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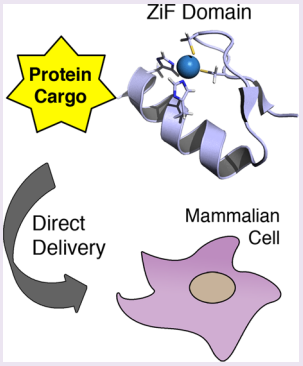
Protein Delivery Using Cys_2–His_2 Zinc-Finger Domains

Thomas Gaj,[†,*] Jia Liu, Kimberly E. Anderson, Shannon J. Sirk,[‡] and Carlos F. Barbas, III

The Skaggs Institute for Chemical Biology and the Departments of Chemistry and Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: The development of new methods for delivering proteins into cells is a central challenge for advancing both basic research and therapeutic applications. We previously reported that zinc-finger nuclease proteins are intrinsically cell-permeable due to the cell-penetrating activity of the Cys_2–His_2 zinc-finger domain. Here, we demonstrate that genetically fused zinc-finger motifs can transport proteins and enzymes into a wide range of primary and transformed mammalian cell types. We show that zinc-finger domains mediate protein uptake at efficiencies that exceed conventional protein transduction systems and do so without compromising enzyme activity. In addition, we demonstrate that zinc-finger proteins enter cells primarily through macropinocytosis and facilitate high levels of cytosolic delivery. These findings establish zinc-finger proteins as not only useful tools for targeted genome engineering but also effective reagents for protein delivery.



The efficient delivery of proteins and enzymes into cells is an integral step for disease treatment[1] and many basic research applications.[2] Among the safest approaches for delivering these macromolecules into cells is direct delivery of purified protein. To date, numerous approaches have been developed that facilitate direct protein entry into the cytoplasm of mammalian cells, including isolated[3−5] or designed[6−9] cell-penetrating peptides, liposomes,[10] protein containers,[11] polymeric microspheres,[12] and nanoparticles,[13,14] naturally occurring[15] and engineered "supercharged" proteins,[16−18] and virus-like particles.[19,20] However, these approaches are routinely confounded by various factors, such as low uptake efficiency,[21] unfavorable endosomal escape properties,[22,23] poor stability,[24] inadvertent cell-type dependency,[25] cytotoxicity,[26] and compromised enzyme activity.[16] As such, the development of new methods that enable the safe and efficient delivery of purified proteins into cells is needed to fulfill the promise of protein delivery as a therapeutic modality.

We previously showed that zinc-finger nucleases (ZFNs)—chimeric enzymes that induce DNA double-strand breaks at targeted genomic loci and thus promote genome editing[27]—are intrinsically cell-permeable.[28] The source of this cell-penetrating activity was shown to be the Cys–His_2 zinc-finger domain. Cys_2–His_2 zinc-fingers are among the most common DNA-binding motifs across all domains of life and consist of approximately 30 amino acid residues situated within a $\beta\beta\alpha$ structure (Figure 1A). Due to their modularity[29] and ability to be reprogrammed to recognize a wide range of DNA sequences,[30] zinc-finger proteins have emerged as powerful tools for genome engineering.[31] In addition, because zinc-fingers are inherently cell-permeable—presumably due to their net positive charge—we hypothesized that they could serve as generalized protein transduction reagents and facilitate direct protein delivery into various mammalian cell types, as observed with ZFN proteins.[28] Here, we show that genetic fusion of zinc-fingers (hereafter referred to as ZiF domains) onto proteins imparts a high degree of cell-penetrating activity that exceeds conventional protein transduction systems and that ZiF domains mediate efficient uptake of proteins and enzymes into a variety of primary and transformed mammalian cell types.

In order to determine whether ZiF domains could deliver functional proteins into mammalian cells, we genetically fused one-, two-, three-, four-, five- or six-finger ZiF proteins to the N-terminus of Emerald GFP (EmGFP), an engineered GFP variant that displays improved photostability and brightness compared to enhanced GFP.[32] Unlike "supercharged" GFP,[18] which possesses the innate ability to cross cell membranes due to its high overall charge,[16,33] EmGFP has no cell-penetrating activity. Because each ZiF domain harbors positive charge (theoretical net charge for typical one- and six-finger ZiF domains at physiological pH: +4 and +20, respectively), we anticipated that ZiF proteins composed of extended zinc-finger arrays would demonstrate increased cell-penetrating activity in comparison to those with fewer. To ensure that ZiF proteins could not recognize DNA, we substituted the α-helical DNA-binding residues of each zinc-finger domain (i.e., −1, 2, 3, and 6) with alanine (Figure 1A). Importantly, because these substitutions lie within positions that routinely encode positively charged residues, the overall net charge for each ZiF domain was expected to decrease (theoretical net charge for an alanine-substituted six-finger ZiF protein: +10. We note that theoretical net charges were calculated in the absence of terminal modifications, such as the polyhistidine-tag, which are expected to increase charge). The complete amino acid

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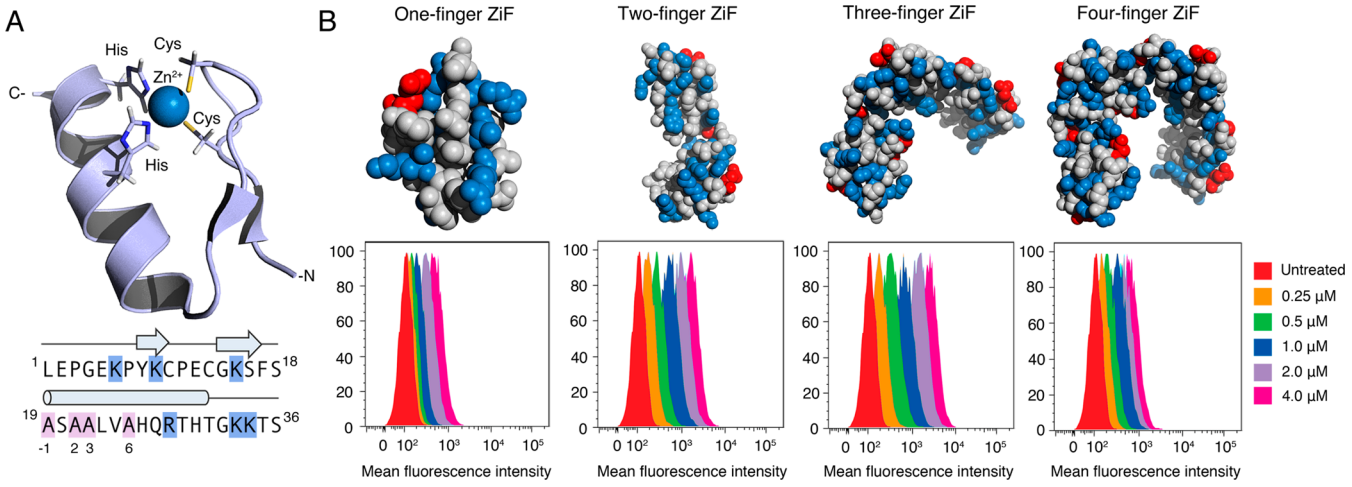

Figure 1. Structure and cellular internalization of zinc-finger proteins. (A) (Top) Structure of a single zinc-finger (ZiF) domain (light blue). The side-chains of the conserved Cys and His residues coordinated with a Zn^{2+} ion (dark blue sphere) are shown as sticks (PDB ID: 2I13).[44] (Bottom) Sequence of the ZiF domain used in this study. Cylinders and arrows indicate α-helix and β-sheet secondary structures, respectively. Alanine-substituted α-helical DNA-binding residues are shaded light pink and numbered according to their position. Positively charged residues are shaded blue (B) (Top) Sphere-representation of one-, two-, three- and four-finger ZiF domains. Positively and negatively charged residues are highlighted blue and red, respectively. Neutral residues are shown as gray spheres. (Bottom) Mean fluorescence intensity as determined by flow cytometry in HeLa cells treated with increasing concentrations of one-, two-, three-, and four-finger ZiF-EmGFP protein.

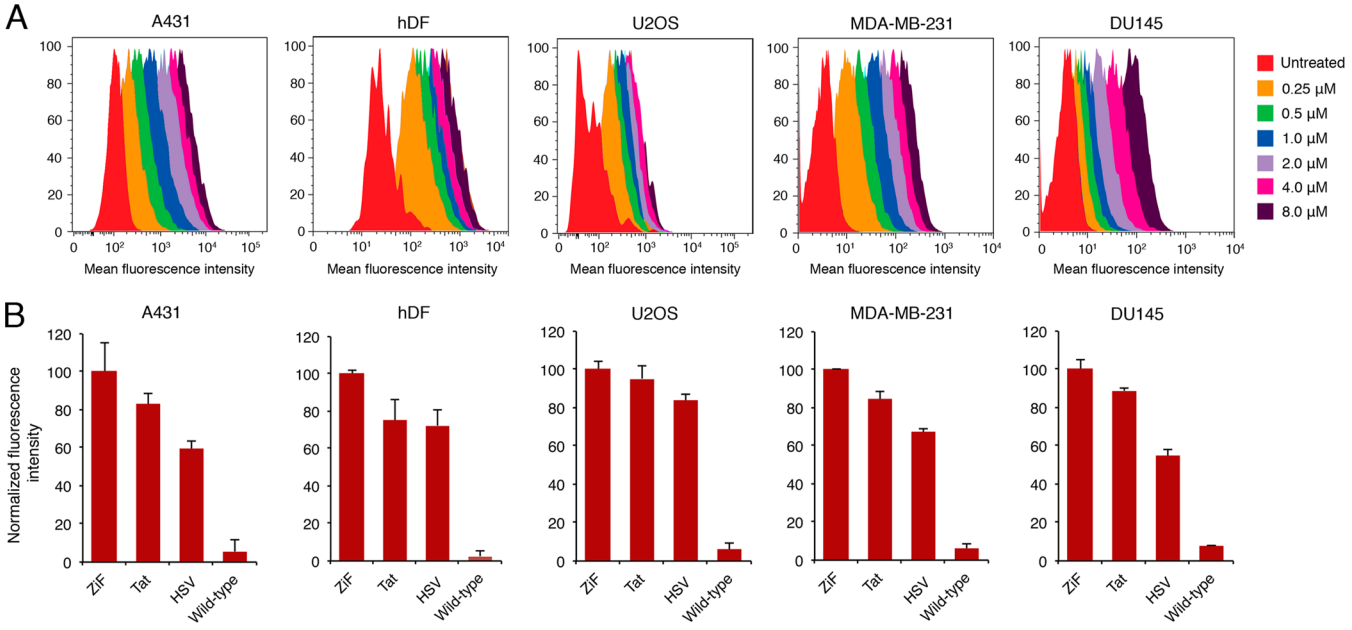

Figure 2. ZiF-mediated protein delivery into mammalian cell types. (A) Mean fluorescence intensity as determined by flow cytometry in A431, human dermal fibroblast (hDF), U2OS, MDA-MB-231, and DU145 cells treated with various concentrations of two-finger ZiF-EmGFP protein and (B) HeLa cells treated with 2.0 μM of either two-finger ZiF-EmGFP, TAT-EmGFP, HSV-EmGFP or unmodified EmGFP (wild-type). Data normalized to untreated cells. Error bars indicate standard deviation (n = 3).

sequence of the zinc-finger domain used in this study is shown in Figure 1A. We expressed each fusion protein in *Escherichia coli* and purified one-, two-, three- or four-finger ZiF-EmGFP proteins to >95% homogeneity (Supporting Information Figure S1) and high yields (>25 mg/L). To our surprise, EmGFP proteins fused to either five- or six-finger ZiF domains were unable to be consistently produced in quantities high enough for downstream analysis, perhaps due to low overall stability (data not shown).

We directly applied 0.25, 0.5, 1, 2, and 4 μM of one-, two-, three-, or four-finger ZiF-EmGFP onto HeLa cells for 90 min at 37 °C and measured EmGFP internalization using flow cytometry. After incubation, cells were washed three times with heparin to remove any surface-bound protein. For each ZiF domain, we observed a dose-dependent increase in EmGFP fluorescence (Figure 1B). Maximum delivery was achieved using both two- and three-finger ZiF domains, indicating that factors other than charge, including protein stability or folding, might influence the efficiency of ZiF-mediated delivery. In particular, parallel substitution of the α-helical DNA-binding residues with alanine might reduce the overall stability of extended ZiF arrays. For two- and three-finger ZiF domains, nearly all cells were positive for EmGFP fluorescence at protein concentrations as low as 0.5 μM (Supporting Information

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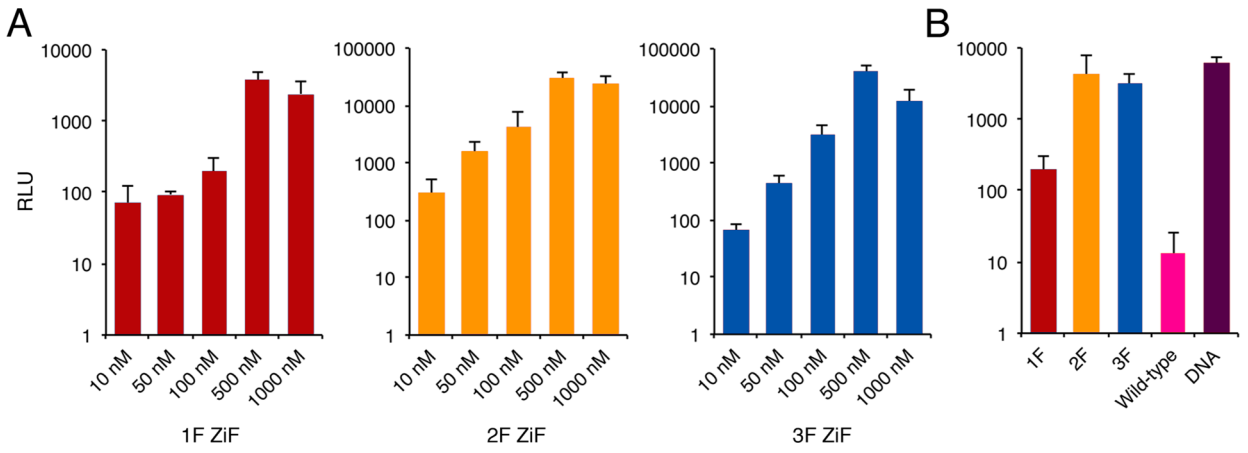

Figure 3. ZiF-mediated delivery of functional enzymes into human cells. (A) Relative luminescence units (RLU) in HeLa cells treated with various concentrations of one-, two- or three-finger ZiF-luciferase protein. (B) RLU in HeLa cells treated with 2.0 μM of either one-, two- or three-finger ZiF-luciferase protein or unmodified (wild-type) luciferase protein, or transfected with 20 ng of luciferase expression vector (pGL3-Prm). All cells were transfected with *Renilla* luciferase (pRL-CMV) 24 h prior to protein treatment. Data normalized to untreated cells transfected with *Renilla* luciferase. Error bars indicate standard deviation (n = 3).

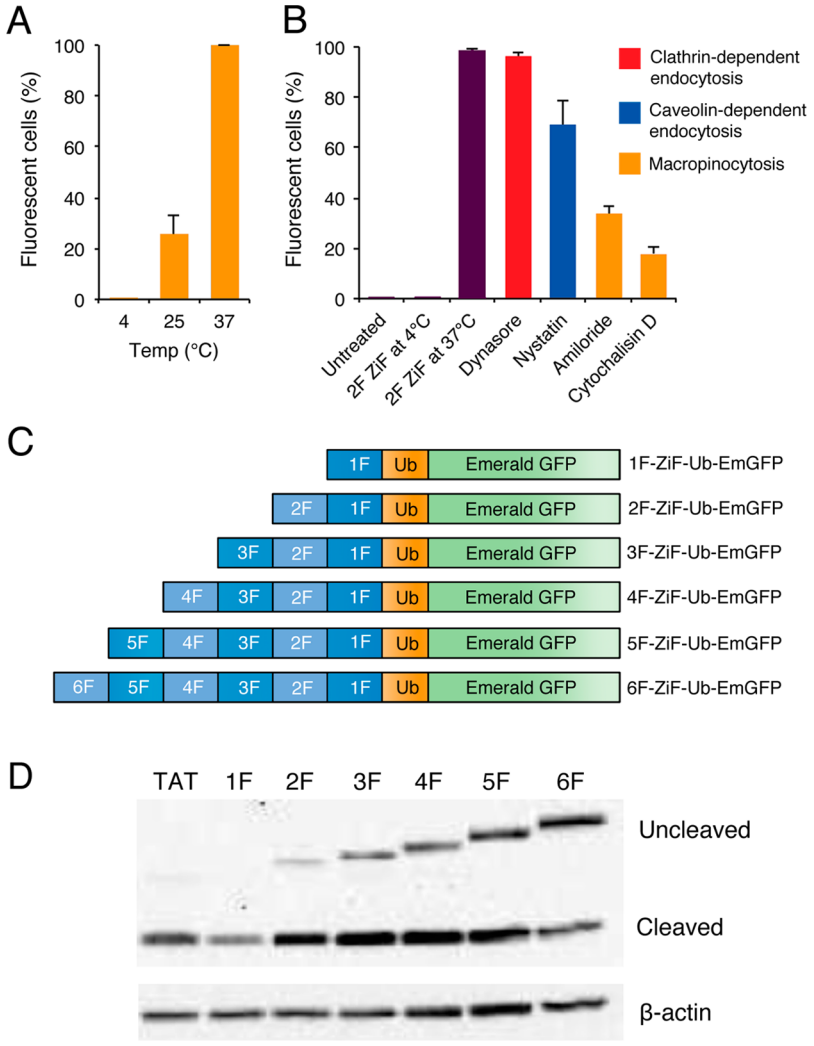

Figure 4. Mechanism of ZiF protein uptake. (A and B) Mean fluorescence intensity of HeLa cells treated with 2 μM of two-finger ZiF-EmGFP protein at (A) 37, 25, or 4 °C or (B) with various endocytic pathway inhibitors. Colors indicate blocked pathway. Error bars indicate standard deviation (n = 3). (C) Diagrams of ZiF-Ub-EmGFP fusion proteins. (D) Western blot of lysate from HeLa cells treated with 4 μM of Ub-EmGFP fusion proteins. Samples were taken immediately after protein treatment and probed with rabbit anti-GFP or rabbit anti-β-actin antibodies, followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies.

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Figure S2), and fluorescence could be further increased by consecutive protein treatments (Supporting Information Figure S3). Critically, no intracellular fluorescence was observed in the absence of any ZiF motif indicating that ZiF domains were responsible for mediating protein uptake. To test the generality of ZiF-mediated protein delivery, we treated multiple cancer cell lines, including A431, U2OS, MDA-MB-231, and DU145 cells, as well as primary human dermal fibroblasts (hDFs) with 0.25, 0.5, 1, 2, 4, and 8 μM of two-finger ZiF-EmGFP protein. As with HeLa cells, we observed dose-dependent EmGFP fluorescence with maximum delivery achieved using 8 μM of two-finger ZiF-EmGFP (Figure 2A). In addition, for each cell type tested, we found that two-finger ZiF proteins were more effective at delivering EmGFP than the TAT[34] and HSV[4] cell-penetrating peptides by approximately 1.5- to 2-fold (Figure 2B). These findings demonstrate that ZiF domains are effective reagents for facilitating protein delivery into various mammalian cell types.

In order to test whether ZiF domains could mediate the entry of functional enzymes into cells, we next fused one-, two- or three-finger ZiF proteins to the N-terminus of firefly luciferase, an enzyme that catalyzes the oxygenation of the small molecule luciferin using ATP and thus is a potentially useful tool for measuring the concentration of intracellular energy metabolites. All proteins were expressed in *E. coli* and purified to >50% homogeneity (Supporting Information Figure S4) at yields similar to those for native protein (~4 mg/L). Critically, we found that fusion of ZiF domains did not affect luciferase activity *in vitro* (Supporting Information Figure S5). We incubated HeLa cells with 10, 50, 100, 500, or 1000 nM of one-, two- or three-finger ZiF-luciferase protein for 90 min at 37 °C and, in order to control for cell number, transfected HeLa cells with plasmid-encoding the *Renilla* luciferase 24 h prior to treatments. Immediately after incubation with protein, cells were washed three times with heparin and lysed to measure the activity of internalized luciferase protein. We again observed a dose- and ZiF-dependent increase in protein internalization with maximum delivery achieved using 500 nM of two-finger ZiF protein (Figure 3A). Compared to untreated cells, we observed a >50 000-fold increase in luminescence using both two- or three-finger ZiF proteins. Most impressively, both two- and three-finger domains delivered luciferase protein into cells as effectively as Lipofectamine-mediated plasmid transfection (Figure 3B). We attempted to compare ZiF-mediated luciferase delivery to both the TAT and HSV cell-penetrating peptides but were unable to express either fusion protein, presumably due to aggregation caused by decreased protein stability. Previous studies, however, have indicated that TAT enhances luciferase uptake by only ~16-fold compared to untreated cells.[35] Together, these findings indicate that ZiF domain attachment does not compromise enzyme activity and facilitates efficient enzyme uptake into cells.

We next sought to determine the mechanism through which ZiF proteins enter cells. For this, we treated HeLa cells with 2 μM of two-finger ZiF-EmGFP protein for 90 min at 37 °C, 25 or 4 °C and measured protein internalization by flow cytometry. No ZiF-fused protein was observed in cells incubated at 25 and 4 °C, indicating that ZiF proteins enter cells through an energy-dependent process (e.g., endocytosis) (Figure 4A). We next evaluated the effects of compounds known to inhibit distinct endocytic pathways on ZiF protein uptake, including dynasore and nystatin, which prevent clathrin- and caveolin-dependent endocytosis, respectively, and amiloride and cytochalasin D, which both block macropinocytosis. We pretreated HeLa cells with each inhibitor for 1 h before incubation with 2 μM of two-finger ZiF-EmGFP protein for 90 min at 37 °C. We then washed cells 3 times consecutively with heparin and measured EmGFP uptake by flow cytometry. Compared to control cells, dynasore had no impact on ZiF protein uptake; however, both amiloride and cytochalasin D inhibited ZiF protein uptake by >60% and 80%, respectively, while nystatin reduced EmGFP fluorescence by ~25% (Figure 4B). These findings indicate that ZiF proteins enter cells primarily through macropinocytosis and, at low frequencies, caveolin-dependent endocytosis.

Finally, we set out to measure the efficiency at which ZiF proteins escape endosomes and enter the cytoplasm. To accomplish this, we utilized an enzymatic assay that relies on processing of ubiquitin (Ub) by deubiquitinating enzymes (DUBs) located exclusively in the cytoplasm.[36] We generated a series of ZiF-Ub-EmGFP proteins that contained a single Ub moiety linked to the N-terminus of EmGFP and the C-terminus of either a one-, two-, three-, four-, five- or six-finger ZiF domain (Figure 4C). Unlike our earlier studies, high levels of five- and six-finger ZiF fusion proteins were produced, indicating that linker composition or neighboring domains might influence ZiF stability. We treated HeLa cells with 4 μM of each ZiF-Ub-EmGFP protein for 90 min at 37 °C and measured the percentage of cleaved versus uncleaved protein by Western blot. All ZiF domains were efficiently deubiquitinated with >80% of one- and two-finger ZiF fusion proteins cleaved after internalization (Figure 4D). In general, deubiquitination decreased with ZiF domain length. Schepartz and co-workers recently reported that as little as 12% of internalized ZiF proteins have access to the cytoplasm.[37] The exact reason behind this discrepancy remains unknown but could be due to our inclusion of additional domains that might protect single ZiF domains from degradation or perhaps differences in protein concentration and incubation time. Taken together, these results indicate that proteins delivered into cells by ZiF domains escape endosomes and enter the cytoplasm with high efficiency.

We show here that cell-penetrating zinc-finger domains are a generalizable class of protein transduction reagents. We demonstrate that proteins genetically fused to either two- or three-finger zinc-finger proteins exhibit potent cell-penetrating activity and that zinc-finger domains are capable of mediating delivery of functional proteins into a wide variety of primary and transformed mammalian cell types. We also show that zinc-finger proteins enter cells primarily through macropinocytosis and, at low frequencies, caveolin-dependent endocytosis. Cell entry is likely attributable to the constellation of one Arg and five Lys residues present on the zinc-finger surface. We suspect that electrostatic interactions between these basic residues and negatively charged lipids might stabilize the zinc-finger $\beta\beta\alpha$ configuration and facilitate its insertion into the membrane interior. Indeed, several studies have indicated the importance of secondary structure for endocytosis-independent cellular internalization,[38,39] particularly for cell-penetrating peptides derived from transcription factors, such as penetratin.[38]

Schepartz and colleagues previously reported the design of a zinc-finger module, ZF5.3, capable of entering cells.[40] Unlike the ZiF domains used in this work, ZF5.3 is based on an Arg-rich α-helical peptide-motif and contains only one basic residue outside this region. Thus, while the ZiF domain and ZF5.3 share similar secondary structures, they exhibit distinct charge

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distribution characteristics and perhaps even cell entry mechanisms. Surprisingly, the charge density of the zinc-finger domains used here are lower than those for many well studied cell-penetrating peptides, including TAT, Hph-1, penetratin, and transportan, as well as naturally occurring cell-penetrating DNA-binding proteins,[15] such as c-Jun and N-DEK. While the relative importance of each positively charged residue within the zinc-finger domain remains unknown, site-directed mutagenesis studies should shed light on their role in cell entry. The cell-penetrating activity of zinc-finger domains could also be leveraged in the future for delivery of DNA or RNA via reversible chemical linkage or transfection through formation of zinc-finger-nucleic acid complexes. Finally, the finding that zinc-finger domains are cell-penetrating suggests the unique possibility that zinc-finger domains might play a role in cell-to-cell communication, as proposed for other cell-penetrating DNA-binding proteins.[15]

METHODS

Plasmid Construction. Alanine-substituted zinc-finger domains were assembled as previously described[41] and released from pSCV by digestion with *Xma*I and SpeI and ligated into the AgeI and SpeI restriction sites of pET-Emerald-GFP (kindly provided by Y. Kato) to generate pET-1-, 2-, 3- and 4-, 5-, and 6-F-ZiF-Emerald-GFP. The firefly luciferase gene was PCR amplified from pRS413-GAL1-luc*(-SKL) (Addgene plasmid 40234)[42] using the primers 5′ AgeI-Luc and 3′ Sac-Luc. PCR products were digested with AgeI and SacI and ligated into the *Xma*I and SacI restriction sites of pET-1-, 2-, 3-, 4-, 5-, and 6-F-ZiF-Emerald-GFP to generate pET-1-, 2-, 3-, 4-, 5-, and 6-F-ZiF-luciferase. Primer sequences are provided in Supporting Information Table S1. TAT and HSV cell-penetrating peptides were fused to the N-terminus of Emerald GFP by PCR using the template pET-Emerald-GFP and the primers 5′ NdeI-TAT-GFP or 5′ NdeI-HSV-GFP and 3′ SacI-GFP. PCR products were ligated into the NdeI and SacI restriction sites of the pET-28 (+) expression vector (Novagen) to generate the plasmids pET-Tat-Emerald-GFP and pET-HSV-emerald-GFP. Ubiquitin was synthesized via overlap PCR using complementary oligonucleotides and subsequently introduced into the AgeI restriction site of pET-ZiF-emerald-GFP to generate pET-1-, 2-, 3-, 4-, 5-, and 6-F-ZiF-Ub-emerald-GFP. Correct construction of each expression cassette was verified by sequence analysis (Supporting Information Table S2).

Protein Expression and Purification. Chemically competent *E. coli* BL21 (DE3) cells (Stratagene) were transformed with ZiF expression plasmid. A single colony was added to 6 mL of lysogeny broth (LB) in the presence of 50 μg/mL kanamycin and 1% glucose and bacteria were grown overnight at 37 °C with shaking. The following day, 100 mL of LB medium supplemented with 50 μg/mL kanamycin, 100 μM $ZnCl_2$ and 1% glucose was inoculated with 2 mL of the overnight culture and incubated at 37 °C with shaking until an OD_{600} of 0.8. Protein synthesis was then induced with 2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells expressing EmGFP or luciferase were then incubated with shaking at 37 °C for 6 h or 25 °C for 10 h, respectively. After incubation, cells were harvested by centrifugation at 2,000 RCF for 15 min at 4 °C and the pellet was resuspended in lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 100 μM $ZnCl_2$, 1 mM DTT, 1 mM $MgCl_2$, 1 mM PMSF, 10 mM imidazole, pH 8.0). Cells were lysed by sonication and the soluble fraction was centrifuged at 25 000 RCF for 30 min at 4 °C. ZiF fusion proteins were purified using Ni-NTA agarose resin (QIAGEN) and eluted with 500 mM imidazole in lysis buffer. All proteins were concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (EMD Millipore). Glycerol was added to the purified protein solution to a final concentration of 10% (v/v) and protein was stored at −80 °C indefinitely. Protein purity and concentration were determined by SDS-PAGE.

Cell Culture and Protein Treatments. HeLa, A431, U2OS, MDA-MB-231, DU145, and primary human dermal fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Life Technologies). Cells were seeded onto 24-well plates at a density of 2×10^5 cells per well and established in a humidified 5% CO_2 atmosphere at 37 °C. ZiF proteins were diluted into serum-free medium containing 100 μM $ZnCl_2$. At 24 h after seeding, cells were washed with PBS and treated with ZiF proteins for 90 min at 37 °C. After treatment, cells were washed with three times with 0.5 mg/mL heparin and harvested for analysis. ZiF-EmGFP-treated cells were resuspended with PBS/1% FBS and EmGFP fluorescence was measured by flow cytometry (FACScan Dual Laser Cytometer; BD Biosciences; FACSDiva software). For each sample, 10 000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc.). ZiF-luciferase-treated cells were lysed with Passive Lysis Buffer (Promega) and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) using a Veritas Microplate Luminometer (Turner Biosystems). *Renilla* luciferase expression from pRL-CMV (Promega) was used to normalize for cell number. Transfections were performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

Western Blots. Treated HeLa cells were lysed with Passive Lysis Buffer (Promega) and 20 μg of total proteins was analyzed by SDS-PAGE with a Novex 4−20% Tris-Glycine Gel (Invitrogen). Samples were transferred onto membrane and visualized by chemiluminescence as previously described.[43] ZiF-EmGFP protein was detected by rabbit anti-GFP antibody (Invitrogen) and horseradish peroxidase conjugated anti-rabbit secondary antibody. β-actin was used as an internal loading control and was detected with peroxidase conjugated anti-β-actin antibody (Sigma).

ASSOCIATED CONTENT

Supporting Information

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AUTHOR INFORMATION

Corresponding Author

*Email: gaj@berkeley.edu.

Present Addresses

†University of California, Berkeley, Berkeley, California, 94720-5800, United States

‡Stanford University, Stanford, California, 94305, United States

Notes

The authors declare no competing financial interest.

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