Enhancing protein expression in single HEK 293 cells

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Abstract

Recombinant proteins are routinely expressed in heterologous expression systems such as human embryonic kidney 293 (HEK 293) cells. The efficiency of the expression is critical when the expressed protein must be characterized at the single-cell level. Here we describe a simple method by which the protein expression efficiency in single HEK 293 cells is enhanced by coexpressing simian virus 40 large T antigen (TAg), a powerful oncoprotein. Using the GluR2 ionotropic glutamate receptor as an example, we found that the receptor expression in single HEK 293S cells increased approximately seven-fold. The ratio of the plasmid amount of TAg to that of the receptor was optimized at 1:10, while the receptor function was unaffected in the presence of TAg. We further used fluorescence imaging from a population of cells as an independent detection method and found a similar increase in expression of green fluorescent protein (GFP) by TAg coexpression. This method is thus applicable for enhancing the expression of both membrane and soluble proteins at the single-cell level. More importantly, the function of a protein can be studied directly in intact cells, a feature particularly useful for studying membrane proteins.
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1. Introduction

The use of human embryonic kidney 293 (HEK 293) cells has been one of the most popular ways for expression of recombinant proteins (Graham et al., 1977). There are a variety of transfection protocols to deliver a recombinant gene to those cells (Chen and Okayama, 1987; Corsaro and Pearson, 1981; Graham and van der Eb, 1973; Luthman and Magnusson, 1983; Washbourne and McAllister, 2002). Generally, those protocols provide a reasonable protein expression yield, especially when the culture volume is not restricted. In such a case, a larger volume of culture from which more cells can be harvested compensates for a low efficiency of protein expression at the single-cell level. However, a low efficiency of single-cell protein expression can be an insurmountable problem when the protein must be characterized by single-cell imaging and/or single-cell recording. This is because the number of protein molecules expressed per cell is generally proportional to the signal strength. For instance, ion channel proteins are expressed routinely in HEK 293 cells and assayed directly using a single cell, either entirely (e.g., for whole-cell recording) or partially (e.g., for recording with membrane patches and for single-channel recording) (Hamill et al., 1981). As such, the number of ion channels expressed per cell is critical to their detection. Furthermore, in the presence of inhibitors, the protein signal is adversely reduced. Thus, these and many other types of studies will benefit from a method by which the efficiency of protein expression in single cells can be enhanced. Developing such a method was the goal of the present study.

The method we established originated from our interest of ionotropic glutamate receptors (Dingledine et al., 1999). These receptors are transmembrane channels that can open upon binding of glutamate, a neurotransmitter in the central nervous system (Dingledine et al., 1999). Glutamate receptors play special roles in brain activities, such as memory and learning, and have been implicated in a variety of neurological diseases, such as post-stroke cellular lesion and amyotrophic lateral sclerosis (Dingledine et al., 1999;

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Heath and Shaw, 2002). To study the structure–function relationship, glutamate receptors are commonly expressed in HEK 293 cells and characterized directly in single cells. By our method, the receptor expression efficiency in single cells can be increased by about seven-fold, compared with a popular expression protocol using calcium phosphate in transfection (Chen and Okayama, 1987). The key to our method is to coexpress simian virus (SV) 40 large T antigen (TAg), a powerful oncoprotein (Ali and DeCaprio, 2001; Chen and Hahn, 2003; Simmons, 2000; Sullivan and Pipas, 2002), with the protein of interest. Specifically, the gene of the protein of interest is harbored in a plasmid containing the SV40 replication origin, and the TAg gene is encoded in a separate vector. Transient coexpression of TAg produces more proteins of interest per cell. This is because, among its functions, SV40 TAg disrupts the cell-cycle checkpoints by binding to and inactivating key tumor suppressors and cell-cycle regulatory proteins such as p53 and pRB (Ali and DeCaprio, 2001; Sullivan and Pipas, 2002). Consequently, the cell turns into a growth-deregulated protein-making factory. Specifically, we characterized TAg enhancement of the single-cell expression of GluR2, a key glutamate receptor subunit (Li et al., 2003b), to establish the optimal plasmid ratio and the most complementing cell line. We further characterized the function of the GluR2 receptor with intact cells, without removing TAg. In addition, using green fluorescence protein (GFP) (Chalfie et al., 1994) as a reporter gene and fluorescence imaging of a population of HEK 293 cells as an independent detection method, we showed that the GFP expression in these cells increased similarly. Together, our results demonstrate that this method represents a significant improvement over conventional protein expression protocols. Furthermore, the method should be general for expressing both soluble and membrane proteins, and for characterizing the protein function directly in single cells.

2. Materials and methods

2.1. Expression of plasmid DNAs and cell culture

The cDNA encoding GluR2 (unedited at the Q/R site, and flip isoform) in a pBlueScript vector (from Prof. Steve Heinemann at Salk Institute) was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) that contained the SV40 replication origin (8.6 kb). To identify cells that might express the GluR2 receptor for recording, the GluR2 plasmid was cotransfected with a GFP plasmid lacking the SV40 replication origin (from Prof. Ben Szaro at SUNY-Albany). As a result, the GFP expression in cells or the resulting green color intensity was not affected by coexpression of TAg (see the text). However, a different GFP construct (pEGFP-C3, 4.7 kb) (Clontech, Palo Alto, CA) that did contain the SV40 replication origin was used in the enhancement of GFP expression by TAg (the TAg plasmid, 6.0 kb, from Prof. Jeremy Nathans at Johns Hopkins University). All the plasmids were

propagated in an *Escherichia coli* host (DH5α) and purified using a kit from QIAGEN (Valencia, CA).

The cell lines used in this study were regular HEK 293 cells (from Prof. Robert Oswald at Cornell University), 293S cells (from Prof. Gobind Khorana at MIT) and 293T cells (American Tissue Culture Collection, Cat. No. CRL-11268, Manassas, VA). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 37 °C, 10% CO_2, humidified incubator.

A standard calcium phosphate method (Chen and Okayama, 1987) was used in most of transient transfections for gene delivery, although lipofectamine (Invitrogen, Cat. No. 18324-111, Carlsbad, CA) was used in some transfections (see text). The GluR2 plasmid used was ~3–6 μg (the plasmid amount used here and below was for a culture in the 35 mm dish). As a cell marker, the GFP plasmid that lacked the SV40 replication origin was cotransfected with the GluR2 with the ratio of the plasmid of GFP to that of GluR2 by weight being 1:10 (Li et al., 2003b). The amount of the TAg plasmid varied in experiments (see Section 3). The amount of pEGFP plasmid was 1–3 μg whereas the weight ratio of pEGFP plasmid to TAg plasmid varied from 2:1 to 15:1. All the cells used for recording or imaging were those that grew in between 48 and 58 h after transfection.

2.2. Whole-cell current recording

Because the expression of the GluR2 gene leads to the formation of functional channels (Boulter et al., 1990), both the expression and the functional properties of this receptor are testable by measuring the magnitude and the time course of the glutamate-induced whole-cell current from an entire cell. The ratio of the current amplitude in the absence and presence of TAg, but at a constant glutamate concentration, was therefore used to represent the effect of TAg in enhancing the receptor expression.

The procedure for recording the GluR2 channel activity was previously described (Li et al., 2003b). Briefly, the recording electrodes were pulled from glass capillaries (World Precision Instruments, Sarasota, FL). The electrode resistance was ~3 MΩ when filled with the electrode solution. The electrode solution contained (in mM) 110 CsF, 30 CsCl, 4 NaCl, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES (pH 7.4 adjusted by CsOH). The external bath solution contained (in mM) 150 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.4 adjusted by HCl). Whole-cell recordings were done at −60 mV, and 22 °C. Specifically, a cell that expressed the GluR2 receptor was bathed in the external solution. Glutamate was applied from a cell-flow device (Udgaonkar and Hess, 1987) to the cell, and the resulting whole-cell current was recorded using an Axopatch-200B amplifier at cutoff frequency of 2 kHz by a built-in, 8-pole Bessel filter, and digitized at 5 kHz sampling frequency using a Digidata 1322A from Axon Instruments (Union City, CA). The data were acquired using pCLAMP 8 (also from Axon). The rise time of

the glutamate-induced whole-cell current response (10–90%) was 2.3 ± 0.1 ms, an average of the measurement from >100 cells. Each data point was an average of at least three measurements collected from at least three cells unless otherwise noted. Origin 7 (Origin Lab, Northampton, MA) was used for plotting. Uncertainties refer to standard error of the fits unless noted otherwise.

2.3. Fluorescence imaging and quantification

The GFP fluorescence in cells was imaged using a confocal laser scanning microscope (ZEISS LSM510 META, Carl Zeiss, Thornwood, NY), and recorded using a RETIGA Ex digital camera (Qimaging, Burnaby, BC, Canada) with the QCapture software (Suite 2.66 from Qimaging, Burnaby, BC, Canada). For bright-field images, 1 ms exposure time was used at gain 3000 and offset 0. For fluorescence images, 15 ms exposure time was used at gain 3500 and offset 2000. Images were digitized using Image J software (version 1.30 from http://rsb.info.nih.gov/ij/).

The fluorescence intensity in a single cell was assumed to be linearly proportional to the amount of the GFP expressed in the cell. Likewise, the accumulated fluorescence intensity per image that included many cells was assumed to be the averaged measure of the GFP expression in those cells. For each 35 mm Petri dish used to express GFP, three bright-field images were taken from three randomly chosen locations, and each image contained about 2000 cells. For the corresponding area, three fluorescence images were also recorded. Each fluorescence image contained 1360×1036 pixels, and each pixel was scored to a value possibly ranging from 7 to 220 (on 8-bit digital scale using Image J). The background value was set to be 35 on this scale; below this value, no cell could be visually recognized. This value was then deducted from the value of each pixel in calculating the fluorescence intensity of a green cell. On average, a green cell was represented by >1000 pixels. The accumulated fluorescence intensity per image was tabulated into a histogram with the bin width of 10. The fluorescence intensity from 85 to 15% range in the histogram was chosen for comparison of the TAg enhancement in 293S cells, excluding those that were too bright and too dim. This was the same practice used qualitatively in choosing green cells for whole-cell recording. The corresponding range of the fluorescence intensity of the cells cotransfected with the TAg gene was compared to that of the control. The ratio of these values was used as the measure of the TAg enhancement of the GFP expression in HEK 293S cells.

3. Results

3.1. TAg enhancement of glutamate receptor expression in single HEK 293 cells assayed by whole-cell recording

The whole-cell current response of the GluR2 receptor to 200 μM glutamate was higher from single HEK 293 cells
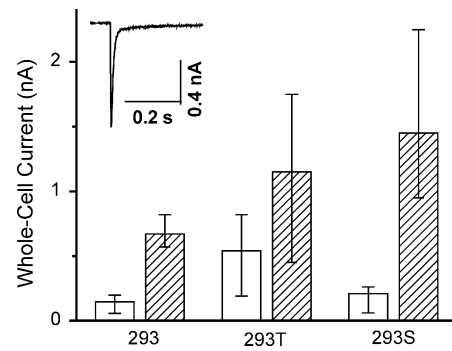

Fig. 1. TAg enhances the GluR2 receptor expression in different HEK 293 cell lines, detected by whole-cell recording. The whole-cell current response to 200 μM glutamate with (dashed column) and without (open column) the coexpression of TAg (the inset is a representative current response) is shown. The height of each column represents the average of the current amplitudes from >40 cells, and the bar represents the range of the current observed for 10–90% of the cells. As compared to the control (open column), the receptor current response, enhanced by TAg coexpression, was increased by 4.5-fold in the regular 293 cell line, 2.1-fold in the 293T cell line and 6.9-fold in the 293S cell line. In these experiments, the amount of TAg, GFP and GluR2 plasmids used for transfection was 0.4, 0.4 and 4 μg, respectively.

cotransfected with TAg than the control or the cell transfected with only GluR2 (Fig. 1). This result suggested that the GluR2 expression in single cells was higher when the cells coexpressed TAg. Moreover, we identified 293S cells (Stillman and Gluzman, 1985) as the most complementing cell type among all three HEK 293 cell lines tested. This was evidenced by a seven-fold increase in glutamate-induced current response in 293S cells (Stillman and Gluzman, 1985), compared with a two- and five-fold increase in 293T cells (Sena-Esteves et al., 1999) and regular 293 cells (Graham et al., 1977), respectively (Fig. 1).

The 293S cell line is a suspension-adapted cell type (Stillman and Gluzman, 1985), whereas the regular 293 and 293T cell lines are known to be suitable for static culture. The 293S cell line, like the regular 293 cell line but unlike the 293T cell line, does not constitutively express TAg (Stillman and Gluzman, 1985). Thus, without introduction by transfection of the TAg gene to these cells, it would be expected and indeed was found (see control values: open columns in Fig. 1) that the efficiency of the receptor expression was higher in the 293T cell. This observation was consistent with the presumed function of TAg, which is to increase the protein expression in that same cell. When TAg was cotransfected with all these cell types, the receptor expression was higher than the corresponding control, as expected, but was the highest in the 293S cell (Fig. 1) for reasons yet not clear. The 293S cells used in our experiments were grown in static culture like other 293 cell lines, rather than in suspension. We found that the 293S cells in static culture were suitable for electrophysiological recording where only the dish-attached cells were picked to make gigaohm seals. In static culture, the majority of 293S cells were indeed attached to Petri dishes, albeit more loosely than regular 293 or 293T cells. Based on these results (Fig. 1), we decided to use 293S cells for the rest of the experiments.
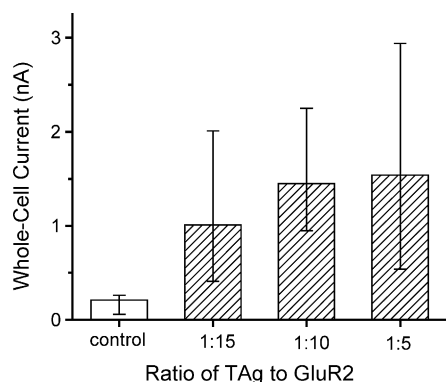
Fig. 2. Enhancement of the GluR2 expression by TAg in HEK 293S cells reaches seven-fold at the 10:1 ratio for the amount of the TAg DNA plasmid used compared to the GluR2 plasmid in transient transfection. Different receptor expression in 293S cells was observed, as shown by the receptor current response to 200 µM glutamate, at different ratios of TAg plasmid amount used for transient transfection, compared to the GluR2 plasmid amount. The GluR2 DNA plasmid was kept to be 4 µg per 35 mm dish. The control was from cells expressing GluR2 only, shown as open column. The height of each column represents the average of the current amplitudes from >40 cells, and the bar represents the range of the current observed for 10–90% of the cells.

3.2. Optimal ratio of TAg plasmid used for transient transfection

Next, we varied the amount of the TAg plasmid relative to that of GluR2 to determine the optimal ratio of TAg for transient expression with HEK 293S cells. This ratio would correspond to a minimal amount of the TAg plasmid that must be used to reach the maximal enhancement of the protein expression. The result of this experiment (Fig. 2) showed that, to reach the seven-fold increase in the receptor expression, the optimal ratio of the amount of the TAg plasmid to the amount of the GluR2 plasmid used in transfection was 1:10.

Notice that we used the amount of the TAg plasmid in microgram (µg) for transient transfection, rather than the amount of the TAg protein actually expressed in the cell, to represent the magnitude of the enhancement of the receptor expression per cell. This is because both the exact amount of TAg sufficient to influence the protein expression in cells and the time it took for the cell to synthesize that amount of TAg after the transient transfection are difficult to assess. After the TAg plasmid was co-introduced into the cell by transfection, the expression of both GluR2 and TAg would presumably occur concomitantly. Given that the presence of TAg led to enhancing the receptor expression in the same cell, we assumed that the expression of TAg must have reached a level sufficient to deregulate the cell-cycle control prior to a significant production of the GluR2 receptor. This assumption was supported by the observation that without TAg, receptor expression was lower at the single-cell level (in both regular 293 cells and 293S cells), compared with the yield in the presence of TAg (either in 293T cells that constitutively expressed TAg or in all three cell types, i.e., regular 293, 293T and 293S cells, but with TAg coexpression) (Figs. 1 and 2). When the plasmid amount of TAg in cotransfection was increased, a

higher expression of the receptor in the cells was observed, compared to the control. Thus, it was assumed that a greater quantity of plasmid proportionally increased the amount of the TAg protein expressed in cells. As an empirical method, using the exact amount of the TAg plasmid for transfection seemed to enable us to control the level of the enhancement of the receptor expression by TAg. Finally, the amount of TAg required to effectively deregulate the cell-cycle control, although unknown, may be low. Such a minimal amount of TAg synthesized in the cell presumably at the early stage of gene expression should be sufficient for the cell to be in a growth-deregulated state, before the expression of the protein of interest is peaked.

3.3. Whole-cell recording assay of the functional properties of glutamate receptors

A method to improve single-cell protein expression should be ideally amenable to the condition where the function of the protein can be assayed in a single cell *directly* or without removing TAg. This is particularly advantageous for studying glutamate ion channels because glutamate channels, like many other membrane proteins, are routinely characterized using electrophysiological recordings in the cell where they are expressed. To test whether our method offered this additional benefit, we measured the properties of the GluR2 channel in the absence and presence of TAg. Our results showed that the coexpression of TAg did not affect GluR2 function, as evidenced by identical desensitization rate constants (Fig. 3A) and current–voltage relationships (Fig. 3B). This finding was also consistent with the identical glutamate dose–response relationship we reported earlier (Li et al., 2003b) for this receptor. Separately, we further tested the desensitization rate constant and the dose–response relationship for other glutamate receptors, such as GluR1 and GluR4 receptors (data not shown). All the results showed that the presence of TAg in the cell expressing the corresponding glutamate receptor did not affect the kinetic properties of the receptor.

3.4. TAg enhancement of GFP expression visualized by fluorescence imaging

We demonstrated, using the GluR2 receptor, that protein expression efficiency was increased at the single-cell level. By inference, protein production efficiency should likewise improve in a population of cells. Therefore, testing the enhancement of protein expression from a large population would serve as a control to validate the conclusions of the single-cell study (Figs. 1 and 2). For this purpose, we examined the TAg enhancement of GFP expression in HEK 293S cells by fluorescence imaging (Fig. 4). There were two notable features in the design of this control experiment using GFP. First, unlike the GluR2 receptor, GFP is a soluble protein and is widely used as a gene expression marker (Chalfie et al., 1994). Second, the use of fluorescence imaging to quan-
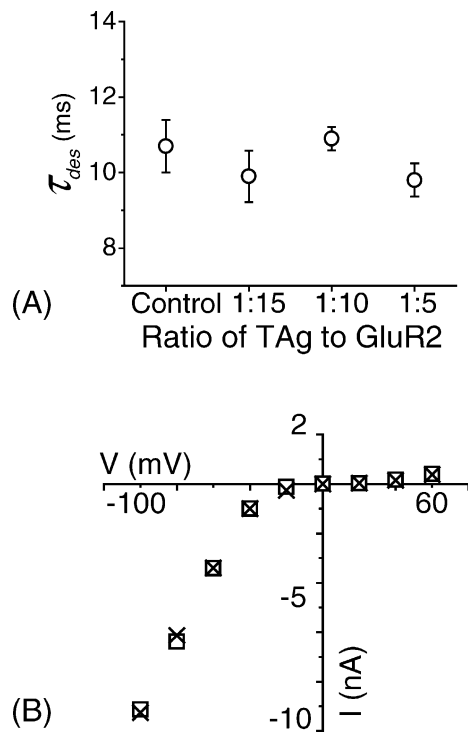
Fig. 3. Coexpression of TAg does not affect the functional properties of the GluR2 receptor expressed in HEK 293S cells. (A) Using whole-cell recording, the desensitization time constant (τ_{des}) was measured at different ratios of the amount of the TAg plasmid used in transient transfection, compared to the amount of the GluR2 plasmid. The desensitization of the GluR2 channel induced by the continuous binding of glutamate at 200 μM was fitted by a single-exponential rate (an example of the receptor desensitization, seen as the fall of the current, is shown in the inset of Fig. 1). (B) Current–voltage relationship, as determined using 200 μM glutamate and whole-cell recording. Each symbol reflects an average of at least three measurements from at least three cells expressing the GluR2 receptor with ($\square$) and without ($\times$) the coexpression of TAg. The ratio of the amount of TAg plasmid (0.4 μg) to that of GluR2 plasmid was 1:10.

tify the GFP expression in a large number of cells was a different detection method, compared to the use of whole-cell recording to assay the GluR2 activity from single cells. Analysis of the fluorescence intensity of GFP expressed in 293S cells with and without TAg showed that TAg enhanced GFP expression (Fig. 4A), as expected, to the maximum of about seven-fold (Fig. 4B), although at the 1:10 plasmid ratio, the enhancement was slightly less than that obtained by single-cell recording (Fig. 1).

It should be noted that GFP was also used as a *cell marker* in the whole-cell recording experiments (Figs. 1–3) because a green-colored cell was easily identifiable, and such a cell would likely express the GluR2 receptor (the ratio of the plasmid used for GFP to GluR2 was 1:10). The plasmid that harbored the GFP gene used in those experiments did not contain the SV40 replication origin, unlike the GFP construct used for the GFP enhancement experiment (Fig. 4). Consequently, the GFP intensity was about the same in the absence and presence of TAg (the fluorescence images are not shown). The use of the GFP plasmid lacking the SV40
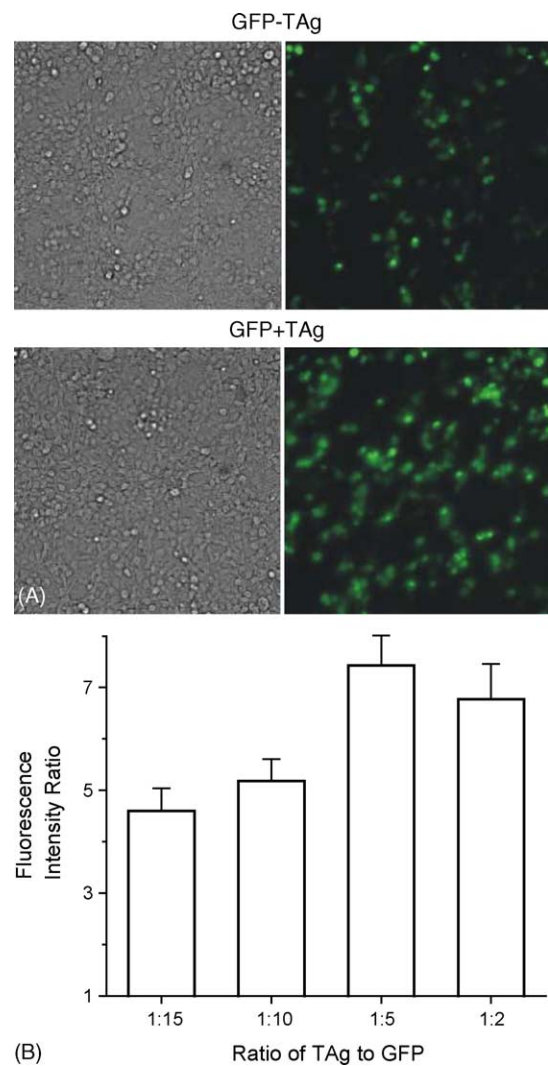

Fig. 4. Coexpression of TAg enhances GFP expression in HEK 293S cells determined by fluorescence imaging and quantification. (A) Comparison of the bright-field (left panel) and green fluorescence images (right panel) for the same number of cells expressing GFP with (lower pair, GFP + TAg) and without (upper pair, GFP − TAg) TAg coexpression. The image acquisition and quantification were described in detail in Section 2. Specifically, the images shown here were from cells transfected with 1 μg GFP and 0.1 μg TAg plasmid. (B) The ratio of the fluorescence intensity, as compared to the control (without TAg coexpression), at different ratios of the amount of TAg used to that of the GFP plasmid. The GFP plasmid used for transfection (i.e., pEGFP-C3) was kept to be 1 μg per 35 mm dish. The absolute fluorescence intensity was determined from the accumulation of about 2000 cells counted from the bright-field image. Three images were taken from each dish, and a total of three dishes were used for image analysis as shown for each column. The fluorescence intensity was averaged and plotted as a ratio to the control, i.e., the fluorescence intensity from the cells expressing GFP without TAg.

replication origin in the single-cell experiments (Figs. 1–3) ensured that only the expression of the GluR2 gene, rather than the expression of both the GluR2 and GFP genes, was affected by the coexpression of TAg. Thus, this experimental design permitted a quantitative comparison of the results of fluorescence imaging of GFP with that of whole-cell recording of GluR2. The lack of the SV40 replication origin in the

GFP plasmid construct used in recordings (Figs. 1–3) and consequently the invariant fluorescent intensity of the cells in the absence and presence of TAg confirmed the function of TAg in deregulating cell growth through the SV40-controlled pathway (Ali and DeCaprio, 2001).

In designing the GFP enhancement experiment by TAg, we considered the difference in assaying protein expression by different techniques. This was because in the whole-cell recording assay, only the GluR2 receptor molecules embedded on the surface of the cellular membrane were detectable. In contrast, the GFP expressed was detectable by fluorescence imaging regardless of its location because GFP is a soluble protein. Fleck et al. (2003) studied glutamate receptor expression/trafficking and reported that only ~40% of the glutamate receptors (e.g., GluR6 kainate receptor subunit) are on the surface of the membrane of HEK 293 cells, whereas 60% of the receptors, once made, are sequestered in the endoplasmic reticulum (ER). For the same receptor, we found previously that the green fluorescence intensity is linearly proportional to the amplitude of the receptor current (Li et al., 2003a). This means that the greener the cell, the larger the current is at a given glutamate concentration. Based on those results, we used 3–6 μg of plasmid encoding the GluR2 gene for transfection in the single-cell experiments (Figs. 1–3). We assumed that 40% of the plasmid amount, or 1–3 μg, would be responsible for producing surface-embedded GluR2 receptors (since glutamate was applied extracellularly and the glutamate-binding site was located in the extracellular face of the receptor) (Armstrong and Gouaux, 2000). In the GFP-enhancement experiment, we used 1–3 μg of the GFP plasmid (Fig. 4). Although a smaller amount of GFP plasmid was used, compared to the total amount of the GluR2 plasmid used for transfection, the entire amount of GFP expressed was detectable by fluorescence imaging. As observed (Fig. 4A), GFP lit up an entire cell with its characteristic green fluorescence color.

3.5. Effect of TAg coexpression on cell growth

The imaging of the GFP fluorescence from a population of cells enabled us to acquire additional information about the effect of TAg coexpression by transient transfection on cellular morphology, viability and growth. First, the cell morphology (Fig. 4A) was unaffected in the presence of TAg. This was true for all 293 cell lines tested. Second, the green fluorescence intensity peaked on day 2 after transfection, although on day 1 the fluorescence intensity of the cell culture subject to the TAg coexpression was visibly higher than controls. After cotransfection of the TAg gene, the doubling time and the cell viability of the culture were no different from controls. These results are consistent with the conclusion that additional amount of protein was made at the single-cell level per unit time in the presence of TAg. Third, the transfection efficiency was slightly higher than control (13% versus ~20% based on 15 transfections and 100% cell count). The transfection efficiency was determined by the percentage of green

cells observed by fluorescence imaging in the total number of cells observed by bright-field microscopy for the same sample (see the comparison of these images in Fig. 4A). One explanation for these results might be that the GFP level in some cells became high enough to be detected but only in the presence of TAg. Together, these results (Fig. 4) indicate that the major TAg enhancement can be accounted for by the increase in protein production at the single-cell level, and such a conclusion agrees with the one previously described for the single-cell assay of the GluR2 receptor expression (Figs. 1 and 2).

4. Discussion

We described a method for improving the single-cell expression efficiency of recombinant proteins by about seven-fold, compared to the efficiency from a popular transfection protocol (Chen and Okayama, 1987). To achieve this enhancement, a powerful oncoprotein, SV40 TAg, was co-expressed with the gene of interest in HEK 293S cells at the optimal ratio of 1:10 in weight for the plasmid amount of TAg used to that of the protein of interest. We demonstrated this method by using two proteins and two detection techniques: a membrane-bound glutamate receptor whose expression level was assayed by electrophysiological recording with single 293S cells, and soluble GFP whose expression was visualized by fluorescence imaging of a population of cells. We further demonstrated that the function of the protein of interest can be assayed directly, if desired, by using an intact cell without removing TAg. Therefore, this method is expected to be useful for single-cell characterization of the protein signal by recordings and/or imaging, because the level of protein expression by a single cell may become high enough to be detected or augmented to achieve a stronger signal. Finally, this method is simple to implement.

The transient transfection method using calcium phosphate precipitation is a very popular method to deliver gene to cells, such as HEK 293 cells. This method is considered a benchmark, compared with many other alternatives, non-viral transfection protocols, because of its relative high transfection efficiency (Chen and Okayama, 1987). The method we described takes advantage of the high transfection efficiency by using the calcium phosphate precipitation for gene delivery, but further improves the yield of protein expression by increasing the expression efficiency per transfected cell. In the presence of TAg, the transfected cell makes more proteins because TAg deregulates the control of the cell growth cycle. Therefore, our method is expected to work in other permissive cell lines, provided that the gene of interest, together with the TAg gene, is first delivered to the host cell. We postulate how the genes are delivered to the host cell may have little influence on the ratio of the enhancement when the TAg is coexpressed. As a test, we alternatively used the lipofection method (Washbourne and McAllister, 2002) to transfect HEK 293S cells with the GluR2 and TAg genes.

We found that the ratio of enhancement of the GluR2 receptor expression was identical to that of the calcium phosphate precipitation method (data not shown). This result suggests that the method we presented may be equally effective for other gene delivery approaches.

The HEK 293 cell is a widely used cell line for mammalian gene expression for both soluble and membrane proteins. The method we described should therefore be generally applicable. Among three different HEK 293 cell lines tested in the present study, namely regular 293, 293T and 293S cells, we found empirically that the suspension-adapted 293S cell line was most complementary to the use of TAg. However, we do not yet know why the 293S cell line is superior. Nevertheless, the advantage of using the suspension-adapted 293S cells further suggests that our method is fully adaptable for suspension culture in shake flasks and bioreactors for a larger scale of production of more proteins per unit volume of culture medium. This may be particularly useful when expensive reagents are needed and when the culture is scaled up. Furthermore, the use of 293S cells may be especially advantageous for preparing membrane proteins, because this cell line is known to express proteins whose activities are modulated by complex post-translation, a feature often observed for membrane proteins (Berg et al., 1993).

How can our method be used to specifically improve studies of ion channel receptors? Membrane proteins such as channels and transporters are routinely expressed in HEK 293 cells and directly assayed in single cells. Thus, our method should be beneficial in general and may be most beneficial in particular for studying proteins whose expression is not efficient or whose signal must be studied at very low concentrations of ligands. For instance, the expression of the GluR2 receptor channel, as used in the present study, is not as efficient as that of GluR6. In some reports, a saturating concentration of glutamate must be used to detect the receptor response (Grosskreutz et al., 2003). In such cases, enhancing the receptor yield in single cells will surely permit a larger current response at the same concentration of glutamate. Furthermore, this method will be useful in inhibitor–receptor studies, because in the presence of inhibitors, the receptor response is less than in their absence. Finally, the use of outside-out patches to achieve a faster receptor response, compared with the use of a whole cell, is a popular method in electrophysiological recording (Hamill et al., 1981). This recording configuration can minimize the distortion of the receptor response due to rapid channel desensitization. However, when outside-out patches are used, only a fraction of the receptor molecules expressed from an entire cell can be sampled because the area of the membrane from an outside-out patch is several hundred times smaller than that of an entire cell. Consequently, greater efficiency of the receptor expression in a single cell from which an outside-out membrane patch is excised is required. The use of TAg to increase the number of receptor molecules expressed in single cells is therefore expected to be helpful for such studies.

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