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RAPID COMMUNICATION

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Direct protein transfer to terminally differentiated muscle cells

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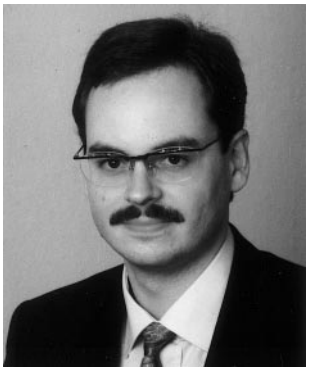

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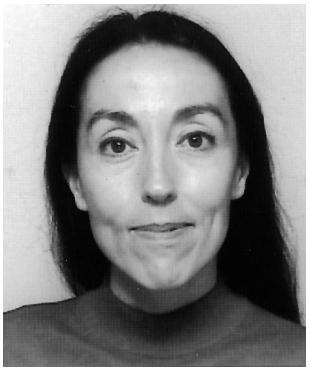

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Abstract Recently, a new approach for direct protein transfer to mammalian cells based on the herpes simplex virus type 1 protein VP22 has been described. This protein has the remarkable property of intercellular trafficking, which is independent of direct cell contacts and is also retained when fused to heterologous proteins. However, the spreading has only been described for proliferating cells and has also been controversially discussed. In this study we describe the generation of a GFP–VP22 fusion protein which is able to spread in COS-7 cells after transient transfection. Moreover, we show in coculture experiments with transfected COS-7 cells and C2C12 myotubes that this fusion protein is also able to spread into terminally differentiated skeletal muscle cells. These results suggest that VP22 might be a novel therapeutic tool for direct protein transfer not only in proliferating but also in terminally differentiated cells.

Key words GFP · Muscle · Protein delivery · Therapy · VP22

Abbreviations *CMV* Cytomegalovirus · *GFP* green fluorescent protein · *HSV-1* herpes simplex virus type 1 · *PCR* polymerase chain reaction

Introduction

Gene therapy is a potentially powerful approach to the treatment of several diseases. Its concept is based on transferring a therapeutic gene into human somatic cells to overcome a malfunction caused by mutated or absent genes. Various vectors and gene delivery systems have been established so far but they are afflicted by several limitations. Until now the applicability of gene therapy approaches has often been limited by problems of sustaining stable transgene expression over long time periods and by side-effects such as the immunogenicity of viral vectors or potential malignant transformation of target cells [1]. The direct transfer of therapeutic proteins to target cells would avoid irreversible genetic modification

(mutation) of targeted cells and would allow better control over dose and treatment duration.

Recently, the herpes simplex virus type 1 (HSV-1) tegument protein VP22 was described as a possible carrier for transporting functional proteins into mammalian cells. The carrier function of this basic phosphoprotein [2] encoded by the UL49 gene [3] is based on its unique property of intercellular trafficking [4]. Following expression in a transfected and/or infected cell, VP22 is exported from the synthesizing cell by a Golgi-independent mechanism to neighboring cells where it is localized to the nucleus. This intercellular transport has been shown to involve the actin cytoskeleton [4].

The ability of VP22 to transport heterologous proteins has already been successfully used to induce apoptosis in a cultured human osteosarcoma cell line by delivering functional p53 fused to VP22 [5]. Moreover, VP22-mediated protein transport has been used in mice to deliver thymidine kinase to neuroblastomas, rendering them sensitive to the prodrug ganciclovir and causing tumor regression [6].

The ability of VP22 to spread in mammalian cell cultures is discussed controversially. Whereas Elliott and O'Hare et al. [4] described VP22 transfer from a small subpopulation of expressing cells to every nontransfected cell in a monolayer culture, Fang et al. [7] could not detect any intercellular VP22 trafficking in cultured mammalian cells.

Furthermore, the spread of VP22 and VP22 fusion proteins has so far only been observed in proliferating, and not in terminally differentiated cells. To test the potential of VP22-mediated protein delivery to differentiated cells as a novel therapeutic tool, we constructed a translational fusion between green fluorescent protein (GFP) and VP22 and assayed its ability to spread into terminally differentiated skeletal muscle cultures. The fusion protein was able to spread in cultures of COS-7 cells after transient transfection. Coculture experiments with transfected COS-7 cells and terminally differentiated C2C12 myotubes indicate that the GFP–VP22 fusion protein is also able to spread into terminally differentiated muscle cells.

Materials and methods

Construction of GFP–VP22 expression plasmid

The UL49 gene(VP22) was amplified by polymerase chain reaction (PCR) from HSV-I strain Angelotti with primers flanking the open reading frame (ORF), which remove the stop codon and add *Eco*RI and *Xba*I sites. The PCR product was digested with *Eco*RI and *Xba*I and ligated into the respective sites of pTRE (Clontech). The VP22 gene was then cut out with *Eco*RI and *Bam*HI and cloned into the respective sites of peGFPLigEB [8] replacing the DNA ligase I insert. The resulting expression construct was named peGVP22. The predicted molecular weight of the GFP–VP22 fusion protein is 61.2 kDa.

Cell culture and transfection

COS-7 cells (African green monkey kidney fibroblast-like cells transformed with SV40T antigen; [9]) were grown in a humidified incubator at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 4.5 g/l glucose, 3.4 g/l $NaHCO_3$, 1 mM sodium pyruvate and 50 µg/ml gentamycin (GIBCO BRL). Mouse C2C12 myoblasts were grown as above except that the medium was supplemented with 20% fetal calf serum.

For differentiation experiments 2×10^5 myoblasts were plated into a p100 culture dish containing coverslips coated with 0.2 mg/ml gelatin (Sigma). Twenty-four hours later the growth medium was changed to differentiation medium (DMEM, 5% horse serum) and after another 72–96 h the myotubes were used for coculture experiments.

For transfection experiments 7.5×10^5 COS-7 cells were plated per p100 culture dish. Six hours later 1 µg of plasmid DNA was transfected using the DEAE-dextran pretreatment method as described previously [10]. Twenty-four hours after transfection, cells were trypsinized and one-third of this cell suspension was transferred onto the C2C12 myotubes. After 1 day of coculture, GFP–VP22 signals were directly analyzed with a fluorescence microscope or, alternatively, cells were fixed either for 10 min in 3.7% formaldehyde or for 5 min in cold methanol followed by counterstaining the DNA with Hoechst 33258 (Sigma).

Immunofluorescence staining and microscopy

Transfected cells were in some cases fixed and stained using rabbit anti-GFP polyclonal antibodies (Clontech and Invitrogen) followed by incubation with biotinylated anti-rabbit Ig (Amersham) and detected with streptavidin-Texas Red (Amersham) as previously described [8].

Fixed or living cells were examined with an Axioplan 2 or Axiovert 100TV microscopes (Zeiss) equipped with phase-contrast and epifluorescence optics, using 20× and 63× Plan Neofluar objectives and band-pass FITC and Hoechst filter sets. Images were collected with a cooled CCD camera (Sensicam) using the Axiovision software (Zeiss) and assembled with Adobe Photoshop and Illustrator software.

Western blot analysis

Transfected COS-7 cell monolayers were washed in phosphate-buffered saline, scraped and extracted for 30 min on ice in RIPA buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), protease inhibitors] as described [8]. The protein extracts were boiled in Laemmli sample buffer, separated on a 12% SDS-PAGE, transferred to a PVDF membrane and probed with anti-GFP antibody (Invitrogen). Reactive proteins were detected with horseradish-peroxidase-conjugated secondary antibody (SIGMA) using ECL+ detection reagents (Amersham) according to the manufacturer's instructions.

Results and discussion

The recent demonstration that the HSV-1 tegument protein VP22 is able to directly transport heterologous proteins into proliferating mammalian cells [4] prompted us to investigate whether this system could also function to deliver proteins to terminally differentiated cells.

In order to test this hypothesis, we prepared an expression plasmid in which VP22 was fused in frame at the C-terminus of an enhanced and codon-optimized

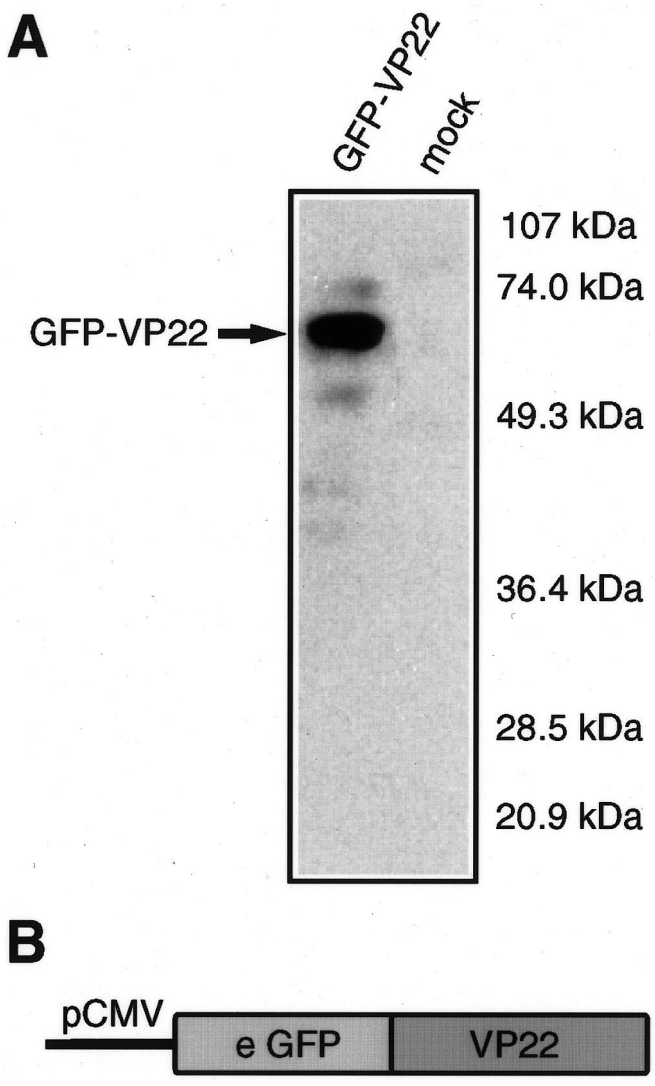

Fig. 1A,B Construction and characterization of a GFP–VP22 fusion protein. **A** Western blot analysis of whole-cell extracts of COS-7 cells transfected with peGVP22 (*left lane*) and mock (*right lane*) transfected using anti-GFP antibody. In the *left lane* a band with an apparent molecular weight of 60–65 kDa corresponding to the fusion protein reacts with the antibody. **B** Schematic representation of the GFP–VP22 fusion construct. Expression in mammalian cells is driven by the CMV promoter. (*CMV* Cytomegalovirus, *GFP* green fluorescent protein, *VP22* herpes simplex virus type 1 protein)

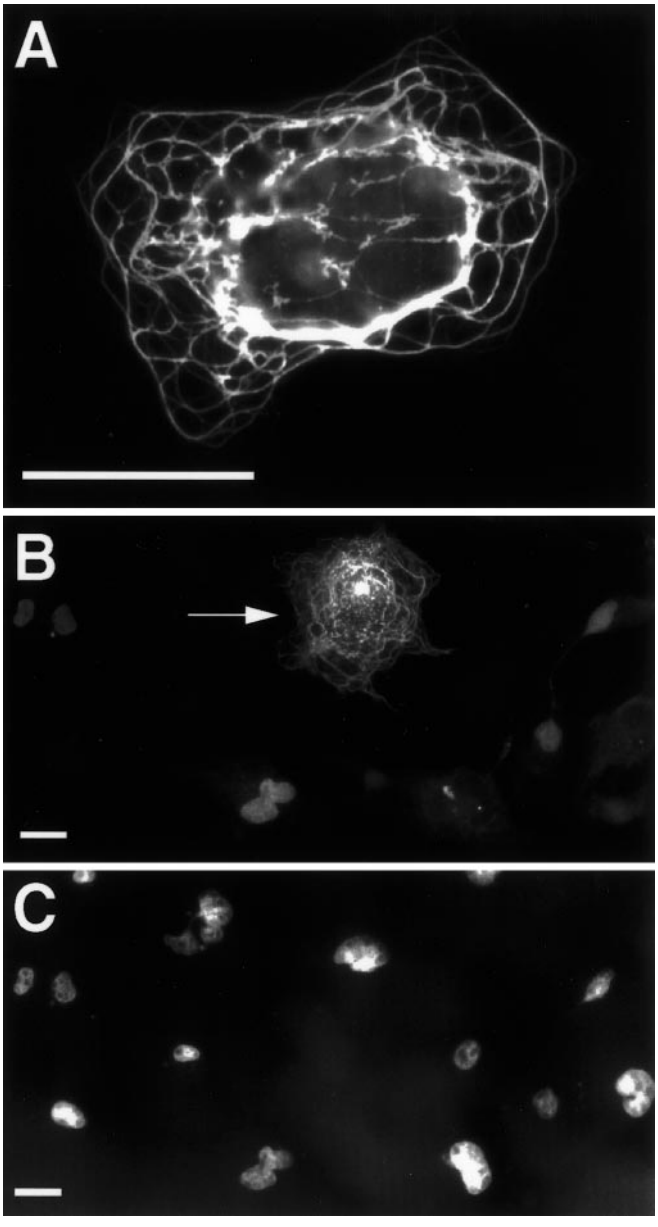

Fig. 2A–C Spreading of GFP–VP22 in COS-7 cells. **A** Transfected cell with cytoplasmic filamentous localization of GFP–VP22. **B** GFP–VP22 synthesized by a transfected cell (*arrow*) spreads into the nuclei of surrounding cells. **C** Nuclear DNA stained with Hoechst 33258 (same field as in **B**). *Scale bar* 20 μm

GFP under the control of the cytomegalovirus (CMV) promoter (Fig. 1B). After transient transfection of COS-7 cells, expression of a fusion protein of the expected size of about 61 kDa was detected by Western blot analysis using an anti-GFP antibody (Fig. 1A).

The spreading ability of VP22 in cultured mammalian cells has been the subject of recent controversy and therefore we first tested the intercellular trafficking properties of our GFP–VP22 fusion protein in proliferating cells. For that purpose, we transfected COS-7 cells with our construct and screened the monolayer for green fluorescence at different times after transfection. We observed a localization pattern similar to that described by Elliot and O'Hare [4]. In transfected cells we could observe fluorescent cytoplasmic filamentous structures, which are caused by the tight association of VP22 to the microtubules (Fig. 2A,B, arrow). This colocalization occurs during the stabilization of the microtubule network in infected or transfected cells by VP22 [11] and might play a role in the export of VP22-containing proteins. The GFP–VP22 fusion protein was also transported to neighboring cells that were not transfected, where it accumulated in the nuclei (Fig. 2B,C). Although we could confirm the intercellular trafficking properties of VP22

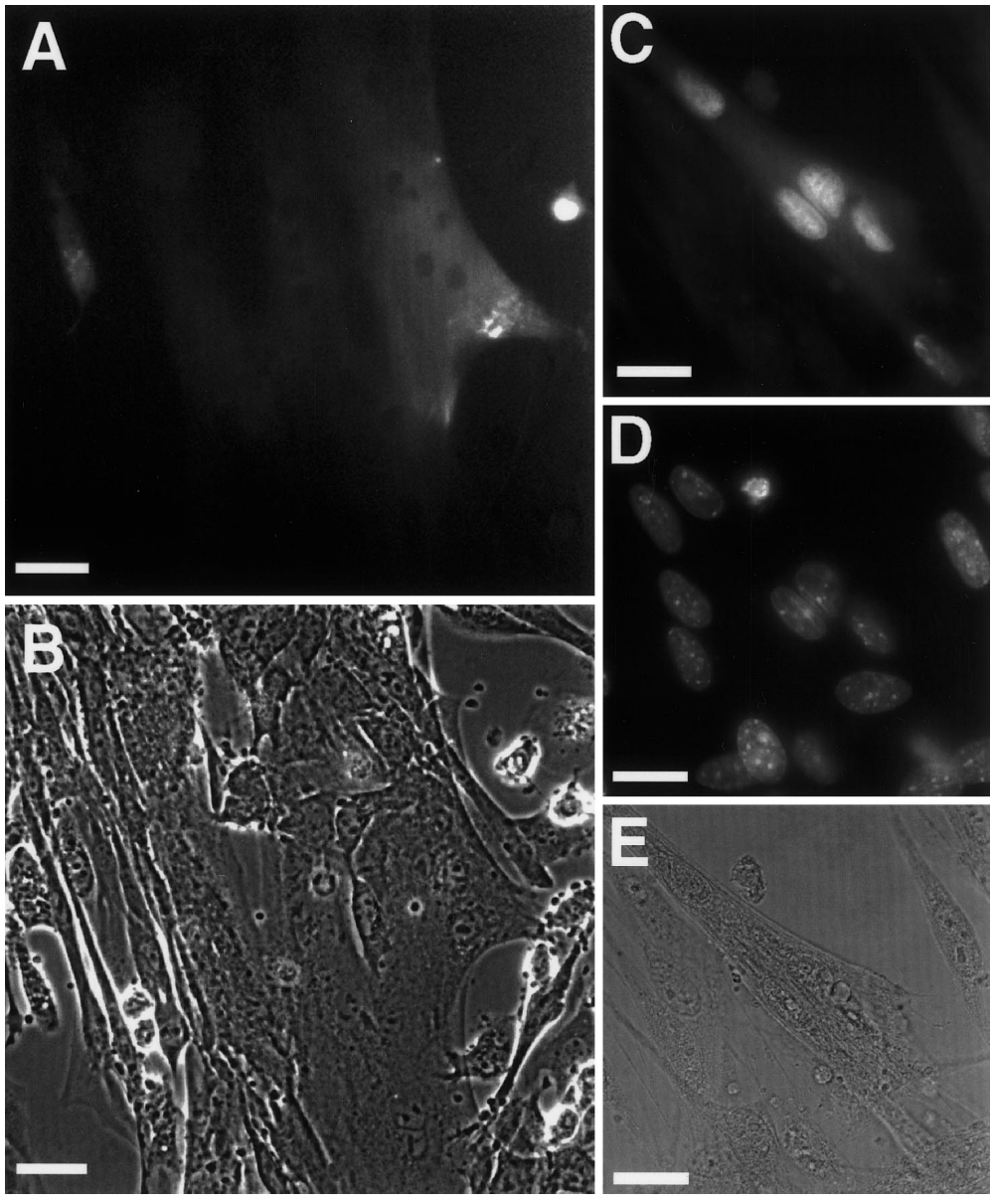

Fig. 3A–E Spreading of GFP–VP22 into terminally differentiated C2C12 myotubes. **A** Transfected COS-7 cells expressing GFP–VP22 fusion protein were plated onto skeletal myotube cultures and the next day myotubes were scanned live under the fluorescence microscope for green fluorescence. This field depicts a large myotube exhibiting a preferentially cytoplasmic localization of the GFP–VP22 fusion protein. **B** Corresponding phase-contrast image showing the multinucleated morphology of the skeletal myotubes. **C** The cocultures were fixed and counterstained with Hoechst 33258. This field shows a myotube with five nuclei that have taken up GFP–VP22 surrounded by other unfused myocytes that do not show detectable fluorescence signals. **D** Corresponding nuclear DNA labeled by Hoechst 33258 depicting the characteristic mouse chromatin organization with the centromeric heterochromatin visible as bright subnuclear dots [10]. All the nuclei visible in this field are from mouse C2C12 cells. **E** Corresponding phase-contrast image. *Scale bar* 20 μm

using GFP as a marker, the efficiency of the spreading was not 100% as reported previously [4, 12]. Since our analysis was mostly done on data from living cells, we next tested whether our detection level was sufficient to visualize most if not all GFP-containing cells. Therefore, we fixed and stained cells with two different anti-GFP antibodies to increase the sensitivity level. In both cases, living or fixed and stained cells, the GFP–VP22 fusion protein was detected in 20–50% of the cells depending on the transfection efficiency (5–15%). The latter was determined by parallel transfection or cotransfection with a plasmid encoding a marker protein not fused to VP22. Under our experimental conditions we could not observe significant differences in detection level between the live cell fluorescence and that of the formaldehyde-fixed and anti-GFP stained cells (data not shown). However, the spreading process differs from that which occurs in traditional transfection or infection methods, as it is a continuous and gradual event, with signals ranging from undetectable to strong. Thus the identification of those cells that have taken up the protein depends not only on the sensitivity of the detection method but also on the encountered background signal, which is often higher in fixed and stained cells.

Skeletal muscle and even more so myotube cultures derived from established cells lines such as C2C12 myoblasts are refractory to most transfection methods as well as to adenoviral infection (our unpublished results and, for example, [13]). To investigate whether the GFP–VP22 fusion protein can also be introduced into terminally differentiated muscle cells, we performed coculture experiments. We collected transfected COS-7 cells and added them to terminally differentiated C2C12 myotube cultures.

As outlined in Fig. 3A,B, the GFP–VP22 protein can spread into myotubes, where it accumulates preferentially in the cytoplasm. In a few myotubes, the GFP–VP22 signal was present in the nuclei (Fig. 3C–E). Unfused myocytes in the same culture dish showed only the nuclear GFP–VP22 signal (data not shown). The predominantly cytoplasmic rather than nuclear localization of the fusion protein in myotubes is probably due to differences in the kinetics of GFP–VP22 nuclear uptake. The VP22 import mechanism requires the actin microfilaments [4], which significantly differ in skeletal myotubes in terms of isoforms and structural organization. Furthermore the cytoplasm-to-nucleus ratio in myotubes is far bigger, which could also contribute to the slower nuclear accumulation kinetics.

The definition and determination of spreading efficiency is complicated by the multinucleated nature of the cells and by the fact that the borders of individual myotubes are very hard to visualize. In addition, the cytoplasm in myotubes is highly autofluorescent, so that weak signals are not distinguishable from background. By comparison, however, the transfer efficiency is clearly higher than that which occurs following traditional transfection and infection protocols.

In summary, our results show that the GFP–VP22 fusion protein can spread in proliferating cells (Fig. 2), and that it can also be taken up by terminally differentiated cells (Fig. 3). Thus the VP22 protein delivery system offers new possibilities for the development of novel therapeutic approaches to the treatment of human diseases. This new protein therapy strategy may prove to be particularly useful in the direct delivery of therapeutic proteins in a transient, dose-controlled manner to differentiated tissues without the risk of permanent alteration of the genome.

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