JOURNAL OF VIROLOGY, Jan. 1997, p. 595–600 Vol. 71, No. 1
0022-538X/97/$04.00+0

Tyrosine Phosphorylation Events during Coxsackievirus B3 Replication

MICHAEL HUBER,* HANS-CHRISTOPH SELINKA, AND REINHARD KANDOLF

Institute for Pathology, Department of Molecular Pathology, University of Tübingen, D-72076 Tübingen, Germany

Received 20 March 1996/Accepted 27 September 1996

In order to study cellular and viral determinants of pathogenicity, interactions between coxsackievirus B3 (CVB3) replication and cellular protein tyrosine phosphorylation were investigated. During CVB3 infection of HeLa cells, distinct proteins become phosphorylated on tyrosine residues, as detected by the use of antiphosphotyrosine Western blotting. Two proteins of 48 and 200 kDa showed enhanced tyrosine phosphorylation 4 to 5 h postinfection (p.i.), although virus-induced inhibition of cellular protein synthesis had already occurred 3 to 4 h p.i. Subcellular fractionation experiments revealed distinct localization of tyrosine-phosphorylated proteins of 48 and 200 kDa in the cytosol and membrane fractions of infected cells, respectively. In addition, in Vero cells infected with CVB3, echovirus (EV)11, or EV12, increased tyrosine phosphorylation of a 200-kDa protein was detected 6 h p.i. Herbimycin A, a specific inhibitor of Src-like protein tyrosine kinases, was shown to inhibit virus-induced tyrosine phosphorylations and to reduce the production of progeny virions. In contrast, in cells treated with the inhibitors staurosporine and calphostin C, the synthesis of progeny virions was not affected. Immunoprecipitation experiments suggested that the tyrosine-phosphorylated 200-kDa protein in CVB3-infected cells is of cellular origin. In summary, these investigations have begun to unravel the effect of CVB3 as well as EV11 and EV12 replication on cellular tyrosine phosphorylation and support the importance of tyrosine phosphorylation events for effective virus replication. Such cellular phosphorylation events triggered in the course of enterovirus infection may enhance virus replication.

Coxsackieviruses of group B (CVB) are enteroviruses of the family *Picornaviridae* and causative agents of a variety of human diseases, from minor common colds to fatal myocarditis and meningitis (20, 22, 34, 35). Distinct variants of CVB have also been associated with insulin-dependent diabetes mellitus (1, 10). Coxsackievirus B3 (CVB3), typically a cytolytic virus, has been shown to be capable of evading immunological surveillance in a host-dependent fashion, inducing persistent heart muscle infection by restricted virus replication (24, 27).

CVB are small, nonenveloped, icosahedral enteroviruses containing a positive-sense, single-stranded RNA genome of about 7,400 nucleotides (28). Infection of a permissive host cell is initiated by attachment of virus to a specific cell surface receptor (37). Following entry into the cell, genomic RNA is replicated by the virus-encoded RNA-dependent RNA polymerase to generate full-length, intermediate negative-strand RNAs. New infectious positive-strand RNAs are subsequently produced by replication of the negative-strand RNAs. Translation occurs by a Cap-independent mechanism, yielding the viral polyprotein which is processed by virus-encoded proteinases. Interactions of viral and cellular components are critically involved in processes necessary for a productive infectious cycle (3, 29). With respect to correct formation of protein-protein and protein-RNA complexes in the course of infection, the question arises as to whether these processes might be modulated by constituents of the cellular signal transduction system itself.

Concerning effects on constitutive cellular tyrosine phosphorylation, different viruses have been described to induce tyrosine phosphorylation of various cellular substrates (6, 9, 11, 31, 33). In astrocytes infected with Newcastle disease virus, increased tyrosine phosphorylation of phosphatidylinositol phospholipase C-γ 1 was demonstrated preceding the activation of protein kinase C (PKC) in tumor necrosis factor alpha gene induction (9). In Epstein-Barr virus-infected human B lymphocytes, the rapid Ca^{2+}-dependent tyrosine phosphorylation of the cytosolic tyrosine kinase $p56^{lck}$ has been shown (5, 31).

This investigation analyzes cellular tyrosine phosphorylation events in the course of CVB3 replication to address the question of whether protein tyrosine kinases might play a role in the regulation of productive virus infection. The following data show time-dependent increases in tyrosine phosphorylation of distinct cellular proteins in enterovirus-infected cells and demonstrate the effect of the specific tyrosine kinase inhibitor herbimycin A on the production of progeny virions.

MATERIALS AND METHODS

Cell lines and viruses. HeLa cells (human cervix carcinoma, CCL 2) and Vero cells (African green monkey kidney cells, CCL 81) were obtained from the American Type Culture Collection. The cells were cultivated as monolayers in Dulbecco's modified Eagle's minimal medium–10% fetal bovine serum. CVB3 (Nancy strain) used in this study was derived by transfection of HeLa cells with infectious recombinant CVB3 cDNA (21, 28), propagated in HeLa cells and maintained in Dulbecco's modified Eagle's minimal medium supplemented with 10% fetal bovine serum. CVB3 adapted to growth in Vero cells was derived by four consecutive passages in this cell line. Echovirus (EV)11 (Gregory strain, VR-41) and EV12 (Travis strain, VR-42) were obtained from the American Type Culture Collection and adapted to growth in Vero cells by six and nine consecutive passages, respectively. CVB3, EV11, and EV12 were purified as described previously (37). Cell cultures were infected with a multiplicity of infection of 10 unless otherwise indicated.

Antibodies and chemicals. Monoclonal antiphosphotyrosine antibody 4G10 (kindly provided by S. Werner, Max Planck Institute for Biochemistry, Martinsried, Germany) was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 μg/μl containing 0.2% bovine serum albumin (BSA). Antiserum recognizing CVB3 polymerase (anti-Pol) was raised against a fusion protein consisting of 99 amino acids of MS2 replicase and 350 amino acids of CVB3 polymerase (40). Protein kinase inhibitors herbimycin A, staurosporine, and calphostin C (Calbiochem) were dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml. Incubations with calphostin C were carried out at room temperature (RT) under white fluorescent light for the first 30 min to activate the

* Corresponding author. Mailing address: Institut für Pathologie, Abt. Molekulare Pathologie, Universität Tübingen, Liebermeisterstr. 8, D-72076 Tübingen, Germany. Phone: 49-7071-2984925. Fax: 49-7071-292258.

inhibitor (4). Protein A-Sepharose was dissolved to 20% in Emerson-Schubert buffer containing 0.2% Triton X-100, 25 mM sodium chloride, and 10% glycine in 10 mM Tris, pH 8.0.

Solubilization of cells, electrophoresis, and Western blotting. To stop CVB3 infection, culture medium was removed and cells were resuspended in 400 μl of lysis buffer (1% Triton X-100, 137 mM sodium chloride, 4 mM EDTA, 10% glycerol, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 0.15 trypsin-inhibiting units of aprotinin/ml, 10 mM sodium orthovanadate in 20 mM Tris, pH 8.0). After lysis at 4°C, nuclei were pelleted at 1,000 × *g* and postnuclear supernatants were normalized for equal protein content (as measured in a Bradford protein assay, Bio-Rad). Samples were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5 or 15% polyacrylamide) (SDS-PAGE) under reducing conditions, using a Mini-Protean II system (Bio-Rad). Proteins were transferred to a polyvinylidene difluoride (PVDF) transfer membrane. The membranes were rinsed twice with PBS for 5 min at RT, incubated for 1 h at 4°C in blocking buffer containing 3% BSA in PBS, and subsequently washed twice with PBS for 5 min at RT. The blots were incubated overnight at 4°C with the first antibody (monoclonal antibody 4G10, 1 μg/ml) in PBS containing 0.1% BSA. The blots were rinsed six times with PBS containing 0.2% Tween 20 for 5 min at RT and incubated for 1 h at 4°C with horseradish peroxidase-conjugated immunoglobulins (anti-mouse immunoglobulin), dissolved 1:6,000 in PBS containing 0.1% BSA. After incubation, the blots were washed five times with PBS containing 0.2% Tween 20, visualized by enhanced chemiluminescence according to the manufacturer's recommendations (Amersham), and exposed to Biomax MR films (Kodak).

Analysis of virus-induced host cell protein shutoff. HeLa cells infected with CVB3 at a multiplicity of infection of 20 were washed twice with PBS and incubated for 1 h with methionine-free medium supplemented with 15 μCi of [^{35}S]methionine (NEN) per ml. At the times indicated, the radiolabeled cell monolayers were washed three times with PBS and treated with 100 μl of lysis buffer, and the radioactivity of postnuclear supernatants was measured in a liquid scintillation counter. Samples of 10^6 cpm were separated by SDS–13.5% PAGE, and the gels were dried and subsequently exposed to X-ray films (NEN).

Plaque assay. HeLa cells were incubated in the presence or in the absence of 5 or 10 μM herbimycin A, 75 nM staurosporine, or 100 nM calphostin C. Cells were infected with CVB3 as outlined above, and virus was recovered from the cell suspensions at different times by three cycles of freezing and thawing. Virus titers were determined by plaque assay on HeLa monolayer cells as previously described (23).

Subcellular fractionation. After CVB3 infection, HeLa cells (10-cm-diameter dishes) were washed with ice-cold buffer A (250 mM sucrose, 2.5 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 25 μg of leupeptin/ml, 0.15 trypsin-inhibiting units of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride in 25 mM Tris-HCl, pH 8.0) and scraped from the plate into 700 μl of buffer A–5 mM EDTA. The cells were disrupted by 30 strokes of a tight-fitting Dounce homogenizer, and homogenates were depleted of cell debris at 10,000 × *g* for 10 min at 4°C. Supernatants were centrifuged at 200,000 × *g* at 4°C for 30 min, and cytosol fractions (supernatants) were used in Western blots. Membrane fractions were extracted with 300 μl of buffer A–5 mM EDTA–1% Triton X-100–0.1% Nonidet P-40 at 4°C for 60 min and recentrifuged. The detergent extracts of these membrane fractions were used in Western blots.

Immunoprecipitation reactions. Postnuclear supernatants from 2 × 10^6 cells were precleared with 20 μl of 20% protein A–Sepharose for 20 min at 4°C. After centrifugation at 10,000 × *g*, the supernatants were incubated overnight at 4°C with 5 μg of the antiphosphotyrosine antibody 4G10 or 5 μl of anti-Pol antiserum. Sixty microliters of 20% protein A–Sepharose was added for 1 h at 4°C, followed by subsequent centrifugation. Pellets were washed seven times with 200 μl of lysis buffer and incubated for 5 min at 98°C in 30 μl of SDS loading buffer. Samples were subjected to SDS-PAGE, and proteins were transferred onto a PVDF membrane and immunoblotted. Following incubation with secondary horseradish peroxidase-conjugated antibodies, the blots were developed by use of an enhanced chemiluminescence reagent kit.

RESULTS

CVB3 infection is associated with intracellular tyrosine phosphorylation. To investigate whether CVB3 induces intracellular tyrosine phosphorylation events in the course of infection, HeLa cells were either infected with CVB3 or mock infected for time intervals from 1 to 7 h. After lysis, postnuclear supernatants were separated by SDS-PAGE and transferred onto PVDF membranes, and tyrosine-phosphorylated proteins were detected in Western blots with antiphosphotyrosine antibodies as shown in Fig. 1. Interestingly, modulations in tyrosine phosphorylation of various proteins (e.g., the 65-, 70-, and 100-kDa proteins) were observed in the first 3 h postinfection (p.i.), in contrast to the constant level of tyrosine phosphorylation of other proteins, like the 200-kDa protein.

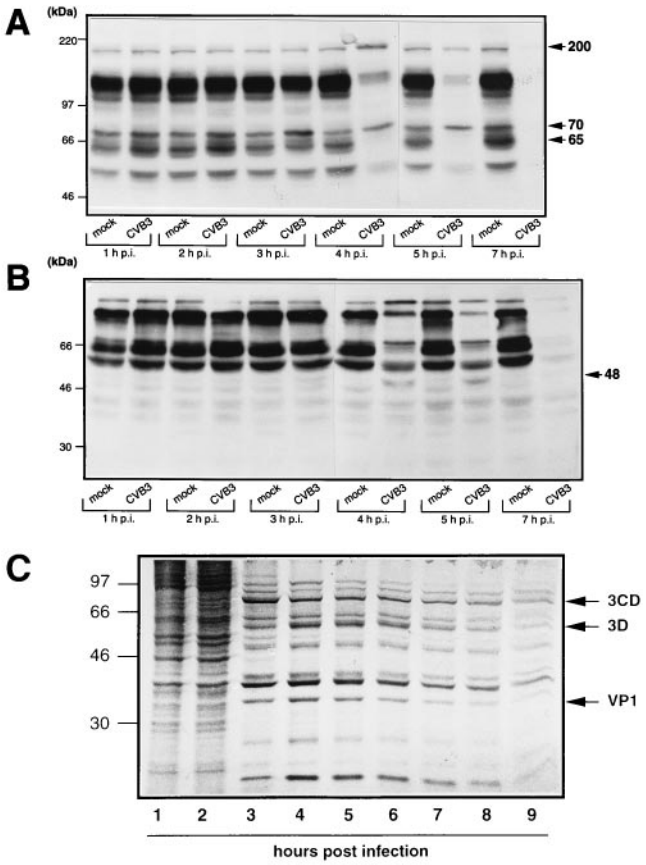

FIG. 1. Kinetics of constitutive tyrosine phosphorylation and host cell protein shutoff in CVB3-infected HeLa cells. (A) Postnuclear supernatants from CVB3- and mock-infected HeLa cells were prepared 1 to 7 h p.i., and equal amounts of protein were subjected to SDS–7.5% PAGE. Proteins were transferred onto PVDF membranes and subjected to antiphosphotyrosine immunoblotting as described in Materials and Methods. The positions of proteins with molecular masses of 65, 70, and 200 kDa are indicated by arrows. (B) Identical protein preparations were separated on SDS–15% polyacrylamide gels and subjected to antiphosphotyrosine Western blotting. The position of the 48-kDa protein is marked by an arrow. Exposure times were 30 s (A) and 3 min (B). (C) To monitor the host cell protein shutoff, CVB3-infected HeLa cells were labeled with 15 μCi of [^{35}S]methionine per ml for 1 h at hourly intervals, and proteins were analyzed by SDS-PAGE with subsequent autoradiography. The numbers at the bottom represent time points when pulse-labeling was stopped; those on the left are sizes, in kilodaltons. The positions of coxsackievirus polypeptides 3CD, 3D, and VP1 are indicated by arrows.

Although most tyrosine phosphorylations had disappeared by 4 h p.i., a 70-kDa protein remained in the tyrosine-phosphorylated state until the late phase of infection. The protein with a molecular mass of 200 kDa, however, showed increased tyrosine phosphorylation at 4 h p.i. To examine proteins with molecular masses smaller than 60 kDa, identical protein preparations were separated on SDS-polyacrylamide gels of higher concentration. As shown in Fig. 1B, a tyrosine-phosphorylated protein with a molecular mass of 48 kDa was induced at 4 to 5 h p.i. To correlate these data with the virus-induced host cell protein shutoff, CVB3-infected HeLa cells were radioactively pulse-labeled at different time intervals, and the resulting protein preparations were analyzed by SDS-PAGE with subsequent autoradiography. As depicted in Fig. 1C, synthesis of viral proteins was detected starting at 3 h p.i. Since the marked decrease in tyrosine phosphorylation was observed at 4 to 5 h p.i. (Fig. 1A and B), production of viral proteins preceded this effect (Fig. 1C). These results indicate that during CVB3 in-

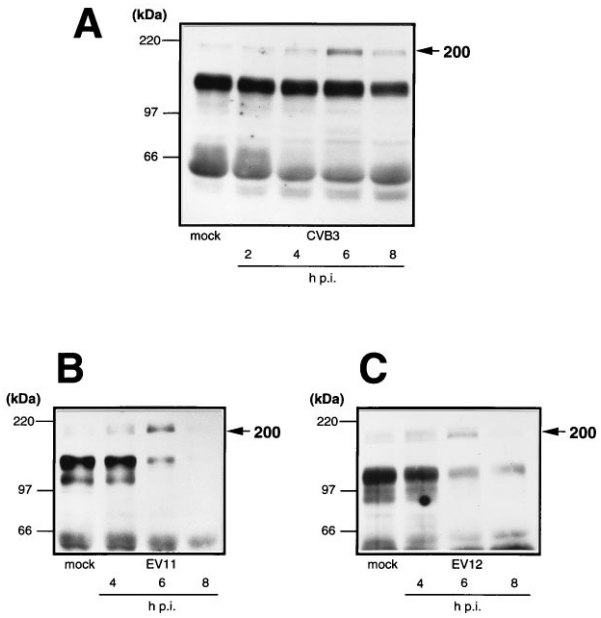

FIG. 2. Tyrosine phosphorylation events in CVB3-, EV11-, and EV12-infected Vero cells. (A) CVB3 infection of Vero cells. Postnuclear supernatants from CVB3- and mock-infected Vero cells were prepared at the indicated times and subjected to antiphosphotyrosine immunoblotting. (B) Infection of Vero cells with EV11. Vero cells were either infected with EV11 or mock infected. Postnuclear supernatants were subjected to Western blotting with phosphotyrosine-specific antibodies. (C) EV12 replication in Vero cells. Protein preparations from EV12- and mock-infected Vero cells were analyzed for phosphotyrosine-containing proteins by immunoblotting. In each panel, the position of the 200-kDa protein is indicated by an arrow.

fection of HeLa cells, certain proteins become phosphorylated on tyrosine residues in a time-dependent fashion, despite efficient virus-induced shutoff of host protein synthesis.

Tyrosine phosphorylation events induced during CVB3, EV11, or EV12 infection of Vero cells. It was further determined whether tyrosine phosphorylation of specific proteins upon CVB3 infection of HeLa cells was cell specific or a rather ubiquitous event in CVB3-susceptible cells. Therefore, in addition to HeLa cells, African green monkey kidney (Vero) cells were infected with CVB3 and the effects on cellular tyrosine phosphorylation patterns were examined. As shown in Fig. 2A, replication of CVB3 in Vero cells resulted in a prominent increase in tyrosine phosphorylation of a 200-kDa protein 6 h p.i. To extend this study to other enteroviruses, tyrosine phosphorylations in Vero cells infected with two echoviruses, EV11 and EV12, were examined. As observed with CVB3-infected Vero cells, a 200-kDa protein exhibiting enhanced tyrosine phosphorylation was detected in EV11- and EV12-infected Vero cells 6 h p.i. by Western blotting with phosphotyrosine-specific antibodies (Fig. 2B and C). In contrast to the case for HeLa cells, phosphorylation of the 48-, 65-, and 70-kDa proteins was not observed with CVB3-, EV11-, or EV12-infected Vero cells. These results suggest a role for tyrosine-phosphorylated proteins, in particular, the 200-kDa protein, in the course of enterovirus replication.

Effect of herbimycin A treatment on CVB3-induced tyrosine phosphorylation. To analyze the nature of specific tyrosine phosphorylation events induced upon CVB3 infection of HeLa cells, inhibition assays were performed by use of herbimycin A. This substance is a widely used tyrosine kinase inhibitor that is considered to be a selective inhibitor of Src-like kinases by irreversibly binding to sulfhydryl groups in the vicinity of the kinase's active center (12, 38). HeLa cells were incubated in the presence or in the absence of 5 μM herbimycin A, followed by CVB3 or mock infection. Four hours p.i., the cells were lysed, and postnuclear supernatants were separated by SDS-PAGE and subjected to Western blotting with phosphotyrosine-specific antibodies (Fig. 3). As shown in Fig. 3A, enhanced tyrosine phosphorylation of the 200-kDa protein was detected 4 h p.i. in the absence of herbimycin A but was reduced to normal levels in herbimycin A-treated HeLa cells. Furthermore, induction of the 48-kDa phosphoprotein in CVB3-infected HeLa cells was detected only in the absence of herbimycin A. These observed decreases of tyrosine phosphorylations in CVB3-infected cells after herbimycin A treatment are not due to general protein degradation induced by herbimycin A. As shown exemplarily, a tyrosine-phosphorylated protein of 70 kDa did not show any decrease in the presence of herbimycin A 4 h p.i. (Fig. 3A). Enhanced tyrosine phosphorylation of the 200-kDa protein was also prevented in Vero cells infected with EV11 in the presence of herbimycin A (Fig. 3B). Intensities of other protein bands were not changed by herbimycin A treatment, as shown exemplarily by the proteins in the range of 130 kDa. These results indicate that Src-like protein tyrosine kinases may be responsible for this increase in tyrosine phosphorylations upon CVB3 and EV11 replication.

Effect of various phosphorylation inhibitors on the production of progeny virions. With respect to the inhibitory effects observed with herbimycin A, it was of interest to study the influence of this inhibitor on CVB3 replication. If tyrosine phosphorylation is important for CVB3 replication, the level of progeny virus should be reduced in CVB3-infected cells treated with herbimycin A. In order to investigate this hypothesis, HeLa cells were incubated with 5 or 10 μM herbimycin A and infected with CVB3, and virus was recovered by freeze-thawing of cells at 3, 5, and 24 h p.i. Subsequently, virus titers were determined by plaque assays on HeLa monolayer cells. As shown in Fig. 4, infection in the presence of 5 or 10 μM herbimycin A resulted in a significant reduction of produced progeny virions at all time points. A similar reduction of produced virions in the presence of herbimycin A was obtained in EV11-infected Vero cells (data not shown). In contrast, virus production was not affected in the presence of 75 nM staurosporine, a broad-range kinase inhibitor, and 100 nM calphostin C, a PKC-specific kinase inhibitor (Fig. 4). These results suggest that activated Src-like protein tyrosine kinases may con-

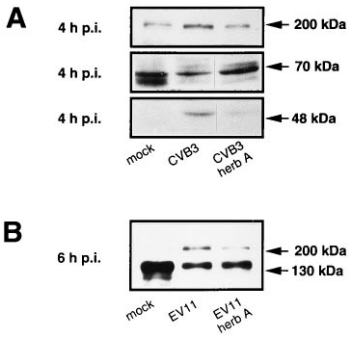

FIG. 3. Effect of herbimycin A on tyrosine phosphorylation events. (A) CVB3 infection of HeLa cells. Cells were incubated with 5 μM herbimycin A or left untreated and subjected to CVB3 or mock infection. Four hours p.i., the cells were lysed and the postnuclear supernatants were analyzed by antiphosphotyrosine Western blotting. (B) EV11 replication in Vero cells. Cells treated with 5 μM herbimycin A or left untreated were either CVB3 or mock infected. Six hours p.i., the cells were lysed and the protein preparations were subjected to antiphosphotyrosine immunoblotting.

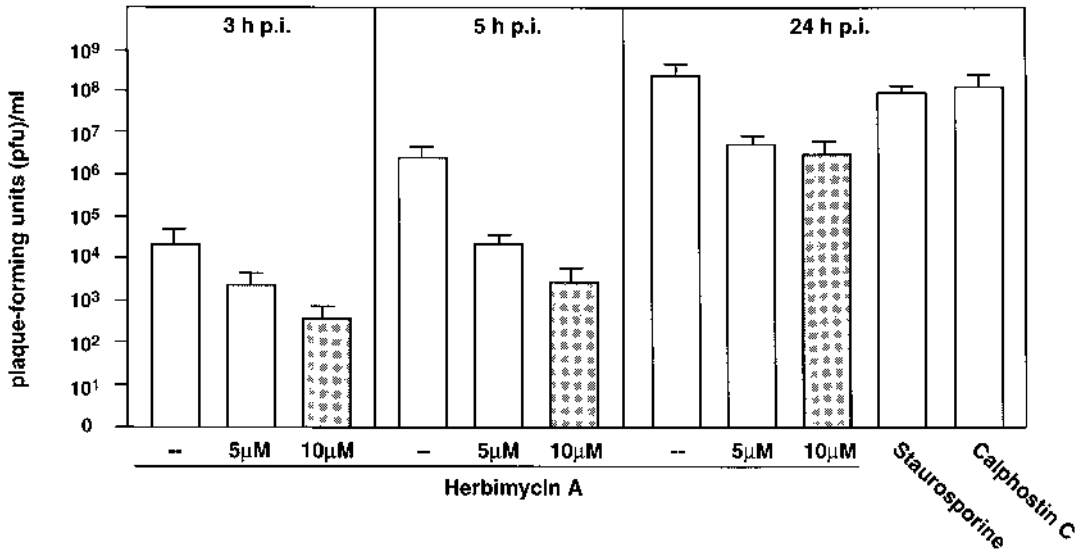

FIG. 4. Decrease in production of progeny virions in the presence of herbimycin A. HeLa cells were left untreated or treated with 5 or 10 μM herbimycin A, 75 nM staurosporine, or 100 nM calphostin C prior to infection with CVB3. At the indicated times, progeny virions were recovered from cell suspensions by freeze-thawing and virus titers were determined by plaque assay on HeLa monolayer cells (data are means ± standard deviations of three separate experiments).

tribute to the enhancement in the production of progeny virions.

Subcellular fractionation of CVB3-infected HeLa cells. We further determined the localization of tyrosine-phosphorylated proteins induced or enhanced during CVB3 replication in HeLa cells. For that purpose, subcellular fractionation was performed 4 h p.i., at a time when increased tyrosine phosphorylation of the 48- and 200-kDa proteins was detected. Figure 5 shows the distribution of tyrosine-phosphorylated proteins in the cytosol and membrane fractions of CVB3-infected cells. The 48-kDa CVB3-induced phosphoprotein was detected only in the cytosol of CVB3-infected cells, whereas the tyrosine-phosphorylated protein with a molecular mass of 200 kDa was predominantly localized in the membrane fractions of CVB3-infected HeLa cells. The 70-kDa tyrosine-phosphorylated protein, which was detected in the tyrosine-phosphorylated state until the late phase of infection, was mainly localized in the cytosol of CVB3-infected as well as mock-infected HeLa cells.

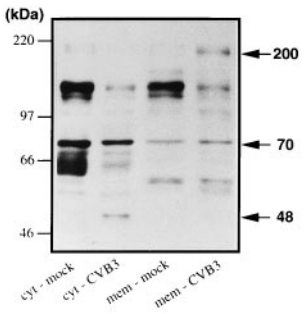

FIG. 5. Subcellular fractionation of CVB3-infected HeLa cells. CVB3- and mock-infected HeLa cells were separated into cytosol (cyt) and membrane (mem) fractions 4 h p.i. Subsequently, the fractions were analyzed for the presence of tyrosine-phosphorylated proteins by antiphosphotyrosine Western blotting. The positions of tyrosine-phosphorylated proteins with molecular masses of 48, 70, and 200 kDa are indicated by arrows.

Immunoprecipitation experiments. In order to analyze whether the 200-kDa protein which shows enhanced tyrosine phosphorylation in the course of CVB3 infection is of cellular or viral origin, antiphosphotyrosine and anti-Pol immunoprecipitations were performed with lysates of CVB3- and mock-infected cells. HeLa cells were lysed 4 h p.i., and postnuclear supernatants were subjected to immunoprecipitations with antiphosphotyrosine antibodies and subsequent antiphosphotyrosine Western blotting. As shown in Fig. 6, CVB3 infection resulted in enhanced tyrosine phosphorylation of the 200-kDa protein. However, the 200-kDa protein was not detected when lysates were immunoprecipitated with a virus-specific antiserum (anti-Pol) and subsequently subjected to antiphosphotyrosine immunoblotting, indicating that the 200-kDa protein is of cellular origin (Fig. 6).

DISCUSSION

Tyrosine phosphorylation events play important roles in the cellular processes of proliferation, differentiation, and malignant transformation. Such phosphorylation events also appear to be relevant for efficient and controlled virus infection (6, 8,

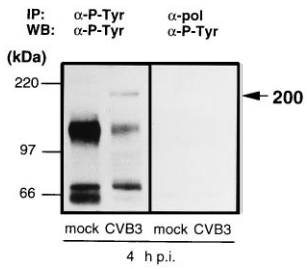

FIG. 6. Immunoprecipitation (IP) analysis of the tyrosine-phosphorylated 200-kDa protein. CVB3- or mock-infected HeLa cells were lysed 4 h p.i., and postnuclear supernatants were immunoprecipitated with antiphosphotyrosine antibodies (α-P-Tyr) or CVB3 polymerase-specific antibodies (α-pol) followed by antiphosphotyrosine immunoblotting. The position of the tyrosine-phosphorylated 200-kDa protein is indicated by an arrow. WB, Western blotting.

17). In this paper, the question of whether CVB3 interferes with cellular tyrosine phosphorylation during infection of host cells has been addressed. Analysis of CVB3 replication in HeLa cells revealed time-dependent changes in the phosphorylation pattern of distinct proteins, in particular, a cytosolic 48-kDa protein and a membrane-bound 200-kDa protein, which showed increased tyrosine phosphorylation 4 to 5 h p.i., a time when virus-induced shutoff of host cell protein synthesis had already occurred (Fig. 1). Extending the study to further enteroviruses, EV11 and EV12 infection of a monkey cell line (Vero cells) also revealed enhanced tyrosine phosphorylation of a 200-kDa protein 6 h p.i. (Fig. 2), suggesting that this protein may play a certain role in the course of infection with these viruses.

When herbimycin A, a specific inhibitor of Src-like tyrosine kinases, was used, inhibition of virus-induced tyrosine phosphorylations (Fig. 3) and reduction of production of progeny virions was observed (Fig. 4). This inhibitor is relatively nontoxic and does not interfere with protein synthesis or signaling events via tyrosine kinase-independent pathways (13, 19, 32). In contrast, calphostin C, a PKC-specific kinase inhibitor, and staurosporine, a broad-range kinase inhibitor, had almost no effect on virus replication compared to the results obtained with herbimycin A (Fig. 4). Herbimycin A treatment did not lead to a reduction in virus binding, demonstrating that this inhibitor does not exert its activity on the level of virus-receptor interactions by modulating the receptor protein (data not shown). This indicates that Src-like kinases may be involved at later stages of infections, e.g., in the process of enterovirus replication. However, the participation of further protein tyrosine kinases during infection cannot be excluded.

The 200-kDa protein, which showed increased tyrosine phosphorylation during infection, was demonstrated to be of cellular origin (Fig. 6). This raised the question of whether this protein may be identical to the cellular protein p220 (eIF-4γ), a eukaryotic initiation factor of protein synthesis known to be cleaved in the early phase of picornavirus infection (30). Protein p220 is predominantly phosphorylated on serine residues, whereas tyrosine phosphorylation of this protein has not been observed (7). This is in agreement with our data, which show that p220 was not present in immunoprecipitates obtained with phosphotyrosine-specific antibodies (data not shown). Furthermore, the complete cleavage of p220 could be monitored in the first 3 h of CVB infection (25). Since the 200-kDa protein showed enhanced tyrosine phosphorylation as late as 4 to 5 h p.i., this tyrosine-phosphorylated 200-kDa protein is not identical to p220.

A role for phosphorylation and dephosphorylation on serine and threonine residues has been observed with poliovirus-infected cells. With respect to dephosphorylation events, it has been reported that poliovirus infection inhibits transcription of a CAT construct from the adenovirus promoter E3 by dephosphorylation of transcription factor CREB/ATF on a serine residue (26). Holsey et al. have reported that poliovirus infection of HeLa cells led to an increase in intracellular pH which promoted viral replication (16). Protein phosphorylation inhibitors of PKC inhibited virus-specific alkalinization, suggesting that PKC-mediated phosphorylation on serine and/or threonine residues activates the mechanism responsible for the increase in intracellular pH in poliovirus-infected cells (15). A further signal transduction system has also been implicated to be important during poliovirus infection of host cells. Guinea et al. have investigated the role of cellular lipases during poliovirus replication (14). An increase in phospholipase C (PLC) activity has been observed, starting 3 h p.i., with a maximum at 5 h p.i., as measured by the intracellular level of inositol trisphosphate. This inositol trisphosphate releases calcium ions from the endoplasmic reticulum which, in turn, activates PLC and phospholipase D (2). In addition, it has been shown by Irurzun et al. that a specific PLC acting on phosphatidylcholine is also activated in the late stages of poliovirus infection (18). Interestingly, all of these effects of poliovirus infection on host cell signaling occur after virus-induced shutoff at late stages of infection. This is in good correlation with our data for tyrosine phosphorylation events in CVB3-, EV11-, and EV12-infected cells. The PLC-γ isoform requires tyrosine phosphorylation for activation, and different tyrosine phosphorylation events precede this activation (36, 39). Analysis of a possible association between virus-induced tyrosine phosphorylation and PLC activation will be addressed in further studies.

The study presented in this paper focused on tyrosine phosphorylation events during CVB3 infection of host cells and led to the identification of proteins which showed enhanced tyrosine phosphorylation during infection of host cells, even after virus-induced shutoff of cellular protein synthesis. The analysis of tyrosine phosphorylation patterns in the course of enterovirus infections should prove to be useful for further characterization of yet-unknown cellular determinants involved in virus replication. We hypothesize that subtle differences in regulation of kinase and phosphatase activities may contribute to tissue-specific replication events, thereby modulating viral pathogenicity.

ACKNOWLEDGMENTS

This work was supported by the BMBF-Programm Klinische Forschungszentren 2000 and the Fortüne-Programm des Tübinger Universitätsklinikums. M.H. is a Kekulé Stipend Fellow of the Stiftung Stipendien-Fonds des Verbandes der Chemischen Industrie.

We thank K. Pfauth-Fischbach and G. Janke for excellent technical assistance.

REFERENCES

1. **Barret-Connor, E.** 1985. Is insulin-dependent diabetes mellitus caused by coxsackievirus B infection? A review of the epidemiologic evidence. Rev. Infect. Dis. **7:**207–215.
2. **Billah, M. M., and J. C. Anthers.** 1990. The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem. J. **269:**281–291.
3. **Borovjagin, A. V., M. V. Ezrokhi, V. M. Rostapshov, T. Y. Ugarova, T. F. Bystrova, and I. N. Shatsky.** 1991. RNA-protein interactions within the internal translation initiation region of encephalomyocarditis virus RNA. Nucleic Acids Res. **19:**4999–5005.
4. **Bruns, R. F., F. D. Mileer, R. L. Merriman, J. J. Howbert, W. F. Heath, E. Kobayashi, I. Takahashi, T. Tamaoki, and H. Nakano.** 1991. Inhibition of protein kinase C by calphostin C is light-dependent. Biochem. Biophys. Res. Commun. **176:**288–293.
5. **Cheung, R. K., and H. M. Dosch.** 1991. The tyrosine kinase lck is critically involved in the growth transformation of human B lymphocytes. J. Biol. Chem. **266:**8667–8670.
6. **Cohen, D. I., Y. Tani, H. Tian, E. Boone, L. E. Samelson, and H. C. Lane.** 1992. Participation of tyrosine phosphorylation in the cytopathic effect of human immunodeficiency virus 1. Science **256:**542–545.
7. **Donaldson, R. W., C. H. Hagedorn, and S. Cohen.** 1991. Epidermal growth factor or okadaic acid stimulates phosphorylation of eukaryotic initiation factor 4F. J. Biol. Chem. **266:**3162–3166.
8. **Filvaroff, E., D. Stern, and G. P. Dotto.** 1990. Tyrosine phosphorylation is an early and specific event involved in primary keratinocyte differentiation. Mol. Cell. Biol. **10:**1164–1173.
9. **Fisher, S. N., Y. U. Kim, and M. L. Shin.** 1994. Tyrosine kinase activation by Newcastle disease virus is required for TNF-alpha induction in astrocytes. J. Immunol. **153:**3210–3217.
10. **Foulis, A. K., M. A. Farquharson, S. O. Cameron, M. McGill, H. Schönke, and R. Kandolf.** 1990. A search for the presence of the enteroviral capsid protein VP1 in pancreases of patients with type 1 (insulin-dependent) diabetes and pancreases and hearts of infants who died of coxsackieviral myocarditis. Diabetologia **33:**290–298.
11. **Fukami, Y., T. Nakamura, A. Nakayama, and T. Kanehisa.** 1986. Phosphorylation of tyrosine residues of calmodulin in Rous sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA **83:**4190–4193.

12. **Fukazawa, H., S. Mizumo, and Y. Uehara.** 1990. Effects of herbimycin A and various SH-reagents on p60v-src kinase activity in vitro. Biochem. Biophys. Res. Commun. **173:**276–282.
13. **Graber, M., C. H. June, L. E. Samelson, and A. Weiss.** 1992. The protein tyrosine kinase inhibitor herbimycin A, but not genistein, specifically inhibits signal transduction by the T cell antigen receptor. Int. Immunol. **4:**1201–1210.
14. **Guinea, R., A. Lopez-Rivas, and L. Carrasco.** 1989. Modification of phospholipase C and phospholipase A2 activities during poliovirus infection. J. Biol. Chem. **264:**21923–21927.
15. **Holsey, C., and C. N. Nair.** 1993. Poliovirus-induced intracellular alkalinization involves a proton ATPase and protein phosphorylation. J. Cell. Physiol. **155:**606–614.
16. **Holsey, C., E. J. Cragoe, Jr., and C. N. Nair.** 1990. Evidence for poliovirus-induced cytoplasmic alkalinization in HeLa cells. J. Cell. Physiol. **142:**586–591.
17. **Hunter, T., and J. A. Cooper.** 1985. Protein tyrosine kinases. Annu. Rev. Biochem. **54:**897–930.
18. **Irurzun, A., L. Pérez, and L. Carrasco.** 1993. Enhancement of phospholipase activity during poliovirus infection. J. Gen. Virol. **74:**1063–1071.
19. **June, C. H., M. C. Fletcher, J. A. Ledbetter, G. L. Schieven, J. N. Siegel, A. F. Phillips, and L. E. Samelson.** 1990. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. Proc. Natl. Acad. Sci. USA **87:**7722–7726.
20. **Kandolf, R.** 1988. The impact of recombinant DNA technology on the study of enteroviral heart disease, p. 293–318. *In* M. Bendinelli and H. Friedmann (ed.), Coxsackieviruses—a general update. Plenum Publishing Corp., New York, New York.
21. **Kandolf, R., and P. H. Hofschneider.** 1985. Molecular cloning of the genome of a cardiotropic coxsackie B3 virus: full-length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells. Proc. Natl. Acad. Sci. USA **82:**4818–4822.
22. **Kandolf, R., and P. H. Hofschneider.** 1989. Viral heart disease. Springer Semin. Immunopathol. **11:**1–13.
23. **Kandolf, R., A. Canu, and P. H. Hofschneider.** 1985. Coxsackie B3 virus can replicate in cultured human foetal heart cells and is inhibited by interferon. J. Mol. Cell. Cardiol. **17:**167–181.
24. **Kandolf, R., D. Ameis, P. Kirschner, A. Canu, and P. H. Hofschneider.** 1987. In situ detection of enteroviral genomes in myocardial cells by nucleic acid hybridization: an approach to the diagnosis of viral heart disease. Proc. Natl. Acad. Sci. USA **84:**6272–6276.
25. **Kirchweger, R., E. Ziegler, B. J. Lamphear, D. Waters, H.-D. Liebig, W. Sommergruber, F. Sobrino, C. Hohenadl, D. Blaas, R. E. Rhoads, and T. Skern.** 1994. Foot-and-mouth disease virus leader proteinase: purification of the Lb form and determination of its cleavage site on eIF-4γ. J. Virol. **68:**5677–5684.
26. **Kliewer, S., C. Muchardt, R. Gaynor, and A. Dasgupta.** 1990. Loss of a phosphorylated form of transcription factor CREB/ATF in poliovirus-infected cells. J. Virol. **64:**4507–4515.
27. **Klingel, K., C. Hohenadl, A. Canu, M. Seemann, G. Mall, and R. Kandolf.** 1992. Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: quantitative analysis of virus replication, tissue damage, and inflammation. Proc. Natl. Acad. Sci. USA **89:**314–318.
28. **Klump, W. M., I. Bergmann, B. C. Müller, D. Ameis, and R. Kandolf.** 1990. Complete nucleotide sequence of infectious coxsackievirus B3 cDNA: two initial 5′ uridine residues are regained during plus-strand RNA synthesis. J. Virol. **64:**1573–1583.
29. **Kräusslich, H.-G., M. J. H. Nicklin, C.-K. Lee, and E. Wimmer.** 1988. Polyprotein processing in picornavirus replication. Biochimie **70:**119–130.
30. **Kräusslich, H.-G., M. J. H. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer.** 1987. Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220. J. Virol. **61:**2711–2718.
31. **Lacy, J., A. Bartiss, and D. L. Coleman.** 1991. Conversion of a human B cell lymphoma line by Epstein-Barr virus is associated with increased tyrosine phosphorylation of a 50 kilodalton cytosolic protein. Virus Res. **20:**85–96.
32. **Ledbetter, J. A., and P. S. Linsley.** 1992. CD28 receptor crosslinking induces tyrosine phosphorylation of PLC gamma 1. Adv. Exp. Med. Biol. **323:**23–27.
33. **Majumder, S., P. Ray, and P. Besmer.** 1990. Tyrosine protein kinase activity of the HZ4-feline sarcoma virus P80gag-kit-transforming protein. Oncogene Res. **5:**329–335.
34. **McManus, B. M., and R. Kandolf.** 1991. Evolving concepts of cause, consequence, and control in myocarditis. Curr. Opin. Cardiol. **6:**418–427.
35. **Melnick, J. L.** 1985. Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses, p. 739–794. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York, New York.
36. **Mohammadi, M., C. A. Dionne, W. Li, N. Li, T. Spivak, A. M. Honegger, M. Jaye, and J. Schlessinger.** 1992. Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. Nature (London) **358:**681–684.
37. **Raab de Verdugo, U., H.-C. Selinka, M. Huber, B. Kramer, J. Kellermann, P. H. Hofschneider, and R. Kandolf.** 1995. Characterization of a 100-kilodalton binding protein for the six serotypes of coxsackie B viruses. J. Virol. **69:**6751–6757.
38. **Uehara, Y., H. Fukazawa, Y. Murakami, and S. Mizuno.** 1989. Irreversible inhibition of v-src kinase activity by herbimycin A and its abrogation by sulfhydryl compounds. Biochem. Biophys. Res. Commun. **163:**803–809.
39. **Vetter, M. L., D. Martin-Zanca, L. F. Parada, J. M. Bishop, and D. R. Kaplan.** 1991. Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene. Proc. Natl. Acad. Sci. USA **88:**5650–5654.
40. **Werner, S., W. M. Klump, H. Schönke, P. H. Hofschneider, and R. Kandolf.** 1988. Expression of coxsackievirus B3 capsid proteins in Escherichia coli and generation of virus-specific antisera. DNA **7:**307–316.