the plasma membrane (Salomone et al., 2012; Salomone et al., 2013; Erazo-Oliveras et al., 2014; Fasoli et al., 2014). Thus, there is a need for improved shuttle agents capable of increasing the transduction efficiency of polypeptide cargos, and delivering the cargos to the cytosol of target eukaryotic cells.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY

The present description stems from the surprising discovery that synthetic peptides comprising an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD) and optionally a histidine-rich domain, have the ability to increase the proportion of cells that can be transduced with a polypeptide cargo of interest, without the synthetic peptide being covalently bound to the polypeptide cargo. Following successful transduction, the synthetic peptides may facilitate the ability of endosomally-trapped polypeptide cargos to gain access to the cytosol, and optionally be targeted to various subcellular comparts (e.g., the nucleus).

In one aspect, the present invention refers to a polypeptide-based shuttle agent when used in increasing the transduction efficiency of an independent polypeptide cargo to the cytosol of target eukaryotic cells, wherein the polypeptide-based shuttle agent comprises an amphiphilic endosome leakage domain (ELD) having endosomolytic activity operably linked to a cell penetrating domain (CPD), or an amphiphilic ELD having endosomolytic activity operably linked to a histidine-rich domain and a CPD, wherein the polypeptide-based shuttle agent has an overall length of at least 20 amino acids, has a net charge of at least +6 at physiological pH, and is soluble in aqueous solution at physiological pH, and wherein the polypeptide-based shuttle agent is used at a concentration sufficient to increase the transduction efficiency of the independent polypeptide cargo to the cytosol of the target eukaryotic cells.

In another aspect, the present invention refers to an *in vitro* method for protein transduction, said method comprising contacting target eukaryotic cells with an independent polypeptide cargo and a concentration of a polypeptide-based shuttle agent sufficient to increase the transduction efficiency of the independent polypeptide cargo to the cytosol of the target eukaryotic cells, wherein the polypeptide-based shuttle agent comprises an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD), or an ELD operably linked to a histidine-rich domain and a CPD.

In a further aspect, the present invention refers to a synthetic peptide comprising an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD), wherein the synthetic peptide (a) comprises a minimum length of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acid residues and a maximum length of 35, 40, 45, 50, 55, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 amino acid residues; (b) has a predicted net charge of at least +6, +7, +8, +9, +10, +11, +12, +13, +14, or +15 at physiological pH; (c) is soluble in aqueous solution; or (d) any combination of (a) to (c), wherein the ELD is as disclosed herein, and wherein the CPD is or is from: a cell-penetrating peptide or the protein transduction domain from a cell-penetrating peptide; PTD4; Penetratin (Antennapedia); Xentry; arginine stretch; transportan; SynB3; or any combination thereof.

In a further aspect, the present invention refers to a composition when used in increasing the transduction efficiency of an independent polypeptide cargo to the cytosol of target eukaryotic cells, the composition comprising the polypeptide-based shuttle agent as disclosed herein, and the independent polypeptide cargo for intracellular delivery, wherein the concentration of the polypeptide-based shuttle agent upon use of the composition is sufficient to increase the transduction efficiency of the independent polypeptide cargo to the cytosol of the target eukaryotic cells.

Accordingly, the present description describes to the following examples:

(1) A synthetic peptide comprising an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD), or an ELD operably linked to a histidine-rich domain and a CPD.

Claims

1. A polypeptide-based shuttle agent when used in increasing the transduction efficiency of an independent polypeptide cargo to the cytosol of target eukaryotic cells, wherein the polypeptide-based shuttle agent comprises an amphiphilic endosome leakage domain (ELD) having endosomolytic activity operably linked to a cell penetrating domain (CPD), or an amphiphilic ELD having endosomolytic activity operably linked to a histidine-rich domain and a CPD, wherein the polypeptide-based shuttle agent has an overall length of at least 20 amino acids, has a net charge of at least +6 at physiological pH, and is soluble in aqueous solution at physiological pH, and wherein the polypeptide-based shuttle agent is used at a concentration sufficient to increase the transduction efficiency of the independent polypeptide cargo to the cytosol of the target eukaryotic cells.

2. The polypeptide-based shuttle agent when used according to claim 1, wherein said polypeptide-based shuttle agent:

(a) comprises a minimum length of 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acid residues and a maximum length of 35, 40, 45, 50, 55, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 amino acid residues;

(b) has a predicted net charge of at least +7, +8, +9, +10, +11, +12, +13, +14, or +15 at physiological pH; or

(c) a combination of (a) and (b).

3. The polypeptide-based shuttle agent when used according to claim 1 or 2, wherein:

(a) said ELD is or is from: an antimicrobial peptide (AMP); a linear cationic alpha-helical antimicrobial peptide; a Cecropin-A/Melittin hybrid (CM) peptide; pH-dependent membrane active peptide (PAMP); a peptide derived from the N terminus of the HA2 subunit of influenza hemagglutinin (HA); CM18; Diphtheria toxin T domain (DT); GALA; PEA; INF-7; LAH4; HGP; H5WYG; HA2; EB1; VSVG; *Pseudomonas* toxin; melittin; KALA; JST-1; $C(LLKK)_3C$; $G(LLKK)_3G$; or any combination thereof;

(b) said CPD is or is from: a cell-penetrating peptide or the protein transduction domain from a cell-penetrating peptide; TAT; PTD4; Penetratin (Antennapedia); pVEC; M918; Pep-1; Pep-2; Xentry; arginine stretch; transportan; SynB1; SynB3; or any combination thereof;

(c) said histidine-rich domain is a stretch of at least 3, at least 4, at least 5, or at least 6 amino acids comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% histidine residues; and/or comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, or at least 9 consecutive histidine residues; or

(d) any combination of (a) to (c).

4. The polypeptide-based shuttle agent when used according to any one of claims 1 to 3, wherein said polypeptide-based shuttle agent comprises:

(a) an ELD comprising the amino acid sequence of any one of **SEQ ID NOs: 1-15, 63, or 64**, or a variant or fragment thereof having endosomolytic activity;

(b) a CPD comprising the amino acid sequence of any one of **SEQ ID NOs: 16-27 or 65,** or a variant or fragment thereof having cell penetrating activity;

(c) a histidine-rich domain having at least 2, at least 3, at least 4, at least 5, or at least 6 consecutive histidine residues; or

(d) of any combination of (a) to (c).

5. The polypeptide-based shuttle agent when used according to any one of claims 1 to 4, wherein said domains are operably linked via one or more linker domains.

6. The polypeptide-based shuttle agent when used according to any one of claims 1 to 5, wherein said shuttle agent comprises at least two different types of CPDs and/or ELDs.

7. The polypeptide-based shuttle agent when used according to any one of claims 1 to 6, wherein said shuttle agent comprises:

(a) an ELD which is CM18, KALA, or $C(LLKK)_3C$ having the amino acid sequence of **SEQ ID NO: 1, 14, or 63**, or a variant thereof having at least 85%, 90%, or 95% identity to **SEQ ID NO: 1, 14, or 63,** and having endosomolytic activity;

(b) a CPD which is TAT or PTD4 having the amino acid sequence of **SEQ ID NO: 17 or 65**, or a variant thereof having at least 85%, 90%, or 95% identity to **SEQ ID NO: 17 or 65** and having cell penetrating activity; or Penetratin having the amino acid sequence of **SEQ ID NO: 18**, or a variant thereof having at least 85%, 90%, or 95% identity to **SEQ ID NO: 18** and having cell penetrating activity;

(c) a histidine-rich domain comprising at least 6 consecutive histidine residues; or

(d) any combination of (a) to (c).

8. The polypeptide-based shuttle agent when used according to any one of claims 1 to 7, wherein said shuttle agent comprises or consists of the amino acid sequence of any one of **SEQ ID NOs: 57-59, 66-72, or 82-102**, or a functional variant thereof having at least 85%, 90%, or 95% identity to any one of **SEQ ID NOs: 57-59, 66-72, or 82-102**.

9. The polypeptide-based shuttle agent when used according to any one of claims 1 to 8, wherein said polypeptide cargo lacks a CPD.

10. The polypeptide-based shuttle agent when used according to any one of claims 1 to 9, wherein said polypeptide cargo:

(a) is a recombinant protein;

(b) comprises a subcellular targeting domain;

(c) is complexed with a DNA and/or RNA molecule; or

(d) any combination of (a) to (c).

11. The polypeptide-based shuttle agent when used according to claim 10, wherein said subcellular targeting domain is:

(a) a nuclear localization signal (NLS);

(b) a nucleolar signal sequence;

(c) a mitochondrial signal sequence; or

(d) a peroxisome signal sequence.

12. The polypeptide-based shuttle agent when used according to claim 11, wherein:

(a) said NLS is from: E1a, T-Ag, c-myc, T-Ag, op-T-NLS, Vp3, nucleoplasmin, histone 2B, Xenopus N1, PARP, PDX-1, QKI-5, HCDA, H2B, v-Rel, Amida, RanBP3, Pho4p, LEF-1, TCF-1, BDV-P, TR2, SOX9, or Max;

(b) said nucleolar signal sequence is from BIRC5 or RECQL4;

(c) said mitochondrial signal sequence is from Tim9 or Yeast cytochrome c oxidase subunit IV; or

(d) said peroxisome signal sequence is from PTS1.

13. The polypeptide-based shuttle agent when used according to any one of claims 1 to 12, wherein said polypeptide cargo is a transcription factor, a nuclease, a cytokine, a hormone, a growth factor, or an antibody.

14. The polypeptide-based shuttle agent when used according to claim 13, wherein:

(a) said transcription factor is: HOXB4, NUP98-HOXA9, Oct3/4, Sox2, Sox9, Klf4, c-Myc, MyoD, Pdx1, Ngn3, MafA, Blimp-1, Eomes, T-bet, FOXO3A, NF-YA, SALL4, ISL1, FoxA1, Nanog, Esrrb, Lin28, HIF1-alpha, Hlf, Runx1t1, Pbx1, Lmo2, Zfp37, Prdm5, Bcl-6, or any combination thereof; and/or

(b) said nuclease is: an RNA-guided endonuclease, a CRISPR endonuclease, a type I CRISPR endonuclease, a type II CRISPR endonuclease, a type III CRISPR endonuclease, a type IV CRISPR endonuclease, a type V CRISPR endonuclease, a type VI CRISPR endonuclease, CRISPR associated protein 9 (Cas9), Cpf1, a zinc-finger nuclease (ZFNs), a Transcription activator-like effector nucleases (TALENs), a homing endonuclease, a meganuclease, or any combination thereof.

15. The polypeptide-based shuttle agent when used according to any one of claims 1 to 12, wherein said polypeptide cargo is a CRISPR endonuclease.

16. The polypeptide-based shuttle agent when used according to any one of claims 1 to 15, wherein said target eukaryotic cells are stem cells, primary cells, immune cells, T cells, or dendritic cells.

17. An *in vitro* method for protein transduction, said method comprising contacting target eukaryotic cells with an independent polypeptide cargo and a concentration of a polypeptide-based shuttle agent sufficient to increase the transduction efficiency of the independent polypeptide cargo to the cytosol of the target eukaryotic cells, wherein the polypeptide-based shuttle agent is as defined in claim 1.

18. The *in vitro* method of claim 17, wherein:

(i) the polypeptide-based shuttle agent is as defined in any one of claims 2 to 8;

(ii) the polypeptide cargo is as defined in any one of claims 9 to 15; and/or

(iii) the target eukaryotic cells are as defined in claim 16.

19. A synthetic peptide comprising an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD), wherein the synthetic peptide:

(a) comprises a minimum length of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acid residues and a maximum length of 35, 40, 45, 50, 55, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 amino acid residues;

(b) has a predicted net charge of at least +6, +7, +8, +9, +10, +11, +12, +13, +14, or +15 at physiological pH;

(c) is soluble in aqueous solution; or

(d) any combination of (a) to (c),

wherein the ELD is as defined in claim 3, 4, or 7, and

wherein the CPD is or is from: a cell-penetrating peptide or the protein transduction domain from a cell-penetrating peptide; PTD4; Penetratin (Antennapedia); Xentry; arginine stretch; transportan; SynB3; or any combination thereof.

20. The synthetic peptide of claim 19, wherein said domains are operably linked via one or more linker domains.

21. The synthetic peptide of claims 19 or 20, wherein said shuttle agent comprises at least two different types of CPDs and/or ELDs.

22. The synthetic peptide of any one of claims 19 to 21, which comprises or consists of the amino acid sequence of any one of **SEQ ID NOs: 57-59, 66-72, or 82-102**, or a functional variant thereof having at least 85%, 90%, or 95% identity to any one of **SEQ ID NOs: 57-59, 66-72, or 82-102**.

23. A composition when used in increasing the transduction efficiency of an independent polypeptide cargo to the cytosol of target eukaryotic cells, the composition comprising the polypeptide-based shuttle agent as defined in any one of claims 1 to 8, and the independent polypeptide cargo for intracellular delivery, wherein the concentration of the polypeptide-based shuttle agent upon use of the composition is sufficient to increase the transduction efficiency of the independent polypeptide cargo to the cytosol of the target eukaryotic cells.

24. The composition of claim 23, wherein the polypeptide cargo is as defined in any one of claims 9 to 15; and/or the target eukaryotic cells are as defined in claim 16.

25. The composition of claim 23 or 24, wherein the independent polypeptide cargo is a biologically active polypeptide.