Viral Fusion Peptides Induce Several Signal Transduction Pathway Activations That Are Essential for Interleukin-10 and Beta-Interferon Production

Mariateresa Vitiello[a]   Emiliana Finamore[a]   Annarita Falanga[b]   Katia Raieta[a]
Marco Cantisani[b]   Francesco Galdiero[a]   Carlo Pedone[b]   Marilena Galdiero[a]
Stefania Galdiero[b]

Departments of [a]Experimental Medicine, Section of Microbiology and Clinical Microbiology,
Second University of Naples, and [b]Biological Sciences, Division of Biostructures, University of Naples
'Federico II' and Institute of Biostructures and Bioimaging, CNR, Naples, Italy

Abstract

Objectives: The deciphering of intracellular signaling pathways that are activated by the interaction between viral fusion peptides and cellular membranes are important for the understanding of both viral replication strategies and host defense mechanisms. ***Methods:*** Fusion peptides of several enveloped viruses belonging to different virus families were prepared by standard 9-fluorenylmethoxycarbonyl polyamine solid-phase synthesis and used to stimulate U937 cells in vitro to analyze the phosphorylation patterns of the signaling pathways (PKC, Src, Akt, and MAPK pathways). Immunoprecipitation and Western blotting were carried out by using phosphospecific antibodies. All samples were also assayed for the presence of IL-10 and IFN-β by ELISA and activation of nuclear factors (AP-1 and NF-κB). ***Results:*** We have demonstrated that hydrophobic domains of fusion proteins are able to induce several transduction pathways that lead to cytokine (IFN-β and IL-10) production, an event that appears to be dependent on early activation of AP-1 and NF-κB. ***Conclusions:*** The results obtained on the signaling activity of fusion peptides from different viruses enabled us to shed some light on the complex mechanism of viral entry and more precisely we focused on the exact signaling event induced by hydrophobic domains characteristic of fusion peptides interacting with the cell membrane.

Copyright © 2010 S. Karger AG, Basel

Introduction

Host cell responses to pathogenic viruses are commonly mediated by phosphorylation-regulated signaling pathways affecting, for example, changes in gene expression patterns. These signaling processes can be initiated by the cell as a defense against a viral pathogen, but can also be used by the virus to support its replication.

Interaction of viral surface proteins with host cellular surfaces has been shown to initiate a cellular reaction [12, 13] that leads to the first wave of cytokines production such as interleukins (ILs), tumor necrosis factor-α (TNF-α), and interferons (IFNs).

The anti-inflammatory cytokine IL-10 is a pleiotropic cytokine playing an important role as a regulator of lymphoid and myeloid cell function. Due to its ability to block

Marilena Galdiero
Department of Experimental Medicine, Section of Microbiology and
Clinical Microbiology, Second University of Naples
IT–80138 Naples (Italy)
Tel. +39 081 566 7646, Fax +39 081 566 5662, E-Mail marilena.galdiero@unina2.it

cytokine synthesis and several accessory cell functions of macrophages, IL-10 is a potent suppressor of the effector functions of macrophages, T cells and NK cells [22].

Many viruses as well as several viral envelopes or particles can induce the activation of innate host defense pathways that results in the production of type I IFNs, among these IFN-α and IFN-β. In particular, induction of IFN-β gene expression is a tightly regulated process, and previous studies have identified the signal transduction pathway TANK-binding kinase-1 (TBK-1)/IFN regulatory factor-3 (IRF-3) as essential to the activation of IFN-β gene expression [33].

In addition to IRF-3 activation, efficient induction of IFN-β usually requires the activation of the nuclear transcription factor-κB (NF-κB) [11] and of the transcription factor-2 (ATF-2/cJun) [5], suggesting that it is an essential component in the innate immune response to virus infection.

The activator protein-1 (AP-1) transcription factor belongs to a large family of structurally related transcription factors that includes c-Fos, c-Jun and c-Myc. AP-1 consists of various combinations of Fos and Jun family members (i.e. c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD) that dimerize via a leucine zipper domain and bind to DNA via a specific target DNA sequence [10].

In particular, these transcription factors become activated by tyrosine and serine/threonine phosphorylation, and involve primarily non-receptor protein tyrosine kinase (NT-PTK), protein kinase C (PKC), and Janus-activated kinase (JAK), initiating further downstream signaling cascades, such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) that regulate several responses including mitosis, apoptosis, motility, proliferation, differentiation and many others. It is not surprising, therefore, that many viruses target the PI3K and MAPK pathways as a means to manipulate cellular function [4, 24].

Four different members of the MAPK family, which are organized in separate cascades, have been identified to date. Three of them, extracellular-signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 have been reported to be activated upon viral infection [24, 30]. ERKs are activated by many viruses including HCMV, Kaposi sarcoma-associated herpesvirus (KSHV), hepatitis B and C viruses, papilloma virus, adenovirus, influenza virus, respiratory syncytial virus (RSV), and human immunodeficiency virus (HIV). P38/JNK MAPKs activation has been documented for infections with rhinovirus, herpesviruses, HIV, adenovirus, influenza virus, and hepatitis B virus.

Many enveloped viruses trigger upon binding to their specific cellular receptors a fusion reaction between the viral envelope and the cell membrane, a process that involves structural modifications and exposure of small stretches of hydrophobic amino acids. The fusion reactions are directed by fusion proteins that undergo consistent structural modifications that lead to the exposure of small stretches of hydrophobic aminoacids, the fusion peptides, which interact with the opposing lipid bilayer and are involved in the initial stages of virus penetration.

Three classes of fusion proteins have been identified so far. Class I comprises many unrelated virus families such as paramyxoviruses, orthomyxoviruses, retroviruses and filoviruses class II applies to the Flaviviridae family, which comprises the tick-borne encephalitis virus (TBE), the dengue virus, the yellow fever virus, the West Nile virus, and the hepatitis C virus and the Togaviridae family, of which the best known are the Semliki Forest virus and the rubella virus; class III fusion proteins comprises rhabdoviruses and herpesviruses [7, 34].

In this study, the effect of the binding on the cell surface of peptides with known fusogenic activity on the activation of signaling pathways was examined. A set of known fusion peptides from different virus families was synthesized and the activation of several signal transduction pathways investigated.

Materials and Methods

Cell Lines

U937 monocytes (ATCC CRL-1593.2) were cultured at 37° in 5% CO_2 in RPMI 1640 (Gibco) with 10% heat-inactivated fetal calf serum, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml) and differentiated as previously described [6]. Before treatment of the cells, the serum concentration was reduced to 5% for 24 h at 37° and then further reduced to serum-free media for at least 10–12 h. This should prevent most of the interference from serum factors on the phosphorylation state of the proteins in the signaling cascade.

Peptide Synthesis and Purification

Fusion peptides corresponding to a set of different viruses were prepared by standard 9-fluorenylmethoxycarbonyl polyamine solid-phase syntheses, using a PSSM8 multispecific peptide synthesizer (Shimadzu Corporation Biotechnology Instruments Department, Kyoto, Japan). The TGA resin (substitution 0.3 mmol/g) was used as the solid-phase support, and syntheses were performed on a scale of 100 μmol. All amino acids, 4 equivalents relative to resin loading, were coupled according to the TBTU/HOBT/DIEA method: 1 equivalent of Fmoc-amino acid, 1 equivalent of TBTU, 1 equivalent of HOBT (1 M HOBT in DMF) and 2 equivalents of DIEA (2 M DIEA in DMF). The Fmoc-protecting group was removed with 30% piperidine in DMF (v/v).

Table 1. Fusion peptide sequences

Peptide	Virus family	Virus name	Sequence
Class I			
FP-HIV-1	Retroviridae	human immunodeficiency virus 1	NH_2-AVGIGALFLGFLGAA-$CONH_2$
FP-SeV	Paramyxoviridae	Sendai virus	NH_2-FFGAVIGTIALG-$CONH_2$
FP-MeV	Paramyxoviridae	measles virus	NH_2-FAGVVLAGAALG-$CONH_2$
FP-NDV	Paramyxoviridae	Newcastle disease virus	NH_2-LIGAIIGGVALG-$CONH_2$
FP-RSV	Paramyxoviridae	respiratory syncytial virus	NH_2-FLGFLLGVGSAI-$CONH_2$
FP-IV	Orthomyxoviridae	influenza virus	NH_2-GLFGAIAGFIEN-$CONH_2$
FP-EBOV	Filoviridae	Ebola virus	NH_2-GAAIGLAWIPYFGPAAE-$CONH_2$
Class II			
FP-SFV	Togaviridae	Semliki forest virus	NH_2-DYQCKVYTGVYPFMWGGAYCFCD-$CONH_2$
FP-TBEV	Flaviviridae	tick-borne encephalitis virus	NH_2-DRGWGNHCGLFGKGSIVACVKAA-$CONH_2$
Class III			
FP-VSV	Rhabdoviridae	vesicular stomatitis virus	NH_2-QGTWLNPGFPPQSCGYATV-$CONH_2$

Peptides were fully deprotected and cleaved from the resin by hydrofluoric acid treatment (89% TFA solution containing 5.5% thioanisole, 3.3% ethandithiol and 2.2% anisole as scavengers); the crude peptides were precipitated with ice-cold ethyl ether, filtered, re-dissolved in water and lyophilised. The crude peptides were purified to homogeneity by preparative reverse-phase high-pressure liquid chromatography (HPLC) on a Waters Delta Prep 3000 chromatographic system, equipped with an UV Lambda Max Mod. 481 detector. The samples were injected on a Jupiter (Phenomenex) C18 column (21.20 mm × 25 cm, 15 μm) eluted with a H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) solvent mixture. A linear gradient from 20 to 75% of B over 55 min at a flow rate of 20 ml min^{-1} was employed. The collected fractions were lyophilised to dryness and analyzed by analytical reverse-phase HPLC on a Shimadzu class-LC10 equipped with a diode array detector SPD-M10AV using a Phenomenex C18 analytical column (10 × 250 mm, 10 μm); a linear gradient from 20 to 75% of B over 55 min at a flow rate of 1 ml/min was used. The identity of purified peptides was confirmed by Maldi spectrometry. All purified peptides were obtained with high yields (50–60%). A scrambled peptide of HIV-1 fusion peptide was also synthesized as a control to evaluate the sequence dependency of results. Table 1 shows the sequences of all the synthesized peptides.

All the peptides were detoxified before being tested on cells. Detoxification was performed by using Detoxy Gel Affinity Pak columns supplied by Pierce (Rockford, Ill., USA).

Peptide Cell Stimulation and Preparation of Cell Lysates
The peptides have been used to stimulate cells in vitro to analyze the phosphorylation patterns of the signaling pathways. U937 cells (3×10^6 cells/ml) were stimulated with each fusion peptides at concentrations comprised between 2 and 20 μM and at different time points. The time points and concentrations have been determined in preliminary experiments. In the further analysis we tested peptides (10 μM) for 10 min. After incubation, the cells were washed twice with ice-cold PBS without Ca^{2+} and Mg^{2+}, and resuspended in 150 μl of appropriate kinase lysis buffer. Lysates were transferred to ice-cold microcentrifuge tubes, vortexed, incubated for 10 min in ice and centrifuged at 16,000 g for 2 min at 4° to remove insoluble components.

Kinase Assays
Cell lysates were precleared with protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc., Calif., USA) (20 μl) for 45 min. Immunoprecipitation was done with the appropriate antibodies and beads at 4° overnight with gentle rotation. After incubation, the beads were pelleted (6,000 g), washed three time with 500 μl of specific lysis buffer, and boiled in 20 μl of Laemmli sample buffer with 5% β-mercaptoethanol for 5 min. Lysate samples containing 50 μg of protein were separated on SDS 15% PAGE gels as described by Laemmli [17].

Following electrophoresis, the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 5 min, and then the proteins were transferred to polyvinylidene difluoride membranes (pore size, 0.45 μm) overnight at 30 V and 4°. Blots were blocked for 1 h at room temperature in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris-HCl [pH 7.5]) containing 1% bovine serum albumin (BSA) plus 1% blotting-grade blocker nonfat milk (Bio-Rad Laboratories), and subsequently membranes were washed twice with TBS containing 0.05% Tween 20 (TTBS) before incubation with anti-phosphorylated kinase antibodies diluted 1:2,000 in TBS containing 1% BSA for 1 h at room temperature. After being washed six times with TTBS for 3 min, the polyvinylidene difluoride membranes were incubated at room temperature for 2 h with specific immunoglobulin G (IgG)-horseradish peroxidase secondary antibodies diluted 1:3,000. They were then washed six times with TTBS and twice with PBS for 5 min. Thereafter, proteins were visualized with enhanced chemiluminescence on Kodak X-OMAT LS film.

Immunoprecipitation and western blotting were carried out by using phospho-specific antibodies as follows: anti-phospho-PKC antibody detects endogenous levels of PKC α, βI, βII, γ, δ, ε, η and θ isoforms only when phosphorylated at a residue homologous to Thr514 of human PKCα (Cell Signaling Technology, Inc.);

anti-phospho-Src family antibody recognizes a synthetic phosphopeptide corresponding to residues surrounding Tyr416 of human Src (Cell Signaling Technology, Inc.); anti-phospho-Akt, which recognizes phosphorylation on Ser473 (Cell Signaling Technology, Inc.); anti-phospho-p44/p42 which is a mouse monoclonal antibody raised against Thr202 of p44 (ERK1) and Tyr204 of p42 (ERK2) (Cell Signaling Technology, Inc.); anti-phospho-p38 antibody (Santa Cruz Biotechnology, Inc.) which is a rabbit polyclonal antibody raised against a peptide mapping at the amino terminus of p38 of mouse origin identical to the corresponding human sequence and is directed against Thr180 and Tyr182-phosphorylated p38 (Santa Cruz Biotechnology, Inc.); and anti-phospho-JNK antibody, which is a mouse monoclonal IgG1 antibody raised against a peptide corresponding to a short amino acid sequence phosphorylated on Thr183 and Tyr185 of JNK of human origin (Santa Cruz Biotechnology, Inc.).

Measurement of Cytokine Concentration

All assays were carried out using U937 cells (3×10^6 cells/ml) stimulated with viral fusion peptides (10 μM) and incubated for 24 h at 37° in 5% CO_2. After incubation, samples were centrifuged at 1,800 rpm at 4° for 10 min and the supernatants were collected and stored at –70°. All samples were assayed for the presence of IL-10 and IFN-β by ELISA, according to the manufacturer's instructions. We used Human Interferon-β (Hu-IFN-β) ELISA kit from PBL Biomedical Laboratories and Human IL-10 ELISA kit from Bender MedSystem.

Standard and sample dilutions were performed at least four times for each individual cell-stimulation assay.

AP-1 and NF-κB Activation Analysis

To detect and quantify AP-1 and NF-κB activation in U937 cells, we used ELISA-based Trans-Am transcription factor kits (Active Motif, Carlsbad, Calif., USA) according to the manufacturer's recommendations. 1 μg of protein was added to the wells of a 96-well plate coated with immobilized oligonucleotide containing a TPA-responsive element (TRE; 5'-TGAGTCA-3') or the NF-κB consensus site (5'-GGGACTTTCC-3') according to the transcription factors analyzed [3, 25]. After a 1-hour incubation period at room temperature, the wells were washed 3 times with the washing buffer included on the kit and 100 μl of the provided anti c-Fos and anti c-Jun or anti-p65 and anti-p50 antibodies was added at a 1:1,000 dilution. The plate was incubated at room temperature for 1 h and the wells were washed 3 times. HRP-conjugated anti-rabbit IgG was added at a 1:1,000 dilution and incubated for 1 h at room temperature. Cells were washed 4 times and developing solution was added, followed by the stop solution. The amount of AP-1 or NF-κB activation was measured at 405 nm in an HTS 700 BioAssay reader (Perkin Elmer, Norwalk, Colo., USA).

In preliminary experiments, the Trans-Am kits showed a good correlation with an EMSA in detecting the DNA binding capacity of AP-1 and NF-κB. The optimal time of stimulation (1 h) and peptides concentration (10 μM) used in the AP-1 and NF-κB ELISA were determined in pilot assays.

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) assay was carried out according to the manufacturer's instructions by using a cytotoxicity detection kit (Roche Diagnostic GmbH, Roche Molecular Biochemicals). LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into cell culture supernatant when the plasma membrane is damaged. LDH activity was determined by a coupled enzymatic reaction whereby the tetrazolium salt (INT) was reduced to formazan. An increase in the number of dead or damaged cells resulted in an increase in LDH activity in the culture supernatant. The amount of LDH shows that treated and untreated cells are healthy.

Endotoxin Contamination

All solutions and peptide preparations used in our experiments were tested for the presence of endotoxin using a Limulus amoebocyte lysate (LAL) gel-clot assay (Associates of Cape Cod, Inc.; distributed by PBI International, Milan, Italy) as described by Yin et al. [35]. The lower detection limit of this assay was 0.1 IU/ml.

Reproducibility

Gels were scanned for densitometry analysis by Sigma Gel software and the results shown are an average of triplicate experiments. The results were expressed as mean values ± SEs of three independent observations.

Results

Peptide Selection

Specific ligand-receptor interactions result in activation of signaling pathways; thus, we selected fusion peptides belonging to each of the known viral classes of fusion glycoproteins in order to verify whether during the interaction with the cell surface, they were sufficient to induce phosphorylation of PKC, Src, Akt, and MAPK pathways, activation of nuclear factor (AP-1 and NF-κB), and cytokine release (IL-10 and IFN-β). To address this question we synthesized fusion peptides of several viruses which had already been identified and their sequences are reported in table 1. In all experiments, we tested a scrambled sequence of the HIV-1 fusion peptide (scrambled: NH2-FLAGIVGALGAAFGL-CONH) and the lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, St. Louis, Mo., USA) in order to verify the reliability of our experiments demonstrating that the activation of signal transduction pathways and cytokine release were only inducted by active sequences.

In addition, a peptide derived from the porin P2 of *Haemophilus influenzae* type b (peptide L1: NH_2-NS-TVDNQKQQHGALR-$CONH_2$) and a peptide derived from a non-fusion viral glycoprotein, namely Sendai virus glycoprotein HN (peptide non-FP: NH_2-NSEVDLDHPF-SALYP-$CONH_2$) were included in our study.

By using the LAL test, endotoxin levels of cell culture and peptide solutions resulted lower than 0.1 IU/ml (data not shown).
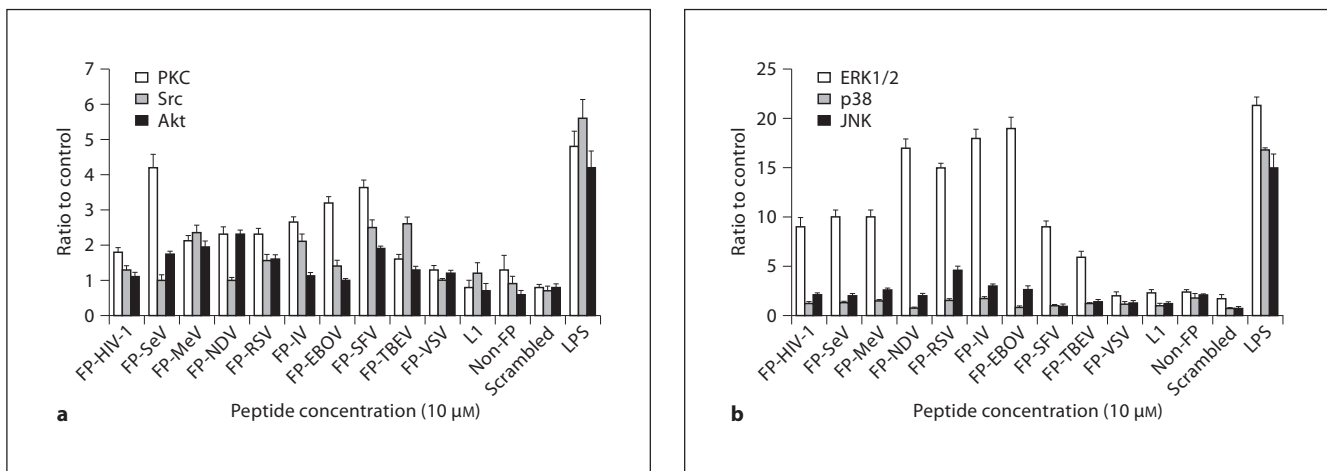
Fig. 1. Signal transduction pathway activations by viral fusion peptides. PKC, Src, Akt (**a**) and MAPKs activation (**b**) in U937 cells in response to peptides (10 μM) after 10 min of stimulation. Cellular lysates were harvested, immunoprecipitated by phosphospecific antibodies, analyzed on SDS-PAGE and immunoblotted with appropriate phosphospecific antibodies. Gels were scanned for densitometry analysis by Sigma Gel software, and the ratio of the value for each peptide stimulation to the value for a non-stimulated control is shown. The data are presented as an average from three separate experiments, and the error bars indicate the standard error of the means. Band intensity was quantified densitometrically.

Signal Transduction Pathway Activation by Viral Fusion Peptides

In order to investigate peptide-induced protein phosphorylation in whole-cell lysates from U937 cells, we assayed immunoprecipitation of phosphorylated proteins followed by Western blot analysis. To determine the dose response and kinetics, U937 cells (3×10^6 cells/well) were treated with peptides at a concentration comprised between 2 and 20 μM and for different time periods (3, 10, 30, 60 min).

The duration of the treatment with peptides at the concentrations used was not toxic for the cells; in fact, the treatment did not induce any significant release of LDH in the cell supernatants (data not shown). Concentrations of 50 μM were still not toxic for cells, as verified by LDH release, and did not further increase the enzyme phosphorylation.

PKC activation being an early marker of cell response to interacting external molecules, we assayed PKC phosphorylation after cell treatment with viral fusion peptides. All fusion peptides studied induced rapid PKC phosphorylation as early as 3 min after treatment. We followed the phosphorylation status of PKC for up to 60 min (data not shown) after treatment and found that the response reached its maximum after 10 min. We next examined PKC phosphorylation at different peptide concentrations (data not shown). The dose-response experiments showed that the optimal peptide concentration to induce kinase phosphorylation resulted 10 μM (data not shown). A standard concentration of 10 μM and stimulation time of 10 min were chosen for subsequent experiments (fig. 1a, b).

We observed that PKC phosphorylation was induced by almost all peptides (except FP-VSV) and that FP-SeV caused the most significant increase. Moreover, a significant phosphorylation of Akt (PKB) was evident after treatment with FP-SeV, FP-MeV, FP-NDV, FP-RSV and FP-SFV.

To assess the eventual activation of NT-PTK cascade as a consequence of the interaction of fusion peptides with the lipid domain of cell surface, we assayed Src phosphorylation. As shown in figure 1a, Src was significantly activated by FP-HIV-1, FP-MeV, FP-RSV, FP-IV, and FP-EBOV, but mainly by FP-SFV, FP-TBEV and FP-MeV.

To further determine the involvement of MAPK cascades, we performed a kinase assay using cell lysates upon fusion peptide treatment. The results indicate that ERK1/2 is the most activated kinase. All peptides (except FP-VSV) strongly increased ERK1/2 phosphorylation. In particular FP-NDV, FP-IV and FP-EBOV caused an activation ~20-fold higher than the control (fig. 1b).
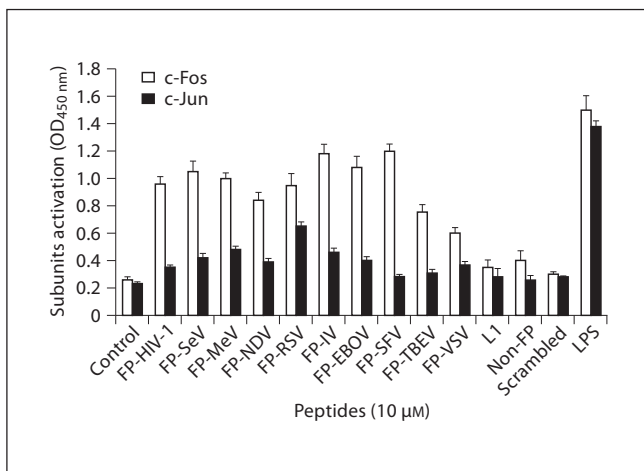
Fig. 2. AP-1 activation by viral fusion peptides. Effect of peptides on binding of AP-1 subunits to an AP-1-binding consensus sequence. U937 cells were stimulated with viral peptides at 10 μM. Cell lysates (10 μg/ml) were tested for binding of the activated c-Fos or c-Jun subunits to an AP-1 consensus sequence using the Trans-Am AP-1 ELISA kit. The experiment was performed in the presence of soluble wild-type or mutated consensus oligonucleotides. The results are expressed as specific binding (absorbance measured in the presence of the mutated oligonucleotide minus that measured in the presence of the wild-type oligonucleotide). The results are shown as means ± SEs of triplicate determinations.
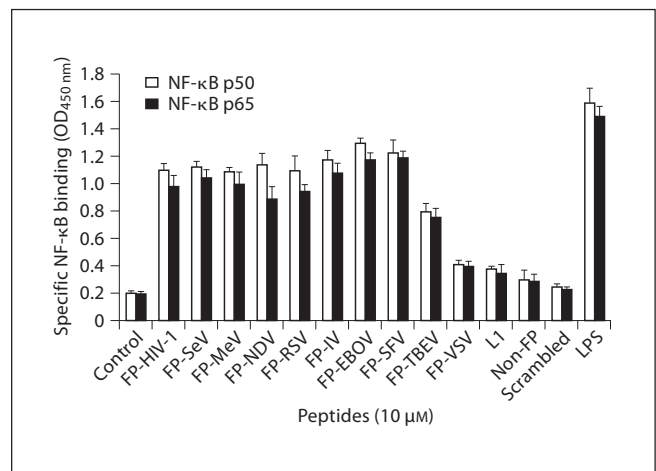

Fig. 3. NF-κB activation by viral fusion peptides. Effect of peptides on binding of NF-κB subunits to an NF-κB-binding consensus sequence. U937 cells were stimulated with viral peptides at 10 μM. Cell lysates were tested for binding of the activated p50 or p65 subunits to an NF-κB consensus sequence using the Trans-Am NF-κB ELISA kit. The experiment was performed in the presence of soluble wild-type or mutated consensus oligonucleotides. The results are expressed as specific binding (absorbance measured in the presence of the mutated oligonucleotide minus that measured in the presence of the wild-type oligonucleotide). The results are shown as means ± SEs of triplicate determinations.

Moreover, as shown in figure 1b, JNK was slightly activated by all tested peptides, and only FP-RSV resulted in an activation 5-fold higher than the control. Similar assays to determine p38 activity indicated no change in the levels of its phosphorylation as a results of peptide stimulations (fig. 1b).

AP-1 and NF-κB Activation by Viral Fusion Peptides in U937 Cells

To selectively analyze the regulation of virus-induced AP-1 and NF-κB activation, an ELISA-based Trans-Am technology from nuclear lysates of U937 cells stimulated by viral fusion peptides was performed.

These transcription factors are supposed to be involved in the expression of proinflammatory cytokine genes. Therefore, in order to demonstrate AP-1 and NF-κB activation, we investigated fusion peptides induction of AP-1 c-Fos/c-Jun subunits and NF-κB p50/p65 subunits in whole-cell extracts using Abs specific for epitopes that are accessible only when the nuclear factors are phosphorylated and bound to their target DNA. Following treatment of U937 cells with synthesized viral fusion

peptides at 10 μM, AP-1 and NF-κB binding significantly increased by 30 min, was maintained at the same level by 60 min, and returned to background levels by 120 min (data not shown).

In particular, we found that all the peptides were able to activate significantly both AP-1 (fig. 2) and NF-κB (fig. 3).

IL-10 and IFN-β Release by U937 Cells Treated with Viral Fusion Peptides

Supernatants from by viral fusion peptides stimulated U937 cells were collected at 24 h and IL-10 and IFN-β measured by ELISA. In figure 4, we report data obtained using U937 cells (3×10^6 cells/ml) stimulated with a concentration of 10 μM for each peptide; the results obtained show that all the peptides were able to induce a significant release of IFN-β and IL-10.

The concentrations of peptides used as well as the duration of the treatment were not toxic for the cells; in fact, the treatment did not induce any significant release of LDH in the cell supernatants.
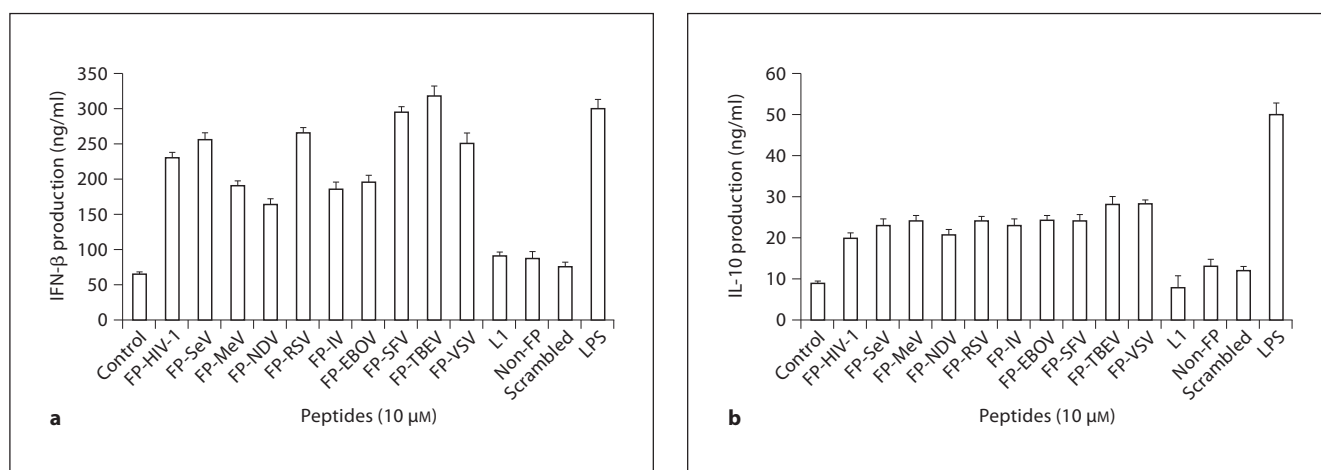
Fig. 4. IFN-β (**a**) and IL-10 (**b**) release by viral fusion peptides. U937 cells (3 × 10⁶ cells/ml) were stimulated with peptides (10 μM) and incubated for 24 h at 37° in 5% CO_2. All samples were assayed for the presence of IFN-β by ELISA, using human interferon-β (Hu-IFN-β) ELISA kit from PBL Biomedical Laboratories (**a**), and IL-10 by ELISA, using human IL-10 ELISA kit from Bender MedSystem (**b**). The results shown are averages from three independent experiments, and the error bars indicate the SEMs.

Discussion

Among the first consequences of viral binding to surface receptors there are changes in the structure of many virus particles. For many enveloped viruses, including retroviruses and herpesviruses, receptor binding can trigger a fusion reaction between the viral membrane and the plasma membrane; for other viruses, including influenza virus, vesicular stomatitis virus and Semliki forest virus, the penetration starts with the fusion of viral membranes with cellular endosomes upon exposure to low pH. As a consequence, fusion glycoproteins undergo consistent structural modifications that lead to the exposure of small stretches of aminoacids, the fusion peptides, which interact with the opposing lipid bilayer and initiate the fusion reaction [7, 34].

To our knowledge, the present study is the first one addressing the role of fusion peptides in the complex events of signaling that are activated by viral infections. The analysis of the role played by fusion peptides from different virus families may also help in understanding the complex mechanism of viral entry.

The experimental results demonstrated that hydrophobic domains of fusion proteins are able to induce several transduction pathways and cytokine production (such as IL-10 and IFN-β) during the earliest events of the viral life cycle. Activation of these pathways seem to be directly related to the sequence and conformation of the viral fusion peptides, in fact the HIV-scrambled peptide did not induce activation of any of the analyzed pathways.

Several viral proteins, such as hepatitis B virus HBx [32], Epstein-Barr virus latent membrane protein-1 [36], HCMV IE1 [14], HSV-2 ICP10 PK [29], and SARS coronavirus nucleocapsid protein [8], have been shown to activate AP-1.

Also for NF-κB, the expression of a single viral protein is sufficient to its activation as seen with Tax from HTLV-1 [2], E3/19K from adenovirus [23], and HBx from hepatitis B virus [15]. However, the underlying molecular mechanisms appear to be different among them and, hence, previous works on signaling cascades during viral entry have mainly focused on either whole viruses or viral proteins.

Pleschka [24] showed that several human pathogenic RNA viruses including influenza, Ebola, hepatitis C and SARS corona virus induced the Raf/MEK/ERK signal transduction cascade. ERK pathway activation is required at different levels during HIV infection [18] as well as in RSV-induced early gene expression [16]. Monick et al. [19] reported that RSV infection caused two separate peaks of ERK activity both immediately following viral binding (10–30 min) and later during active viral replication (24–48 h) matched by activation of multiple PKC isoforms. Among such kinases, the PKC superfamily is responsible for diverse regulatory roles in many cellular

processes and has also been implicated in virus entry. In fact, the entry of several enveloped viruses, including rhabdoviruses, alphaviruses, herpesviruses and influenza virus, requires PKC activation upon binding to host cell surface receptors [1, 28].

Taken together, all literature data highlight the role of PKC and ERK pathways in the efficient infection and replication of certain viral species and are consistent with the results reported in this study of fusion peptides.

The results show that immediately following treatment of U937 cells with peptides of class I (FP-HIV, FP-SeV, FP-MeV, FP-NDV, FP-RSV, FP-IV, FP-EBOV) and class II (FP-SFV, FP-TBEV) PKC and ERK1/2 are activated; moreover, class I peptides also induce the activation of JNK. Our results show that none of the peptides was able to induce activation of p38.

Moreover, fusion peptides of both class I and II are able to induce activation of AP-1; in particular all the peptides were able to activate more significantly c-Fos and less significantly c-Jun. Though the predominant mechanism of activation of the transcription factor AP-1 is usually through phosphorylation of c-Jun by JNK, recently ERK was also shown to phosphorylate c-Fos and thereby activate AP-1 [20, 21].

All the peptides significantly activate the Src signaling pathway. Src family kinases are proto-oncogenic enzymes that were initially characterized in the context of cell growth and differentiation. The activation of Src should result in the activation of downstream cellular targets and their physiological effects. Here a higher ERK1/2 activity was demonstrated in U937 cells according to other investigators that have shown that activation of MAPK in cells follow the Src-dependent pathway.

Akt affects multiple cellular targets that increase metabolism, growth, synthetic processes and proliferation and suppress apoptosis. All of these processes are beneficial to viral lytic replication, thus it is not surprising that viruses have developed means to activate Akt during lytic infections. Sun et al. [31] have recently reported that Akt activity is required for optimal replication of paramyxovirus and other nonsegmentated negative-strand viruses; moreover, Akt has been demonstrated to play a fundamental role also in other viruses such as herpesviruses, adenoviruses, polyomaviruses and poxviruses [4]. The results show that Akt phosphorylation is evident following treatment with FP-MeV, FP-NDV and FP-RSV fusion peptides (all members of the Paramyxoviridae family).

Particularly interesting appear the results obtained for a segment of VSV glycoprotein G, which has the characteristic of a class III fusion protein. The peptide is not able to induce significantly the activation of any of the kinases studied here, although it is able to induce the release of cytokines.

FP-VSV correspond to one of the two predicted fusion loops of VSV glycoprotein G [26, 27]. The resolution of the crystal structure of the protein resembles the structure of glycoprotein B of HSV-1 and points out to a possible cooperation of two nonconsecutive segments both on the tip of their relative loop in order to induce lipid destabilization during the event of membrane fusion [9, 26]. Class III of fusion proteins seems to operate with a different mechanism from the other two, therefore it is interesting to observe that cytokine production is obtained through different signaling pathway.

The results strongly suggest that the analyzed fusion peptides activate a signaling cascade that leads to the activation of different components, regulating the transcription factors AP-1 and NF-κB. It has been well established that these transcription factors are a hallmark of most infections including viral infections and play a central role in virus-dependent cytokine expression and pathology. Thus, the release of IL-10 and IFN-β, following treatment of U937 cells with peptides, has also been analyzed. The results demonstrate that both cytokines are significantly released after stimulation with all the selected peptides.

The data reported show the presence of a strong correlation between the fusion peptides, and the activation of signaling pathways and the release of cytokines, and further support the fundamental role played by fusion peptides in viral infections.

References

1 Ahmad R, Ennaciri J, Cordeiro P, El Bassam S, Menezes J: Herpes simplex virus-1 up-regulates IL-15 gene expression in monocytic cells through the activation of protein tyrosine kinase and PKC zeta/lambda signaling pathways. J Mol Biol 2007;367:25–35.

2 Azran-Shaish I, Tabakin-Fix Y, Huleihel M, Bakhanashvili M, Aboud M: HTLV-1 tax-induced NF-kappaB activation is synergistically enhanced by 12-O-tetradecanoylphorbol-13-acetate: mechanism and implications for Tax oncogenicity. J Mol Med 2008;86: 799–814.

3 Basbous J, Arpin C, Gaudray G, Piechaczyk Devaux C, Mesnard JM: The HBZ factor of human T-cell leukaemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity. J Biol Chem 2003;278:43620–43627.

4 Cooray S: The pivotal role of phosphatidylinositol 3-kinase-Akt signal transduction in virus survival. J Gen Virol 2004;85:1065–1076.

5 Falvo JV, Parekh BS, Lin CH, Fraenkel E, Maniatis T: Assembly of a functional beta interferon enhanceosome is dependent on ATF-2-c-jun heterodimer orientation. Mol Cell Biol 2000;20:4814–4825.

6 Galdiero M, Tortora A, Damiano N, Vitiello M, Longanella A, Galdiero E: Induction of cytokine mRNA expression in U937 cells by *Salmonella typhimurium* porins is regulated by different phosphorylation pathways. Med Microbiol Immunol 2005;194:13–23.

7 Harrison SC: Viral membrane fusion. Nat Struct Mol Biol 2008;15:690–698.

8 He R, Leeson A, Andonov A, Li Y, Bastien N, Cao J, Osiowy C, Dobie F, Cutts T, Ballantine M, Li X: Activation of AP-1 signal transduction pathway by SARS coronavirus nucleocapsid protein. Biochem Biophys Res Commun 2003;311:870–876.

9 Heldwein EE, Lou H, Bender FC, Cohen GH, Eisenberg RJ, Harrison SC: Crystal structure of glycoprotein B from herpes simplex virus 1. Science 2006;313:217–220.

10 Hess J, Angel P, Schorpp-Kistner M: AP-1 subunits: quarrel and harmony among siblings. J Cell Sci 2004;117:5965–5973.

11 Hiscott J: Convergence of the NF-kappaB and IRF pathways in the regulation of the innate antiviral response. Cytokine Growth Factor Rev 2007;18:483–490.

12 Huang CF, Lin SS, Ho YC, Chen FL, Yang CC: The immune response induced by hepatitis B virus principal antigens. Cell Mol Immunol 2006;3:97–106.

13 Irshad M, Khushboo I, Singh S, Singh S: Hepatitis C virus (HCV): a review of immunological aspects. Int Rev Immunol 2008;27:497–517.

14 Kim S, Yu SS, Lee IS, Ohno S, Yim J, Kim S, Kang HS: Human cytomegalovirus IE1 protein activates AP-1 through a cellular protein kinase(s). J Gen Virol 1999;80:961–969.

15 Kim SY, Kim JC, Kim JK, Kim HJ, Lee HM, Choi MS, Maeng PJ, Ahn JK: Hepatitis B virus X protein enhances NFkappaB activity through cooperating with VBP1. BMB Rep 2008;41:158–163.

16 Kong X, San Juan H, Behera A, Peeples ME, Wu J, Lockey RF, Mohapatra SS: ERK-1/2 activity is required for efficient RSV infection. FEBS Lett 2004;559:33–38.

17 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.

18 Mettling C, Desmetz C, Fiser AL, Réant B, Corbeau P, Lin YL: Galphai protein-dependant extracellular signal-regulated kinase-1/2 activation is required for HIV-1 reverse transcription. AIDS 2008;22:1569–1576.

19 Monick M, Staber J, Thomas K, Hunninghake G: Respiratory syncytial virus infection results in activation of multiple protein kinase C isoforms leading to activation of mitogen-activated protein kinase. J Immunol 2001;166:2681–2687.

20 Monje P, Marinissen MJ, Gutkind JS: Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. Mol Cell Biol 2003;23:7030–7043.

21 Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J: Molecular interpretation of ERK signal duration by immediate early gene products. Nat Cell Biol 2002;4:556–564.

22 O'Garra A, Barrat FJ, Castro AG, Vicari A, Hawrylowicz C: Strategies for use of IL-10 or its antagonists in human disease. Immunol Rev Jun 2008;223:114–131.

23 Pahl HL, Sester M, Burgert HG, Baeuerle PA: Activation of transcription factor NF-kappaB by the adenovirus E3/19K protein requires its ER retention. J Cell Biol 1996;132:511–522.

24 Pleschka S: RNA viruses and the mitogenic Raf/MEK/ERK signal transduction cascade. Biol Chem 2008;389:1273–1282.

25 Renard P, Ernest I, Houbion A, Art M, Le Calvez H, Raes M, Remacle J: Development of a sensitive multi-well colorimetric assay for active NFkB. Nucl Acids Res 2001;29:E21.

26 Roche S, Bressanelli S, Rey FA, Gaudin Y: Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science 2006;313:187–191.

27 Roche S, Rey FA, Gaudin Y, Bressanelli S: Structure of the perfusion form of the vesicular stomatitis virus glycoprotein G. Science 2007;315:843–848.

28 Sieczkarski SB, Brown HA, Whittaker GR: Role of protein kinase C betaII in influenza virus entry via late endosomes. J Virol 2003;77:460–469.

29 Smith CC: The herpes simplex virus type 2 protein ICP10PK: a master of versatility. Front Biosci 2005;10:2820–2831.

30 Sumbayev VV, Yasinska IM: Role of MAP kinase-dependent apoptotic pathway in innate immune responses and viral infection. Scand J Immunol 2006;63:391–400.

31 Sun M, Fuentes SM, Timani K, Sun D, Murphy C, Lin Y, August A, Teng MN, He B: Akt plays a critical role in replication of nonsegmented negative-stranded RNA viruses. J Virol 2008;82:105–114.

32 Tanaka Y, Kanai F, Ichimura T, Tateishi K, Asaoka Y, Guleng B, Jazag A, Ohta M, Imamura J, Ikenoue T, Ijichi H, Kawabe T, Isobe T, Omata M: The hepatitis B virus X protein enhances AP-1 activation through interaction with Jab1. Oncogene 2006;25:633–642.

33 Unterstab G, Ludwig S, Anton A, Planz O, Dauber B, Krappmann D, Heins G, Ehrhardt C, Wolff T: Viral targeting of the interferon-{beta}-inducing Traf family member-associated NF-{kappa}B activator (TANK)-binding kinase-1. Proc Natl Acad Sci USA 2005; 102:13640–13645.

34 White JM, Delos SE, Brecher M, Schornberg K: Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme. Crit Rev Biochem Mol Biol 2008;43:189–219.

35 Yin ET, Galanos C, Kinsky S, Bradshaw RA, Wessler S, Lüderitz O, Sarmiento ME: Picogram-sensitive assay for endotoxin: gelation of Limulus polyphemus blood cell lysate induced by purified lipopolysaccharides and lipid A from gram-negative bacteria. Biochim Biophys Acta 1972;261:284–289.

36 Zheng H, Li LL, Hu DS, Deng XY, Cao Y: Role of Epstein-Barr virus encoded latent membrane protein 1 in the carcinogenesis of nasopharyngeal carcinoma. Cell Mol Immunol 2007;4:185–196.