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Arginine Grafting to Endow Cell-Permeability

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Abstract

We report on a means to endow proteins with the ability to permeate mammalian cells without appending an exogenous domain. Our approach is to install a cationic patch on the surface of a target protein by the grafting of arginine residues. Doing so with green fluorescent protein (GFP) did not compromise conformational stability but enabled efficient cellular uptake that was dependent on cell-surface glycosaminoglycans. We anticipate that this cell-permeable variant of GFP, which obviates the need for transfection, will be useful for numerous applications in cell biology, and that the method of arginine grafting will be broadly applicable.

The plasma membrane is a natural barrier that excludes most molecules. Breaching this barrier is a limiting factor in the development of proteins and other biomolecules as therapeutics and diagnostic tools (1). Accordingly, there is much interest in developing new means to deliver proteins and other macromolecules into cells.

Small cationic peptides are capable of cellular entry, promoted best by the guanidinium group on the side chain of arginine residues (2–4). Appending polyarginine to proteins can enable uptake, but the transducing domain increases the size of a target protein and is readily susceptible to proteolysis (5). Chemical modification to supply a protein with cationic functional groups can likewise enable cellular entry (6,7), but at the expense of homogeneity. Some ribonucleases are known to invade mammalian cells (8), perhaps due to a natural cluster of cationic residues on their surface (9). These precedents inspired us to use site-directed mutagenesis to modify a three-dimensional scaffold with the intent of endowing cell permeability.

As a model, we chose to employ the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. GFP is a well-characterized protein (10–13) with a convenient signal for detecting cellular uptake—intrinsic fluorescence (14,15). More specifically, we made amino-acid substitutions in enhanced GFP (eGFP), which is the F64L/S65T variant and has desirable fluorescence properties (16).

GFP is an acidic protein, having a net charge (*i.e.*, Arg + Lys − Asp − Glu) of $Z = -9$ at neutral pH. We noted that one face of GFP is variegated with acidic and basic residues (Figure 1). We chose to replace the five acidic residues (Glu17, Asp19, Asp21, Glu111, and Glu124) on this face with arginine. These acidic residues reside on three, adjacent β-strands, proximal to five basic residues (Lys107, Arg109, Lys113, Lys122, and Lys126). Hence, these five substitutions

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Supporting Information Available: This material is available free of charge *via* the Internet.

created a highly cationic patch on the surface of eGFP (Figure 1), yielding a nearly neutral (Z = +1) variant that we refer to as cell-permeable GFP (cpGFP).

We produced cpGFP in *Escherichia coli* (17). Cation-exchange chromatography was especially efficacious in the purification of cpGFP, affording nearly homogeneous protein. The fluorescence properties of cpGFP were found to be nearly identical to those of eGFP (see Supporting Information).

Formation of the GFP fluorophore (18,19) requires its proper folding (20). Moreover, use of GFP requires the retention of its conformational stability in biological assays. Replacing anionic residues with cationic ones can alter protein stability, though this effect is not readily predictable (21). Hence, we used chemical denaturation to ascertain the effect of arginine grafting on the stability of eGFP. We observed that both cpGFP and eGFP have unfolding midpoints at $C_{1/2}$ = (3.1 ± 0.3) M guanidine–HCl (see Supporting Information). Thus, the creation of a cationic patch did not have a deleterious effect on conformational stability.

Cellular internalization of GFP can be visualized by fluorescence microscopy (14,15). Hence, we incubated HeLa cells with increasing concentrations of either cpGFP or eGFP for known times at 37 °C. Prior to visualization, cells were placed in fresh medium for 1 h to allow for the internalization of any protein bound to the cell surface. We observed fluorescence within living cells incubated with cpGFP and found its intensity to be dose-dependent, increasing at high concentration of cpGFP (Figure 2a–c). No cytotoxicity was observed, even upon incubation with 50 μM cpGFP. Although a small amount was detectable in the cytosol, cpGFP was observed primarily in vesicles. This localization is similar to that observed with cationic peptides, such as polyarginine (22). Insignificant fluorescence intensity was observed in cells incubated with eGFP (Figure 2d) or eGFP with a polyarginine appendage (23), though this latter experiment used neuronal cells and a much shorter incubation period.

Glycosaminoglycans (GAGs) such as heparan sulfate (HS) and condroitin sulfate (CS) on the cell surface can mediate the binding of cationic peptides and proteins (22,24,4). To probe for a role for GAGs in cpGFP internalization, we compared cell-surface binding and cellular internalization of cpGFP in wild-type Chinese hamster ovary (CHO) cells (CHO-KI) to that in a CHO cell line that is deficient in GAG biosynthesis. In wild-type CHO-K1 cells, cpGFP was observed to bind to the cell surface and undergo internalization (Figure 2e). In CHO-745 cells (which are deficient in HS and CS), there is little internalization of cpGFP (Figure 2f). At a 10-fold higher protein concentration, cpGFP is internalized in the GAG-deficient cell line (see Supporting Information). Similar results were obtained with another GAG-deficient cell line, CHO-677 (data not shown). Apparently, cpGFP internalization relies largely, but not exclusively, on the interaction with cell-surface GAGs, and is efficient in both human and rodent cells displaying GAGs.

GFP and its variants are in widespread use in cell biology (10,12,13). Among these variants, cpGFP is unique in obviating a need for transfection or chemical additives to infuse mammalian cells with a fluorescent protein (23), and hence could have numerous applications, both *in vitro* and *in vivo* (25). For example, some GFP variants respond to changes in the solution pH or reduction potential (17,26). Merging such variants with cpGFP could provide a useful sensor for important physicochemical parameters within living cells. Likewise, cpGFP could serve as a component of a FRET-based substrate for assays of proteolytic or other enzymatic activities (27,28). More generally, our data demonstrate that an extraneous transduction domain (2–4) is not a necessary component of a cell-permeable protein. Accordingly, we anticipate that arginine grafting could become a useful means to endow many proteins with cell-permeability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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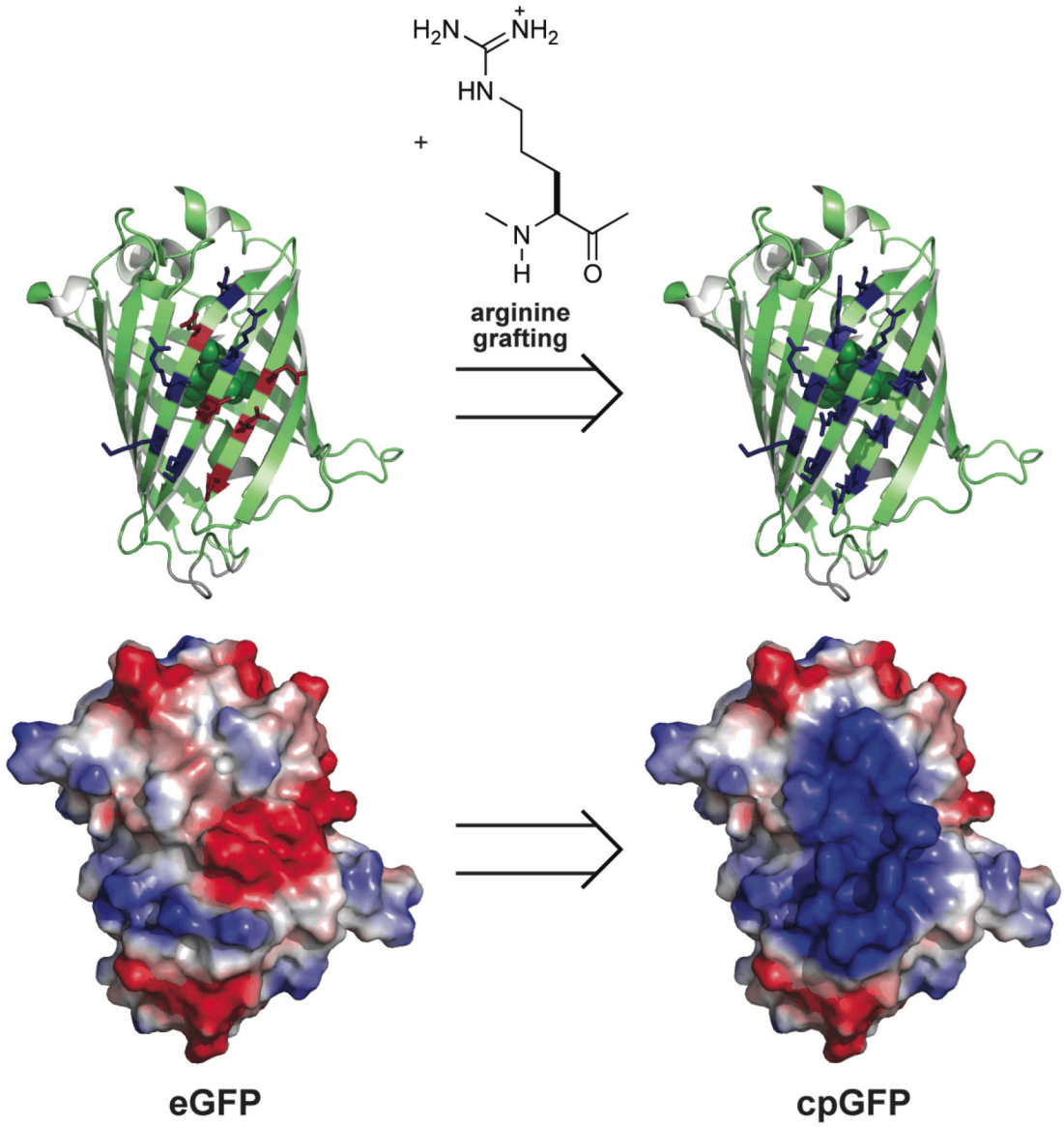

Figure 1.
Scheme for arginine grafting to create a cell-permeable variant of GFP (cpGFP). (**top**) Ribbon model depicting the location of the five anionic residues in GFP that were replaced with arginine to yield a surface comprised of ten cationic residues. The fluorophore is depicted in space-filling mode. (**bottom**) Space-filling model depicting the effect of the arginine substitutions on the electropotential surface (blue: cationic; red: anionic).

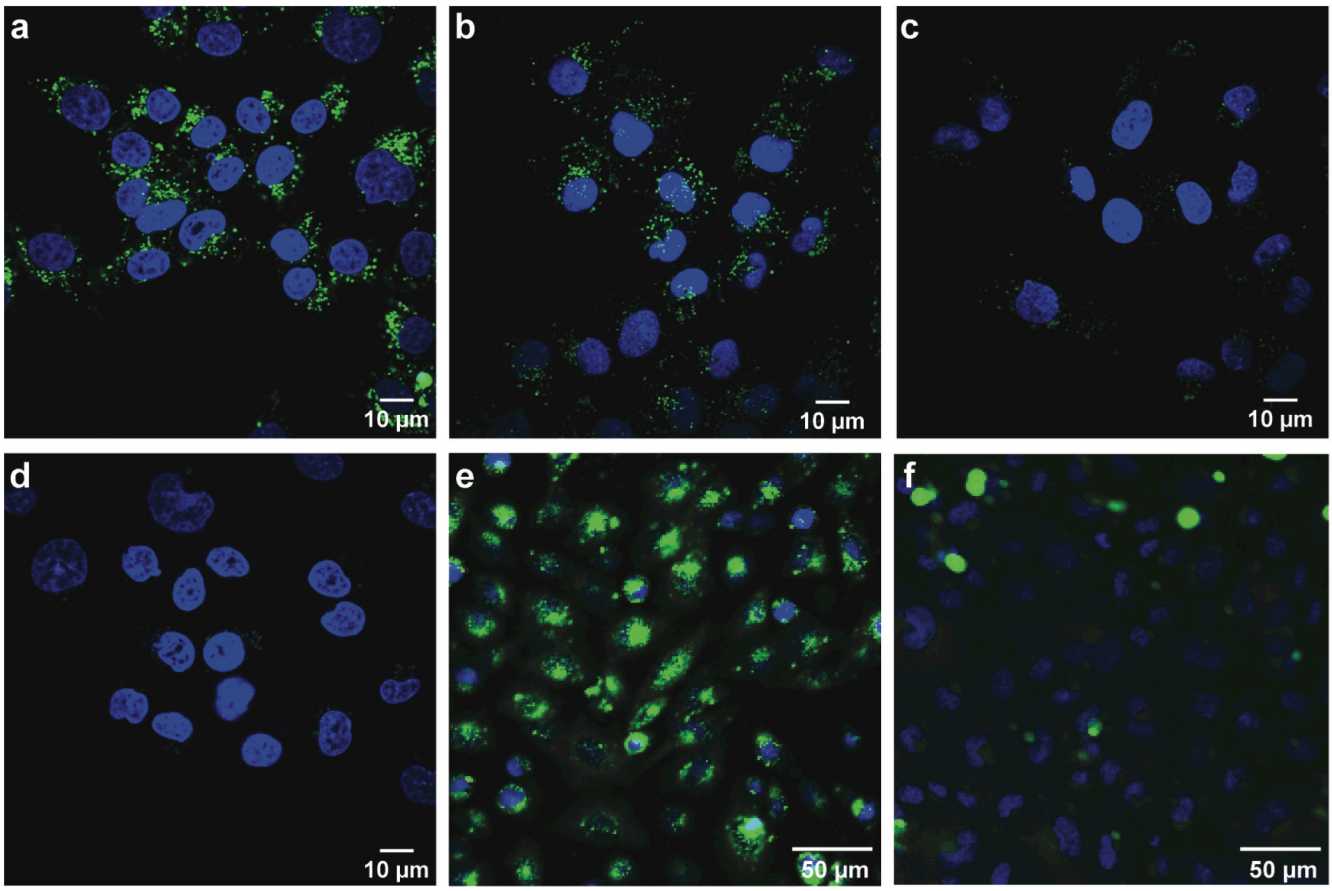

Figure 2.

Images of the internalization of GFP variants into living human and rodent cells. HeLa cells were incubated with cpGFP (**a**, 10 μM; **b**, 1 μM; **c**, 0.1 μM) and eGFP (**d**, 10 μM) for 3 h in Opti-MEM medium at 37 °C. Cells were then placed in fresh medium for 1 h and stained with Hoescht 33342 (blue) and propidium iodide (red) for 15 min prior to visualization by confocal microscopy. (**e**) CHO-K1 and (**f**) CHO-745 cells (which are GAG-deficient) were incubated with cpGFP (2 μM) for 3 h at 37 °C in Opti-MEM medium. Cells were then placed in fresh medium for 1 h and stained with Hoescht 33342 (blue) and propidium iodide (red) for 15 min prior to visualization.