RICK Activates a NF-κB-dependent Anti-human Cytomegalovirus Response*

Jan Eickhoff‡, Miriam Hanke‡, Matthias Stein-Gerlach‡, Tan Poi Kiang‡, Katrin Herzberger‡, Peter Habenberger‡, Stefan Müller‡, Bert Klebl‡, Manfred Marschall§, Thomas Stamminger§, and Matt Cotten¶

From ‡Axxima Pharmaceuticals AG, 81377 München, Germany and §Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, 91054 Erlangen, Germany

The adapter kinase receptor interacting protein-like interacting caspase-like apoptosis regulatory protein kinase (RICK, also called RIP2 and CARDIAK) was found to be elevated at both the protein and RNA levels during human cytomegalovirus (HCMV) replication, suggesting either that the virus may require RICK for replication or that RICK is part of an unsuccessful host attempt to inhibit HCMV replication. It is demonstrated here that forced expression of RICK in either a kinase active or inactive form activates nuclear factor (NF)-κB by means of its intermediate domain and potently blocks HCMV replication in human fibroblasts. Importantly, NF-κB activation, which exerted a modestly positive effect on the early phase of infection, clearly had a strongly negative impact during later viral steps. A stable inhibitor of NF-κB (IκB) reverses the RICK inhibitory effect, and the activation of NF-κB by IκB kinase-β expression is inhibitory to HCMV, demonstrating that NF-κB activation is part of a potent anti-HCMV response. Supernatant transfer experiments identified interferon-β as a downstream component of the RICK inhibitory pathway. RICK expression was found to synergize with HCMV infection in the induction of interferon-β expression. This study identifies an endogenous RICK-activated, NF-κB- and interferon-β-dependent antiviral pathway that is either inhibited or faulty under normal HCMV replication conditions; efforts to bolster this pathway may lead to novel anti-viral approaches.

Alterations in host gene expression can be a useful starting point in efforts to identify novel anti-viral strategies. Up-regulated genes can be deliberately induced and used by pathogens for their own functions. Alternately, these novel gene expression events can be components of the host response to pathogen entry and replication, the innate immune response (reviewed in Refs. 1–3). Because of the long evolutionary interplay between host and pathogen, these factors are complex and not trivial to unravel. One major transcriptional program that has recently become clear is the innate immune response. This is an evolutionarily conserved, potent anti-pathogen response comprising a set of pattern recognition receptors including the toll-like receptors on the cell surface as well as the nucleotide-binding oligomerization domain (NOD) molecules intracellularly that recognize pathogen-associated molecular patterns. Upon engagement of a receptor with the appropriate pathogen-associated molecular pattern (lipopolysaccharide, peptidoglycan, double-stranded RNA, flagellin, etc.), this binding results in the recruitment and activation of several kinase complexes. Importantly, the scaffold protein NEMO (IKKγ) interacts with one of several kinases, IKKα, IKKβ, IKKe, and tank binding kinase, resulting in the activation of nuclear factor-κB (NF-κB) (via IKK phosphorylation of IκB) or activation of IRF3 (via IKKe or tank-binding kinase; Refs. 4, 5). In turn, this selection of transcription factors activates anti-pathogen response genes, including the interferons and other cytokines, and host defense genes, such as NOS, IDO, PKR, various nucleases, and proteases. Thus, the innate immune response comprises three types of molecules: pathogen sensors on both the cell surface and intracellularly which respond to the presence of pathogen components by oligomerization and activation of a kinase complex; transcription factors, which are either directly or indirectly activated by the kinase complexes; and the activated host genes, whose composition is determined by the variety and extent of transcription factors activated. The susceptibility to bacterial and viral infection of human patients with genetic lesions in components of this pathway (e.g. NOD2, Ref. 6; NEMO, Ref. 7) or of mice with these pathways disrupted (e.g. IRF, Ref. 8; RICK, Ref. 9; TLR4, Ref. 10) demonstrates the importance of the innate immune response as a primary barrier to a number of different pathogens.

In an analysis of the host response to human cytomegalovirus (HCMV) replication, the cellular kinase RICK (also known as RIP2, CARDIAK, Refs. 11–14) was found to be up-regulated at both the transcriptional and protein levels during infection. RICK is a broadly expressed serine/threonine kinase consisting of an N-terminal kinase domain, an intermediate domain, and a C-terminal CARD. RICK is implicated in the activation of a number of signaling pathways including NF-κB, p38, c-Jun NH2-terminal kinase, and extracellular signal-regulated kinase activation (11–14). Surprisingly, the activation of these

* This work was supported in part by a grant from the German Bundesministerium für Bildung und Forschung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Axxima Pharmaceuticals AG, Max-Lebsche-Platz 32, 81377 München, Germany. Tel.: 49-89550-65472; Fax: 49-89550-65461; E-mail: Matt.Cotten@axxima.com.

This paper is available on line at http://www.jbc.org
RICK Activates an Anti-viral Response

downstream events by RICK occurs independently of the RICK kinase activity but requires either an intact full-length molecule (11) or oligomerization of the kinase plus an intermediate domain (14). Studies with mice homozygous for a disrupted RICK allele demonstrate an essential role for RICK in activating NF-κB, p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase downstream of several toll-like receptors and the intracellular NOD1 and NOD2 molecules as well as playing an important role in the activation of interferon-γ expression in response to a variety of stimuli (9, 15). Given this broad range of cellular events activated by RICK, we sought to determine whether the observed RICK up-regulation is stimulatory to HCMV replication or, alternately, is activated by the host in response to HCMV infection and is part of a host defense program.

An analysis of the role of RICK in HCMV replication revealed that modest overexpression of the RICK molecule is inhibitory to HCMV replication; the inhibition requires NF-κB activation, and the essential downstream components of the inhibition are NF-κB activation and the induction of interferon-β (IFN-β) production. Thus, it seems that RICK is contributing to the innate immune response of the HCMV infection, a response that under normal conditions is not sufficient to prevent HCMV replication.

EXPERIMENTAL PROCEDURES

Quantification of RNA Induction—For analysis of RICK expression levels by real-time PCR, human foreskin fibroblasts (HFFs) were infected with different HCMV isolates at the indicated m.o.i.s, harvested 24 or 72 h after infection, and the total RNA was isolated by the TRIzol method (Invitrogen), followed by a DNase I digest. 5 μg of RNA was used for reverse transcription with oligo dT primers. RICK expression was determined by real-time PCR and normalized with GAPDH expression. Real-time PCR was performed with SYBR Green on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Darmstadt, Germany) with the following primers: RICK: 5'-AGCTCT- GCAGCCCTGTTAGCC-3' and 5'-AGGGGACTTGCAGCTGGTATAA-3'; GAPDH: 5'-GGCTTCTAGTTGCAACAATATCT-3' and 5-TCGGA-GTCAACGGATTTGG-3'. IFN-β induction was measured with real-time PCR on an ABI PRISM 7000 sequence detection system using the following primers: IFN-β, 5'-GACATCCTGGAGAGATAGGCAAC-3' and 5'-GGACACTC-TAGATGTGCTGAAATTT-3'; GAPDH was quantified by using an established TaqMan assay kit (ABI Prism predeveloped TaqMan assay reagent for human GAPDH, Applied Biosystems).

Cell Culture—HFF and 293 cell cultures (24) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. NF-κB reporter cells and NF-κB-dependent clinical isolates of HCMV (isolate 3, isolate 4, and isolate 5) were maintained by serial passage in 293 cells (25). RICK plasmids were transfected with Lipofectamine 2000 (Invitrogen). EGFP-expressing cell lines were stained with crystal violet, and the number of viral plaques per well was counted.

RESULTS

RICK mRNA and Protein Levels Are Increased during HCMV Infection—In a broad screen of cellular signaling molecules influenced by HCMV replication, the serine/threonine adapter kinase RICK was found to be up-regulated during productive HCMV replication in primary HFFs. Initial results using the DNA filter array revealed a consistent increase in RICK mRNA levels (results not shown). Infection with the HCMV laboratory strain AD169 resulted in an increase of RICK on both the protein and RNA levels (Fig. 1a). Infection with three independent clinical isolates of HCMV (isolate 3, isolate 4, and isolate 5) also resulted in an increase of RICK protein and mRNA levels (Fig. 1b). In the clinical HCMV strains, up-regulation of RICK was less pronounced on the protein than on the RNA level.

RICK Inhibits the Replication of HCMV—A major concern in interpreting elevated levels of host protein or RNA during virus infection is to determine whether the increase is directly activated by the pathogen and used to promote the course of infection, or if the up-regulated protein is part of a host defense pathway. To address this important question, an assay system based on replication-defective adenovirus vectors was developed to monitor the effects of specific cellular protein expression on HCMV replication. HCMV replication was measured by using HCMV AD169-GFP (27). Infection, replication, and...
spread of HCMV over a 7-day period resulted in a reliable EGFP signal that could be quantitated (Fig. 2a, compare lanes 1 and 2). Adenovirus vectors were prepared that direct expression of test proteins throughout an entire culture of fibroblasts (e.g. luciferase or EGFP with >90% of the cells expressing the test protein, results not shown). Transduction of HFFs with a control adenovirus AdJ5 (E1/E3 negative, no expression cas-

![Image](https://example.com/image1.png)

**FIG. 1.** RICK is induced upon infection of HFFs with HCMV. a, RICK up-regulation by HCMV strain AD169. HFFs were infected with an m.o.i. of 3 and lysed with RIPA buffer 7, 24, 48, and 72 h after infection (top panel), or with increasing m.o.i.s as indicated and lysed 72 h after infection (middle panel). Lysates were separated by SDS-10% PAGE and transferred to nitrocellulose. Samples were probed with an anti-RICK-antibody (OPA, Dianova) and as a loading control, anti-α-tubulin (Sigma); probing was followed by ECL. For analysis of RICK up-regulation at the RNA level, RNA from HFFs infected with the indicated m.o.i.s AD169 was prepared at 72 h after infection and monitored for levels of the RICK mRNA by using real-time PCR (lower panel). b, RICK up-regulation by clinical HCMV strains. HFFs were infected with the HCMV strains TB40E and two different clinical isolates with the indicated m.o.i.s and lysed with RIPA buffer 72 h after infection (top). Lysates were treated as described above for AD169. For analysis of RICK up-regulation at the RNA level, RNA from HFFs infected with the indicated HCMV strains at an m.o.i. of 0.5 or 2 was prepared at 72 h after infection and monitored for levels of the RICK mRNA by using real-time PCR (lower panel).
RICK Activates an Anti-viral Response

sette) had no significant effect of HCMV replication (Fig. 2a, lanes 3 and 4). However, transduction with a RICK-expressing adenovirus at the same viral particle/cell number severely impaired the replication of HCMV (Fig. 2a, lanes 5 and 6). Transduction with an adenovirus-expressing RICK ki had the same inhibitory effect on HCMV replication as RICK wt (Fig. 2a, lanes 7 and 8). RICK has an intermediate domain as well as a C-terminal CARD (Caspase Recruitment Domain; Refs. 12–14) which is an important signal for protein-protein interaction and is thought to target molecules to specific signaling complexes (28). Deletion of the CARD (plus 1/2 of the IM) of either RICK wt or RICK ki led to increased expression of the proteins (Fig. 2b) but largely destroyed the HCMV inhibitory activity (Fig. 2a, lanes 9–12). Thus, the forced expression of RICK inhibits HCMV replication, the inhibition does not require the serine/threonine kinase activity of RICK but is sensitive to mutations that disrupt the intermediate domain and remove the CARD. The slightly reduced mobility of the RICK protein...
The inhibition of HCMV replication by RICK correlates with NF-κB activation.

**a.** To monitor NF-κB activation, 3 x 10^4 3T3 NF-κB Luc cells/well were seeded in 96-well plates. The cells were transduced the following day with either control AdJ5 (30,000 p/c) or 3000, 10,000, or 30,000 p/c of adenoviruses directing the expression of various RICK molecules. All adenovirus transductions were adjusted to equal virus levels by adding control adenovirus AdJ5. After 4 h, the medium was replaced with fresh Dulbecco's modified Eagle's medium without fetal calf serum and cultured for another 24 h. As a positive control, cells were stimulated with 20 ng/ml TNF-α 2 h before lysis. Luciferase activity was determined by using a Luclite Plus luciferase detection system from Packard BioSciences.

**b.** IκBα expression reverses the inhibitory effect of RICK on HCMV replication. Subconfluent monolayers of HFFs were transduced with 300 or 1000 p/c of adenoviruses expressing either RICK wt or RICK ki and, in addition, 1000 p/c of adenovirus expressing IκBα, as indicated. All adenovirus transductions were adjusted to equal virus levels (2000 p/c) by adding control AdJ5. At 24 h after adenoviral transduction, cells were infected with HCMV AD169-GFP. Cells were lysed 7 days later and analyzed for EGFP content. All reported values are derived from duplicate infections with mean and standard deviation values shown.

**c.** For RICK expression controls, cells were lysed in RIPA buffer. The lysates were separated by SDS-10% PAGE, transferred to nitrocellulose, and probed with an anti-HA-antibody (Roche Applied Science). IκBα expression blocks the RICK activation of NF-κB. The analysis was performed as in Fig. 3. Cells were transduced with 30,000 p/c of adenoviruses directing the expression of RICK wt and 30,000, 10,000, or 3000 p/c of adenoviruses directing the expression of IκBα. All adenovirus transductions were adjusted to equal virus levels by adding control adenovirus AdJ5. Note that 3T3 cells express very low levels of the adenovirus receptor CAR; thus, the quantities of adenovirus used were adjusted to generated levels of protein expression similar to those obtained with lower m.o.i.s in HFFs.

**d.** Dissection of NF-κB activation by RICK. Upper panel, diagram of the RICK domain.
caused by the HA-epitope allows one to estimate that the viral expressed RICK was ~5-fold over endogenous levels (Fig. 2c). Expression of the RICK CARD (plus 1/2 of the IM, the portion of RICK removed in the \( \Delta \)CARD molecule) fused to an EGFP molecule, also did not impair HCMV replication (Fig. 3a, lanes 8 and 9). Furthermore, transduction of HFFs with an adenovirus directing synthesis of the kinase RIP, which belongs to the same kinase family, was found to have no effect on HCMV replication (Fig. 3a, lanes 10 and 11, RIP wt), although the two kinases, RICK and RIP, were expressed to similar levels (Fig. 3a, lower panel). Furthermore, no inhibition of HCMV replication was observed when cells were transduced with the same quantities of adenovirus expressing the cellular kinases SRPK2, NIK (Nck interacting kinase), or mitogen-activated protein kinase kinase (results not shown). Thus, the observed effect of full-length RICK on HCMV replication was not due to trivial consequences of overexpressing a mammalian kinase with adenovirus.

As an additional measure of the RICK inhibition of HCMV replication, cells were transduced with the various RICK adenoviruses or control adenovirus and infected with HCMV AD169; HCMV replication was monitored directly by a conventional plaque assay (Fig. 3b). Consistent with the results obtained with the GFP assay, expression of full-length RICK in either the kinase active or kinase inactive forms blocked the replication of HCMV (Fig. 3b, lanes 4–9). The removal of the RICK CARD and 1/2 of the IM was again found to disrupt the HCMV inhibitory effect (Fig. 3b, lanes 10–12).

Under some conditions such as co-overexpression of caspase (13) or expression in a tumor cell line (12), RICK overexpression results in increased apoptosis. We found that in HFFs, cell viability was not influenced by transduction with adenovirus directing the expression of RICK in either the absence of HCMV replication (Fig. 3c, upper panel) or in the presence of HCMV replication (Fig. 3c, lower panel). Thus, the impairment of HCMV replication cannot be accounted for by an increased cell death promoted by RICK.

The Inhibition of HCMV Replication by RICK Correlates with Its Ability to Activate NF-\( \kappa \)B—RICK activates the NF-\( \kappa \)B and mitogen-activated protein kinase pathways (11–14). To identify downstream effectors mediating HCMV inhibition, we analyzed the RICK constructs tested against HCMV for NF-\( \kappa \)B activation. RICK with wild-type kinase activity (Fig. 4a, lanes 4–6) as well as kinase inactive RICK (Fig. 4a, lanes 7–9) activated NF-\( \kappa \)B to levels comparable with that obtained with TNFa (Fig. 4a, lane 2). Transduction with the control adenovirus J5 at the same concentrations did not activate NF-\( \kappa \)B (Fig. 4a, lane 3 shows 10,000 particles/cell (p/c) AdJ5). Removal of amino acids 354–540, which remove the CARD and part of the intermediate domain of either kinase wt or ki forms, disrupted the ability of RICK to activate NF-\( \kappa \)B (Fig. 4a, lanes 10–15). The activation of NF-\( \kappa \)B signaling by the different RICK constructs correlated closely with their ability to inhibit HCMV replication (Fig. 2).

Blocking NF-\( \kappa \)B Activation Prevents the RICK Inhibition of HCMV—The correlation between RICK molecules that activate NF-\( \kappa \)B and inhibit HCMV replication suggested that the NF-\( \kappa \)B activation may be an essential part of activating an intracellular anti-viral response. To test the hypothesis that RICK inhibits HCMV replication by means of the activation of NF-\( \kappa \)B, we examined the consequences of expressing an IxB\( \alpha \)S32A/S36A mutant (19, 20) on NF-\( \kappa \)B activation by RICK. IxB\( \alpha \) lacks the serine residues phosphorylated by IxB kinases during NF-\( \kappa \)B activation. These phosphorylation events must occur to promote IxB ubiquitination, degradation, and the ensuing NF-\( \kappa \)B activation; thus IxB\( \alpha \) is a potent inhibitor of NF-\( \kappa \)B activation (19, 20). Expression of IxB\( \alpha \) reversed to a substantial extent the inhibitory effect of both RICK wt (Fig. 4b, lanes 5 and 7) and RICK ki (Fig. 4b, lanes 9 and 11) on HCMV, demonstrating an essential role for NF-\( \kappa \)B activation in the RICK inhibitory response. Expression controls demonstrated that the RICK expression itself was not impaired by coexpression of IxB\( \alpha \) (Fig. 4c). Coexpression of IxB\( \alpha \) clearly blocked the NF-\( \kappa \)B activation triggered by RICK expression (Fig. 4d) and also that provided by TNFa, IFN-\( \gamma \), and HCMV infection (results not shown), demonstrating the function of this inhibitor. Thus, the inhibitory mechanism of RICK on HCMV replication requires an essential step of activating the transcription factor NF-\( \kappa \)B. That blocking NF-\( \kappa \)B activation does not impair HCMV replication has been observed previously by Benedict et al. (39) and supports the idea that NF-\( \kappa \)B, although modestly important for HCMV transcriptional events, plays a more critical role in driving an anti-viral cellular response.

The Intermediate Domain of RICK Is Essential for NF-\( \kappa \)B Activation—To dissect the activation of NF-\( \kappa \)B by RICK, deletion mutants of RICK altering the different modules of the protein were analyzed for their ability to activate NF-\( \kappa \)B (Fig. 4e). It is clear that the full-length RICK activates, and a deletion that removes the entire CARD and 1/2 of the IM domain disrupts this activation (compare RICK wt with RICK\( \Delta \)CARD, Fig. 4e). A RICK molecule deleted of CARD but expressing the complete IM (RICK KIM) has nearly wild-type NF-\( \kappa \)B activity, demonstrating that the portion of the IM removed in the \( \Delta \)CARD molecules is essential for NF-\( \kappa \)B activation. The IM expressed alone, however, fails to activate NF-\( \kappa \)B and, surprisingly, adding CARD to the IM restores the activity. We conclude that the intermediate domain, especially the region between amino acids 353–425 is essential for NF-\( \kappa \)B activation. However, this domain must interact with other components of the signaling complex, and it can do this only if it is fused with CARD or the kinase domain. Previously, a RICK molecule spanning amino acids 1–425 was shown to interact with IKB\( \gamma \) (NEMO) (14).

Changes in HCMV Protein Expression Coincide with HCMV Replication Block—Previous studies had reported a role for NF-\( \kappa \)B in activating the HCMV immediate early gene promoter (29, 30). HCMV encodes at least two types of gene products demonstrated to promote NF-\( \kappa \)B activation (31–37), suggesting that NF-\( \kappa \)B activation might promote HCMV replication. We examined the consequences of RICK and IxB\( \alpha \) expression on NF-\( \kappa \)B-activated immediate early gene products, IE1p72 and IE2p86. We found that early during HCMV infection, blocking NF-\( \kappa \)B activation with IxB\( \alpha \) resulted in a modest decline in IE1p72 expression (Fig. 5a, lane 2, 6 h p.i.), which is consistent with NF-\( \kappa \)B activation promoting IE1p72 expression. However, at 24 and 72 h after infection, RICK expression resulted in lower levels of IE1p72 expression, and this RICK-mediated decline was blocked by IxB\( \alpha \) (compare Fig. 5a, lanes 5 and 6, 24h and 72h p.i.). RICK represses both IE1p72 and IE2p86 to a comparable extent in an NF-\( \kappa \)B-dependent manner (Fig. 5b), ruling out a possible decline of IE1p72 because of an induction
of IE2p86, which has been reported to repress IE1p72 after the IE phase. The level of IE1p72 expressed in this system is a composite of initial gene expression from the infecting HCMV (which might be modestly stimulated by NF-κB activation), as well as amplification because of virus replication (which is dependent on the health of the infected cell as well as the consequences of host anti-viral activities). However, as measured by IE1p72 and IE2p86 gene expression late in infection (Fig. 5c), as well as by total virus replication (Figs. 2a, 35, 46), the dominant effect of NF-κB activation is clearly to impair HCMV infection.

Further demonstration that the dominant role of NF-κB activation is inhibitory to HCMV replication derives from an analysis of an HCMV late protein, glycoprotein B (gB). RICK expression resulted in the depression of gB levels at 72 h after infection (Fig. 5c, lanes 3 and 5), with the decline blocked by the co-expression of IκB (Fig. 5c, lanes 4 and 6).

**IKKβ Activation of NF-κB Also Blocks HCMV Replication—**

Stimuli that activate NF-κB commonly activate one of the IκB phosphorylating kinases, which subsequently phosphorylate IκB, leading to its degradation and releasing NF-κB for transcriptional function. Overexpression of an IκB kinase, especially the IKKβ isoform, has been shown to be sufficient to activate the pathway (38). An adenovirus directing the synthesis of IKKβ (Fig. 6c) provided the expected NF-κB activation (Fig. 6b). Transduction of HFFs with this adenovirus was inhibitory to HCMV replication (Fig. 6a), further demonstrating that NF-κB activation is inhibitory to HCMV. Note that in comparison to the inhibition obtained with AdRICK, the quantity of AdIKKβ required for similar HCMV inhibition is 3- to 10-fold higher. Although it is difficult to directly compare the two gene products, these results suggest that, in addition to NF-κB activation, the inhibition promoted by RICK expression may involve additional factors.

**RICK and HCMV Cooperate in the Induction of a Soluble Inhibitory Factor—**

The previous results suggest that the RICK elevation is part of a host anti-pathogen mechanism rather than a mechanism employed by HCMV to promote its replication. Experiments in RICK knockout mice demonstrated a failure to produce interferon-γ in response to activation of toll-like receptors or intracellular NOD molecules (9, 15). We suspected that the RICK inhibition of HCMV replication might involve the secretion of inhibitory cytokines. To test the idea that either RICK alone, or RICK plus HCMV, would induce the production of inhibitory factors, HFFs were treated with either control adenovirus J5, adenovirus expressing RICK, or the same adenoviruses plus HCMV. The supernatants were collected and tested for inhibition of HCMV replication (Fig. 7a). Supernatants from RICK-expressing cells gave only a slight

---

**Fig. 5. The consequences of RICK expression on CMV gene expression.** 

**a.** IE1p72, RICK, IκBα analysis. Subconfluent monolayers of HFFs were transduced with 300, 1000, or 3000 p/c of adenoviruses expressing RICK wt, or with 1000 p/c of adenovirus expressing IκBα. Adenovirus transductions were adjusted to equal virus levels (3000 p/c) by adding AdJ5. At 24 h after adenoviral transduction, the cells were infected with an HCMV strain AD169 with an m.o.i. of 0.1 or left uninfected as control. Cells were lysed 6, 24, or 72 h after HCMV infection with RIPA buffer. Lysates were separated by SDS-10% PAGE, transferred to nitrocellulose, and probed for the immediate early HCMV gene IE1p72. The blot was stripped and reprobed for RICK expression with anti-HA-antibody (Roche Applied Science), for IκBα expression (Santa Cruz Biotechnology), or as control (α-tubulin). 

**b.** IE2p86, IE1p72 analysis. Cells were treated as in a: HFFs were transduced with the indicated amounts of adenoviruses expressing RICK or IκBα. All adenovirus transductions were adjusted to equal virus levels (4000 p/c) by adding AdJ5. 24 h after transduction, cells were infected with AD169 (m.o.i. = 0.2). 48 h after HCMV infection, cells were lysed in RIPA buffer, and lysates were separated by SDS-10% PAGE, transferred to nitrocellulose, and probed for the HCMV major envelope protein glycoprotein B (gB). The blot was stripped and reprobed as control with an antibody against α-tubulin.

---

**Fig. 6. The consequences of AdRICK infection on CMV late gene expression.** 

A composite of initial gene expression from the infecting HCMV (which might be stimulated by NF-κB activation) is clearly to impair HCMV infection.

---

**Fig. 7. The consequences of RICK expression on CMV late gene expression.** 

A composite of initial gene expression from the infecting HCMV (which might be stimulated by NF-κB activation) is clearly to impair HCMV infection.
inhibition in comparison to the control J5-treated cells (Fig. 7a, lanes 2 and 4). Supernatants from cells infected with HCMV plus RICK-expressing cells (Fig. 7a, lane 5) but not HCMV alone (lane 3) produced a substantial inhibition of HCMV replication when transferred to freshly infected cells. (Note that supernatants were added only once during the 7-day replication assay.) Thus, an inhibitory factor is produced in the supernatant because of the combined effects of HCMV replication and RICK expression.

**IFN-β is an Essential Part of the HCMV Inhibition Induced by RICK—**As NF-κB-activating stimuli can induce the production of the antiviral cytokine IFN-β in HCMV-infected fibroblasts (39), IFN-β was an obvious candidate for a component of the inhibitory supernatant. Type 1 interferon transcription is controlled by NF-κB, AP-1 (ATF2/Jun), and members of the IRF family (40, 41), and at least two of these transcriptional pathways (NF-κB and AP-1) are known to be stimulated by RICK. When examined directly by real-time PCR, the changes in IFN-β transcript levels were consistent with the inhibitory action of the supernatant: IFN-β was increased modestly by HCMV alone (Fig. 7b, lane 4), but the combination of HCMV replication plus RICK expression resulted in a 20-fold increase in IFN-β transcription, compared with infection with HCMV alone (Fig. 7b, lane 6). To test directly whether IFN-β is responsible for the inhibitory activity, supernatant transfer experiments were performed in the presence of a control antibody (recognizing EGFP) or an antibody directed against human IFN-β. Cells treated with supernatants from RICK plus HCMV-treated cells in the presence of a control antibody recognizing EGFP (Fig. 7a, lane 6) or a TNFα neutralizing antibody (data not shown) did not influence the inhibitory activity. However, when incubated with an antibody against IFN-β, the same supernatant was no longer inhibitory to HCMV replication (lane 7). We conclude that an essential component of the inhibitory supernatant generated by RICK expression plus HCMV infection is IFN-β. It has been demonstrated clearly that HCMV can interfere with the viral-induced interferon synthesis (54, 55, 56). Indeed, the interferon response to UV-inactivated HCMV infection is robust, whereas during the course of active HCMV replication, this response is largely suppressed (55, 56). To test whether RICK is able to override these mechanisms of HCMV interferon suppression, we analyzed IFN-β induction in the presence of very high m.o.i. of HCMV, conditions which ensure that a high proportion of the cell population is infected with HCMV. RICK is able to induce interferon expression even at HCMV m.o.i.s of 10, although a strong IFN-β induction occurs already at lower m.o.i.s, which resemble more physiological infection conditions (Fig. 7c). As NF-κB-signaling was essential for the RICK inhibition of HCMV replication, it was next determined whether IFN-β induction by RICK was dependent on NF-κB signaling. Indeed, 1xΔ blocked the induction of IFN-β by RICK used at similar levels as in the HCMV replication assay (300 p/c of Ad RICK; Fig. 7d, lanes 4 and 6). However, at high levels of RICK expression (3000 p/c of Ad RICK; lanes 6 and 7), 1xΔ was no longer able to block the induction of IFN-β, perhaps because of the strength of the RICK signaling or because of RICK influencing other transcription pathways.

**DISCUSSION**

We demonstrate here that RICK, a serine/threonine kinase, CARD-containing adapter protein, is elevated during HCMV replication. To determine whether RICK is a cellular protein required by HCMV for replication or part of a cellular anti-viral response, a method of assessing the consequences of expression of a test protein on HCMV replication was developed, employing transduction with replication-defective adenovirus to direct expression of a test protein in a large proportion of a cell population, followed by infection with HCMV. We clearly demonstrate that expression of RICK leads to the inhibition of HCMV replication. Thus, the elevation of RICK during infection is most likely part of an incomplete host defense response.

There is a strict correlation between RICK molecules that activate NF-κB and the inhibition of HCMV replication. Furthermore, a potent inhibitor of the NF-κB pathway (1xΔ) reversed the HCMV inhibition by RICK, demonstrating that NF-κB activation is required for the inhibitory program driven by RICK. In efforts to understand the mechanism of HCMV inhibition downstream of RICK and NF-κB, we find that treating HCMV-infected cells with supernatants from HCMV-infected, RICK-expressing cells recapitulates the HCMV inhibition. The soluble inhibitory factor was found to be IFN-β.

The concept that RICK signaling leads to an anti-HCMV state is consistent with the established biology of RICK. Mice lacking RICK have defects in LPS-stimulated activation of p38, NF-κB, c-Jun NH2-terminal kinase, and extracellular signal-regulated kinase (15), impaired toll-like receptor (2,3,4 but not 9) stimulation of NF-κB activation (15), interferon-γ production by means of a variety of stimuli, and T cell receptor signaling...
RICK has also been implicated in intracellular pathogen recognition by linking the pathogen-sensing molecules NOD1 and NOD2 with NF-κB activation (42–44). Where it has been examined, the downstream signaling functions of RICK do not require the RICK kinase activity, but do require the intact molecule (kinase domain, intermediate domain, and CARD domain; Refs. 11, 13). Thus a model is emerging of RICK serving an essential scaffolding function in the communication between pathogen-sensing pathways, either extracellularly by means of the Toll-like receptors, or intracellularly by means of NOD1 and NOD2 molecules and downstream activation of transcriptional events that can lead to protection against the pathogen.

The antiviral effects of RICK shown here as well as most of the known downstream events (e.g. NF-κB, mitogen-activated protein kinase activation) occur independently of the RICK kinase activity. However, the kinase domain is expected to have some function (e.g. a modulatory function) in the biology of RICK. The mammalian cell normally has evolved strict control mechanisms to prevent chronic activation of such inflammatory responses. Thus, failing to find a role for the kinase domain in the positive downstream events activated by RICK, it is quite possible that the RICK kinase domain plays a role in down-regulating the RICK response. Indeed, we find that kinase inactive RICK activates NF-κB stronger than wild-type RICK (Fig. 4a).

It has long been known that HCMV immediate early gene expression is increased by NF-κB activation (29, 30).
HCMV infection itself can activate NF-κB. Several distinct mechanisms have been proposed for this activation, including up-regulation of p105/p50, p65, and relB expression at the transcriptional level by HCMV early gene expression (31–34). HCMV, like other herpes viruses, encodes several G-protein-coupled receptors, and it has been demonstrated that one, the US28 gene product, can activate NF-κB (35, 36), possibly by means of activation of the phosphatidylinositol 3-kinase/Akt pathway (37). Inhibition of this function by pertussis toxin suggests a role for G-protein activation (45). Thus, one interpretation of these results is that NF-κB activation is a positive factor for HCMV replication, and that inhibiting NF-κB activation could be a useful strategy to block the virus. However, NF-κB is an important transcription factor for inflammatory and anti-viral cytokine genes and genes whose products are part of the host innate immune response. Because of the evolutionary potential of viruses, it is quite possible that NF-κB is primarily a transcription factor activated in response to pathogen entry and important for driving anti-pathogen responses, but viruses have evolved to take advantage of the strong and rapidly induced transcription factors available during infection. However, in most work examining the role of NF-κB in HCMV replication, the ultimate consequences on HCMV replication of activating this pathway were not examined. We demonstrate here that blocking NF-κB does not impair HCMV replication (Fig. 4b) but rather has the opposite effect of blocking anti-viral responses from the cell. One should point out that there is a series of publications (46–52) describing the ability of potent NF-κB inhibitory agents, glucocorticoids, to promote HCMV or murine CMV replication in cell culture as well as in patients and in mice. We demonstrate here that blocking NF-κB reverses the inhibitory effects of RICK, and these results provide a molecular mechanism for these older literature reports on the stimulatory effects of glucocorticoids on HCMV replication and pathology.

The results presented here are also consistent with and extend the results from other studies examining the host response to HCMV infection. It was clearly demonstrated by Benedict et al. (39) that HCMV infection is not impaired in the presence of a non-phosphorylatable IkB, that lymphotixin receptor activation combined with HCMV infection synergize in the activation of IFN-β, and that this activation was blocked by this inhibitor. Furthermore, HCMV entry has been found to activate NF-κB and the innate immune response via viral envelope protein engagement of Toll-like receptor 2 (TLR2) and CD14 (53). RICK functions downstream of TLR2 in this pathway (9, 15), and it is possible that the synergy between HCMV and RICK in IFN-β induction is due to a synergy in stimulating this pathway. This, together with recent data showing that HCMV virions are recognized by TLR2, leading by means of induction of NF-κB to the release of inflammatory cytokines (53), make TLR2 a promising candidate for mediating RICK activation during HCMV infection. HCMV has been shown to have functions that down-regulate the type-1 interferon response (54). An extensive analysis of the cellular gene expression changes in response to HCMV replication compared with infection with UV-inactivated virus or in the presence of cycloheximide revealed that a large number of genes, including IFN-β as well as interferon-activated genes, were clearly activated by HCMV entry, but functions encoded by the virus down-modulate this response (54–56). The results presented here demonstrate that forced RICK expression allows the HCMV-infected cells to override the anti-interferon functions encoded by HCMV. The boost in IFN-β induction that we observed with combined HCMV infection and forced RICK expression shifts the balance in favor of the cell.

Future efforts will include a detailed analysis of the upstream events leading to RICK signaling. It is possible that the TLR2 or NOD1 pathway functions as a pattern-recognition molecule to alert cells to the presence of a virus, and efforts to enhance this pathway may lead to potent anti-viral strategies. The results presented here identify IFN-β as an effector gene product of this pathway. Further efforts will determine whether IFN-β is the single essential component in the inhibitory supernatant, or if additional factors contribute to the response. It will also be of great interest to determine whether the pathway activated by RICK is inhibitory to other viral and bacterial pathogens.

Acknowledgments—We thank Allegra Ritscher, Alexandra Baumgartner, and Kerstin Stegmueller for technical assistance and Thomas Herget and Ursula Wellenbrock for establishing the NF-κB-luciferase cell line.

REFERENCES

1. Janeway, C. A., Jr., and Medzhitov, R. (2002) Annu. Rev. Immunol. 20, 197–216
2. Inohara, N., and Nunez, G. (2003) Nat. Rev. Immunol. 3, 371–382
3. Barton, G. M., and Medzhitov, R. (2003) Science 306, 1524–1525
4. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, P., Layton, B., and Beutler, B. (1998) Science 282, 2085–2088
5. Thome, M., Hofmann, K., Burns, K., Martinon, F., Bodmer, J. L., Mattmann, J., Lammert, C., and Tschopp, J. (1998) Curr. Biol. 8, 885–888
6. Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G., Girardin, S. E., Boneca, I. G., Chamaillard, M., Labigne, A., Thomas, G.
E. S. (1997) J. Virol. 71, 4638–4648
34. Jiang, H. Y., Petrovas, C., and Sonenshein, G. E. (2002) J. Virol. 76, 5737–5747
35. Casarosa, P., Bakker, R. A., Verzijl, D., Navis, M., Timmerman, H., Leurs, R., and Smit, M. J. (2001) J. Biol. Chem. 276, 1133–1137
36. Waldheer, M., Kledal, T. N., Farrell, H., and Schwartz, T. W. (2002) J. Virol. 76, 8161–8168
37. Johnson, R. A., Wang, X., Ma, X. L., Huang, S. M., and Huang, E. S. (2001) J. Virol. 75, 6022–6032
38. Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997) Science 278, 866–869
39. Benedict, C. A., Banks, T. A., Senderowicz, L., Ko, M., Britt, W. J., Angulo, A., Ghazal, P., and Ware, C. F. (2001) Immunity 15, 617–626
40. Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000) Cell 103, 667–678
41. Falvo, J. V., Parekh, B. S., Lin, C. H., Fraenkel, E., and Maniatis, T. (2000) Mol. Cell. Biol. 20, 4814–4825
42. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J., and Nunez, G. (1999) J. Biol. Chem. 274, 14560–14567
43. Inohara, N., Ogura, Y., Chen, F. F., Muto, A., and Nunez, G. (2001) J. Biol. Chem. 276, 2551–2554
44. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamanka, S., and Nunez, G. (2001) J. Biol. Chem. 276, 4812–4818
45. Carlquist, J. F., Edelman, L., Bennion, D. W., and Anderson, J. L. (1999) J. Infect. Dis. 179, 1094–1100
46. Jordan, M. C., Shanley, J. D., and Stevens, J. G. (1977) J. Gen. Virol. 37, 419–423
47. Velasco, N., Catto, G. R., Edward, N., Engeset, J., and Moffat, M. A. (1984) J. Infect. 9, 69–78
48. Tanaka, J., Ogura, T., Kamiya, S., Sato, H., Yoshie, T., Ogura, H., and Hatano, M. (1984) J. Gen. Virol. 65, 1729–1767
49. Koment, R. W. (1985) J. Med. Virol. 15, 149–156
50. Koment, R. W. (1989) J. Med. Virol. 27, 44–47
51. Forbes, B. A., Bonville, C. A., and Dock, N. L. (1990) J. Infect. Dis. 162, 39–45
52. Lathey, J. L., and Spector, S. A. (1991) J. Virol. 65, 6371–6375
53. Compton, T., Kurt-Jones, E. A., Boehme, K. W., Belko, J., Latz, E., Golenbock, D. T., and Finberg, R. W. (2003) J. Virol. 77, 4588–4596
54. Miller, D. M., Zhang, Y., Rabill, B. M., Waldman, W. J., and Sedmak, D. D. (1999) J. Immunol. 162, 6107–6113
55. Browne, E. P., Wing, B., Coleman, D., and Shenk, T. (2001) J. Virol. 75, 12319–12330
56. Browne, E. P., and Shenk, T. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11439–11444