CellPress

Engineering therapeutic proteins for cell entry: the natural approach

Sandrine Guillard, Ralph R. Minter, and Ronald H. Jackson

Department of Antibody Discovery and Protein Engineering, MedImmune, Milstein Building, Granta Park, Cambridge CB21 6GH, UK

Owing to the challenges of cell entry, protein-based therapies have so far been restricted to extracellular targets, whereas intracellular targets have been almost exclusively addressed by small molecules. The specificity and potency of proteins would enable them to be effective intracellular drugs, provided that the proteins are delivered efficiently to appropriate intracellular compartments within specific cell types. By mimicking the natural mechanisms of toxins and other natural proteins, new intracellular delivery systems are being developed, the first of which are showing clinical efficacy. This review highlights a range of ingenious approaches designed to adapt natural entry mechanisms to facilitate delivery of proteins and open up a range of validated intracellular targets to modulation by potent and specific therapeutic drugs.

Nature provides templates for engineering therapeutic proteins

The attractive specificity, potency, and pharmacokinetics attained with engineered proteins has enabled the generation and marketing of numerous antibodies and other protein drugs that target extracellular proteins [1]. Small-molecule drugs have continued to be the modality of choice for addressing intracellular targets owing to the formidable barriers to cell entry that proteins face. However, despite considerable advances, there remain many protein–protein interactions that small molecules cannot modulate effectively [2] and engineered proteins have a lower propensity for off-target effects. Nature has evolved protein molecules, such as toxins [3] that target specific cells and enter the cell in sufficient quantities to effectively modulate target molecules. Similarly, viruses encode proteins that can enter cells and modulate host cell function [4–6]. These natural mechanisms are beginning to be adapted for therapeutic modulation of important intracellular targets using engineered delivery molecules inspired by toxins and other natural proteins.

To achieve efficacy, engineered delivery systems need to satisfy key parameters for targeting, uptake, translocation, and activity of the payload (Box 1). Trafficking across cell membranes commonly occurs through endocytosis into an endosome via, for example, clathrin-coated pits (Figure 1). Release from intracellular vesicles is the most challenging step to achieve efficiently, with endosomal entrapment being widely reported as an issue for delivery of biologics [7,8]. Toxins often use the changing pH, redox potential, and protease concentrations within the cell to trigger release from endosomes or for retrograde transport from the Golgi apparatus. We review how protein engineering has been used to generate molecules with receptor targeting, translocation, and payload domains that utilize or mimic toxin, viral, and other natural mechanisms to deliver payloads that are also stable and potent within the cell. We identify the key areas where improvements in efficiency are necessary to enable delivery of molecules to modulate the large number of validated but, to date, undruggable intracellular targets, such as Ras [9] and Myc [10].

How bacterial AB toxins have evolved to deliver proteins to cells

The bacterial AB toxins [11] have evolved intricate strategies to access the cytoplasm of host cells and disrupt intracellular processes (Table 1), and they thus provide a useful template for drug design. These toxins derive their name from their enzymatically 'active' A moiety and a separate 'binding/translocation' B moiety. AB toxins have evolved attributes to take advantage of host cell biology and address the key challenges of intracellular delivery (Box 1).

The cell surface receptors utilized by AB toxins offer the initial entry route into the endocytosis pathway of the cell, and determine both cell selectivity and uptake efficiency. Botulinum toxin utilizes neuron-specific receptors enabling entry through the synaptic vesicle recycling pathway [12]. By contrast, *Pseudomonas* exotoxin A utilizes the widely expressed CD91 protein, which internalizes efficiently as part of its normal function in endocytosing α2-macroglobulin and lipoproteins [13]. In this way, receptor choice influences the range of cell types which can be targeted by the toxin. Once the toxins have entered the endosome–lysosome pathway, the increasingly acidic pH, generally reducing environment, and presence of highly active proteases are exploited by the toxins to enable translocation to the cytoplasm. Diphtheria toxin, for example, escapes directly from the endosome–lysosome system (Figure 2). By contrast, toxins such as *Pseudomonas* exotoxin A mainly employ an entry strategy known as retrograde trafficking [14], traveling via endosomes and Golgi to the endoplasmic reticulum (ER) using sequences

Corresponding author: Jackson, R.H. (jacksonr@medimmune.com).
Keywords: intracellular; protein; toxin; virus.

0167-7799/
 http://dx.doi.org/10.1016/j.tibtech.2014.12.004

Box 1. Key parameters for functional therapeutic intracellular delivery of engineered proteins and nanoparticles

Targeting

Efficient biodistribution to the cell: evading the immune system, clearance from the circulation, and sequestration by irrelevant targets in the extracellular environment.

Binding to the target cell: association with surface through targeted interactions (e.g., with anti-receptor antibody) or untargeted interactions (e.g., charged residues with cell surface proteoglycans).

Uptake and Translocation

Uptake into the cell: efficient uptake through for example endocytic mechanisms, mediated through clathrin.

Release from intracellular vesicles: through release from or disruption of endosomes or translocation from the ER following retrograde transport.

Payload Activity

Stability of functional 'payload': a functional protein element, such as an enzyme or inhibitory antibody, will need to be stable in both the extracellular oxidizing environment and the intracellular reducing environment in the cytoplasm.

Potency of functional 'payload': the potency relative to the attainable intracellular concentration will be important, especially for proteins modulating stoichiometrically rather than catalytically.

related to the ER-localizing motif KDEL before translocating through the ER membrane to the cytoplasm (Figure 1).

The final stage of AB toxin function is to inactivate a key host cell protein or process. Without exception, the A subunits of all AB toxins employ catalytic rather than stoichiometric mechanisms to inhibit their target protein in the host cell (Table 1). By employing catalytic turnover, a single toxin molecule can inactivate multiple target molecules, thereby enabling dramatic phenotypic effects with relatively few successfully delivered toxin molecules [15].

Applications of immunotoxins in therapy

By far the best-explored approach to exploiting toxin biology for therapy has been the development of immunotoxin drugs in which a toxin is redirected to a tumor cell by replacement of the binding 'B' moiety of the toxin with an antibody or other cell binding ligand [16]. To date over 46 different immunotoxins have been tested clinically, with some showing impressive therapeutic benefit (Box 2). The general design principle of the immunotoxin is to create a genetic fusion between the antibody or cell binding ligand and the catalytic and translocation domains of the toxin (Figure 3; Table 1). Following the clinical proof of concept for immunotoxin therapy, a series of *Pseudomonas* exotoxin A fusion proteins were designed to redirect the toxin to at least 18 other internalizing cell surface targets expressed on tumor cells, including mesothelin [17], Lewis Y antigen [18], epidermal growth factor receptor [19], and Her2 receptor [20].

Recent work in the immunotoxin field has focused in three main areas: reducing the immunogenicity of protein toxins [21], exploring synergies with other therapies [22], and improving our understanding of toxin translocation to the cytoplasm [23].

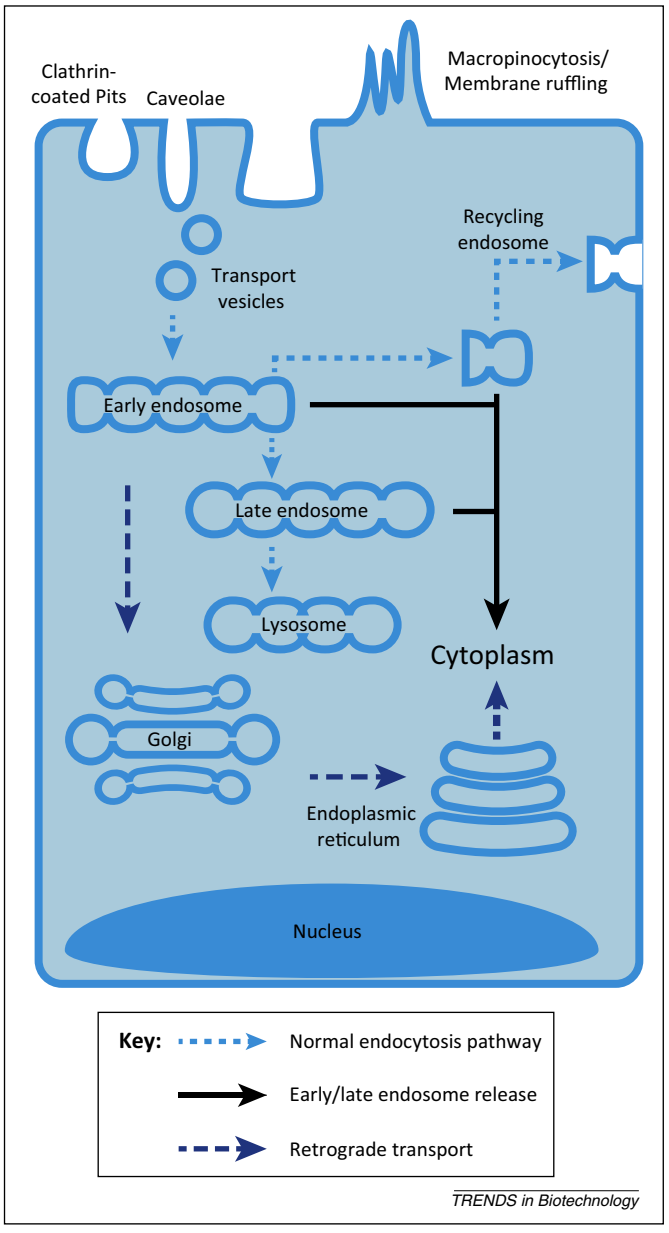

Figure 1. Pathways for intracellular delivery of engineered therapeutic proteins. Engineered protein therapeutics are taken up through the plasma membrane via clathrin, caveolae, or macropinocytosis mechanisms (usually following receptor binding), and are largely transported to the early endosome. As the endosome matures it acidifies, leading to the release of molecules (black arrow) through pH-induced conformation changes in proteins, such as diphtheria toxin domains. Proteins containing specific sequences related to the endoplasmic reticulum (ER)-localizing motif KDEL, such as the *Pseudomonas* exotoxin A catalytic domain, are routed to the ER by retrograde transport from where they are translocated into the cytoplasm. Proteins targeting nuclear elements may transition to the nucleus, a process favored by the addition of a nuclear localization sequence.

Further development of intracellular delivery using toxin domains

The range of intracellular delivery systems available is being expanded by modular use of toxin translocation or catalytic domains targeted to specific cells by natural or engineered protein ligands. This includes systems based on two protein chain components from *Clostridium botulinum* C2 toxin [24] or anthrax toxin [25]. The protective antigen (PA) of anthrax toxin, which forms a heptameric pore in endosomes, has been coadministered with the N-terminal