JOURNAL OF VIROLOGY, May 1999, p. 3587–3594 Vol. 73, No. 5
0022-538X/99/$04.00+0

Cleavage of RasGAP and Phosphorylation of Mitogen-Activated Protein Kinase in the Course of Coxsackievirus B3 Replication

MICHAEL HUBER,[1]† KATHLEEN A. WATSON,[2] HANS-CHRISTOPH SELINKA,[1] CHRISTOPHER M. CARTHY,[2] KARIN KLINGEL,[1] BRUCE M. MCMANUS,[2] AND REINHARD KANDOLF[1]*

Department of Molecular Pathology, Institute for Pathology, University of Tübingen, D-72076 Tübingen, Germany,[1] and Department of Pathology and Laboratory Medicine, University of British Columbia-St. Paul's Hospital, Vancouver, British Columbia, Canada[2]

Received 1 September 1998/Accepted 26 January 1999

Recently, we reported on tyrosine phosphorylation of distinct cellular proteins in the course of enterovirus infections (M. Huber, H.-C. Selinka, and R. Kandolf, J. Virol. 71:595–600, 1997). These phosphorylation events were mediated by Src-like kinases and were shown to be necessary for effective virus replication. That study is now extended by examination of the interaction of the adapter protein Sam68, a cellular target of Src-like kinases which has been shown to interact with the poliovirus 3D polypeptide, with cellular signaling proteins as well as the function of the latter during infection. Here, we report that the RNA-binding and protein-binding protein Sam68 associates with the p21^{ras} GTPase-activating protein RasGAP. Remarkably, RasGAP is cleaved during infections with different strains of coxsackievirus B3 as well as with echovirus 11 and echovirus 12, yielding a 104-kDa protein fragment. This cleavage event, which cannot be prevented by the general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, may promote the activation of the Ras pathway, as shown by the activating dual phosphorylation of the mitogen-activated protein kinases Erk-1 and Erk-2 in the late phase of infection. Moreover, downstream targets of the mitogen-activated protein kinases, i.e., the p21^{ras} exchange factor Sos-1 and cytoplasmic phospholipase A_2, are phosphorylated with parallel time courses during infection. Activation or inhibition of cellular signaling pathways may play a general role in regulating effective enterovirus replication and pathogenesis, and the results of this study begin to unravel the molecular cross talk between enterovirus infection and key cellular signaling networks.

Coxsackieviruses (CV) are important human pathogens, causing a remarkable variety of diseases, from minor common colds to fatal myocarditis, neurological disorders, and possibly acute-onset diabetes (21, 35, 37, 45). CV group A (CVA) and CV group B (CVB), together with echoviruses and polioviruses, are enteroviruses of the family *Picornaviridae*. The genetic material of CVB is encoded in a single-stranded RNA molecule of positive polarity and about 7,500 nucleotides in length (36, 38). Infection of permissive host cells is initiated by the attachment of the virus to the specific cell surface receptor molecule CAR (3, 63). Following entry into the cell, genomic viral plus-strand RNA serves as a template for transcription by the virus-encoded RNA-dependent RNA polymerase $3D^{pol}$ to yield minus-strand RNA, an intermediate in the life cycle of enteroviruses which is subsequently transcribed by $3D^{pol}$ into large amounts of genomic plus-strand RNA (52). Translation of genomic viral RNA occurs by a Cap-independent mechanism, yielding the viral precursor polyprotein of 243 kDa. The virus-encoded proteinases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$ cleave the polyprotein co- and posttranslationally into mature proteins which exhibit multiple functions (39). Due to their presence in RNA replication complexes and their biochemical properties, enterovirus nonstructural polypeptides 2B, 2C, 3A, and 3B as well as their precursor proteins 2BC, 3AB, and $3CD^{pro}$ also appear to be involved in viral RNA replication (70).

In order to study the influence of virus replication on cellular signal transduction systems and vice versa, we have recently examined the effects of enterovirus infections on cellular tyrosine phosphorylation events. Distinct proteins were found to be tyrosine phosphorylated upon infection of cells with CVB or echoviruses (29, 55). Inhibition of virus-induced phosphorylation by herbimycin A, an inhibitor of Src-like tyrosine kinases, resulted in a significant reduction of progeny virions, suggesting that cellular phosphorylation events triggered in the course of enterovirus infections may enhance virus replication (29).

Interestingly, McBride et al. (44) have reported on the specific interaction of poliovirus $3D^{pol}$ with the C terminus of the cellular adapter protein Sam68, a target for Src-like tyrosine kinases during mitosis (22, 61). Sam68 is found on poliovirus-induced membranes and relocalizes during the course of infection (44). Furthermore, Sam68 is capable of both binding to RNA and interacting with signaling proteins containing Src homology 3 (SH3) and SH2 domains via its SH3 domain-binding motifs and multiple tyrosine phosphorylation sites, respectively (49, 72). Meanwhile, Sam68 has been reported to interact with various Src family tyrosine kinases; the adapter proteins Grb2, Grap, Cbl, and Nck; phospholipase C γ-1; the regulatory p85 subunit of phosphatidylinositol 3-kinase; the tyrosine kinases Jak3 and Itk; $p47^{phox}$; and the tyrosine phosphatase SHP-1 (8, 20, 23, 32, 40, 49, 65).

Concerning its RNA-binding capability, Sam68 contains a K homology domain, a small protein module that consists of 70 to 100 amino acids and that is thought to enable direct protein-RNA contacts (57). Interestingly, this K homology domain has been shown to mediate the self-association of Sam68, which requires the presence of RNA (10). Moreover, binding of the Src kinase SH3 domain to Sam68 inhibits its association with

* Corresponding author. Mailing address: Abteilung fuer Molekulare Pathologie, Universität Tübingen, Liebermeisterstr. 8, D-72076 Tübingen, Germany. Phone: 49-7071-2982264. Fax: 49-7071-295334. E-mail: reinhard.kandolf@med.uni-tuebingen.de.

† Present address: The Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada.

RNA (61, 62), indicating mutual dependence of the RNA-binding and protein-binding domains of Sam68. Since enterovirus replication takes place within protein-RNA complexes, the cellular protein Sam68, due to its protein- and RNA-binding properties, may be an adapter protein that directs multiple cellular signaling proteins to the viral replication complex both to support and to regulate viral replication. These interesting facts prompted us to investigate the possible role of Sam68 in the course of CVB3 replication.

Here, we present evidence for the association of Sam68 with the p21^{ras} GTPase-activating protein RasGAP. Furthermore, we demonstrate the proteolytic cleavage of RasGAP in the course of CVB and echovirus infections as well as the dual phosphorylation of the mitogen-activated protein kinases (MAPK) Erk-1 and Erk-2, resulting in the phosphorylation of MAPK target proteins.

MATERIALS AND METHODS

Cell lines and viruses. HeLa cells (human cervix carcinoma cells; CCL 2) and Vero cells (African green monkey kidney cells; CCL 81) were obtained from the American Type Culture Collection. MO7e cells (human megakaryocytic leukemia cells) were a gift from G. Krystal, Terry Fox Laboratory, Vancouver, British Columbia, Canada. Cells were cultivated as monolayers in Dulbecco's modified Eagle's minimal medium (DMEM)–10% fetal bovine serum (FBS). The CVB3 strain (Nancy strain) used in this study was generated by transfection of HeLa cells with infectious recombinant CVB3 cDNA (36, 38), propagated in HeLa cells, and maintained in DMEM supplemented with 10% FBS. Unless stated otherwise, CVB3 (Nancy strain) was used throughout this study. CVB3 (Gauntt strain) was a gift from Charles Gauntt and was adapted for growth in HeLa cells by five passages. Echovirus 11 (EV11) (Gregory strain; VR-41) and EV12 (Travis strain; VR-42) were obtained from the American Type Culture Collection and adapted for growth in Vero cells by six and nine consecutive passages, respectively. Cell cultures were infected with a multiplicity of infection of 10 throughout the study.

Antibodies and chemicals. Monoclonal antibody B4F8, detecting the N-terminal noncatalytic region of RasGAP, and a polyclonal antibody with specificity for the p21^{ras} exchange factor Sos-1 were purchased from Upstate Biotechnology. Monoclonal antibodies recognizing Sam68 were obtained from Transduction Laboratories (P20120) and Santa Cruz Biotechnology (sc333). These antibodies were described as recognizing RasGAP-associated protein hump62, but Sam68 was recently identified as the true antigen (42). Antibodies P20120 and sc333 were used for immunoprecipitations and Western blotting, respectively. Polyclonal antibodies recognizing MAPK and phosphorylated MAPK were purchased from Upstate Biotechnology and New England BioLabs, respectively. Polyclonal rabbit anti-cytoplasmic phospholipase A_2 (cPLA$_2$) antibody and monoclonal anti–caspase-3 antibody were obtained from Santa Cruz Biotechnology and Transduction Laboratories, respectively. 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). The general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD .fmk) was obtained from Bachem. A stock solution (100 mM in dimethyl sulfoxide) of ZVAD.fmk was diluted in DMEM to a final concentration of 100 μM, and HeLa cells were incubated with the inhibitor for 30 min prior to infection. After CVB3 infection, the cells were washed with phosphate-buffered saline (PBS) and placed in DMEM containing 10% FBS and fresh ZVAD.fmk.

Virus purification. Confluent monolayer cells in 10-cm culture dishes were infected for 45 min at 37°C with an input multiplicity of 1 to 5 PFU per cell. Cells were washed twice with PBS and incubated in DMEM supplemented with 5% FBS for 18 h. To release intracellular virus particles, cells were subjected to three cycles of freezing and thawing. After centrifugation at 700 × *g* for 15 min to remove cell debris, the virus was pelleted at 190,000 × *g* for 90 min at 4°C. For further purification, the pellet was resuspended in 4 ml of PBS, loaded onto a 30% sucrose cushion, and centrifuged at 190,000 × *g* for 3.5 h at 4°C. The virus pellet was resuspended in PBS with 10 mM $MgCl_2$, and aliquots were stored at −80°C.

Solubilization of cells, electrophoresis, and Western blotting. To stop virus replication, medium was removed and cells were resuspended in 400 μl of lysis buffer (0.5% Nonidet P-40, 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, 5 mM $Na_4P_2O_7$, 5 mM sodium orthovanadate in 20 mM Tris [pH 8.0]). After lysis at 4°C, nuclei were pelleted at 1,000 × *g*, and nucleus-free supernatants were normalized for equal protein content (as measured with Bradford protein assay kits; Bio-Rad). Samples were electrophoresed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 7.5 or 10% polyacrylamide under reducing conditions. Subsequently, proteins were transferred to polyvinylidene difluoride (PVDF) transfer membranes (Dupont, NEN Research Products). Membranes were rinsed twice with PBS for 5 min each time at room temperature (RT), incubated for 1 h at 4°C in blocking buffer containing 5% bovine serum albumin (BSA) in PBS, and washed twice with PBS for 5 min each time. Blots were incubated for 90 min at RT with the primary antibody in PBS containing 0.1% BSA. Subsequently, the blots were rinsed six times for 5 min each time with PBS containing 0.2% Tween 20 and incubated for 1 h at 4°C with horseradish peroxidase-conjugated secondary antibodies (Dakopatts) 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 with an enhanced chemiluminescence reagent kit in accordance with the manufacturer's recommendations (Amersham), and exposed to BIOMAX MR films (Kodak).

Immunoprecipitations. Postnuclear supernatants from 10^6 cells were precleared overnight at 4°C with 20 μl of 20% protein A-Sepharose or 30 μl of anti-mouse immunoglobulin G (IgG) antibodies coupled to agarose (Sigma). After centrifugation at 10,000 × *g*, supernatants were incubated for 3 h at 4°C with precipitating antibodies. Fifteen microliters of 20% protein A-Sepharose (or, in the case of anti-Sam68 antibody [P20120], 30 μl of anti-mouse IgG antibodies coupled to agarose) was added for 1 h at 4°C, followed by centrifugation. Pellets were washed six times with 200 μl of lysis buffer and incubated for 5 min at 98°C in 30 μl of 1× SDS loading buffer. Samples were subjected to SDS-PAGE, and proteins were transferred to PVDF membranes and immunoblotted. Following incubation with secondary antibodies (horseradish peroxidase-conjugated antibodies), blots were developed with an enhanced chemiluminescence reagent kit.

Silver staining of SDS-polyacrylamide gels. A modification of the protocol of Ohsawa and Ebata (47) was used for gel staining. All steps were carried out at RT. Briefly, gels were fixed in 25% methanol–10% acetic acid for 30 min, placed directly in 10% glutaraldehyde solution in H_2O for 30 min, and rinsed six times with approximately 500 ml of H_2O. A diamine solution (0.2% NaOH, 2% concentrated NH_4OH, 0.2% $AgNO_3$) was added for 15 min, and the gels were rinsed two times with H_2O and placed in a solution containing 0.005% citric acid and 0.0185% formaldehyde. After protein spots developed, the gels were washed twice with H_2O and once with 10% acetic acid before being dried on Whatman paper.

RESULTS

RasGAP is a Sam68-associated protein. The adapter protein Sam68, exerting various protein- and RNA-binding capabilities, may represent an important link between the enterovirus replication complex and proteins involved in cellular signal transduction. Therefore, we wanted to detect any new Sam68-associated proteins and their possible role in regulating virus replication. Silver staining of proteins which coimmunoprecipitated with Sam68 showed a characteristic triplet in the range of 120 kDa in HeLa cells as well as in cells of hemopoietic origin (MO7e) (Fig. 1A). These proteins were not precipitated by anti-mouse IgG coupled to agarose alone (Fig. 1A). A test of various antibodies detecting proteins with molecular masses of approximately 120 kDa ultimately revealed that the p21^{ras} GTPase-activating protein RasGAP was capable of associating with Sam68. As shown in Fig. 1B, Sam68 was coimmunoprecipitated with RasGAP-specific antibodies in both CVB3-infected and mock-infected HeLa cells. To verify the association between RasGAP and Sam68, postnuclear supernatants of mock-infected and CVB3-infected HeLa cells were immunoprecipitated with Sam68-specific antibodies 5 h postinfection (p.i.) and subsequently probed with RasGAP-specific antibodies. As shown in Fig. 1C, RasGAP was coprecipitated with Sam68 in both mock-infected and CVB3-infected HeLa cells. However, in contrast to the results for mock-infected HeLa cells, the 120-kDa RasGAP band appeared very faint in CVB3-infected HeLa cells. Interestingly, a major protein of approximately 104 kDa and cross-reacting with RasGAP-specific antibodies was detected in the anti-Sam68 antibody immunoprecipitates, suggesting the presence of a proteolytic cleavage product of RasGAP (Fig. 1C). To further prove the specificity of the coimmunoprecipitation reactions, Fig. 1D shows that neither Sam68 nor RasGAP was precipitated by the precipitating reagent itself.

RasGAP expression in CVB3-infected HeLa cells. Since a second protein cross-reacting with the anti-RasGAP antibodies was coimmunoprecipitated with Sam68 in CVB3-infected cells, it was of interest to determine whether it was a virus-in-

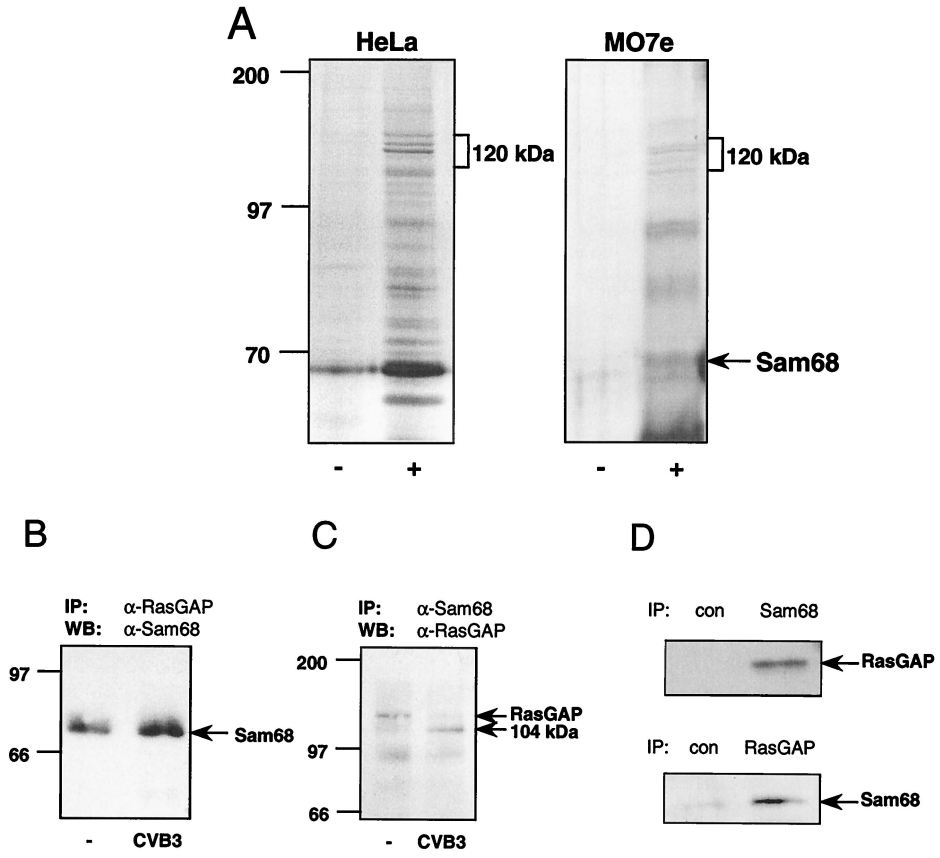

FIG. 1. Association of Sam68 and RasGAP in HeLa cells. (A) Multiple proteins associate with Sam68 in cells of human origin. Postnuclear supernatants of HeLa and MO7e cells were precleared and subsequently immunoprecipitated with anti-Sam68 antibodies (+). The precipitating agent (anti-mouse IgG antibodies coupled to agarose) served as a control (−). The precipitates were separated by SDS-PAGE and subjected to a silver staining procedure. Coprecipitated proteins in the range of 120 kDa are indicated. (B) Coimmunoprecipitation of Sam68 with antibodies specific for RasGAP. CVB3-infected (CVB3) or mock-infected (−) HeLa cells were lysed 5 h p.i., and postnuclear supernatants were immunoprecipitated (IP) with RasGAP-specific antibodies. The immunoprecipitates were analyzed on Western blots (WB) with Sam68-specific antibodies. Protein Sam68 is indicated. (C) RasGAP is coprecipitated with Sam68-specific antibodies. Postnuclear supernatants were immunoprecipitated with Sam68-specific antibodies and examined by Western blotting with RasGAP-specific antibodies. RasGAP and the 104-kDa cleavage product are indicated. (D) HeLa cell lysates were subjected to immunoprecipitation with anti-Sam68 antibodies or precipitating reagent (con) and with anti-RasGAP antibodies or precipitating reagent (con) and probed for RasGAP and Sam68, respectively. Sam68 and RasGAP are indicated.

duced cleavage product of RasGAP. Therefore, a time course experiment was performed with HeLa cells for the period from 1 h to 7 h p.i. CVB3-infected HeLa cells were lysed at 60-min intervals, and postnuclear supernatants were subjected to Western blot analysis with RasGAP-specific antibodies. As shown in Fig. 2A, the 120-kDa RasGAP protein was detected in mock-infected cells as well as in CVB3-infected cells. At 5 to 7 h p.i., an additional protein of 104 kDa was recognized by RasGAP-specific antibodies in CVB3-infected cells. The decrease in the amount of immunoprecipitated RasGAP protein between 5 h and 7 h p.i. correlated with the increase in the expression of the 104-kDa protein, suggesting the proteolytic conversion of RasGAP to a 104-kDa protein during the course of CVB3 replication. Furthermore, a time course experiment with the Gauntt strain of CVB3 showed evidence that RasGAP cleavage was not a quality limited to infection with the Nancy strain of CVB3 (Fig. 2B).

Cleavage of RasGAP in echovirus-infected Vero cells. We examined whether the appearance of the 104-kDa RasGAP cleavage product is restricted to CVB3-infected cells or is generally observed in cells infected with different enteroviruses. Vero cells were therefore infected with EV11 or EV12 and analyzed for RasGAP expression as described above. The 104-kDa RasGAP cleavage product was also detected in EV11-

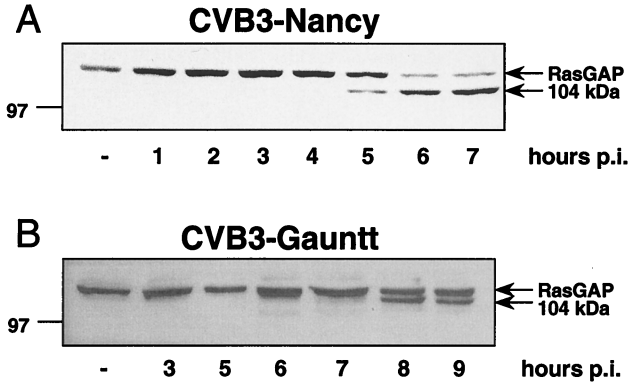

FIG. 2. Expression of RasGAP during the course of CVB3 infection. (A) RasGAP cleavage during the course of infection with CVB3 (Nancy strain). HeLa cells were infected with CVB3 (Nancy strain) or mock infected and lysed at 60-min intervals from 1 h to 7 h p.i. Protein preparations were subjected to Western blotting with RasGAP-specific antibodies. (B) RasGAP cleavage during the course of infection with CVB3 (Gauntt strain). HeLa cells were infected with CVB3 or mock infected and lysed from 3 h to 9 h p.i. Postnuclear supernatants were subjected to Western blotting with RasGAP-specific antibodies. RasGAP (120 kDa) and the 104-kDa protein are indicated.

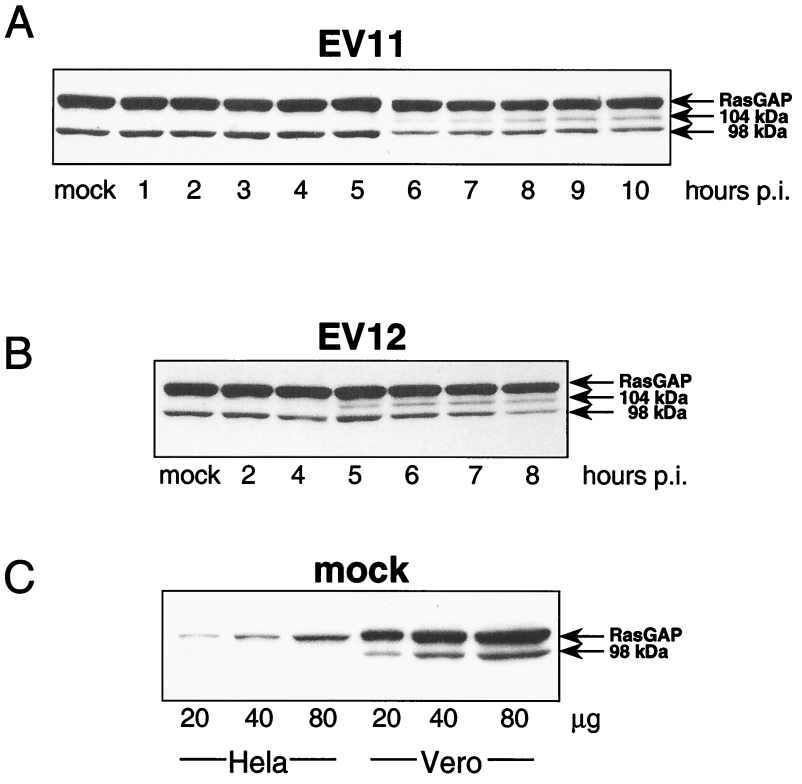

FIG. 3. RasGAP expression in EV11- and EV12-infected Vero cells. (A) Time course of expression of RasGAP in EV11-infected Vero cells. Vero cells were infected with EV11 and lysed at 60-min intervals from 1 h to 10 h p.i. Postnuclear supernatants were analyzed for RasGAP expression by Western blotting with RasGAP-specific antibodies. RasGAP, the 104-kDa protein, and the additional Vero cell-specific 98-kDa protein are indicated. (B) Time course of expression of RasGAP in EV12-infected Vero cells. Protein preparations of EV12- and mock-infected Vero cells were subjected to Western blotting with RasGAP-specific antibodies. RasGAP and RasGAP-related proteins of 104 and 98 kDa are indicated. (C) Comparison of RasGAP expression in noninfected HeLa and Vero cells. Lysates from HeLa and Vero cells (20, 40, and 80 μg) were separated by SDS–10% PAGE and probed with RasGAP-specific antibodies. RasGAP and the Vero cell-specific, RasGAP-related 98-kDa protein are indicated.

infected (Fig. 3A) and EV12-infected (Fig. 3B) Vero cells. Starting at 5 h p.i., increased expression of the 104-kDa cleavage product was observed during the course of infection with both viruses. In Vero cells, however, the intensity of the 120-kDa RasGAP protein did not decrease significantly during infection, due to the very high level of RasGAP expression in Vero cells compared to HeLa cells (Fig. 3C). In these Vero cells, an additional, 98-kDa protein was observed to cross-react with RasGAP-specific antibodies. This 98-kDa protein was not affected significantly by enterovirus replication (Fig. 3A and B).

RasGAP cleavage is not due to caspase activation. Recently, we have reported on the activation of caspases and the cleavage of specific caspase substrates during the course of CVB3 infection (9). Furthermore, Widmann et al. (68), using different apoptotic stimuli, have shown that RasGAP can serve as a caspase substrate, yielding an 85-kDa processing product. Therefore, we were interested in determining whether the RasGAP cleavage observed in our CVB3-infected HeLa cells was also due to caspase activation. For that purpose, HeLa cells were pretreated with 100 μM ZVAD.fmk (a general caspase inhibitor) and infected with CVB3 (Gauntt strain) for 3, 6, and 9 h. As shown in Fig. 4A, RasGAP was still cleaved at 9 h p.i. in the presence of the inhibitory peptide. However, the caspase inhibitor ZVAD.fmk was capable of preventing the apoptosis-mediating autoprocessing of caspase-3 and its consequent 12-kDa cleavage product (Fig. 4B) (9). This result suggests that the observed RasGAP cleavage event was not a result of caspase activation during CVB3 infection of HeLa

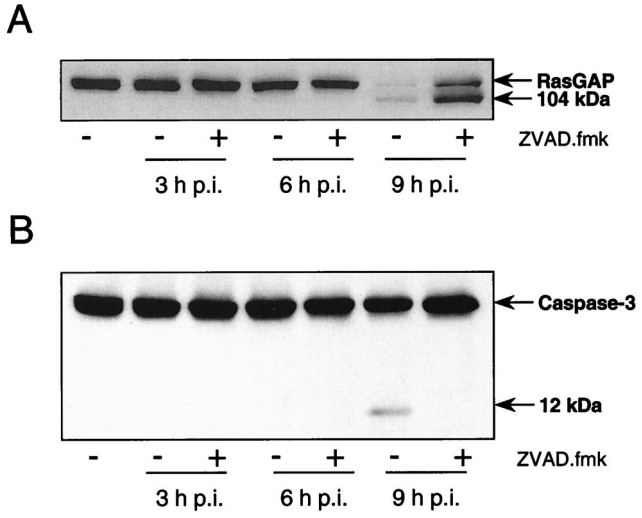

FIG. 4. CVB3-induced RasGAP cleavage in the presence of the general caspase inhibitor ZVAD.fmk. (A) Effect of the caspase inhibitor ZVAD.fmk on RasGAP cleavage. HeLa cells were mock infected (leftmost lane) or infected with CVB3 for 3, 6, and 9 h in the presence (+) or absence (−) of 100 μM ZVAD.fmk. The cells were lysed, and postnuclear lysates were separated by SDS-PAGE. After transfer to a PVDF membrane, RasGAP was detected by Western blotting. RasGAP and the 104-kDa cleavage product are indicated. (B) Caspase-3 processing is prevented by ZVAD.fmk. The postnuclear lysates described for panel A were examined for caspase-3 expression by Western blotting. Caspase-3 is indicated.

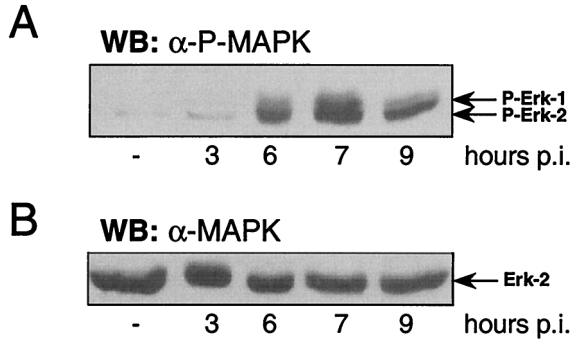

FIG. 5. Activation of MAPK during CVB3 infection. (A) MAPK are dually phosphorylated in CVB3-infected cells. HeLa cells were CVB3 (Gauntt strain) infected or mock infected (leftmost lane) and lysed at various times p.i. Postnuclear supernatants were separated by SDS-PAGE and subjected to Western blot (WB) analysis with antibodies specific for the dually phosphorylated MAPK Erk-1 and Erk-2. Phosphorylated Erk-1 and phosphorylated Erk-2 (P-Erk-1 and P-Erk-2, respectively) are indicated. (B) MAPK expression during CVB3 replication. The postnuclear lysates described for panel A were subjected to anti-MAPK Western blot analysis to confirm the presence of equal amounts of MAPK in these preparations. Erk-2 is indicated.

cells but rather was the effect of the presence of active viral proteinases.

Activation of MAPK during CVB3 infection. RasGAP is a multidomain signaling protein (15) and, depending on the site of cleavage, its function could be impaired in several ways. Since RasGAP was not processed by infection-induced caspases (Fig. 4), the RasGAP protein sequence was searched for putative cleavage sites of enterovirus proteinases. A potential cleavage site (Gln938/Asn939) for the $3CD^{pro}$ proteinases of CV and echoviruses was found in the C-terminal, catalytic region of RasGAP, and cleavage at this dipeptide would result in a protein of 104 kDa, coinciding with the RasGAP fragment detected in enterovirus-infected cells (Fig. 2 and 3). Cleavage of RasGAP within the catalytic region might result in an imbalance between $p21^{ras}$ activators and inhibitors. Thus, the virus-induced proteolysis of RasGAP might favor the activation of $p21^{ras}$. To test this hypothesis, the phosphorylation state of the MAPK Erk-1 and Erk-2 was examined during the course of CVB3 (Gauntt strain) infection of HeLa cells as a direct measure of the induction of $p21^{ras}$ activity (34, 43). Dually phosphorylated MAPK (phospho-Thr202–phospho-Tyr204) were detected by Western blotting with a phosphorylation-specific antibody directed against dually phosphorylated MAPK. As shown in Fig. 5A, weak basal MAPK activity was detected in mock-infected HeLa cells; this activity increased strongly between 6 and 9 h p.i. Equal loading was assessed by probing with an anti-MAPK antibody which detects MAPK independent of their phosphorylation state (Fig. 5B). The data indicate activation of the Ras pathway during CVB3 infection of host cells and suggest a role for RasGAP cleavage in that activation.

Downstream targets of MAPK are phosphorylated in CVB3-infected cells. MAPK are located in the cytosol of mammalian cells but have the capability of translocating to the nucleus in response to their activation. For this reason, their substrates include various cytosolic proteins as well as transcription factors (16). To verify the dual phosphorylation and activation of MAPK during CVB3 infection and to gain further insight into the regulation of enterovirus infection, the phosphorylation states of two known MAPK targets, the $p21^{ras}$ exchange factor Sos-1 and $cPLA_2$, were examined (41, 60). Since both targets are known to respond to MAPK phosphorylation with a characteristic band shift upon SDS-PAGE separation (11, 41, 67), the electrophoretic mobilities of these proteins were analyzed during the course of CVB3 infection (Gauntt strain). As depicted in Fig. 6A, infection of HeLa cells with CVB3 caused a shift in the electrophoretic migration of Sos-1 proteins starting at 6 h p.i., coinciding with the dual phosphorylation of MAPK (Fig. 5A). Furthermore, from 7 to 9 h p.i., the entire pool of $cPLA_2$ proteins was represented by the more slowly migrating form, indicating phosphorylation and activation of $cPLA_2$ during CVB3 replication (Fig. 6B). These results verify the dual phosphorylation and activation of MAPK and suggest the specific regulation of the signaling proteins Sos-1 and $cPLA_2$ in CVB3-infected cells.

DISCUSSION

Recently, we have shown that distinct cellular proteins become tyrosine phosphorylated during infections with the enteroviruses CVB3, EV11, and EV12 (29). The specific inhibition of these phosphorylation events by an inhibitor of Src family kinases resulted in the reduced production of progeny virions, indicating the importance of intracellular signaling events for effective enterovirus infection (29). In this study, we extended our findings by showing that the cellular adapter protein and target for Src-like kinases, Sam68, which is known to interact with poliovirus polypeptide 3D (44), associates with the $p21^{ras}$ GTPase-activating protein RasGAP. RasGAP itself is cleaved during enterovirus infection, potentially promoting the activation of a Ras-activated kinase cascade (Ras pathway) as well as the phosphorylation of specific MAPK target proteins.

Originally, the cellular protein Sam68 was detected as a target of Src kinase by association with Src through its SH2 and SH3 domains (22, 61). Sam68 is also capable of binding single- and double-stranded RNAs. Binding of the Src kinase SH3 domain to Sam68 inhibits the association with poly(U) (61, 62), indicating a mutual dependence of the RNA- and protein-binding domains of Sam68. Furthermore, Sam68 is thought to represent a multifunctional adapter protein for cellular signaling proteins because it contains SH3 domain-binding motifs and multiple tyrosine phosphorylation sites for binding to proteins with SH2 domains (49, 72). With regard to these properties, Sam68 has been reported to interact with the following signaling proteins: the tyrosine kinases Jak3 and Itk; various Src family members; phospholipase C γ-1; $p85^{PI3K}$; SHP-1; the

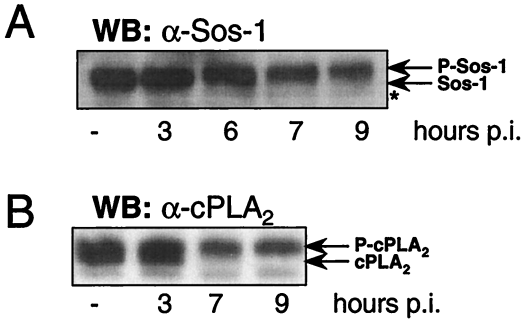

FIG. 6. CVB3 replication induces the phosphorylation of MAPK substrates. (A) Hyperphosphorylation of Sos-1 during CVB3 infection. CVB3 (Gauntt strain)-infected and mock-infected (leftmost lane) HeLa cells were lysed at various times p.i., and postnuclear lysates were separated by SDS–8% PAGE and subjected to anti–Sos-1 Western blotting (WB). Sos-1 and phosphorylated Sos-1 (P-Sos-1) are indicated. Note the increasing distance between the Sos-1 protein and a cross-reacting protein (asterisk), which does not show a change in its electrophoretic mobility. (B) Mobility shift of $cPLA_2$ during the course of CVB3 replication. The lysates described for panel A were analyzed by Western blotting with antibodies specific for $cPLA_2$. $cPLA_2$ and phosphorylated $cPLA_2$ (P-$cPLA_2$) are indicated.

adapter proteins Grb2, Grap, Nck, and c-Cbl; and the 47-kDa subunit of the NADPH oxidase system, $p47^{phox}$ (8, 20, 23, 32, 40, 49, 65).

Enterovirus replication takes place within protein-RNA complexes on virus-induced membrane vesicles originating from the rough endoplasmic reticulum. In addition to the polymerase $3D^{pol}$ and the proteinase $3CD^{pro}$, which is also essential for the initiation of viral RNA replication (1, 73), the viral polypeptides 2B, 2C, 2BC, and 3AB are located within these complexes (4). Interestingly, McBride et al. (44) have observed a specific interaction of Sam68 with poliovirus polymerase $3D^{pol}$ by using the yeast two-hybrid system. Based on this report and what is further known about the properties of Sam68, we were interested in assessing what other signaling proteins Sam68 binds to and their potential role in the course of enterovirus replication. The association of Sam68 with viral polypeptides may reflect the presence of Sam68 within the viral replication complex (44). Moreover, immunoprecipitation of the polypyrimidine tract-binding protein, a cellular constituent within the viral replication complex (27), revealed Sam68 as one of the coprecipitating proteins (data not shown; 25). Therefore, due to its protein- and RNA-binding properties, Sam68 may direct multiple cellular signaling proteins to the replication complex (22, 23, 49, 61). This putative complex, consisting of cellular signaling molecules and viral polypeptides, could function as a large replication unit, promoting and controlling enterovirus replication.

For this reason, we were interested in new Sam68-associated proteins and their potential role during CVB3 infection. Using a silver staining and Western blotting approach, we identified RasGAP in association with Sam68 in mock- as well as virus-infected cells (Fig. 1). Interestingly, Sam68 was first cloned as hump62, a RasGAP-associated protein, but has been shown not to coimmunoprecipitate with RasGAP in NIH 3T3 cells (42). Later, Guitard et al. (26) reported that the interaction between Sam68 and RasGAP in NIH 3T3 cells was specific for the G_2-M transition and suggested that the interaction between these proteins is cell cycle dependent. However, very recently, Jabado et al. (31) observed the association of RasGAP and Sam68 in nonsynchronized human mature T cells and in a T-cell line, $HUT78CD4^+$. The strong association of the two proteins was even noted in nonactivated cells, a finding comparable to that of our study in which the RasGAP-Sam68 association was even detectable in uninfected HeLa cells. Intriguingly, RasGAP was cleaved during infections with different strains of CVB3, resulting in the appearance of a 104-kDa cleavage product (Fig. 2). When the study was extended to different enteroviruses, EV11 and EV12 infection of host cells also resulted in the cleavage of RasGAP, yielding the 104-kDa fragment (Fig. 3) and indicating that proteolytic cleavage of RasGAP is an event common to enterovirus infections.

So far, only a few cellular proteins have been described to be cleaved during picornavirus infections. The human TATA-binding protein, a transcription factor required for the transcription of RNA polymerase I, II, and III genes, has been shown to be directly cleaved by poliovirus proteinase $3C^{pro}$. Cleavage results in the inhibition of RNA polymerase-mediated transcription in poliovirus-infected cells (13, 17). A further direct substrate of proteinase $3C^{pro}$ is the cyclic AMP-responsive element-binding protein, CREB (74). Furthermore, transcription by RNA polymerase III during poliovirus infection is affected by $3C^{pro}$-mediated proteolysis of the transcription factor TFIIIC (14). Besides the impairment of components of the cellular transcription system, cleavage of microtubule-associated protein 4 as well as an as-yet-unidentified host protein has been reported (33, 51). Moreover, specific $2A^{pro}$-induced proteolysis of eIF-4γ, an initiation factor for protein synthesis, occurs during infection of host cells with enteroviruses, rhinoviruses, and aphthoviruses, leading to the inability of ribosomes to bind cellular capped mRNAs and promoting host cell protein shutoff (18, 58, 59). Furthermore, poliovirus induces the specific degradation of P68/PKR, the interferon-induced double-stranded RNA-activated protein kinase, to avoid a decrease in the rate of protein synthesis during infection (5, 6).

Although we do not provide direct proof for the involvement of a viral proteinase in RasGAP cleavage, there are several indications that the viral proteinase $3CD^{pro}$ may be responsible for the observed cleavage event. First, through interactions of Sam68 with both $3CD^{pro}$ (data not shown) and RasGAP, the spatial proximity is provided for cleavage to occur. Second, a potential cleavage site (Gln938/Asn939) for the coxsackievirus and echovirus proteinases $3CD^{pro}$ was found in the C-terminal catalytic region of RasGAP. Third, the use of this potential cleavage site would result in a RasGAP fragment of 104 kDa, as was observed in this study. Further proof of C-terminal RasGAP cleavage is provided by the use of the monoclonal anti-RasGAP antibody B4F8 throughout this study; this antibody was raised to recognize the N-terminal noncatalytic region of RasGAP but still is capable of detecting the 104-kDa RasGAP cleavage product. Based on the three-dimensional model of the RasGAP catalytic domain recently published by Scheffzek et al. (54), it can be concluded that $3CD^{pro}$-mediated proteolysis may lead to the impairment of RasGAP catalytic activity during enterovirus replication. Interestingly, it has been reported previously that RasGAP can serve as a substrate for apoptosis-mediating caspases, yielding protein fragments of approximately 40 and 80 kDa (68). Since in our system a highly abundant cleavage product of 104 kDa was observed and this processing of RasGAP could not be prevented by the general caspase inhibitor ZVAD.fmk (Fig. 4), the possibility that RasGAP is cleaved by a viral proteinase during enterovirus infection is reinforced.

The $p21^{ras}$ GTPase-activating protein RasGAP is an important cellular signaling protein catalyzing the inactivation of $p21^{ras}$, which itself plays a substantial role in the signal transduction of normal and transformed cells (2, 7). In association with GDP, $p21^{ras}$ is inactive, and the exchange of GDP with GTP results in the activation of $p21^{ras}$ (64). The binding of nucleotides to $p21^{ras}$ is controlled by certain cytosolic proteins. Whereas nucleotide exchange factors such as Sos-1 promote activation, RasGAP catalyzes the inactivation of $p21^{ras}$ by stimulating its weak GTPase activity (24, 64, 71). Enterovirus infection-induced cleavage of RasGAP may therefore lead to its inactivation as well as to disturbed regulation of the Ras pathway in response to infection-relevant stimuli, such as interferons and cytokines (19), thereby influencing the progression of enterovirus infection.

In corroboration of the foregoing concept, we found dual phosphorylation of the MAPK Erk-1 and Erk-2 during CVB3 infection of host cells (Fig. 5). Moreover, downstream of $p21^{ras}$ and upstream of MAPK, the protein kinases Raf-1 and Mek-1 were activated, as assessed by band shift assays and inhibitory studies (data not shown); these findings verified the activation of the Ras pathway during CVB3 replication. Although MAPK dual phosphorylation resulted in the hyperphosphorylation of the $p21^{ras}$ exchange factor Sos-1 (Fig. 6A), which is known to uncouple the Ras pathway from upstream activators (11, 67), MAPK dual phosphorylation remained present during the entire late phase of infection (Fig. 5). This permanent MAPK activation may be due to the observed RasGAP cleavage during replication, potentially leading to an imbalance between $p21^{ras}$ activation and inactivation and promoting prolonged

MAPK phosphorylation. Interestingly, Muszynski et al. (46) have reported recently that both polycythemia- and anemia-inducing strains of Friend spleen focus-forming virus induce constitutive activation of the Raf-1/MAPK signal transduction pathway. Furthermore, it has been shown previously that Erk-1 and Erk-2 are activated in a sustained manner during human cytomegalovirus infection (50), suggesting a more general mechanism of cellular activation during virus infections.

Besides Sos-1, MAPK are known to phosphorylate other cytosolic proteins and nuclear transcription factors. $cPLA_2$ is one of these MAPK substrates. $cPLA_2$ releases fatty acid from the sn-2 position of phospholipids. This hydrolysis is of particular significance when arachidonic acid is liberated, since this fatty acid represents the rate-limiting precursor for the synthesis of prostaglandins, leukotrienes, and thromboxanes, three classes of potent inflammatory mediators (53). $cPLA_2$ is activated by increased cytosolic Ca^{2+} concentrations, which cause the translocation of $cPLA_2$ from the cytosol to membranes, where its phospholipid substrate is localized (12). Furthermore, phosphorylation of $cPLA_2$ by MAPK has been reported to be important for full activation of $cPLA_2$ (41). During CVB3 infection, cytosolic Ca^{2+} concentrations gradually increase, due to the release of Ca^{2+} from the endoplasmic reticulum as well as the influx of extracellular Ca^{2+} (66). Moreover, CVB3 has been shown to induce the production and secretion of prostaglandin E_2 during infection of freshly isolated human monocytes (28). In this study, we report the first evidence for the dual phosphorylation of the MAPK Erk-1 and Erk-2 during the course of CVB3 infection, providing an important link between CVB3-induced Ca^{2+} flux and CVB3-stimulated prostaglandin E_2 release. Furthermore, Huttunen et al. (30) reported on the induction of the immediate-early genes c-*jun*, *junB*, and c-*fos* during echovirus replication, a process in which activated MAPK are known to be capable of playing a key role (16). Since the upregulation of c-*jun*, c-*fos*, and *junB* has been related to apoptosis (48) and since we have recently shown that CVB3 replication induces apoptotic pathways (9), activated MAPK might be an important mediator for CVB3-induced apoptosis and cytopathic effects.

According to the observed time courses of RasGAP cleavage (Fig. 2) and MAPK activation (Fig. 5), RasGAP cleavage and inactivation might be involved in the prolongation of MAPK dual phosphorylation rather than in the dual phosphorylation itself, an idea which leads to the question of the initial $p21^{ras}$-activating event. In the cellular system used throughout this study, the activation of Src-like kinases during the replication of CVB3 may be an important inducer of the Ras pathway (29, 69). In a target organ infection, CVB3-infected cells are in contact with a wide variety of cellular stimuli, such as growth factors, cytokines, and hormones. These factors could initiate the activation of $p21^{ras}$. In this respect, we found that epidermal growth factor is still capable of inducing cellular tyrosine phosphorylation events until the very late phase of CVB3 infection (data not shown).

Virus-infected cells still receive signals from and are capable of sending signals to surrounding cells. Therefore, it seems important to determine the possible interactions between virus replication and cellular signaling mechanisms as a means of establishing host-specific determinants of effective virus replication.

ACKNOWLEDGMENTS

This work was supported by the Federal Ministry of Education and Research and the Interdisciplinary Clinical Research Center (IKFZ, 01 KS 9602), Tübingen, Germany, the Heart and Stroke Foundation of British Columbia and Yukon Territory, and the Medical Research Council of Canada. M.H. was supported by the fortüne-Programm des Tübinger Universitätsklinikums.

The excellent technical assistance of G. Janke is acknowledged. We thank G. Krystal for support throughout the study.

REFERENCES

1. **Andino, R., G. E. Rieckhof, P. L. Achacoso, and D. Baltimore.** 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. EMBO J. **12:**3587–3598.
2. **Barbacid, M.** 1987. Ras genes. Annu. Rev. Biochem. **56:**779–827.
3. **Bergelson, J. M., J. A. Cunningham, G. Droguett, E. A. Kurt-Jones, A. Krithivas, J. S. Hong, M. S. Horwitz, R. L. Crowell, and R. W. Finberg.** 1997. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science **275:**1320–1323.
4. **Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990. Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region. J. Virol. **64:**1156–1163.
5. **Black, T. L., B. Safer, A. Hovannessian, and M. G. Katze.** 1989. The cellular 68,000-M_r protein kinase is highly autophosphorylated and activated yet significantly degraded during poliovirus infection: implications for translational regulation. J. Virol. **63:**2244–2251.
6. **Black, T. L., G. N. Barber, and M. G. Katze.** 1993. Degradation of the interferon-induced 68,000-M_r protein kinase by poliovirus requires RNA. J. Virol. **67:**791–800.
7. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature (London) **348:** 125–132.
8. **Bunnell, S. C., P. A. Henry, R. Kolluri, T. Kirchhausen, R. J. Rickles, and L. J. Berg.** 1996. Identification of Itk/Tsk Src homology 3 domain ligands. J. Biol. Chem. **271:**25646–25656.
9. **Carthy, C. M., D. Granville, K. A. Watson, D. R. Anderson, J. E. Wilson, D. C. Yang, D. A. Hunt, and B. M. McManus.** 1998. Caspase activation and cleavage of substrates following coxsackievirus B3-induced cytopathic effect in HeLa cells. J. Virol. **72:**7669–7675.
10. **Chen, T., B. B. Damaj, C. Herrera, P. Lasko, and S. Richard.** 1997. Self-association of the single-KH-domain family members Sam68, GRP33, GLD-1, and Qk1: role of the KH domain. Mol. Cell. Biol. **17:**5707–5718.
11. **Cherniack, A. D., J. K. Klarlund, B. R. Conway, and M. P. Czech.** 1995. Disassembly of son-of-sevenless proteins from Grb2 during $p21^{ras}$ desensitization by insulin. J. Biol. Chem. **270:**1485–1488.
12. **Clark, J. D., L.-L. Lin, R. W. Kriz, C. S. Ramesha, L. A. Sultzman, A. Y. Lin, N. Milona, and J. L. Knopf.** 1991. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. Cell **65:**1043–1051.
13. **Clark, M. E., P. M. Lieberman, A. J. Berk, and A. Dasgupta.** 1993. Direct cleavage of human TATA-binding protein by poliovirus protease 3C in vivo and in vitro. Mol. Cell. Biol. **13:**1232–1237.
14. **Clark, M. E., T. Hämmerle, E. Wimmer, and A. Dasgupta.** 1991. Poliovirus proteinase 3C converts an active form of transcription factor IIIC to an inactive form: a mechanism for inhibition of host cell polymerase III transcription by poliovirus. EMBO J. **10:**2941–2947.
15. **Cohen, G. B., R. Ren, and D. Baltimore.** 1995. Modular binding domains in signal transduction proteins. Cell **80:**237–248.
16. **Cohen, P.** 1997. The search for physiological substrates of MAP and SAP kinases in mammalian cells. Trends Cell. Biol. **7:**353–361.
17. **Das, S., and A. Dasgupta.** 1993. Identification of the cleavage site and determinants required for poliovirus $3C^{pro}$-catalyzed cleavage of human TATA-binding transcription factor TBP. J. Virol. **67:**3326–3331.
18. **Devaney, M. A., V. N. Vakharia, R. E. Lloyd, E. Ehrenfeld, and M. J. Grubman.** 1988. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. J. Virol. **62:**4407–4409.
19. **Downward, J., J. Graves, and D. Cantrell.** 1992. The regulation and function of $p21^{ras}$ in T cells. Immunol. Today **13:**89–92.
20. **Finan, P. M., A. Hall, and S. Kellie.** 1996. Sam68 from an immortalised B-cell line associates with a subset of SH3 domains. FEBS Lett. **389:**141–144.
21. **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.
22. **Fumagalli, S., N. F. Totty, J. J. Hsuan, and S. A. Courtneidge.** 1994. A target for Src in mitosis. Nature (London) **368:**871–874.
23. **Fusaki, N., A. Iwamatsu, M. Iwashima, and J.-I. Fujisawa.** 1997. Interaction between Sam68 and Src family tyrosine kinases, Fyn and Lck, in T cell receptor signaling. J. Biol. Chem. **272:**6214–6219.
24. **Gibbs, J. B., M. D. Schaber, W. J. Allard, I. S. Sigal, and E. M. Scolnick.** 1988. Purification of ras GTPase activating protein from bovine brain. Proc. Natl. Acad. Sci. USA **85:**5026–5030.
25. **Grossman, J. S., M. I. Meyer, Y. C. Wang, G. J. Mulligan, R. Kobayashi, and D. M. Helfman.** 1998. The use of antibodies to the polypyrimidine tract binding protein (PTB) to analyze the protein components that assemble on

alternatively spliced pre-mRNAs that use distant branch points. RNA **4:**613–625.
26. **Guitard, E., I. Barlat, F. Maurier, F. Schweighoffer, and B. Tocque.** 1998. Sam68 is a Ras-GAP-associated protein in mitosis. Biochem. Biophys. Res. Commun. **245:**562–566.
27. **Hellen, C. U., T. V. Pestova, M. Litterst, and E. Wimmer.** 1994. The cellular polypeptide p57 (pyrimidine tract-binding protein) binds to multiple sites in the poliovirus 5′ nontranslated region. J. Virol. **68:**941–950.
28. **Henke, A., H. P. Spengler, A. Stelzner, M. Nain, and D. Gemsa.** 1992. Lipopolysaccharide suppresses cytokine release from coxsackie virus-infected human monocytes. Res. Immunol. **143:**65–70.
29. **Huber, M., H.-C. Selinka, and R. Kandolf.** 1997. Tyrosine phosphorylation events during coxsackievirus B3 replication. J. Virol. **71:**595–600.
30. **Huttunen, P., J. Heino, and T. Hyypiä.** 1997. Echovirus 1 replication, not only virus binding to its receptor, VLA-2, is required for the induction of cellular immediate-early genes. J. Virol. **71:**4176–4180.
31. **Jabado, N., S. Jauliac, A. Pallier, F. Bernard, A. Fischer, and C. Hivroz.** 1998. Sam68 association with p120GAP in CD4+ T cells is dependent on CD4 molecule expression. J. Immunol. **161:**2798–2803.
32. **Jabado, N., A. Pallier, F. Le Deist, F. Bernard, A. Fischer, and C. Hivroz.** 1997. CD4 ligands inhibit the formation of multifunctional transduction complexes involved in T cell activation. J. Immunol. **158:**94–103.
33. **Joachims, M., K. S. Harris, and D. Etchison.** 1995. Poliovirus protease 3C mediates cleavage of microtubule-associated protein 4. Virology **211:**451–461.
34. **Johnson, G. L., and R. R. Vaillancourt.** 1994. Sequential protein kinase reactions controlling cell growth and differentiation. Curr. Opin. Cell. Biol. **6:**230–238.
35. **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, N.Y.
36. **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.
37. **Kandolf, R., K. Klingel, R. Zell, H.-C. Selinka, U. Raab, W. Schneider-Brachert, and B. Bültmann.** 1993. Molecular pathogenesis of enterovirus-induced myocarditis: virus persistence and chronic inflammation. Intervirology **35:**140–151.
38. **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.
39. **Kräusslich, H.-G., M. J. H. Nicklin, C.-K. Lee, and E. Wimmer.** 1988. Polyprotein processing in picornavirus replication. Biochimie **70:**119–130.
40. **Lawe, D. C., C. Hahn, and A. J. Wong.** 1997. The Nck SH2/SH3 adaptor protein is present in the nucleus and associates with the nuclear protein SAM68. Oncogene **14:**223–231.
41. **Lin, L.-L., M. Wartmann, A. Y. Lin, J. L. Knopf, A. Seth, and R. J. Davis.** 1993. $cPLA_2$ is phosphorylated and activated by MAP kinase. Cell **72:**269–278.
42. **Lock, P., S. Fumagalli, P. Polakis, F. McCormick, and S. A. Courtneidge.** 1996. The human p62 cDNA encodes Sam68 and not the RasGAP-associated p62 protein. Cell **84:**23–24.
43. **Marshall, C. J.** 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. **4:**82–89.
44. **McBride, A. E., A. Schlegel, and K. Kirkegaard.** 1996. Human protein Sam68 relocalization and interaction with poliovirus RNA polymerase in infected cells. Proc. Natl. Acad. Sci. USA **93:**2296–2301.
45. **Melnick, J. L.** 1996. Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses, p. 655–712. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 3rd ed. Raven Press, New York, N.Y.
46. **Muszynski, K. W., T. Ohashi, C. Hanson, and S. K. Ruscetti.** 1998. Both the polycythemia- and anemia-inducing strains of Friend spleen focus-forming virus induce constitutive activation of the Raf-1/mitogen-activated protein kinase signal transduction pathway. J. Virol. **72:**919–925.
47. **Ohsawa, K., and N. Ebata.** 1983. Silver stain for detecting 10-femtogram quantities of protein after polyacrylamide gel electrophoresis. Anal. Biochem. **135:**409–415.
48. **Pandey, S., and E. Wang.** 1995. Cells en route to apoptosis are characterized by the upregulation of *c-fos*, *c-myc*, *cdc2*, and RB phosphorylation, resembling events of early cell-cycle traverse. J. Cell. Biochem. **58:**135–150.
49. **Richard, S., D. Yu, K. J. Blumer, D. Hausladen, M. W. Olszowy, P. A. Connelly, and A. S. Shaw.** 1995. Association of p62, a multifunctional SH2- and SH3-domain-binding protein, with src family tyrosine kinases, Grb2, and phospholipase C gamma-1. Mol. Cell. Biol. **15:**186–197.
50. **Rodems, S. M., and D. H. Spector.** 1998. Extracellular signal-regulated kinase activity is sustained early during human cytomegalovirus infection. J. Virol. **72:**9173–9180.
51. **Roehl, H. H., T. B. Parsley, T. V. Ho, and B. L. Semler.** 1997. Processing of a cellular polypeptide by 3CD proteinase is required for poliovirus ribonucleoprotein complex assembly. J. Virol. **71:**578–585.
52. **Rueckert, R. R.** 1996. Picornaviridae: the viruses and their replication, p. 609–654. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology, 3rd ed. Raven Press, New York, N.Y.
53. **Samuelsson, B., S. E. Dahlen, J. A. Lindgren, C. A. Rouzer, and C. N. Serhan.** 1987. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Science **237:**1171–1176.
54. **Scheffzek, K., A. Lautwein, W. Kabsch, M. R. Ahmadian, and A. Wittinghofer.** 1996. Crystal structure of the GTPase-activating domain of human p120GAP and implications for the interaction with Ras. Nature (London) **384:**591–596.
55. **Selinka, H.-C., K. Klingel, M. Huber, B. Kramer, U. Kämmerer, and R. Kandolf.** 1997. Molecular pathogenesis of myocarditis and cardiomyopathy: analysis of virus-receptor interactions and tyrosine phosphorylation events, p. 17–26. *In* H. P. Schultheiss and P. Schwimmbeck (ed.), The role of immune mechanisms in cardiovascular disease. Springer-Verlag KG, Berlin, Germany.
56. **Settleman, J., V. Narasimhan, L. C. Foster, and R. A. Weinberg.** 1992. Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from ras to the nucleus. Cell **69:**539–549.
57. **Siomi, H., M. Choi, M. C. Siomi, R. L. Nussbaum, and G. Dreyfuss.** 1994. Essential role for KH domain in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell **77:**33–39.
58. **Sommergruber, W., H. Ahorn, A. Zoephel, I. Maurer-Fogy, F. Fessl, G. Schnorrenberg, H.-D. Liebig, D. Blaas, E. Kuechler, and T. Skern.** 1992. Cleavage specificity on synthetic peptide substrates of human rhinovirus 2 proteinase 2A. J. Biol. Chem. **267:**22639–22644.
59. **Sommergruber, W., H. Ahorn, H. Klump, J. Seipelt, A. Zoephel, F. Fessl, E. Krystek, D. Blaas, E. Kuechler, H.-D. Liebig, and T. Skern.** 1994. 2A proteinases of coxsackie- and rhinovirus cleave peptides derived from eIF-4 gamma via a common recognition motif. Virology **198:**741–745.
60. **Stokoe, D., F. B. Caudwell, P. T. W. Cohen, and P. Cohen.** 1993. The substrate specificity and structure of mitogen-activated protein (MAP) kinase-activated protein kinase-2. Biochem. J. **296:**843–849.
61. **Taylor, S. J., and D. Shalloway.** 1994. An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. Nature (London) **368:**867–871.
62. **Taylor, S. J., M. Anafi, T. Pawson, and D. Shalloway.** 1995. Functional interaction between c-Src and its mitotic target, Sam 68. J. Biol. Chem. **270:**10120–10124.
63. **Tomko, R. P., R. Xu, and L. Philipson.** 1997. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. USA **94:**3352–3356.
64. **Trahey, M., and F. McCormick.** 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science **238:**542–545.
65. **Trueb, T., J. D. Frantz, M. Miyazaki, H. Band, and S. E. Shoelson.** 1997. The role of a lymphoid-restricted, Grb2-like SH3-SH2-SH3 protein in T cell receptor signaling. J. Biol. Chem. **272:**894–902.
66. **van Kuppeveld, F. J. M., J. G. J. Hoenderop, R. L. L. Smeets, P. H. G. M. Willems, H. B. P. M. Dijkman, J. M. D. Galama, and W. J. G. Melchers.** 1997. Coxsackievirus protein 2B modifies endoplasmic reticulum membrane and plasma membrane permeability and facilitates virus release. EMBO J. **16:**3519–3532.
67. **Waters, S. B., K. H. Holt, S. E. Ross, L.-J. Syu, K.-L. Guan, A. R. Saltiel, G. A. Koretzky, and J. E. Pessin.** 1995. Desensitization of Ras activation by a feedback disassociation of the Sos-Grb2 complex. J. Biol. Chem. **270:**20883–20886.
68. **Widmann, C., S. Gibson, and G. L. Johnson.** 1998. Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. J. Biol. Chem. **273:**7141–7147.
69. **Williams, N. G., and T. M. Roberts.** 1994. Signal transduction pathways involving the Raf proto-oncogene. Cancer Metastasis Rev. **13:**105–116.
70. **Wimmer, E., C. U. T. Hellen, and X. Cao.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353–436.
71. **Wolfman, A., and I. G. Macara.** 1990. A cytosolic protein catalyzes the release of GDP from $p21^{ras}$. Science **248:**67–69.
72. **Wong, G., O. Mueller, R. Clark, L. Conroy, M. F. Moran, P. Polakis, and F. McCormick.** 1992. Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell **69:**551–558.
73. **Xiang, W., K. S. Harris, L. Alexander, and E. Wimmer.** 1995. Interaction between the 5′-terminal cloverleaf and 3AB/3CDpro of poliovirus is essential for RNA replication. J. Virol. **69:**3658–3667.
74. **Yalamanchili, P., U. Datta, and A. Dasgupta.** 1997. Inhibition of host cell transcription by poliovirus: cleavage of transcription factor CREB by poliovirus-encoded protease 3Cpro. J. Virol. **71:**1220–1226.