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PERSPECTIVE

Fusion Polypeptides That Inhibit Exocytosis: Fusing Aptamer and Cell-Penetrating Peptide Technologies and Pharmacologies

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ABSTRACT

Cell-penetrating peptides are amphipathic or cationic oligopeptides able to transport covalently attached cargoes across cell membranes. Peptide aptamers are polypeptide fragments of endogenous proteins that mimic and thus perturb interactions with other cellular proteins. Combining aptamer and CPP technology can generate pharmacological reagents effective in cell culture models and in vivo.

This issue of *Molecular Pharmacology* contains a contribution from Matsushita et al. (2005) describing a novel class of pharmacological inhibitors of regulated secretion. They have designed polypeptides that possess an N-terminal sequence with cell penetrating activity and occlude the secretory machinery via a second unique C-terminal sequence. These workers have thus joined two emerging areas, peptide aptamers and cell-penetrating peptides (CPPs), to create a peptide-based pharmacological reagent that acts within cells.

The term 'aptamer' has been largely co-opted to refer to oligonucleotides selected by the SELEX technology to bind to specific protein targets (Brody and Gold, 2000). However, peptide aptamers are fragments of endogenous polypeptides that bind to cognate protein/polypeptide binding sites and act as 'perturbogens' to occlude assembly, trafficking, signaling, or metabolic or other enzymatic processes (Juliano et al., 2001). As more is learned about protein-protein interactions structurally through X-ray crystallography and functionally through yeast two-hybrid and coimmunoprecipitation-style interaction experiments, attempts to create pharmacological reagents based on the small peptide fragments that comprise protein-protein contact domains to mask interaction sites and perturb protein-protein interactions are proceeding with intensity in many laboratories (Geyer and Brent, 2000). Initially, peptide aptamers were applied mainly to extracellular targets, including pseudosubstrates for protein cleavage enzymes (Hertting and Meyer, 1974) and so-called 'receptor decoy' or 'antireceptor' peptides: fragments of receptors such as the cholinergic nicotinic receptor, to bind cholinergic neurotoxins, or the HIV receptor CD4 to bind viral envelope and decrease infectivity (Gershoni and Aronheim, 1988; Lifson et al., 1988). Directing peptide aptamers, as pharmacological agents, to intracellular targets requires the ability to penetrate the cell membrane, a property most peptides do not possess. Thus, aptamer modification is required. Peptide permeability can be enhanced by myristoylation (O'Brian et al., 1990) or covalent attachment of additional peptide sequences that facilitate cell entry.

CPPs from a number of sources have been covalently attached to oligonucleotides, fluorescent probes, and other peptides to bring these cargoes into mammalian cells (Green et al., 2003; Trehin and Merkle, 2004). CPPs are now used fairly extensively, although, as is typical of such tools during early development, details of their full cellular range, caveats for utilization, off-target effects, efficacy, and pharmacodynamics are still fairly incomplete. This is partly because

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ABBREVIATIONS: CPP, cell-penetrating peptide; NSF, *N*-ethyl maleimide-sensitive factor; SNARE, soluble NSF attachment protein receptor; SNAP, soluble NSF attachment protein; vWF, von Willebrand factor.

important details of their molecular mechanisms of action are still lacking. Three major peptide carriers can be briefly described. The antennapedia peptide RQIKIWFQNRRMKWKK, or penetratin, was identified in 1994 as a segment of the antennapedia homeodomain protein that allows its penetration across biological membranes (Derossi et al., 1994). A recent intriguing use is in a fusion peptide to a nuclear export protein fragment that occludes mRNA export from the nucleus (Gallouzi and Steitz, 2001). VP22, DAATATRGRSAASRPTERPRAPARSASRPRRPVD, lends itself to expression vectors because it is relatively large and has been used to create fusion proteins via expression during in vivo transduction to increase the spread of genetically expressed cargo to neighboring cells (Schwarze and Dowdy, 2000). An 11-amino acid fragment of the HIV transactivator of transcription (Tat) protein (YGRKKRRQRRR) was first shown to penetrate cell membranes in the context of its mediation of transcellular effects of HIV (Frankel and Pabo, 1988) then harnessed, like the lentiviral genome itself, for use in vectorial delivery of nonviral cargo to mammalian cells. More recently, because of the basic amino acid-rich composition of the three naturally occurring cell-permeant peptides, polyarginine has also been used as a CPP that can carry covalently attached cargo into cells (Futaki et al., 2001). How do CPPs work? Surprisingly little is known. Initial evidence seemed to favor a passive entry unassisted by cellular membrane transport mechanisms. It now appears that many cationic CPPs gain entry to cells via an endocytotic mechanism—cellular adsorption preceding actual membrane translocation may have obfuscated earlier experimental conclusions (Green et al., 2003).

Leaving aside the problem of peptide entry, the SNARE complex would seem to be an ideal target for pharmacological modulation by aptamers, though perhaps an unlikely one for specifically targeting any one secretory system in vivo. The SNARE complex, whose cyclic assembly and disassembly is required for regulated secretory vesicle fusion with the plasma membrane, consists of a vesicular membrane-bound protein called synaptobrevin (or VAMP), and two target membrane-bound proteins on the plasma membrane called SNAP-25 (for synaptosome-associated protein of 25 kDa) and syntaxin. The binding of all three to each other, followed by their tight coiling together, brings the vesicle and plasma membranes close enough to fuse, allowing exocytosis and release of soluble cargo. Two molecules that give the SNARE proteins their names are the SNAPs (soluble NSF attachment proteins) and NSF (*N*-ethyl maleimide-sensitive factor). (By a nomenclatural coincidence, the previously named plasma membrane protein SNAP-25 is a SNARE, not a SNAP). NSF, associating with the SNARE complex through α-SNAP, hydrolyzes ATP to disassociate the very stable VAMP/SNAP-25/syntaxin/α-SNAP/NSF complex, thus allowing the vesicular membrane-bound and target membrane-bound-SNAREs to return to their respective compartments (synaptobrevin/VAMP to the now endocytosing or newly formed secretory vesicles and SNAP-25 and syntaxin to the plasma membrane) and participate in additional cycles of exocytosis. Endothelial cells express a closely related SNARE called SNAP-23 (Matsushita et al., 2005) that substitutes for SNAP-25 during secretion of von Willebrand factor (vWF) and other cargo from the secretory vesicles (Weibel-Palade bodies) of endothelial cells.

Detailed X-ray crystallographic and other structural work has provided a wealth of information about the precise contact points among syntaxin, SNAP-23/25, and synaptobrevin that allow them to contact, complex, tighten their interaction through coiling, and draw the vesicular and plasma membranes together to induce fusion and exocytosis (Bruns and Jahn, 2002) and also about the ATP-dependent roles of NSF and SNAPs and their attachment points to the SNARE complex, whose assembly they regulate (Jahn and Südhof, 1999). Much of the work on functional delineation of the role and mechanisms of interaction of SNARE proteins in exocytotic secretion has employed toxins whose protease functionality is brought into cells by separate permeabilization polypeptide subunits (which are themselves not easily converted to CPPs apart from the holotoxin, for various reasons) or via physical permeabilization of the cell membrane allowing peptide entry through small aqueous pores without impairment of exocytotic secretion via fusion of the secretory vesicle with the plasma membrane (Lin and Scheller, 2000).

In their report, Matushita et al. (2005) have identified NSF-derived aptamers that inhibit ATP hydrolysis by NSF in SNARE complexes, and therefore ATP-dependent, NSF-mediated SNARE complex disassembly. To get these aptamers into secretory cells where they can occlude the SNARE assembly/disassembly cycle, and thus block exocytosis, they have been fused to the tat peptide through a flexible linker. The chimeric peptides are taken up by cells both in culture and in vivo and inhibit the secretion of vWF from endothelial cells in culture and insulin from a pancreatic β-cell line. Administered in vivo, the most potent of the NSF-tat fusion peptides decrease clotting time in vivo, presumably because of inhibition of Weibel-Palade body secretion of vWF. The work is noteworthy for two reasons. First, the precise role of SNARE proteins in exocytotic secretion of vWF in cultured cell models has been extended to physiological secretion in vivo, an intellectually satisfying demonstration. Second, the work provides proof-of-principle for the aptamer-CPP fusion peptide as a pharmacological agent with in vivo efficacy. However, the studies necessarily leave several very intriguing questions unanswered, which will probably be the subjects of additional experimentation by this and other laboratories.

For example, if the SNARE mechanism for vesicular fusion is virtually universal (and much evidence exists that it is, at least in endocrine, neuronal, and exocrine secretory systems) (Eiden, 2003) then why are the effects of NSF-tat fusion peptides apparently so limited in vivo? Although this is advantageous for the therapeutic purpose proposed, it would be good to know why it happens. One can imagine pharmacodynamic factors leading to selective effects on endothelial cells, and Matsushita et al. (2005) present some evidence that the fusion peptides have a limited volume of distribution within the vasculature—they are effective when administered intravenously, but not parenterally, and have a long plasma half-life. The lack of effects on SNARE-dependent platelet secretion in vivo is ascribed to lack of penetration of the tat-aptamer peptides across this particular cell's plasma membrane. Paradoxically, if the NSF-tat approach is to be generalized to other secretory cells, or even to be exploited for anti-clotting therapy via a route more convenient than intravenous injection, the problem of getting these reagents beyond the vasculature in vivo will need to be addressed. When it is, though, cell specificity of the particular SNARE

interaction pathway leading to exocytotic secretion in endothelial compared with neuronal, endocrine, or exocrine cells, will be a significant issue. Generating NSF-tat fusion peptides (or CPP fusion peptides with aptamers targeting other components of the SNARE machinery) with specificity in inhibiting vWF secretion from endothelial cells without grossly affecting endocrine, neuronal, or exocrine SNARE-dependent regulated exocytotic secretion will be an interesting challenge.

While the present report is certainly paradigmatic for the deployment of aptamer-CPPs in other contexts, some caveats might be listed in attempting to generalize these results and similar reports from other laboratories. It needs to be assumed that a given aptamer-CPP will have its own 'signature' for cellular entry coupled with subsequent intracellular distribution. Thus, not all the aptamers worked exactly when presented to cells as aptamer-CPP fusion peptides as predicted by the efficacy of aptamer alone in cell-free SNARE interaction assays (Matsushita et al., 2005). In addition, the reliability of labeled peptide fate data needs to be weighed with caution, given the potential for fluorophores to alter the behavior of CPPs (Trehin and Merkle, 2004), to avoid artifactually uncoupling cell fate and biological activity. The use of actual tracers (i.e., hydrogen or carbon isotopic labeling) could resurge in the further development of CPP biotechnology and pharmacology.

The potential for this class of pharmacological agent in research and therapeutics development is immense and scarcely tapped. Dominant-negative peptides will probably be more informative, dosage-controllable, and precise than dominant-negative proteins administered via transcription from expression vectors and subsequent translation. The latter are precision reagents from the standpoint of cellular and molecular biology but are rather clumsy from the standpoint of pharmacological principles of mass action, as drugs. In this particular case, determining whether all SNARE-mediated secretion would come to a screeching halt upon administration of tat-NSF CPP-aptamers with greater bioavailability to brain, autonomic nervous system, and endocrine organs in vivo will be necessary to direct this approach to secretory systems other than endothelial cells. Mastering the mechanistic details of CPP entry and aptamer action for this class of reagents might in future produce safe and predictable therapeutic agents for inhibiting mast cell versus platelet, chromaffin cell versus neuronal, or excitatory versus inhibitory neuronal exocytosis, without serious side effects, in settings such as asthma, malignant hypertension, or epilepsy. The conclusions of the recent and thoughtful review by Trehin and Merkle (2004) are worth quoting in regard to future applicability of cell penetrating peptide-aptamer chimeras as pharmacological reagents: ". . . what appeared to be an event of broad applicability for many types of cells and biological barriers now appears to be an individual cellular feature that needs to be investigated case-by-case rather than under a general concept". The success of Matsushita et al. (2005) may be unique to the endothelial cell because of idiosyncrasies of both plasma membrane composition and secretory vesicle fusion complex formation in this cell. If so, its very lack of cellular generality may, with careful experimentation, make CPP-aptamer fusion peptides the biological deliverable of choice in selective aptamer targeting of specific types of mammalian cells in vivo.

References

Brody EN and Gold L (2000) Aptamers as therapeutic and diagnostic agents. *J Biotechnol* **74:**5–13.

Bruns D and Jahn R (2002) Molecular determinants of exocytosis. *Pflueg Arch Eur J Physiol* **443:**333–338.

Derossi D, Joliot AH, Chassaing G, and Prochaintz A (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269:**10444–10450.

Eiden LE (2003) Signaling during exocytosis, in *Handbook of Cell Signaling* (Bradshaw R and Dennis E eds) pp 375–392, Academic Press, New York.

Frankel AD and Pabo CO (1988) Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55:**1189–1193.

Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, and Sugiura Y (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* **276:**5836–5840.

Gallouzi I-E and Steitz JA (2001) Delineation of mRNA export pathways by the use of cell-permeable peptides. *Science (Wash DC)* **294:**1895–1901.

Gershoni JM and Aronheim A (1988) Molecular decoys: ligand-binding recombinant proteins protect mice from curaremimetic neurotoxins. *Proc Natl Acad Sci USA* **85:**4087–4089.

Geyer CR and Brent R (2000) Selection of genetic agents from random peptide aptamer expression libraries. *Methods Enzymol* **328:**171–208.

Green I, Christison R, Voyce CJ, Bundell KR, and Lindsay MA (2003) Protein transduction domains: are they delivering? *Trends Pharmacol Sci* **24:**213–215.

Hertting G and Meyer DK (1974) Effect of converting enzyme blockade on isoprenaline- and angiotensin I-induced drinking. *Br J Pharmacol* **52:**381–386.

Jahn R and Südhof TC (1999) Membrane fusion and exocytosis. *Annu Rev Biochem* **68:**863–911.

Juliano RL, Astriab-Fisher A, and Falke D (2001) Macromolecular therapeutics: emerging strategies for drug discovery in the postgenome era. *Mol Interv* **1:**40–53.

Lifson JD, Hwang KM, Nara PL, Fraser B, Padgett M, Dunlop NM, and Eiden LE (1988) Synthetic CD4 peptide derivatives that inhibit HIV infection and cytopathicity. *Science (Wash DC)* **241:**712–716.

Lin RC and Scheller RH (2000) Mechanisms of synaptic vesicle exocytosis. *Annu Rev Cell Dev Biol* **16:**19–49.

Matsushita K, Morrell CN, and Lowenstein CJ (2005) A novel class of fusion polypeptides inhibits exocytosis. *Mol Pharmacol* **67:**1137–1144.

O'Brian CA, Ward NE, Liskamp RM, de Bont DB, and van Boom JH (1990) N-myristyl-Lys-Arg-Thr-Leu-Arg: a novel protein kinase C inhibitor. *Biochem Pharmacol* **39:**49–57.

Schwarze SR and Dowdy SF (2000) In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol Sci* **21:**45–48.

Trehin R and Merkle HP (2004) Chances and pitfalls of cell penetrating peptides for cellular drug delivery. *Eur J Pharm Biopharm* **58:**209–223.

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