Cts28-encoded F10 is inactive in vitro (32). The fact that the Cts28-encoded F10 remains stable during infection may explain the partial rescue that we observed, because it may retain the ability to bind to F10-interacting proteins (48, 59) and exert a modest dominant-negative effect.

Upon induction of the viral F10 kinase in both 293-F10 and CV1-F10 cells, clear changes in cell morphology were seen (Fig. 3 and 4). In 293 cells, the impact of F10 occurred within 12 h of induction and caused the cells to retract their protrusions and become rounded and refractile. In CV-1 cells, the changes were evident at 24 h postinduction and were more pronounced after 48 h postinduction; the most striking change was the loss of the well-defined colony morphology seen in this cell line, although cell rounding was also seen. Although both 293 and CV-1 cell lines are derived from kidney epithelia, their morphology in cell culture is strikingly different, and so it is perhaps not surprising that the phenotype seen upon F10 expression is also different. The impact of F10 on cell morphology was also seen in the prolonged and enhanced cell rounding that accompanies infection with a virus that overexpresses F10 (supplemental Fig. S4). Cumulatively these data support the hypothesis that F10 targets cellular proteins in a manner that can impact cell architecture.

Phosphoproteomic Analysis Identifies 27 Cellular Targets of F10-mediated Phosphorylation—Our SILAC phosphoproteomic comparison analysis of post-nuclear supernatants prepared from uninduced and induced 293-F10 cells identified 27 cellular proteins whose phosphostatus changed upon F10 induction in a significant manner. These proteins have been associated with roles in diverse cellular functions, including actin cytoskeleton remodeling, membrane trafficking and metabolism (Table I).

Of the 27 proteins identified, 18 proteins showed an increase in their H/L ratios, whereas 9 proteins showed a decrease in their H/L ratios. An increase in H/L ratio reflects increased phosphorylation upon expression of F10. The simplest interpretation of this phenotype is that F10 phosphorylates these protein targets directly. It is formally possible that F10 may activate another kinase (or inactivate a phosphatase), and that its impact on some or all of these 18 proteins is indirect. However, no kinases or phosphatases were identified in our analysis (Table I). The 30 phosphopeptides identified in the 18 proteins showing an increased H/L ratio were analyzed using the WebLogo algorithm, and we identified a consensus sequence in which the central pS>>pT>>pY residue was found immediately upstream of a P residue (Fig. 5C). Charged residues were also seen preferentially both upstream and downstream of this motif. This consensus sequence is in agreement with one previously seen when F10-
mediated phosphorylation events were analyzed in a proteomic characterization of purified vaccinia virions (57), increasing our confidence that F10 is directly responsible for phosphorylating these 18 cellular proteins. Interestingly, the central [S/T/Y]-P motif found within the F10 substrates that we identified is also found within the consensus for the CDK and p38 families of cellular kinases (57, 60, 61). The observation that the [S/T]-P motif can be found in >80% of the human proteome (60), suggests there must be additional requirements for determining the specificity of F10 for its substrates. Examples of these additional requirements is seen in the CDK family, which require a positively charged reside at the +2 position (57, 60) as well as the p38 family, which require a DEF motif downstream of the phosphorylation site (61). Further refinement of the consensus sequence for the F10 kinase could be resolved by additional biochemical analyses that are beyond the scope of this study.

Nine proteins showed a decrease in their H/L ratio, reflecting diminished phosphorylation upon expression of F10. One possible explanation is that F10 may phosphorylate and activate a cellular phosphatase, although none was identified as a target in our screen (Table I). Our previous comparison of metabolically labeled cell lysates prepared from WT or F10-deficient infections also showed a comparable result (32). An alternative explanation would be that F10-mediated phosphorylation alters cellular localization, leading to decreased abundance in the postnuclear supernatant. As a precedent, the replication factor MCM3 can shuttle into and out of the nucleus; a particular phosphorylation event is known to disrupt its NLS and induces proteosomal degradation of the cytosolic form (62, 63). Although the target residue within MCM3 that was identified by our study is different, F10-mediated phosphorylation may similarly alter the subcellular localization of phosphorylated proteins, reducing their levels in the postnuclear supernatant that we examined. A third possibility is that some of these phosphoproteins may be rendered unstable upon F10 expression, which would also give a reduced H/L ratio; this possibility should be evaluated in future studies.

**Pathway Analysis of Proteins Whose Phosphostatus Changes Upon F10 Expression**—STRING analysis ([www.string-db.org](http://www.string-db.org)) was used to investigate possible interactions among the F10 substrates identified in the SILAC phosphoproteomic screen (Fig. 5B, left); the GeneCards human gene database was consulted for further insight into protein function ([www.genecards.org](http://www.genecards.org)). For most of the proteins, no evidence of interaction was seen, suggesting that F10 may modulate components of multiple pathways. GBF1 (Go1gi-specific brefeldin A resistance factor 1) is a guanine nucleotide exchange factor (GEF) for Arf1 and perhaps Arf5. It’s most highly validated role is in the Arf1-mediated assembly of the COPI coat for Golgi-to-ER retrograde transport (64). Interestingly, the formation of vaccinia virus mature virions (MV) is resistant to brefeldin A (65). ARHGAP1, the prototypic member of the Rho GTPase activating protein (GAP) family, activates RhoA, Rac and Cdc42 (66), each of which modulate the actin cytoskeleton and other determinants of cell morphology. GBF1 was hypophosphorylated upon F10 induction; previous work has shown that GBF1 is phosphorylated by CDK1/Cyclin B and AMPK during mitosis and Golgi fragmentation, respectively (67, 68). Further investigation of how phosphorylation might modulate GBF1 is clearly of future interest.

Clathrin light chain A (CLTA) provides the scaffold for pits and vesicles involved in endocytosis. PIK3C2A, another protein identified, is a lipid kinase that participates in the formation and distribution of clathrin-coated endocytic vesicles. Interestingly, another target identified in our screen is Hook1, which is thought to link endocytic membrane trafficking to the microtubule cytoskeleton. Together, these proteins may contribute to virion or core trafficking, or to membrane acquisition.

Another interesting node identified by STRING associates the ADRM1 - RAD23B - Sec23IP proteins. ADRM1 is a component of the proteasome; it serves as a ubiquitin receptor and recruits a deubiquitinating enzyme (69, 70). In addition to a canonical role in nucleotide excision repair, Rad23B has been shown to interact with the proteasome as well as with ubiquitinated proteins (71, 72) and may play a role in ER associated degradation (ERAD) (73). Interestingly, RAD23’s association with the proteasome is regulated by phosphorylation on Ser75, which prevents RAD23’s association with the proteasome and leads to a reduction in protein turnover (74). This site of phosphorylation is different from that identified in our analysis of F10-regulated proteins (Thr155). Sec23IP interacts with components of the COPII coat complex, where it marks and organizes ER exit sites and mediates ER to Golgi transport (75, 76). Cumulatively, these proteins may enable membrane diversion from the ER in a manner that supports viral membrane biogenesis. They may manipulate ER exit sites through phosphorylation of Sec23IP, and/or prevent degradation of ER membrane proteins through phosphorylation of ADRM1 and RAD23B. Further investigation into these proteins may prove invaluable in understanding the mechanism by which vaccinia virus membrane proteins exit the ER.

Another intriguing protein that may relate to membrane biogenesis is NACA, a subunit of the nascent polypeptide associated complex. NACA prevents the inappropriate targeting of non-secretory polypeptides to the ER by binding to nascent polypeptide chains as they emerge from the ribosomes and preventing their interaction with the SRP. The vaccinia virus transmembrane proteins A14 and A17 are inserted into the ER membrane although they do not contain a traditional signal sequence (33); it is interesting to speculate that phosphorylation of NACA may diminish its activity and allow nascent A14 and A17 polypeptides to bind SRP and translocate into the ER membrane.

**A Focus on Formins: Validation of F10-mediated Phosphorylation of mDia and Discovery of a Role for Ser22 as a Regulator of Stress Fiber Formation**—The SILAC screen identified
several proteins that are involved in regulating the actin cytoskeleton including ARHGAP1 (described above), ARHGAP17 and DIAPH1. DIAPH1 (diaphanous related formin 1), also known as mDia was one of the phosphoproteins showing the most dramatic increase in H/L ratio (18.8), and was chosen for more intensive follow-up studies. mDia mediates apical F-actin polymerization by both stimulating actin polymerization and impairing the binding of capping proteins that terminate polymerization (77). mDia’s function in modulating actin stress fiber formation is tightly regulated. In the cytoplasm, mDia adopts an intramolecular autoinhibitory conformation in which the N-terminal Dia-inhibitory domain (DID) binds to the C-terminal Dia autoregulatory domain (DAD), precluding the two formin homology domains (FHs) from nucleating and elongating actin (50, 51). This inhibition is relieved through the binding of activated Rho and induces the formation of actin stress fibers (45, 52). As illustrated by studies of the interface between vaccinia virus and the cytoskeleton, mDia has also been associated with the modulation of cortical actin and microtubule networks (78, 79).

The activation of mDia through the binding of Rho is well documented; however, there is little data describing mDia regulation through phosphorylation. One report has shown that another formin family member, FHOD1, is phosphorylated at three residues at the C terminus by ROCK (80). These phosphorylation events relieve the intramolecular autoinhibition resulting in activation of FHOD1 and stress fiber formation.

As shown in Fig. 6A and 7, we successfully validated robust F10-dependent phosphorylation (~9–12-fold increase) of mDia in cultured cells using metabolic labeling and immunoprecipitation. Mutation of this residue to alanine or aspartic acid greatly diminished the extent of F10-mediated phosphorylation in cell culture (reduced to ~2-fold) (Fig. 7). Using these alleles of mDia, we were able to demonstrate that phosphorylation of Ser22 plays an important and previously unknown role in mDia-mediated regulation of actin stress fibers (Fig. 8). In the absence of F10, expression of mDia Ser22Ala increased the percentage of cells with stress fibers from ~60 to ~80%, whereas exogenous expression of WT mDia had no impact. These data indicate that there are cellular kinases that negatively regulate mDia by phosphorylation of mDia on Ser22, because the expression of mDia that cannot be phosphorylated on this residue has a striking impact on stress fiber formation. Conversely, expression of mDia Ser22Asp, a phosphomimetic variant, reduces the percentage of cells with stress fibers from ~60% to ~34%. These data probably underrepresent the impact of mDia’s phosphorylation on stress fibers, because the mutant alleles of mDia were introduced into cells by transfection, which occurs with an efficiency that is certainly significantly less than 100%. In sum, however, these data reveal for the first time that phosphorylation of a formin family member negatively regulates stress fiber formation.

The data obtained in F10-expressing cells confirms that F10-mediated phosphorylation of mDia has a similar effect. Upon expression of F10, the percentage of cells with stress fibers is reduced from the ~60% seen in control cells to ~23–28%; expression of the phosphomimetic variant had no additional effect. Conversely, expression of the Ser22Ala protein raised the percentage of cells with stress fibers to ~54.8%. Thus, our proteomic screen of F10-mediated phosphorylation targets has had the unexpected benefit of uncovering a cellular mechanism of regulation that had not herefore been known.

Although RhoA-mediated activation of mDia has been well studied, little is known about the mechanisms by which the activity of mDia is downregulated. The liprin-α protein has been shown to bind to the N-terminal mDia DID (inhibitory) and DD (dimerization) domains, thereby dislodging mDia from the plasma membrane, reducing the amount of active RhoA-mDia, and impairing stress fiber formation (81). Our data reinforce the conclusion that the N terminus of mDia is critical in negatively regulating actin stress fibers. The other two isoforms of mDia, mDia2 and mDia3, do not contain a serine at position 22. Therefore it is unlikely that these isoforms are playing redundant roles in regulating actin stress fibers. Furthermore, mDia2 is involved in filopodia formation as well as cytokinesis, whereas mDia3 participates in spindle alignment and endocytosis (82).

We propose three models for how the phosphorylation of Ser22 might regulate mDia: 1) phosphorylation may reduce the binding of RhoA and thus impair the activation of mDia, 2) phosphorylation may increase the affinity of the DID domain for the DAD domain, stabilizing the autoinhibitory conformation, or 3) phosphorylation may recruit liprin-α and mediate stress fiber dissolution. Interestingly, the prolyl isomerase Pin1 binds to pS/T - P motifs, where it catalyzes protein isomerization in a manner that can change protein binding partners (reviewed (83–85)). The F10 consensus motif identified in the SILAC screen contained a central pS/T - P motif (Fig. 5C). Therefore, Pin1 may facilitate a change in protein:protein interactions that leads to a diminution in active mDia and hence to stress fiber dissolution.

Vaccinia Virus Infection Relies Upon and Modulates Several Pathways That Regulate the Cytoskeleton—Vaccinia virus induces many changes in both the microtubule network and actin cytoskeleton (13). Early during infection, microtubules facilitate the movement of cores to the cell center (86); later, nascent virions move toward the periphery on microtubules. The viral F11 protein leads to an increase in microtubule dynamics and a loosening of the cortical actin network; F11 impairs the ability of Rho to signal to mDia and ROCK (87). As a consequence, cortical actin is less of a barrier to virion egress. Finally, vaccinia stimulates the polymerization of actin tails underneath the sites of EV exocytosis; these actin tails propel EV toward neighboring cells (13). Interestingly, the
latter process also involves the activation of the formin family member FHOD1 at the cell surface.

Our current work adds to the complex framework by which vaccinia virus manipulates the actin cytoskeleton. In addition to revealing that F10-mediated phosphorylation of the formin mDia reduces stress fiber formation in uninfected cells, we show that the phosphorylation of mDia also increases significantly during vaccinia virus infection (~6-fold) (Fig. 9A). Future studies, beyond of the scope of this work, will allow us to further dissect the temporal and spatial impact of F10 on mDia during infection, and the impact of this modification on the viral life cycle.

Vaccinia virus exhibits complex and exquisitely regulated control of cellular architecture. As the work described herein and elsewhere has demonstrated, vaccinia virus modulates the function of mDia in at least two ways, modeled in Fig. 9B. As Michael Way’s group has nicely demonstrated, the viral F11 protein inhibits Rho signaling to mDia; the consequence of this inhibition is a loosening of the cortical actin network to facilitate virion transit to the cell surface (78). As we have reported here, phosphorylation of mDia by the viral F10 protein kinase leads to a significant loss in actin stress fibers. This reduction in stress fibers may allow vaccinia virus to establish organelle-free zones for virion assembly (see Fig. 1). It will be of great interest in the future to determine the effect that the Ser22Ala and Ser22Asp mDia mutants would have on virion assembly. Moreover, the consequence of mDia phosphorylation on the microtubule network warrants further investigation.

In conclusion, we report here, for the first time, an unbiased proteomic screen focused on the identification of cellular targets of F10-mediated phosphorylation. Validation and further study of these phosphorylation events will enhance our understanding of how vaccinia virus manipulates cytoskeleton dynamics, membrane trafficking and metabolism in a manner essential to productive infection. These insights may identify new targets for therapeutic intervention in poxvirus infections. Furthermore, as evidenced by our discovery that cellular kinases can regulate stress fibers by phosphorylating mDia on Ser22, analysis of viral strategies will also elucidate cellular regulatory mechanisms.

Acknowledgments—We thank Kate Noon, Xiaogang Wu and Michael Pereckas at the Medical College of Wisconsin’s Mass Spectrometry facility for their invaluable help in processing and analyzing MS samples; this work is dedicated to Kate Noon in memoriam. A special thanks goes to Heidi Meeks for her invaluable help in scoring actin stress fibers. We would also like to thank past and present members of the Traktman laboratory for their lively discussions and interest in this project.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the data set identifier PXD005246.

* This work was supported by an NIH grant awarded to PT (R01 AI 107123). SST and DC were supported by grant R01AI083281 awarded to STT. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

[5] This article contains supplemental material.

† To whom correspondence should be addressed: Departments of Biochemistry & Molecular Biology and Microbiology & Immunology, Hollings Cancer Center, Medical University of South Carolina. Tel.: 843-876-2414 or 843-876-2405; E-mail: traktman@musc.edu.

REFERENCES

1. Damon, I. K. (2013) Poxviruses. In: Knipe, D. M., and Howley, P. M., eds. Fields Virology, 6th Ed., pp. 2160–2184, Lippincott Williams & Wilkins
2. Moss, B. (2013) Poxviridae. In: Knipe, D. M., and Howley, P. M., eds. Fields Virology, 6th Ed., pp. 2129–2159, Lippincott Williams & Wilkins
3. Sanchez-Sampedro, L., Perdiguero, B., Mejias-Perez, E., Garcia-Ariaza, J., Di Pilato, M., and Esteban, M. (2015) The evolution of poxvirus vaccines. Viruses, 7, 1726–1803
4. Sampath, P., and Thorne, S. H. (2014) Arming viruses in multi-mechanistic oncolytic viral therapy: current research and future developments, with emphasis on poxviruses. Oncolytic Virother, 3, 1–9
5. Schmidt, F. I., Bleck, C. K., Reh, L., Novy, K., Wollscheid, B., Helenium, A., Stahlberg, H., and Mercer, J. (2013) Vaccinia virus entry is followed by core activation and proteasome-mediated release of the immunomodulatory effector VH1 from lateral bodies. Cell Rep. 4, 464–476
6. Tolonen, N., Doglio, L., Schieich, S., and Krijsne-Locker, J. (2001) Vaccinia virus DNA replication occurs in endoplasmic reticulum-enclosed cytoplasmic mini-nuclei. Mol. Biol. Cell, 12, 2031–2046
7. Greseth, M. D., Boyle, K. A., Bluma, M. S., Unger, B., Wiebe, M. S., Soares-Martins, J. A., Wickramasekera, N. T., Wahlberg, J., and Traktman, P. (2012) Molecular genetic and biochemical characterization of the vaccinia virus I3 protein, the replicative single-stranded DNA binding protein. J. Virol. 86, 6197–6209
8. Condit, R. C., Moussatche, N., and Traktman, P. (2006) In a nutshell: structure and assembly of the vaccinia virion. Adv. Virus Res 66, 31–124
9. Boyle, K. A., Greseth, M. D., and Traktman, P. (2015) Genetic Confirmation that the H5 Protein Is Required for Vaccinia Virus DNA Replication. J. Virol. 89, 6312–6327
10. Chang, C. W., Li, H. C., Hsu, C. F., Chang, C. Y., and Lo, S. Y. (2009) Increased ATP generation in the host cell is required for efficient vaccinia virus production. J. Biomed. Sci. 16, 80
11. Greseth, M. D., and Traktman, P. (2014) De novo fatty acid biosynthesis contributes significantly to establishment of a bioenergetically favorable environment for vaccinia virus infection. PLoS. Pathog. 10, e1004202
12. Schepsis, A., Schramm, B., de Haan, C. A., and Locker, J. K. (2006) Vaccinia virus-induced microtubule-dependent cellular rearrangements. Traffic 7, 308–323
13. Leite, F., and Way, M. (2015) The role of signalling and the cytoskeleton during Vaccinia Virus egress. Virus Res. 209, 87–99
14. Liu, K., Lemon, B., and Traktman, P. (1995) The dual-specificity phosphatase encoded by vaccinia virus, VH1, is essential for viral transcription in vivo and in vitro. J. Virol. 69, 7823–7834
15. Guan, K. L., Broyles, S. S., and Dixon, J. E. (1991) A Tyr/Ser protein phosphatase encoded by vaccinia virus. Nature 350, 359–362
16. Rempel, R. E., and Traktman, P. (1992) Vaccinia virus B1 kinase: phenotypic analysis of temperature-sensitive mutants and enzymatic characterization of recombinant proteins. J. Virol. 66, 4413–4426
17. Banham, A. H., and Smith, G. L. (1992) Vaccinia virus gene B1R encodes a 34-kDa serine/threonine protein kinase that localizes in cytoplasmic factories and is packaged into virions. Virology 191, 803–812
18. Lin, S., Chen, W., and Broyles, S. S. (1992) The vaccinia virus B1R gene product is a serine/threonine protein kinase. J. Virol. 66, 2717–2723
19. Wang, S., and Shuman, S. (1995) Vaccinia virus morphogenesis is blocked by temperature-sensitive mutations in the F10 gene, which encodes protein kinase 2. J. Virol. 69, 6376–6388
20. Traktman, P., Caligiuri, A., Jesty, S. A., Liu, K., and Sankar, U. (1995) Temperature-sensitive mutants with lesions in the vaccinia virus F10 kinase undergo arrest at the earliest stage of virion morphogenesis. J. Virol. 69, 6581–6587
Molecular & Cellular Proteomics 16 Supplement 4

66. F10 Modulates Actin Stress Fibers by Phosphorylation of mDia

65. Ulaeto, D., Grosenbach, D., and Hruby, D. E. (1995) Brefeldin A inhibits vaccinia virus envelopment but does not prevent normal processing and localization of the putative envelopment receptor P37. J. Gen. Virol 76, 103–111

66. Tcherkezian, J., and Lamarche-Vane, N. (2007) Current knowledge of the large RhoGAP family of proteins. Biol. Cell 99, 67–86

67. Morohashi, Y., Balklava, Z., Ball, M., Hughes, H., and Lowe, M. (2010) Phosphorylation and membrane dissociation of the ARF exchange factor GBF1 in mitosis. Biochem. J. 427, 401–412

68. Miyamoto, T., Oshiro, N., Yoshino, K., Nakashima, A., Eguchi, S., Taka-hashi, M., Ono, Y., Kikkawa, U., and Yonezawa, K. (2008) AMP-activated protein kinase phosphorylates Golgi-specific brefeldin A resistance factor 1 at Thr1337 to induce disassembly of Golgi apparatus. J. Biol. Chem. 283, 4430–4438

69. Schreiner, P., Chen, X., Husnjak, K., Randles, L., Zhang, N., Elsasser, S., Finley, D., Dikic, I., Walters, K. J., and Groll, M. (2008) Ubiquitin docking at the proteasome through a novel pleckstrin-homology domain interaction. Nature 453, 548–552

70. Husnjak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofmann, K., Walters, K. J., Finley, D., and Dikic, I. (2008) Proteasome subunit Rpn13 is a novel ubiquitin receptor. Nature 453, 481–488

71. Chen, L., and Madura, K. (2002) Rad23 promotes the targeting of proteolytic substrates to the proteasome. Mol. Cell. Biol. 22, 4902–4913

72. Hiyama, H., Yokoi, M., Masutani, C., Sugasawa, K., Maekawa, T., Tanaka, K., Hoeijmakers, J. H., and Hanaoka, F. (1999) Interaction of hHR23 with S5a. The ubiquitin-like domain of hHR23 mediates interaction with S5a subunit of 26 S proteasome. J. Biol. Chem. 274, 28019–28025

73. Medicherla, B., Kostova, Z., Schaefer, A., and Wolf, D. H. (2004) A genomic screen identifies Dak2p and Rad23p as essential components of ER-associated degradation. EMBO Rep. 5, 692–697

74. Liang, R. Y., Chen, L., Ko, B. T., Shen, Y. H., Li, Y. T., Chen, B. R., Lin, K. T., Madura, K., and Chuang, S. M. (2014) Rad23 interaction with the proteasome is regulated by phosphorylation of its ubiquitin-like (UbL) domain. J. Mol. Biol. 426, 4049–4060

75. Klinkenberg, D., Long, K. R., Shome, K., Watkins, S. C., and Aridor, M. (2014) A cascade of ER exit site assembly that is regulated by p125A and lipid signals. J. Cell Sci. 127, 1765–1778

76. Ong, Y. S., Tang, B. L., Loo, L. S., and Hong, W. (2010) p125A exists as part of the mammalian Sec13/Sec31 COPII subcomplex to facilitate ER-Golgi transport. J. Cell Biol. 190, 331–345

77. Goode, B. L., and Eck, M. J. (2007) Mechanism and function of formins in the control of actin assembly. Annu. Rev. Biochem. 76, 593–627

78. Arakawa, Y., Cordeiro, J. V., Schleich, S., Newsome, T. P., and Way, M. (2007) The release of vaccinia virus from infected cells requires RhoA-mDia modulation of cortical actin. Cell Host Microbe 1, 227–240

79. Arakawa, Y., Cordeiro, J. V., and Way, M. (2007) F11L-mediated inhibition of RhoA-mDia signaling stimulates microtubule dynamics during vaccinia virus infection. Cell Host Microbe 1, 213–226

80. Takeya, R., Taniguchi, K., Narumiya, S., and Sumimoto, H. (2008) The mammalian formin FHOD1 is activated through phosphorylation by ROCK and mediates thrombin-induced stress fibre formation in endothelial cells. EMBO J. 27, 618–628

81. Sakamoto, S., Ishizaki, T., Okawa, K., Watanabe, S., Arakawa, T., Wat-tanabe, N., and Narumiya, S. (2012) Liprin-alpha controls stress fiber formation by binding to mDia and regulating its membrane localization. J. Cell Sci. 125, 108–120

82. Thumkeo, D., Watanabe, S., and Narumiya, S. (2013) Physiological roles of Rho and Rho effectors in mammals. Eur. J. Cell Biol. 92, 303–315

83. Min, S. H., Zhou, X. Z., and Lu, K. P. (2016) The role of Pin1 in the development and treatment of cancer. Arch. Pharm. Res. 39, 1609–1620

84. Nakatsu, Y., Matsunaga, Y., Yamamotoya, T., Ueda, K., Inoue, Y., Mori, K., Sakoda, H., Fujishiro, M., Ono, H., Kushiyama, A., and Asano, T. (2016) Physiological and Pathogenic Roles of Prolyl Isomerase Pin1 in Metabolic Regulations via Multiple Signal Transduction Pathway Modulations. Int J Mol Sci 17, 1495–1512

85. Zhou, X. Z., and Lu, K. P. (2016) The isomerase Pin1 controls numerous cancer-driving pathways and is a unique drug target. Nat. Rev. Cancer 16, 463–478

86. Carter, G. C., Rodger, G., Murphy, B. J., Law, M., Krauss, O., Hollinshead, M., and Smith, G. L. (2003) Vaccinia virus cores are transported on microtubules. J. Gen. Virol 84, 2443–2458

87. Valderrama, F., Cordeiro, J. V., Schleich, S., Frischknecht, F., and Way, M. (2006) Vaccinia virus-induced cell motility requires F11L-mediated inhibition of RhoA signaling. Science 311, 377–381