11. Huang, J.C., Hsu, D.S., Kazantsev, A., and Sancar, A. (1994). Proc. Natl. Acad. Sci. USA *91*, 12213–12217.
12. Matsunaga, T., Mu, D., Park, C.H., Reardon, J.T., and Sancar, A. (1995). J. Biol. Chem. *270*, 20862–20869.
13. Gunz, D., Hess, M.T., and Naegeli, H. (1996). J. Biol. Chem. *271*, 25089–25098.
14. Mu, D., Wakasugi, M., Hsu, D.S., and Sancar, A. (1997). J. Biol. Chem. *272*, 28971–28979.
15. Hess, M.T., Schwitter, U., Petretta, M., Giese, B., and Naegeli, H. (1997). Proc. Natl. Acad. Sci. USA *94*, 6664–6669.
16. Buschta-Hedayat, N., Buterin, T., Hess, M.T., Missura, M., and Naegeli, H. (1999). Proc. Natl. Acad. Sci. USA *96*, 6090–6095.
17. Tang, J.Y., Hwang, B.J., Ford, J.M., Hanawalt, P.C., and Chu, G. (2000). Mol. Cell *5*, 737–744.
18. Sugasawa, K., Okamoto, T., Shimizu, Y., Masutani, C., Iwai, S., and Hanaoka, F. (2001). Genes Dev. *15*, 507–521.

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Calcium Liberates PNAs from Endosomes

Many peptides are reported to enhance cellular uptake of peptide nucleic acids and other macromolecules. Cellular uptake, however, is not synonymous with cellular activity. In this issue of *Chemistry & Biology*, Nielsen and colleagues [1] examine the traffic of PNAs and investigate protocols for improving recognition of target mRNA inside cells.

Synthetic nucleic acids and nucleic acid mimics have the potential to bind to complementary targets inside cells and control gene expression. In theory, all genes can be targeted, allowing an unlimited array of research tools and providing a starting point for development of therapeutics capable of treating almost any disease.

Currently, it is clear that antisense oligonucleotides, small interfering RNAs (siRNAs), and peptide nucleic acids (PNAs) can bind to nucleic acid targets inside cells and block gene expression [2]. The challenge now is to enhance the potency and specificity of these agents so that the potential of the approach can be fully exploited. Enhanced potency would (1) make protocols simpler and more reliable; (2) reduce off-target effects due to unwanted interactions inside cells; (3) reduce costs; and (4) increase the likelihood of successful translation into clinical use. Nielsen and coworkers attack this challenge at its most fundamental level: understanding how oligomers move within the cells and how to maximize the concentration of oligomer available for recognition of cellular targets [1].

PNA is a nonionic DNA/RNA mimic in which the deoxyribose backbone is replaced by uncharged amide linkages. PNAs bind complementary sequences by Watson-Crick base pairing with excellent affinity and specificity, making them promising agents for in vivo target recognition. To exploit these strengths, however, PNAs must be able to enter cells and bind to their targets. One method for effective cellular delivery involves transfection of PNAs mixed with DNA and cationic lipid [3], but this multistep protocol is complex. Several laboratories have synthesized PNA-peptide conjugates that spontaneously cross cell membranes and are active inside cells [4–6]. This method is more convenient, but inhibition by these conjugates is inefficient relative to lipid-mediated transfection.

Cells have the ability to take up macromolecules directly from the environment through a process known as endocytosis. These macromolecules enter an intracellular compartment known as the endosome. Endosomes are gradually converted into lysosomes, which are specialized compartments capable of degrading macromolecules by a variety of hydrolytic pathways. A PNA inside an endosome or lysosome will appear by microscopy to be inside a cell but will be useless for recognition of RNA or DNA.

The work by Nielson and colleagues begins with recognition that efficient entry into cells can be easily achieved by PNA-peptide conjugates and that the real challenge is converting entry into colocalization with target. They hypothesized that the PNA-peptides are being taken up by endosomes and that endosomal release limits biological activity. A recent report on uptake of plasmid DNA in complex with histones [7] led them to speculate that a simple experimental manipulation—addition of calcium—could enhance endosomal release and activity by PNAs.

Nielson and colleagues coupled a variety of different cationic peptides to PNAs and focused on examining conditions likely to improve endosomal/lysosomal release. They use a luciferase-based reporter assay to examine the efficacy of the PNA conjugates. In this assay, active luciferase is expressed only when a PNA binds to the reporter mRNA and blocks an aberrant splice site. The advantage of this assay is that it produces an unambiguous positive signal and virtually eliminates the possibility of artifactual results.

In all cases, addition of calcium to the media caused significant increases in luciferase activity. Some increases were modest. Addition of calcium increased activity of a PNA tagged with nine arginines by only 2-fold. However, the activity of a PNA attached to a widely-used import peptide derived from HIV TAT protein increased over 30-fold. The authors also tested the effect of addition of chloroquine, a small molecule known to disrupt endosomes and promote release of trapped molecules [8]. This treatment also generated large increases in antisense activity.

The authors used microscopy and intracellular visualization of fluorescent PNA-peptide conjugates to bolster the linkage between calcium addition, endosomal

release, and enhanced activity. In the absence of calcium, the authors observed a punctate distribution consistent with localization to the endosome. Upon addition of calcium, fluorescence appears to increase at the plasma membrane, suggesting that addition of calcium may bring more PNA close to the cell and increase endosome-mediated internalization. Other experiments with fluorescent dextran, a marker for internalization, showed that calcium addition promoted greater endosomal release into the cytoplasm. It will be interesting to further investigate the mechanism of calcium-mediated uptake and to see the results of similar studies using chloroquine.

It is important to note that the authors used confocal microscopy and live cells. Many previous reports of localization of PNAs and oligonucleotides have used fixed cells. Fixatives can cause diffusion of cellular contents, making it essential that live cells be observed for these types of studies [9]. Studies that do not use confocal microscopy cannot readily determine if macromolecules are bound inside cells or on their surface. It is likely that further progress in this area will require increasingly sophisticated techniques for the accurate examination of the localization of PNAs and other macromolecules.

Mammalian cells are complicated biological structures with multiple compartments. There are many opportunities for movement between compartments and between the inside and outside of a cell. Most work with synthetic small molecules, oligonucleotides, PNA, and peptides has ignored this complexity and focused on important but limited questions: Does the compound enter cells? Is the compound active? Shirashi et al. [1] ask the next generation of question: Once the compound is in a cell, what can be done to maximize its ability to colocalize with its target? This is a question that chemists and biologists need to address directly more often.

Chloroquine and calcium are two answers to the question of how to increase the effective concentration of PNA inside cells. It is likely that other agents can also be added to cultured cells to produce similar or even better effects. It would be preferable, however, to avoid the use of additives together. The ideal solution would be a simple chemical modification that could be introduced directly onto a PNA or a PNA-peptide conjugate. Such hypothetical conjugates could be developed for ease of synthesis and use.

The experiments by Nielson and coworkers focused on uptake and release of PNAs in cultured cells, but it is essential to remember that the potential for the application is much larger. These solutions may apply to many other types of synthetic molecules and be important for work in cell culture, animal studies, and therapeutic development.

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Selected Reading

1. Shirashi, T., Pankratova, S., and Nielsen, P.E. (2005). Chem. Biol. *12*, this issue, 923–929.
2. Kaihatsu, K., Janowski, B.A., and Corey, D.R. (2004). Chem. Biol. *11*, 749–758.
3. Janowski, B.A., Huffman, K.E., Schwartz, J.C., Ram, R., Hardy, D., Shames, D.S., Minna, J.D., and Corey, D.R. (2005). Nat. Chem. Biol. *1*, 210–215.
4. Sazani, P., Kang, S.H., Maier, M.A., Wei, C., Dillman, J., Summerton, J., Manoharan, M., and Kole, R. (2001). Nucleic Acids Res. *29*, 3965–3974.
5. Pooga, M., Soomets, U., Bartfai, T., and Langel, U. (2002). Methods Mol. Biol. *208*, 225–236.
6. Kaihatsu, K., Huffman, K.E., and Corey, D.R. (2004). Biochemistry *43*, 14340–14347.
7. Zaitsev, S., Buchwalow, I., Haberland, A., Tkachuk, S., Zaitseva, I., Haller, H., and Bottger, M. (2002). Acta Histochem. *104*, 85–92.
8. Wadia, J.S., Stan, R.V., and Dowdy, S.F. (2004). Nat. Med. *3*, 310–315.
9. Belitsky, J.M., Leslie, S.J., Arora, P.S., Beerman, T.A., and Dervan, P.B. (2002). Bioorg. Med. Chem. *10*, 3313–3318.