Protein Kinase C Recognizes the Protein Kinase A-binding Motif of Nonstructural Protein 3 of Hepatitis C Virus*

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The nonstructural protein 3 (NS3) of hepatitis C virus (HCV) inhibits the nuclear transport and the enzymatic activity of the catalytic subunit of protein kinase A. This inhibition is mediated by an arginine-rich domain localized between amino acids 1487–1500 of the HCV polyprotein. The data presented here indicate that the arginine-rich domain, when embedded in recombinant fragments of NS3, interacts with the catalytic site of protein kinase C (PKC) and inhibits the phosphorylation mediated by this enzyme in vitro and in vivo. Furthermore, a direct binding of PKC to the NS3 fragments leads to an inhibition of the free shuttling of the kinase between the cytoplasm and the particulate fraction. In contrast, a peptide corresponding to the arginine-rich domain (HCV (1487–1500)), despite also being a PKC inhibitor, did not influence the PKC shuttling process and was transported to the particulate fraction by the translocating kinase upon activation with tetradecanoylphorbol-13-acetate. Using the tetradecanoylphorbol-13-acetate-stimulated respiratory burst of NS3-introduced neutrophils as a model system, we could demonstrate that NS3 is able to block PKC-mediated functions within intact cells. Our data support the possibility that NS3 disrupts the PKC-mediated signal transduction.

In more than 50% of the investigated cases, liver infections with the hepatitis C virus (HCV) result in chronic liver disease, which often leads to cirrhosis and hepatocellular carcinoma (1, 2). HCV is a small enveloped RNA virus. Its genome is similarly organized to that of pesti- and flaviviruses (3). The viral genome encodes a polyprotein of 3010 amino acids that is processed by host and viral proteases into core protein, envelope proteins (E1 and E2), and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (4). Although a great amount of data has been accumulated about functional regions in these proteins, relatively little is known about their intracellular targets.

Previously, we have reported that NS3 contains an arginine-rich amino acid sequence: Arg1487-Gly-Arg-Arg-Thyr-Gly-Arg-Gly-Arg-Gly-Ile-Tyr-Arg1500 (5) (numbered according to the HCV polyprotein (6)). This sequence is similar to sequences occurring in the regulatory subunit of the cAMP-dependent protein kinase, in protein substrates of this enzyme and in the heat stable inhibitor of PKA (7). In fact, this NS3 protein sequence mediates the inhibition of PKA activity. The catalytic subunit of the kinase directly binds to the viral protein, and consequently the cytoplasm-to-nucleus translocation of the kinase is slowed down (8).

Arginine residues are also known to be recognition motifs of substrates and autoregulatory regions of numerous serine/threonine specific protein kinases (9). This is the reason for overlapping specificities of the kinases with regard to their substrates (10) or to their inhibition mediated by inhibitory peptides resembling their autoinhibitory domains (11).

Recently we have shown that a peptide (HCV (1487–1500)) that reproduces the PKA binding domain of NS3 mentioned above is recognized by another serine/threonine protein kinase, the PKC. The peptide directly interacts with the kinase and serves as an excellent substrate for the enzyme (12). Therefore, it seems likely that the arginine-rich sequence embedded in a larger fragment of the HCV polyprotein interacts with the catalytic domain of PKC, as observed in the case of PKA in vivo (8). By means of these protein-protein interactions, the PKC functions could be blocked or slowed down. These interactions may be a new important aspect of pathogenesis of the HCV infection.

In the present study we investigate the mechanisms by which PKC interacts with NS3 in vitro. Furthermore, we examine certain biological effects controlled by PKC in cells harboring the viral protein. Our results provide the first evidence that NS3 affects PKC functions by a complex mechanism similar to the one that impairs the functions of PKA.

EXPERIMENTAL PROCEDURES

Materials—Affinity-purified rat brain PKC isolated according to the method described previously (13) was a generous gift of Dr. Hilz (University of Hamburg). Human HCV-positive antisera against NS3 and NS4 of HCV were kindly provided by Dr. Polywka (University of Hamburg). Peptides were synthesized and purified by high pressure liquid chromatography by Dr. Kullmann (Zentrum für Molekulare Neurobiologie of the University of Hamburg). [γ-32P]ATP (3000 Ci/mmol) and 125I-labeled protein A (30 mCi/mg) were obtained from Amersham Pharmacia Biotech, N-Succinimidyl [2,3-3H]propionate and 125I were purchased from DuPont. Antibody (MC5) specific for α-βγ-βγ-PC-isoforms and all other chemicals were obtained from Sigma. Blood from healthy volunteers was kindly provided by Dr. Roos (Abteilung für Transfusionsmedizin, University of Hamburg).

Cell Cultures, Protein Introduction, and Respiratory Burst—Highly purified neutrophils (> 95%) were prepared from freshly drawn blood.
by Ficoll-Hypaque centrifugation, dextrane sedimentation, and hypotonic lysis of red cells (14). Neutrophils were resuspended in complete RPMI 1640 Medium supplemented with 10% fetal calf serum and kept at 37 °C in humidified air containing 10% CO₂. The introduction of proteins or peptides by protease permeabilization was performed as described previously (5, 25). The permeabilized neutrophils were washed with 2.5% fetal calf serum for 20 min. The pellet was resuspended in hypotonic buffer and heated at 95 °C for 5 min (21). Phosphoproteins were resolved by SDS/PAGE and visualized by autoradiography. Heat stable proteins serving as PKC substrates in in vitro reactions were partially purified according to the method described by Mahadevan et al. (22) with the following modifications. Cells were washed with cold 150 mM NaCl and resuspended in hypotonic buffer containing 50 mM Hepes, pH 8.0, 2 mM EDTA, 2 mM EGTA, 5 mM phenylmethanesulfonyl fluoride, 2 mM N-tosyl-L-phenylalanyl chloride, 2 mM N-3-[(4-hydroxyphenyl)imino]-2,5-bi-‘H-benzimidazol (5 µM in H₂O), for 10 min at room temperature. After washing with destilled water the coverslips were placed in glycerol containing 10% (v/v) phosphate-buffered saline, pH 6.5, and 0.1% (w/v) cytochrome c. The cells were viewed with a Zeiss AXIOPLAN microscope equipped with fluorescence illuminator and photographed on Kodak film.

**Other Methods**—The cytosolic and particulate fractions of the neutrophils were prepared as described above (22). The protein concentration was measured according to the method of Lowry et al. (23).

**RESULTS**

**Domains of NS3**—The comparison of the amino acid sequence of the NS3 arginine-rich region: Arg¹⁴⁸⁶-Arg-Gly-Arg-Thr-Gly-Arg-Gly-Arg-Gly-Ile-Tyr-Arg¹⁵⁰⁰ with the sequence of the PKC autoregulatory domain revealed a similarity. A synthetic peptide reproducing the sequence above served as an excellent substrate for the kinase and inhibited the kinase activity competitively in respect to other substrates (12). Therefore, we now examined the possibility that NS3 interacts via its arginine-rich region with PKC in vitro and affects PKC-mediated functions in vivo.

To obtain sufficient amounts of HCV proteins, we cloned and expressed the proteins in E. coli. Because the entire NS3 protein was not found to be readily soluble (Ref. 24 and data not shown), we investigated the interaction between NS3 and PKC, using the well characterized C- and N-truncated form of the NS3 protein (HCV polyprotein-(1189-1525)) (5, 8). A second NS3 fragment that also contains the amino acid sequence HCV polyprotein-(1400-1615) was synthesized to ascertain the specificity of the NS3-PKC interaction. The position of both fragments within the NS3 is schematically represented in Fig. 1.

The assays performed were controlled with a further fragment of HCV polyprotein containing amino acid residues 1923-2043 (HCV polyprotein-(1923-2023)) (5, 8). All proteins were numbered according to their position within the HCV polyprotein (6).

**Interaction between PKC and NS3**—We used the overlay method to analyze the specific physical interactions between NS3 and its derived peptides fragments and PKC. Equimolar amounts of the fusion protein, GST-HCV polyprotein-(1189-1525), GST-HCV polyprotein-(1923-2043), and TRX-HCV polyprotein-(1400-1615) as well as of GST and TRX were immobilized on nitrocellulose sheets, and their binding to ³²P-labeled PKC was determined. Both proteins GST-HCV polyprotein-(1189-1525) and TRX-HCV polyprotein-(1400-1615) were found to bind PKC. A lack of binding of the kinase...
NS3 Interacts with PKC-mediated Phosphorylation

FIG. 1. Schematic representation of the NS3 fragments used in this study. The top bar (open) represents the entire NS3 molecule. The shadowed bars below represent the expressed fragments of NS3: HCV polyprotein-(1189–1525) and HCV polyprotein-(1400–1615). The position of the arginine-rich sequence within NS3 is shown with a solid box. The represents the phosphorylatable threonine residue Thr\(^{1551}\) of the synthetic peptide that reproduces the arginine-rich domain of NS3 (12). The amino acid numbers were taken from Ref. 6.

FIG. 2. Binding of PKC to recombinant fragments of NS3 and inhibition of the binding by synthetic peptides that correspond to the arginine-rich domain of NS3 and to the autoregulatory domain of PKC. A, increasing amounts (0.1, 0.3, and 1 nmol) of GST-HCV polyprotein-(1189–1525), TRX-HCV polyprotein-(1400–1615), GST-HCV polyprotein-(1523–2023), GST, and TRX proteins were immobilized on nitrocellulose and overlaid with \(^{125}\)I-labeled PKC (50 nm). The nitrocellulose was washed, dried, and exposed to Kodak film for 4 h as described under “Experimental Procedures.” B, increasing amounts of immobilized GST-HCV polyprotein-(1189–1525) and TRX-HCV polyprotein-(1400–1615) were overlaid with \(^{125}\)I-labeled PKC as described above in presence of a 5-fold molar excess of the peptides HCV (1487–1500) or PKC-(19–36) in the overlaying buffer. The nitrocellulose was washed, dried, and autoradiographed for 4 h.

Results suggest that the catalytic site of PKC binds NS3 via the arginine-rich region HCV (1487–1500). It is conceivable that such an involvement of the catalytic site of the kinase in protein-protein interactions affects its activity. Indeed, our previous studies indicated that a similar interaction between PKA and NS3 resulted in an inhibition of PKA kinase activity (5). We therefore analyzed the substrate phosphorylation by PKC in the presence of HCV proteins \(in vitro\). Experiments performed with histone IIIA as a substrate revealed a concentration-dependent inhibition of the kinase activity by GST-HCV polyprotein-(1189–1525). When myelin basic protein and histone H2B were used as substrates, the GST-HCV polyprotein-(1189–1525) concentrations that were required to give a 50% inhibition of substrate phosphorylation (IC\(_{50}\)) varied significantly (Fig. 3A). The kinetic analysis of the inhibition revealed a mixed type of inhibition (Fig. 3B). Studies performed with TRX-HCV polyprotein-(1400–1615) also revealed significant differences between the IC\(_{50}\) for the phosphorylation of different substrates and a mixed type of inhibition. This suggests, in analogy to the inhibition of PKA by NS3 observed previously, a complex interaction between PKC, its substrate, and the inhibiting viral protein.

Intracellular Distribution of Introduced HCV polyprotein-(1189–1525) and Peptide HCV (1487–1500)—Previous studies that investigated the subcellular distribution of NS3 in HCV-infected cells demonstrated its cytoplasmatic localization (26). Moreover, NS3 fragments GST-HCV polyprotein-(1189–1525) and TRX-HCV polyprotein-(1400–1615) introduced into HEp-2 cells were also detected in the cytoplasm and in the perinuclear region (Ref. 8 and data not shown). In the following experiments, we examined the subcellular localization of the introduced GST-HCV polyprotein-(1189–1525) and the exclusion of the protein from the nuclei. Immunoblot analysis of subcellular fractions obtained from GST-HCV polyprotein-(1189–1525)-introduced cells revealed that approximately 40% of the introduced protein was associated with the particulate and approximately 60% with the cytosolic fraction. Separation of cell organelles according to the method of Storrie and Madsen (27) did not reveal any association of the introduced protein with cell organelles. These procedures affected the cell viability only marginally, as tested with trypan blue. The evaluated half-life of the introduced GST-HCV polyprotein-(1189–1525) was longer than 12 h (data not shown).

Effects of NS3 on the Subcellular PKC Distribution—Because NS3 binds and inhibits PKC, we investigated these interactions with regard to their ability to inhibit the translocation of PKC from the cytosolic to the particulate fraction. GST-HCV polyprotein-(1189–1525) as well as GST were introduced...
radioactivity was measured as described. The results (Fig. 5 and a transient membrane association of [3H]HCV (1487–1500) demonstrate that TPA treatment induced a rapid translocation of protein substrates by PKC was performed in the presence of increasing concentrations of GST-HCV polyprotein-(1189–1525) as inhibitor. The substrates: histone IIIS (open circles), histone H2B (filled circles), and myelin basic protein (triangles) were phosphorylated at concentrations corresponding to their Km values (4.5 μM for histone IIIS, 2.5 μM for histone H2B, and 25 μM for myelin basic protein). The kinase activity toward each substrate in the absence of the inhibitor was referred to as 100%. The Incorporation into substrates was determined as described under “Experimental Procedures.” B, PKC activity in the presence of GST-HCV polyprotein-(1189–1525) as inhibitor was investigated with histone IIIS. The substrate was phosphorylated at concentrations of 13.5, 4.5, and 1.5 μM corresponding to 3-, 1-, or 1/3-fold of the Km of histone phosphorylation (filled triangles, open triangles, and squares, respectively). The PKC activity was determined as described above, and the data obtained were plotted according to Cornish-Bowden (49). The results shown are representative for three independent experiments.

into the cells, and the effect of the proteins on the subcellular distribution of PKC was evaluated after treatment of the cells with 100 nM TPA for 5 min. Subsequently, the cytosolic and particulate fractions were separated and examined for their PKC content by immunoblot. The result indicates that the NS3 fragment was able to inhibit the TPA-induced redistribution of PKC (Fig. 5A). Very similar results were obtained with HEP-2 cells (results not shown). In contrast the treatment of the cells with HCV (1487–1500) peptide that strongly binds to PKC in vitro does not affect the extent of the TPA-induced cytosol-to-membrane translocation of the kinase. We therefore determined the distribution of the peptide and its interaction with PKC in the cell. The HCV (1487–1500) peptide was labeled with [3H] introduced into neutrophils, and its half-life was determined as described under “Experimental Procedures.” After 12 h approximately 80% of the introduced peptide was found in the cells. Subcellular fractionation followed by measuring the [3H] activity revealed that approximately 97% of the labeled peptide was located in the cytosolic compartment and approximately 2–3% in the nuclear fraction. Only <0.5% of the peptide introduced was associated with the membrane fraction. The [3H]HCV (1487–1500)-introduced cells were then incubated with TPA for 5, 10, 20, or 30 min. Thereafter, the cytosolic and the particulate fractions were separated, and [3H] radioactivity was measured as described. The results (Fig. 5B) demonstrate that TPA treatment induced a rapid translocation and a transient membrane association of [3H]HCV (1487–1500). These events were accompanied by a corresponding increase in immunoreactive PKC in the particulate fraction, when analyzed by counting the [32P]labeled bands obtained in an immunoblot with anti-PKC antibody (Fig. 5B).

NS3 Inhibits TPA-induced Phosphorylation of p80—Because TPA treatment activates PKC and induces the rapid phosphorylation of p80 in numerous cell systems (28), we compared the induction of cellular p80 phosphorylation by TPA in neutrophils harboring the introduced HCV protein. Cells were labeled with [32P] for 2 h and then treated with TPA for 5 and 20 min. SDS/PAGE analysis of heat stable proteins extracted from the cells demonstrated that TPA-induced a rapid increase in the phosphorylation level of p80 and several other proteins in GST-HCV polyprotein-(1923–2043)-introduced neutrophils as well as in control (not introduced) neutrophils. A similar pattern of p80 phosphorylation was observed in GST-introduced cells. GST-HCV polyprotein-(1189–1525) reduced the TPA-induced increase in the phosphorylation level of the cellular heat stable proteins (Fig. 6A).

Surprisingly, no inhibition of TPA-induced p80 phosphorylation in vivo was seen in HCV (1487–1500) peptide-introduced neutrophils (Fig. 6C). A similar failure to inhibit the p80 phosphorylation by HCV (1487–1500) was also observed in other TPA-treated cells, i.e. HEP-2 and 3T3 cells (data not shown). Therefore, an experiment was performed to test the capacity of HCV (1487–1500) to inhibit PKC phosphorylation of p80 in vitro. The heat stable fraction of cellular proteins was prepared from neutrophils and phosphorylated by rat brain PKC in the presence of increasing concentrations of HCV (1487–1500). As shown in Fig. 6B, the phosphorylation of p80 in vitro was not significantly affected by the peptide at concentrations up to 50 μM. We have synthesized two peptides, analogous to HCV (1487–1500), in which the Thr1491 was replaced by alanine or valine. When the substituted derivatives were used for inhibition studies, the analogue [Ala1491]HCV (1487–1500) was

![Fig. 3. Inhibition of the PKC-mediated phosphorylation by GST-HCV polyprotein-(1189–1525) and a plot demonstrating the mixed type of PKC inhibition relative to histone IIIS.](image)

![Fig. 4. Direct fluorescence microscopy to detect the cellular distribution of introduced GST-HCV polyprotein-(1189–1525).](image)
RPMI 1640 medium for 4 h as described under "Experimental Procedures." Thereafter, the introduced as well as control (not introduced) cells were treated with the carrier (Me2SO) or with 100 nM TPA for 5 min. Aliquots of a constant amount of the cells were lysed, and extracts from the cytosolic and the particulate fractions were applied to SDS/PAGE. The proteins were transferred onto nitrocellulose and subjected to immunoblot with an antibody against PKC band (32). The blots were exposed for 14 h. Arrows indicate the positions of PKC. The labeled peptide [3H]HCV (1487–1500) was introduced into neutrophils as described above. The cells were treated with 100 nM TPA for 0, 5, 10, 20, or 30 min and lysed. Aliquots of the extracts from the particulate fraction were removed, and the 3H radioactivity was counted in a liquid scintillation counter. The results shown are representative for three independent experiments.

As the main result of this study we have shown that the enzymatic activity and the cytosol-to-membrane translocation of PKC is inhibited by the nonstructural protein 3 of hepatitis C virus. The arginine-rich sequence of NS3 Arg-Arg-Gly-Arg-Thr-Gly-Arg-Gly-Arg-Gly-Ile-Tyr-Arg, corresponding to amino acids 1487–1500 of HCV polyprotein and resembling the autoregulatory domain of PKC (25), is essential for this interaction. We have demonstrated that a synthetic peptide reproducing this sequence above acts as a PKC inhibitor in vitro and binds to and co-translocates with the kinase in vivo. When this sequence is embedded in a larger fragment of the NS3, it also binds to PKC and functions as an inhibitor of the kinase in vitro and in vivo. However, in contrast to the peptide, the larger recombinant NS3 fragment that contains this sequence prevents the translocation of PKC upon activation with TPA. These findings led us to conclude that PKC is immobilized by NS3 and thus does not translocate toward its intracellular membrane-associated receptor protein(s) RACK (31, 32).

Although the pepide HCV (1487–1500) and the recombinant NS3 fragments bind PKC via its catalytic site, some differences in their interactions with the kinase were observed. The synthetic pepide HCV (1487–1500) serves as a substrate for the kinase and acts as a competitive inhibitor of PKC toward other substrates, whereas the NS3 fragments bearing the sequence are not phosphorylatable and inhibit the kinase in a mixed manner. On the basis of our previous report (8), we suppose that the arginine-rich sequence may only represent a part of the PKC-binding site on NS3 and thus does not translocate toward its intracellular membrane-associated receptor protein(s) RACK (31, 32).

The model of the endogenous receptor protein for PKC suggests that the substrates and RACK may concomitantly bind the kinase (32). This is supported by the observation that the small molecule of the pepide HCV (1487–1500), which binds to the PKC catalytic site, does not inhibit the association of PKC with the particulate fraction. On the other hand, we have not ruled out the possibility that the RACK binding site(s) of PKC may also be involved in the binding to the NS3.

Another possibility is that when the arginine-rich sequence is embedded in a larger fragment of NS3, other sites are exposed to the solvent and could interact with PKC (35). This could contribute to the explanation of why HCV polyprotein-(1189–

Fig. 5. Effect of introduced HCV (1487–1500) peptide and GST-HCV polyprotein-(1189–1525) on the TPA-induced subcellular redistribution of PKC. Membrane association of HCV (1487–1500) peptide in course of TPA-induced PKC translocation. A, HCV (1487–1500) peptide, GST-HCV polyprotein-(1189–1525), and GST protein were introduced into neutrophils, and the cells were suspended in RPMI 1640 medium for 4 h as described under "Experimental Procedures." Thereafter, the introduced as well as control (not introduced) cells were treated with the carrier (Me2SO) or with 100 nM TPA for 5 min. Aliquots of a constant amount of the cells were lysed, and extracts from the cytosolic and the particulate fractions were applied to SDS/PAGE. The proteins were transferred onto nitrocellulose and subjected to immunoblot with an antibody against PKC band (32). The blots were exposed for 14 h. Arrows indicate the positions of PKC. The labeled peptide [3H]HCV (1487–1500) was introduced into neutrophils as described above. The cells were treated with 100 nM TPA for 0, 5, 10, 20, or 30 min and lysed. Aliquots of the extracts from the particulate fraction were removed, and the 3H radioactivity was counted in a liquid scintillation counter. The results shown are representative for three independent experiments.

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found to be a potent inhibitor of the p80 phosphorylation (IC50 = 1.3 μM). Interestingly, the phosphorylation of several other heat stable proteins (of approximately 70 and 62 kDa) was only moderately affected by the peptide (Fig. 6B). No significant inhibition was observed with [Val1493]HCV (1487–1500) (IC50 > 50 μM). In subsequent experiments we compared these two peptides with regard to their ability to inhibit the p80 phosphorylation in vitro. The [Ala1491]HCV (1487–1500)- and [Val1493]HCV (1487–1500)-introduced cells were labeled with [32P] and then treated with TPA for 5 and 20 min as described above. SDS/PAGE analysis of cellular heat stable proteins demonstrated that [Ala1491]HCV (1487–1500), but not [Val1493]HCV (1487–1500), reduced the TPA-induced phosphorylation of p80 (Fig. 6C).

NS3 Fragments Inhibit the Respiratory Burst of Neutrophils—To ascertain the specificity of the interaction between NS3 and PKC, we examined the ability of the NS3 fragment to inhibit a further PKC-mediated process: the TPA-stimulated superoxide burst of neutrophils. The superoxide burst activity was measured in control (not introduced) neutrophils and in the cells that harbored the HCV proteins and HCV (1487–1500) peptide, as well as GST protein. Further experiments were performed with neutrophils treated with the known PKC inhibitors, 1-(5-isouquinolylsulfonyl)-2-methylpiperazine (29) and sangivamycin (30). As shown in Fig. 7, TPA-stimulated superoxide burst activity was inhibited by GST-HCV polyprotein-(1189–1525) and HCV (1487–1500) peptide. The inhibiting effect was comparable with the inhibitory potential of 1-(5-isouquinolylsulfonyl)-2-methylpiperazine and sangivamycin applied at concentrations corresponding to the 5-fold of IC50 determined with rat brain PKC.
and nonphosphorylatable peptide [Ala 1491]HCV (1487–1500) are better inhibitors of p80 phosphorylation than HCV (1487–1500). Finally, it is possible that some domains or fragments of NS3 determine the accessibility of the arginine-rich sequence as an anchoring site for PKC. Such a regulatory mechanism was previously demonstrated for the phosphorylation of full-length and truncated lipocortin and Ras-GAP (36, 37). The changes of the disposition of the arginine-rich sequence by other domains of NS3 could at least partially explain the different binding capacity of HCV polyprotein-(1189–1525) and HCV polyprotein-(1400–1615) toward PKC.

Our results have several important implications for the role of NS3 in the pathogenesis of the disease caused by HCV. First, the free shuttling of PKC between cellular compartments and the phosphorylation of target proteins, which are normal functions of the kinase, are down-regulated. Second, smaller fragments of NS3 containing the kinase binding sequence would be

![Figure 6](http://www.jbc.org/)  
**Figure 6.** Inhibition of the TPA-induced phosphorylation of p80 by GST-HCV polyprotein-(1189–1525), HCV (1487–1500), and HCV (1487–1500)-derived peptides in vivo and in vitro. A, GST-HCV polyprotein-(1189–1525) and GST protein were introduced into neutrophils as described above. The cells were metabolically labeled with 32P and during this labeling a treatment with 100 nM TPA for 0, 5, or 20 min was carried out. Heat stable proteins were extracted and separated by SDS/PAGE. The dried gel was exposed for 12 h. The migration of molecular mass protein standards is indicated at the left. B, the phosphorylation by PKC of heat stable proteins extracted from neutrophils in vitro was performed in the presence of increasing concentrations of the peptides HCV (1487–1500), [Ala 1491]HCV (1487–1500), and [Val 1491]HCV (1487–1500) as indicated. The phosphoproteins were separated by SDS/PAGE, and dried gels were exposed for 12 h. For the determination of the IC50, the 32P-labeled p80 bands were cut out, and the radioactivity was counted. C, peptides HCV (1487–1500), [Ala 1491]HCV (1487–1500), and [Val 1491]HCV (1487–1500) were introduced into the neutrophils, and the cells were labeled with 32P and treated with TPA as described for A. Heat stable proteins were extracted and the phosphorylation of p80 was analyzed as described above. Arrows indicate the positions of p80.

![Figure 7](http://www.jbc.org/)  
**Figure 7.** Inhibition of the TPA-induced respiratory burst of neutrophils by GST-HCV polyprotein-(1189–1525) and HCV (1487–1500) peptide. GST-HCV polyprotein-(1189–1525), GST-HCV polyprotein (1923–2043), GST protein, and HCV (1487–1500) peptide were introduced into neutrophils. The cells were suspended in RPMI 1640 medium for 4 h as described above. The respiratory burst was measured by visible spectroscopy of superoxide dismutase-inhibitable reduction of cytochrome c. Data are normalized to the maximum rate of superoxide production by the control cells (not introduced neutrophils). Further control stimulations were performed on not introduced cells pretreated for 4 h with the PKC inhibitors 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (150 μM) or sangivamycin (30 nM). The results shown are representative for five independent experiments.
transported with the translocating enzyme. Because different
PKC-isofoms translocate to distinct cellular compartments
upon activation, the nuclear presence of biologically active
helicase fragments (transported by the catalytic subunit of PKA
(8) or β-PKC (38)) is not unlikely. Similarly, wild-type p53
forms a complex with fragments of NS3 and transports them
to the nucleus (39). Finally, according to the data presented here
and in our previous works, the kinase inhibition mediated by
the HCV (1487–1500) peptide or by NS3 fragments strongly
depends on the nature of the substrate tested (5, 12). This
observation predicts that NS3 or its fragments do not produce
any uniform effect on the PKC-mediated signal transduction;
rather pleiotropic effects on different signal pathways should
be expected. The explanation of the interaction of NS3 with the
intracellular signal pathways is further complicated by the fact
that NS3 forms complexes with target proteins of protein ki-

REFERENCES
Science 244, 359–362
2057–2061
5. Borowski, P., Heiland, M., Oehlmann, K., Becker, B., Kornezyk, L., Feucht,
6. Takamizawa, A., Mori, C., Puhe, I., Manabe, S., Murakami, S., Fujita, J.,
1105–1113
Acad. Sci. U. S. A. 82, 4379–4383
2838–2843
1837–1840
Chem. Hoppe-Seyler in press
Chem. Hoppe-Seyler 380, 403–412
358–365
33, 620–624
76, 4350–4354
921–926
Chem. 193, 265–275
26. Krawczyński, K., Beach, M., Bradley, D., Kuo, G., di-Bisceglie, A. M., Houghton,
terology 103, 622–629
5036–5041
U. S. A. 88, 2997–4000
492–496
417–422
35. Yao, N., Hesson, T., Cable, M., Hong, Z., Kwong, A. D., Le, H. V., and Weber.
38. Rauge, P., Labourette, G., Masmoudi, A., Yoshida, I., Huang, F. L., Huang,
4161–4165
71, 4954–4961
40. Kaperon, M., Zhang, L., Ramachandra, M., Kusukawa, J., Ehner, K. E., and
1109–1117
45. Matsunami, M., Heisch, T.-Y., Zhu, N., VanArsdale, T., Huang, S. B., Jeng,
J. Virol. 71, 1301–1309
U. S. A. 78, 1067–1071
Protein Kinase C Recognizes the Protein Kinase A-binding Motif of Nonstructural Protein 3 of Hepatitis C Virus
Peter Borowski, Julian Schulze zur Wiesch, Kerstin Resch, Hubert Feucht, Rainer Laufs and Herbert Schmitz

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