Intracellular localization of Herpes simplex virus type 1 thymidine kinase fused to different fluorescent proteins depends on choice of fluorescent tag

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Abstract **Gene therapy employing the suicide gene/prodrug activating system Herpes simplex virus type 1 thymidine kinase (HSV-TK)/ganciclovir (GCV) is effective in killing malignant tumor cells. Labeling of the HSV-TK enzyme with fluorescent proteins makes possible the non-invasive imaging of transduction efficiency, enzyme localization and activity in cell culture and in animal models of human cancers. Here we report the expression of HSV-TK tagged with different fluorescent proteins (EGFP, DSRed1, DsRed2, dsdrFP616) and show that intracellular localization of the fusion products depends on the nature of the fluorescent tag despite the presence of several nuclear targeting signals within the enzyme itself. Coexpression of red fluorescent HSV-TK fusion proteins with TK-EGFP or untagged HSV-TK allowed these proteins to enter the nucleus by inhibiting formation of red fluorescent protein oligomers. As enzyme localization may influence HSV-TK activity, this observation is of potential importance to gene therapy studies.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Brain tumor; Gene therapy; Red fluorescent protein; Green fluorescent protein; Herpes simplex virus type 1 thymidine kinase

1. Introduction

Herpes simplex virus type 1 thymidine kinase (HSV-TK) phosphorylates naturally occuring nucleosides as well as several nucleoside analogs, including ganciclovir (GCV), thereby converting antiherpetic drugs to cytotoxic metabolites in tumor cells transduced with the HSV-TK gene. Although treatment of malignant brain tumor cells with HSV-TK and GCV has shown efficacy in cell culture studies and in animal models of human brain tumors [1–3], clinical trials for treatment of malignant brain tumors employing the HSV-TK/GCV system did not result in any treatment benefit [4]. The failure of this therapeutic strategy in humans may be related to the rather low transduction rate of human tumor cells in vivo. Therefore, imaging the localization, activity and persistence of transgenic HSV-TK in living tumor cells may be a first step towards optimization of gene therapy protocols.

The HSV-TK gene encodes a protein of 375 amino acids and contains several nuclear localization signals. Crystallographic analysis revealed that HSV-TK may form a homodimer [5]. Mutation of the nonapeptide ^{25}RRTALRPRR^{33} was shown to result in loss of the predominant nuclear localization of HSV-TK [6]. However, the mutated enzyme, detected in both the cytosol and the nucleus, retained kinase activity. Two additional positively charged sites, R236–R237 and K317–R318, also seem to be important for nuclear trafficking [7]. Mutation of these sites resulted in predominant enrichment of the enzyme in the cytosol. K317–R318 are located at the interface between the two HSV-TK monomers and R318 seems to be crucial for formation of the HSV-TK dimer. It has been proposed that only dimeric HSV-TK is transported to the nucleus due to a special nuclear localization signal formed only in the HSV-TK dimer [7].

Recently, reports on non-invasive in vivo imaging of HSV-TK activity with positron-emission tomography and radiolabeled nucleoside analogs such as ^{124}I-FIAU (2′-fluoro-2′-deoxy-1β-D-arabino-furanosyl-5-iodo-uracil) have been published [8,9]. The cloning of genes coding for fluorescent proteins has introduced new and sensitive reporter molecules for non-invasive and non-radioactive monitoring of gene expression. Expression of fluorescent reporters alone or as fusion proteins offers the possibility to monitor in real time protein expression and localization in single living cells and in whole organisms. Fluorescent proteins emitting light in the red spectrum are attractive candidates for multicolor imaging as their emission maximum is distant from the widely employed green fluorescent protein (GFP) (λ_{max} 508 nm) and light transmission through tissue is higher when using red shifted probes (>600 nm) [10].

GFP, isolated first from the jellyfish *Aequorea victoria*, has been established as a sensitive and non-invasive reporter system for monitoring transgene expression [11,12]. EGFP, a mutant form of GFP, fluoresces 35-fold more intensely than the wild type protein [13]. The red fluorescent protein (RFP) DsRed has been cloned recently from *Discosoma* coral [14] and is commercially available as DsRed1 (Clontech, Palo Alto, CA, USA). The 28 kDa protein DsRed is excited at 558 nm, shows an emission maximum at 583 nm and a sig-

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nificant light emission above 600 nm. While GFP is monomeric, shows only a weak propensity to dimerize, and acquires bright fluorescence relatively fast, DsRed1 forms a tight tetramer (approx. 115 kDa) and shows a slow chromophore maturation of up to 48 h [15]. Each monomer is structurally very similar to GFP and displays approximately 24% sequence homology. Fusion of DsRed to another protein may impair its function and localization due to formation of tetramers or even higher-order aggregates of the DsRed moiety [16,17]. Furthermore, a direct toxic effect of DsRed on cells has been discussed [16,17]. Basic residues near the N-terminus might lead to formation of salt bridges with adjacent tetramers, thereby promoting aggregation. Recently, Yanushevich et al. introduced mutations of basic residues located N-terminally in DsRed (R2A, K5E, K9T) resulting in a low-aggregating form of the protein [18]. The cDNA coding for this mutagenized protein is available commercially as DsRed2 (Clontech, Palo Alto, CA, USA). A DsRed variant whose emission maximum has been further red-shifted by mutagenesis was described by Fradkov et al. [19]: dsdrFP616 is excited at 583 nm and emits light at λ_{max} of 616 nm while showing identical fluorescence yield as DsRed1. DsdrFP616 is approx. 96% homologous to DsRed2 and has not been mutagenized N-terminally as described above for DsRed2 (Fig. 1).

Here, we have fused EGFP, DsRed1, DsRed2, and dsdrFP616 to HSV-TK in order to use these fusion constructs as reporters for imaging of HSV-TK activity in living glioma cells, and have investigated the subcellular localization of the different fusion proteins.

2. Materials and methods

2.1. Construction of cDNAs

The HSV-TK cDNA was amplified from the retroviral vector G1Tk1SvNa [20] by PCR and ligated to the plasmids pDsRed2-N1 or pdsdrFP616-N1 (both Clontech, Palo Alto, CA, USA), respectively. The HSV-TK gene was inserted in frame upstream of the red fluorescent reporter genes. The 8 amino acid spacer sequence between full length HSV-TK and both RFPs was identical. Before availability of DsRed2, a TK-DsRed1 translational fusion had been constructed by fusing a truncated HSV-TK lacking 6 amino acids at the 3′-end to full length DsRed1. The intervening sequence consisted of 6 amino acids. TK-EGFP (kind gift, C.M. Kramm, Dusseldorf, Germany) was constructed as reported elsewhere [21]. All fusion genes were placed under the control of the CMV promoter. The correct sequence of the fusion constructs was confirmed by sequencing.

2.2. Cell culture and transfection

The human glioblastoma cell lines U87MG and T98MG, as well as human embryonic kidney HEK293 cells, murine embryonic NIH3T3 fibroblasts and rat hepatocytes were cultured under standard conditions. For transfection, cells were seeded in 6-well plates at a density of $3–5\times10^5$ cells/well 16–24 h prior to transfection. Cells were transfected under serum-free conditions with Lipofectamine (Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol. Fluorescence images were taken 24–48 h after transfection using a Nikon TS100 inverted microscope equipped with a Coolpix 990 digital camera (Nikon, Tokyo, Japan). For selection of stable transfectants transfected cells were replated at low density 48 h after transfection and incubated with 1 mg/ml f.c. geneticin (Calbiochem, Bad Soden, Germany) for 4 weeks. Single cell clones were picked and analyzed for transgene expression.

2.3. Cytotoxicity assay

U87MG or HEK293 cells stably transfected with either HSV-TK or the respective HSV-TK fusion constructs were seeded at $4–8\times10^3$ cells/well in a 96-well plate. GCV was added at final concentrations of 0–20 μg/ml and cells were incubated at 37°C/5% CO_2 for 4 days. MTT (Sigma, Deisenhofen, Germany) was added at a final concentration of 0.5 mg/ml for 3 h. Absorbance was measured in a plate reader (Victor2, Perkin Elmer Life Sciences, Turku, Finland) at 590 nm (reference 660 nm). Experiments were performed in quadruplicates.

3. Results and discussion

3.1. Expression of fusion constructs

After transient transfection of human glioblastoma cells, HEK293 cells, NIH3T3 cells and rat hepatocytes with DsRed1, DsRed2, dsdrFP616 and EGFP, fluorescence was visible throughout both the cytoplasm and the nucleus (data not shown).

In the majority of cells expressing TK-DsRed2 and TK-dsdrFP616 fusion proteins tightly packed highly fluorescent aggregates were visible within approximately 30 h after transfection (Fig. 2). Although both fusion proteins contained identical nuclear targeting sequences in their HSV-TK moiety, only TK-DsRed2 was located predominantly as uniformly shaped and strongly fluorescent aggregates in the nucleus while TK-dsdrFP616 was visible as massive polymorphic aggregates in the cytoplasm with virtually no simultaneous staining of the nucleus (Fig. 2). The strong tendency of dsdrFP616 to precipitate from solution has been described before [19] and most likely accounts for the observed intracellular distribution of TK-dsdrFP616. DsdrFP616 alone

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                1                   20                  40
DsRed2          M·ASSENVITEFMRFKVRMEGTVNGHEFEIEGEGEGRPYEGHNTVKLKVTKGGPLPFAWDI
DsRed1          MVR--K---K-----------------------------------------------------
dsdrFP616       MVSC-K---K-----Q--------------K-----------CS-----------------
EGFP            MVSKG-ELF-GVVPIL-ELD-D----K-SVS-----DATY-KL-L-FIC-T-·K--VP-PT

                60                  80                  100
DsRed2          LSPQFQYGSKVYVKHPADIP··DYKKLSFPEGFKWERVMNFEDGGVATVTQDSSLQDGCFI
DsRed1          --------------------··--------------------V--------------------
dsdrFP616       ----------------------··--M----------------V--S-----K-----
EGFP            -VTTLT--VQCFSRY-DHMKQH-FF-SAM---YVQ--TIF-K-D-NYKTRAEVKFEGDTLV

                120                 140                 160
DsRed2          YKVKFIGVNFPSDGPVMQKKTMGWEASTERLYPRDGVLKGETHKALKLK····DGGHYLVE
DsRed1          ----------------------------------------I--------···---------
dsdrFP616       -E----------------RR-R-----S------------DI-M--R-E····G--------
EGFP            NRIELK-ID-KE-NILGH-·-LEYNYNSHNV-IMADKQ-NGIKVNF-IRHNIE--SVQ-AD

                    180                 200                 220
DsRed2          F··KSIYMAKKPVQLPGYYYVDAKLDIT···SHNEDYTIVEQYERTEGRHHLFL
DsRed1          -·--------------------S------···----------------------
dsdrFP616       -·--------------------S------···----------------S---------
EGFP            HYQQNTPIGDG--L--DNH-LSTQSALSKDPNEKR-HMVLLEFVTAA-ITLGMDELYK
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Fig. 1. Alignment of DsRed2, DsRed1, dsdrFP616, and EGFP. Identical residues are represented by dashes, introduced gaps by dots. The numbering refers to the DsRed2 sequence. Sequences of the commercially available fluorescent proteins (Clontech, Palo Alto, CA, USA) are used. Note that amino acid valine has been introduced at position 2 into the wild type forms of DsRed1, dsdrFP616, and GFP by the manufacturer. Amino acids substituted at the N-terminus in DsRed2 leading to a low-aggregating form of DsRed are shaded.

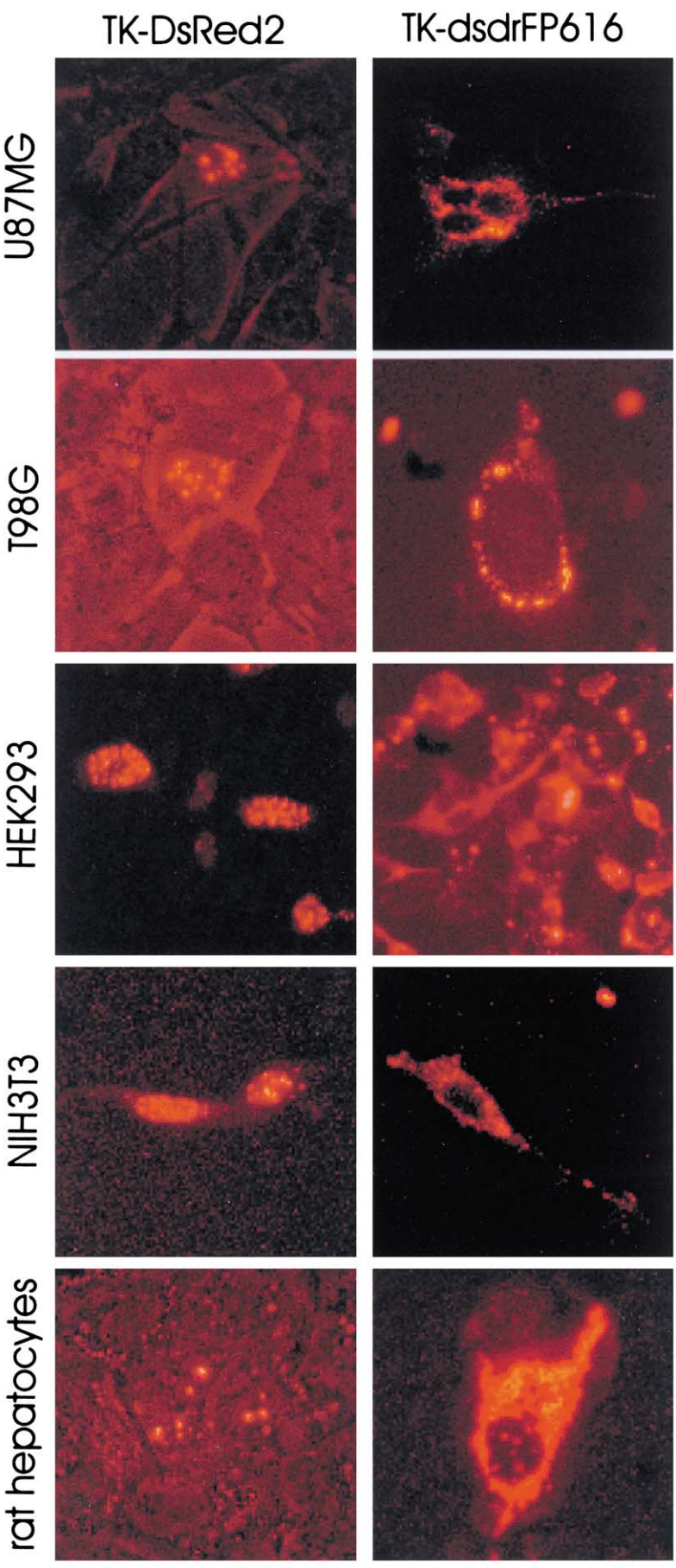

Fig. 2. Expression of dsdrFP616- and DsRed2-tagged HSV-TK in different cell lines. While TK-DsRed2 is transferred to the nucleus, expression of TK-dsdrFP616 results in massive aggregation in the cytoplasm of all cell lines tested.

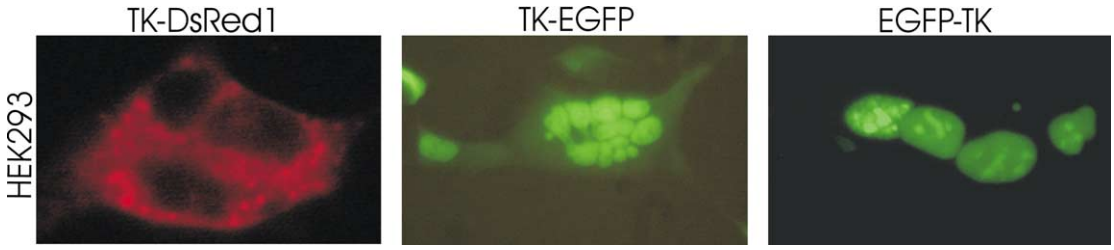

Fig. 3. Expression of TK-DsRed1, TK-EGFP, and EGFP-TK in HEK293 cells. While TK-DsRed1 aggregates in the cytoplasm and is excluded from the nucleus, TK-EGFP and EGFP-TK are able to gain access to it. The homogenous intranuclear structures seen in EGFP-TK transfected cells represent nuclear bodies similar to those seen when tagging fluorescent proteins with a nuclear targeting sequence only ([24], and K. Schlüter, Clontech, personal communication).

tends to aggregate even more than DsRed1, while the N-terminally mutated protein DsRed2 does not. As described above for DsRed2, Yanushevich et al. [18] introduced the mutations K5E and K9T into dsdrFP616 and demonstrated that the mutated protein shows a lower tendency to aggregate. Introducing additionally the mutations S2del and C3del even further improved solubility of dsdrFP616. As seen with TK-dsdrFP616, cells expressing the TK-DsRed1 fusion also fluoresced predominantly in the cytoplasm but showed far less tendency to aggregate as compared with TK-dsdrFP616 (Fig. 3). N-terminally mutated DsRed (DsRed2) seems to facilitate nuclear transport of DsRed-tagged proteins containing nuclear localization signals, most likely by reducing the likelihood of aggregate formation. It is conceivable that transfection of HSV-TK fused to N-terminally mutated dsdrFP616 will also result in nuclear transfer of this fusion protein. While fluorescence in cells transfected with the EGFP-TK construct was homogenous and completely restricted to the nucleus, cells transfected with TK-EGFP showed predominant homogenous labeling of the nucleus as well as staining of the cytoplasm, indicating that the precise position of the nuclear targeting sequences within the fusion gene also influences protein localization (Fig. 3). As often seen in transient transfection experiments [18] aggregates occurred occasionally in brightly fluorescing cells that had been transfected with EGFP-tagged HSV-TK, most likely due to protein overexpression (data not shown). DsRed2-tagged HSV-TK, as well as TK-EGFP and EGFP-TK can obviously pass through nuclear pores. However, unlike TK-EGFP, TK-DsRed2 precipitates intranuclearly, indicating that the N-terminally introduced mutations into DsRed2 are not sufficient to prevent aggregation after transport of the fusion proteins into the nucleus.

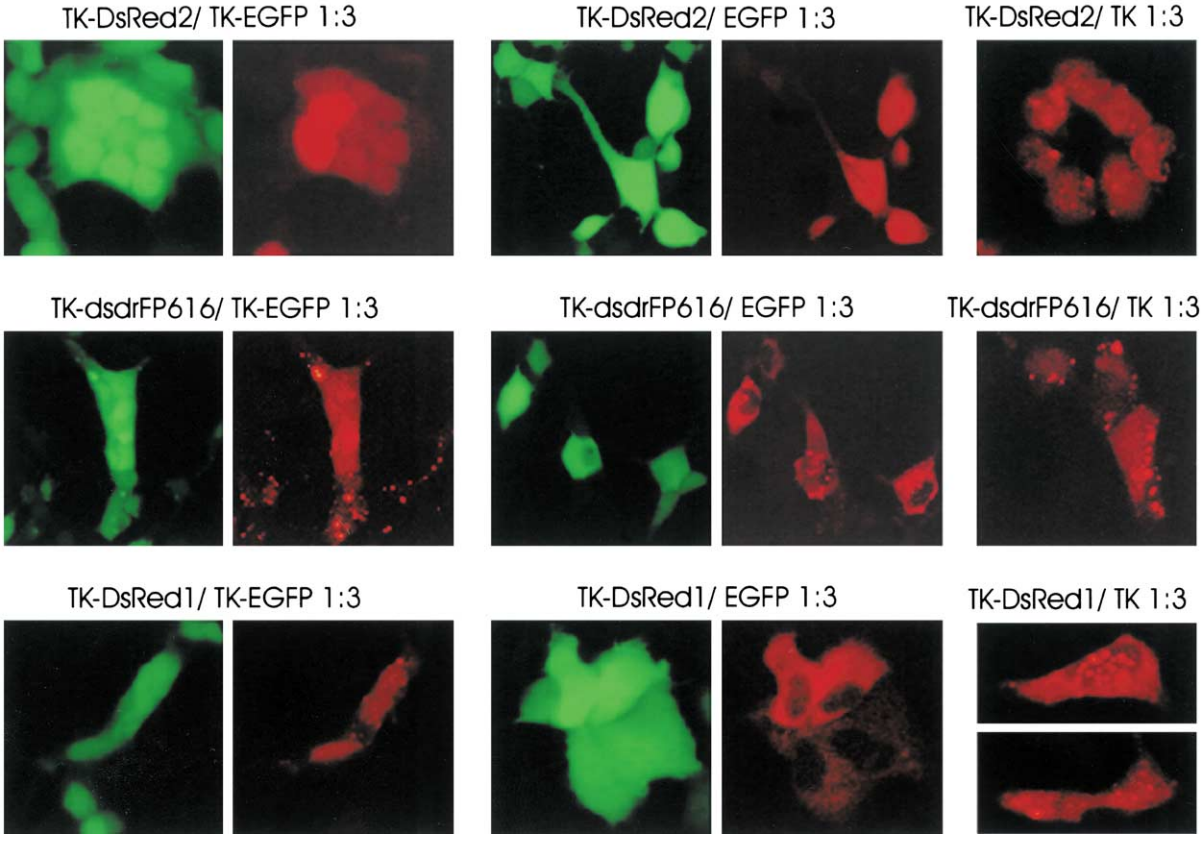

Fig. 4. Cotransfection of DsRed2-, DsRed1- or dsdrFP616-tagged HSV-TK with TK, EGFP or TK-EGFP into HEK293 cells (molar ratio 1 (RFP-TK):3). The strongest effect on solubility and nuclear transport of RFP-tagged HSV-TK can be seen when cotransfecting TK-EGFP.

3.2. Rescue of HSV-TK tagged with different RFPs

We examined whether aggregation could be reduced by employing the 'rescue strategy' described by Lauf et al. [17]. This group had shown that DsRed1-tagged connexins aggregated perinuclearly in opposite to their untagged connexin counterparts which normally assemble as oligomeric gap junction channel proteins in plasma membranes. Correct localization of the DsRed-tagged proteins could be achieved by cotransfecting either untagged or EGFP-tagged connexins and increased with the amount of plasmid added. Cotransfection of DsRed1-tagged connexins with EGFP alone was not described by these authors.

We cotransfected TK-DsRed1, TK-DsRed2, and TK-dsdrFP616 with EGFP, HSV-TK or TK-EGFP at different molar ratios (1:1 and 1:3) into U87MG and HEK293 cells. Cotransfection of TK-DsRed1 and TK-dsdrFP616 with EGFP resulted in improved homogenous bright red staining of the cytoplasm, but nuclear red fluorescence was visible only occasionally when high doses of EGFP were administered (Fig. 4). This effect was more pronounced in TK-DsRed1- than in TK-dsdrFP616-transfected cells. When TK-DsRed2 was cotransfected with EGFP, an increased fraction of cells contained homogenously red fluorescing nuclei (Fig. 4). Solubility increased with the amount of EGFP-plasmid added. Given the virtual identity of the overall fold of GFP and DsRed and the proposed ability of DsRed-like fluorescent proteins to hetero-oligomerize [22,23] it seems reasonable to assume that EGFP can substitute for RFPs in oligomer formation, thereby preventing tetramerization and thus the formation of higher-order aggregates. It would be interesting to see whether aggregation of DsRed1-tagged connexins as described by Lauf et al. [17] could also be prevented at least partially by EGFP-cotransfection. As connexins oligomerize also normally into large connexins, the effect of EGFP alone on solubility of DsRed1-tagged connexins might be less prominent than observed in our system. Nuclear targeting of RFP-tagged HSV-TK is not enhanced significantly by EGFP as EGFP alone does not promote TK-dimerization, which most likely is a prerequisite for nuclear transfer of the fusion protein [6,7]. Cotransfection of all three RFP-TK fusion constructs with HSV-TK alone had a similar but less pronounced positive effect on homogenous intracellular distribution of red fluorescence (Fig. 4), indicating that dimerization of RFP-tagged HSV-TK might still enable the RFP tag to form oligomers or even larger aggregates. On the other hand, HSV-TK cotransfection at a molar ratio of 1:3 facilitated nuclear transfer of RFP-tagged HSV-TK in cells expressing TK-DsRed1 and TK-dsdrFP616, most likely due to dimerization of HSV-TK with RFP-tagged HSV-TK (Fig. 4).

The greatest and most impressive positive effect on solubility and nuclear transfer rate of RFP-tagged HSV-TK could be achieved by cotransfecting TK-EGFP (Fig. 4). This effect could be seen in both U87MG glioblastoma and HEK293 cells and increased with the amount of TK-EGFP added. TK-DsRed2 was now homogenously distributed in the nucleus of virtually all cotransfected cells (Fig. 4). Cotransfection of TK-DsRed1 or TK-dsdrFP616 with TK-EGFP also improved their solubility and resulted in greatly enhanced nuclear transfer of the fusion proteins. Given the strong propensity of dsdrFP616 to form aggregates [19] cotransfection of TK-EGFP into TK-dsdrFP616 expressing cells still did not lead to complete homogenous staining of the cells. The effect

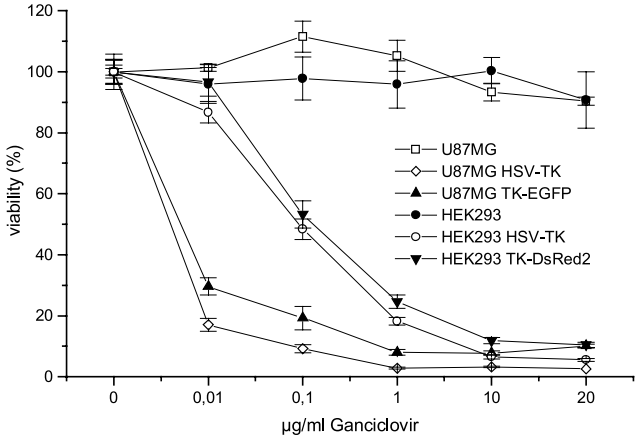

Fig. 5. GCV sensitivity of HEK293 and U87MG cells stably expressing HSV-TK, TK-EGFP or TK-DsRed2, respectively. These clones as well as control cells were exposed to different concentrations of the nucleoside analogue GCV for 4 days. Cytotoxicity was assessed by the MTT assay.

of TK-EGFP on solubility and intracellular distribution of RFP-tagged HSV-TK seems to combine the positive effects seen when cotransfecting either HSV-TK or EGFP alone. While the EGFP moiety increases solubility of all RFP-tagged proteins the HSV-TK part, which has less impact on RFP solubility, supports trafficking of the RFP-TK heterodimer into the nucleus. As large red fluorescent aggregates were not seen intranuclearly in cotransfected cells, heterodimerization seems to prevent cross-linking of RFP-tagged TK into higher-order aggregates.

DsRed is an obligate tetramer in solution [15], but it is unknown at present whether oligomerization is a prerequisite for red fluorescence in living cells. In vitro, DsRed monomers were shown to fluoresce at 583 nm after disassembly of the DsRed tetramer by sodium dodecyl sulfate treatment [17]. As deduced by Lauf et al. [17] from their cotransfection experiments with DsRed-tagged connexin, our data also suggest that red fluorescence can be emitted by oligomers containing less than four RFP tags. Remarkably, cells coexpressing RFP-TK and either HSV-TK, TK-EGFP or EGFP fluoresced more brightly than cells expressing only RFP-tagged HSV-TK. This points out the possibility that fluorescence emission is enhanced in RFPs consisting of less than four (identical) molecules as a consequence of a dequenching effect.

3.3. Stable expression of transgene proteins

Human glioblastoma cells stably expressing DsRed1, DsRed2, EGFP and dsdrFP616 could be established by selection with G418. Establishment of stable glioblastoma cell clones expressing DsRed1 proved to be difficult, as reported for other cell lines [18], indicating that this protein is also toxic for malignant brain tumor cells. We did not succeed in establishing any stable clones expressing TK-DsRed2 and TK-dsdrFP616 (TK-DsRed1 was not tested) in several human glioblastoma cell lines, while TK-EGFP expressing stable clones could be generated. In HEK293 cells, only one stable clone expressing TK-DsRed2 but no clones expressing TK-dsdrFP616 could be established. As expected, HEK293 cells stably expressing TK-DsRed2 were far less fluorescent than their transiently transfected counterparts. Unlike transiently transfected cells, fluorescence in clones stably expressing

TK-DsRed2 was distributed more evenly throughout the cytoplasm. However, fluorescent aggregates were still seen within these cells. Interestingly, the MTT cytotoxicity assay showed that these cells retained HSV-TK activity and were killed by GCV (Fig. 5). Human U87MG glioblastoma cells stably expressing HSV-TK and TK-EGFP were also shown to be sensitive to GCV, thus confirming that expression of the tagged or untagged wild type HSV-TK protein resulted in a functionally active enzyme in human glioblastoma cells (Fig. 5). The reduced sensitivity of HSV-TK expressing HEK293 cells towards GCV compared with HSV-TK expressing U87MG glioma cells can be ascribed most likely to differences in cell-specific properties, as this phenomenon is observed with cell clones expressing either tagged or untagged HSV-TK (Fig. 5).

In conclusion, fusion of HSV-TK to RFPs offers a valuable tool to study transgene expression in human malignant brain tumors. However, the success of this approach requires that oligomerization and aggregation of the RFP moiety is avoided by either using mutated RFPs which have lost the tendency to aggregate or by coexpression of other proteins which limits tetramerization and aggregation of red fluorescent HSV-TK fusion proteins.

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