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Published on Web 11/23/2002

Tandem Ligation of Multipartite Peptides with Cell-Permeable Activity

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Abstract: To prepare multipartite peptides with several functional cargoes including a cell-permeable sequence or transportant for intracellular delivery, tandem ligation of peptides is a convenient convergent approach with the fewest synthetic steps. It links three or four unprotected segments forming two or more regiospecific bonds consecutively without a deprotection step. This paper describes a tandem ligation strategy to prepare multipartite peptides with normal and branched architectures carrying a novel transportant peptide that is rich in arginine and proline to permit their cargoes to be translocated across membranes to affect their biological functions in cytoplasm. Our strategy consists of three ligation methods specific for amino terminal cysteine (Cys), serine/threonine (Ser/Thr), and N^{α}-chloroacetylated amine to afford Xaa-Cys, Xaa-*O*Pro (oxaproline) and Xaa-ψGly (pseudoglycine) at the ligation sites, respectively. Assembly of single-chain peptides from three different segments was achieved by the tandem Cys/*O*Pro ligation to form two amide bonds, an Xaa-Cys and then an Xaa-*O*Pro. Assembly of two- and three-chain peptides with branched architectures from four different segments was accomplished by tandem Cys/ψGly/*O*Pro ligation. These NT-specific tandem ligation strategies were successful in generating cell-permeable multipartite peptides with one-, two-, and three-chain architectures, ranging in size from 52 to 75 residues and without the need of a protection or deprotection step. In addition, our results show that there is considerable flexibility in architectural design to obtain cell-permeable multipartite peptides containing a transportant sequence.

Introduction

Many functionally active peptides or motifs contain continuous sequences embedded in proteins. These peptides vary in lengths that range from 3 to >20 amino acids. They are found in cell adhesion molecules,[1] immunologically relevant B- and T-cell epitopes,[2] and sequences specific for signal transduction,[3] phosphorylation,[4] sulfation,[5] and nuclear localization.[6] However, most of these peptides target intracellular proteins and will require a transportant or cell-permeable peptide for intracellular delivery[7] that has the advantage of being noninvasive.

Two common transportant motifs are known to contain contiguous and characteristic amino acid sequences that range from 8 to 18 amino acid residues. The first type such as Tat peptide is highly cationic and rich in arginine.[8] The second type such as those derived from the signal sequences is highly

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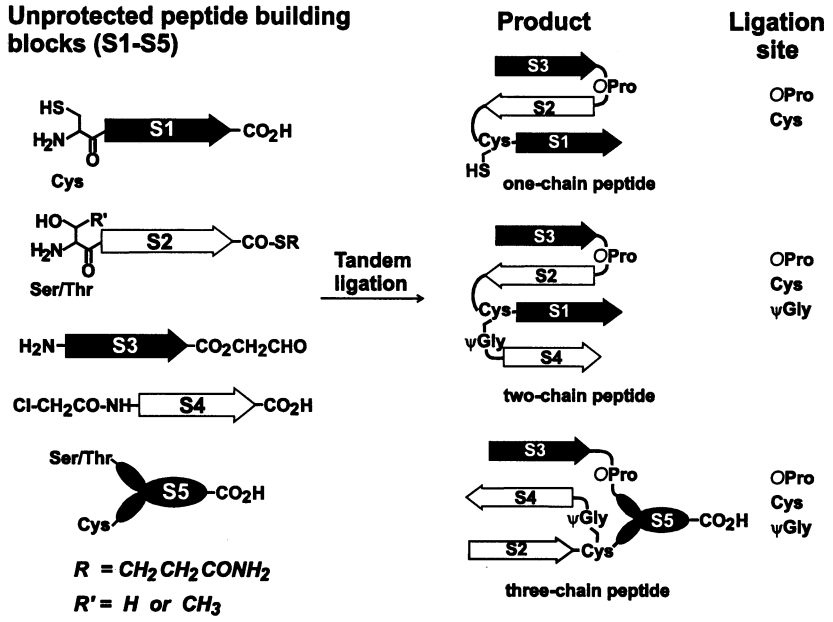

Figure 1. General scheme of NT-specific tandem ligation for the assembly of multipartite peptides to form one-, two-, and three-chain peptides using unprotected functional peptides S1 to S5 where S3 is a transportant for intracellular delivery.

hydrophobic and rich in aliphatic amino acids. Recently, a third type of novel transportant motif has been identified that is rich in arginine and proline.[9]

Arginine and proline-rich peptides (RP-peptide) are found in antimicrobial peptides produced by a range of species.[9] They include PR-39,[10] abaecin,[11] apidaecin,[12] and Bac5 and Bac7.[13] The bactericidal properties of the RP-rich peptides are different from the conventional pore-forming antimicrobial peptides. They act by penetrating the membrane and subsequently kill cells by interfering with the protein synthesis machinery.[14] The membrane-penetrating activity exhibited by RP-rich Bac7 is retained by the 24-residue peptide RG24, RRIRPRPPRLPRPRPRPLPF-PRPG.[15] Recently, we have shown that this RG24 and a series of truncated peptides can translocate across the cell membrane and could act as transportants for functional cargoes. Furthermore, these peptides are not cytotoxic at concentrations up to 100 μM after exposure for 24 h.

Our laboratory is interested in the design and synthesis of intracellularly active peptides that are multipartite to probe cytoplasmic protein−protein interactions. Ideally, such peptides would contain a transportant for intracellular delivery, one or more functional peptide cargoes directed to target cytoplasmic proteins and an additional peptide for providing extracellular specificity. At present, most multipartite peptides containing a transportant are constructed as single-chain peptides that link different motifs in tandem because they are convenient to prepare by stepwise solid-phase or recombinant methods. We reasoned that a multipartite branched design with motifs tethered onto a core might be as effective as the single-chain design because the biological activity of each motif is largely dependent on the content of its sequence (Figure 1). Furthermore, the synthesis of branched peptides has been become achievable by recent advances of chemoselective ligation, particularly the tandem ligation methods that provide the most direct method for assembling heteromeric multipartite peptides.[15] Toward this end, we have exploited tandem ligation methods to prepare multipartite peptides with one-, two-, or three-chain arrangements. Among the methods for chemoselective ligation, orthogonal ligation[16] for coupling two peptide segments is attractive. It is an N-terminal-specific ligation method for forming an amide bond between a particular N-terminal α-amine and a C-terminal α-carboxylic group in the presence of a variety of functional groups found in unprotected peptide segments. Various orthogonal ligation methods have been developed for forming bonds containing thiaproline,[21] oxaproline,[22] cysteine,[23] selenocysteine,[24] methionine,[25] tryptophan,[26] histidine,[27] and NT-

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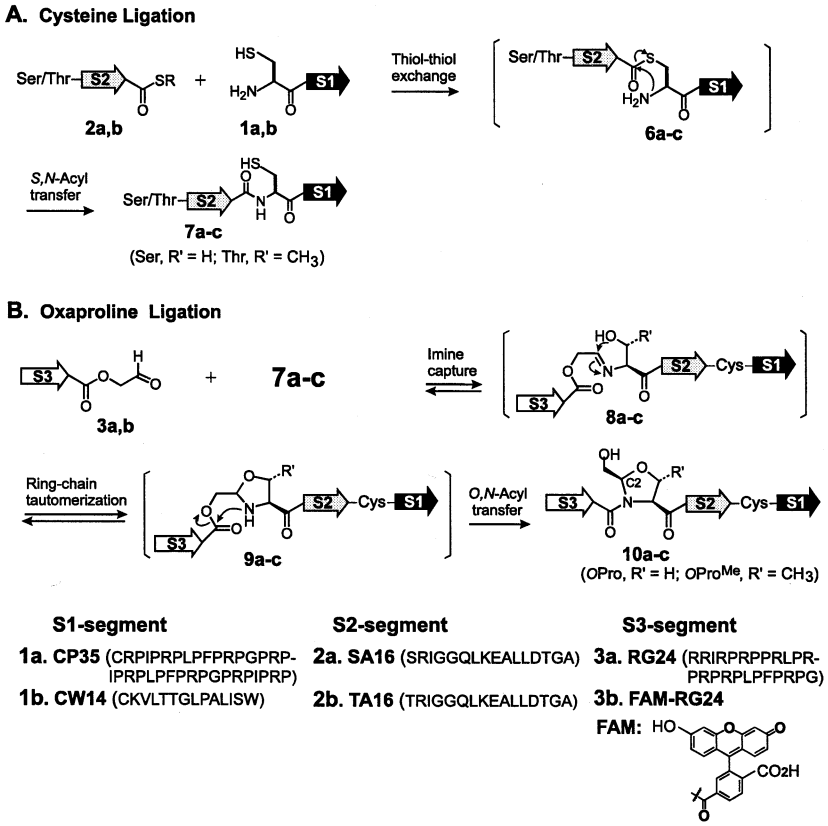

Figure 2. C-to-N tandem ligation of single-chain peptides through (A) cysteine ligation and (B) oxaproline ligation.

amino substituted amino acids.[28] The ligated product retains the amide backbone, but the side chain generated at the ligation site can be either native or modified.[17−20] Furthermore, these methods do not use a protecting group or a coupling reagent. Consequently, peptides of different shapes and sizes can be formed in aqueous solutions by employing building blocks derived from chemical, natural or biosynthetic sources.[29]

Tandem ligation employs several orthogonal methods to permit the consecutive coupling of three or more peptide segments. Because a protecting group scheme is not being used, tandem ligation has the advantage of a convergent synthesis with the fewest synthetic steps. Previously, we have described two tandem ligation schemes for linking three segments. The first scheme is the tandem pseudoproline (XPro) ligation based on the chemoselectivity of a glycoaldehyde ester to two different N-terminal nucleophiles, N-terminal Cys versus an N-terminal Ser/Thr in peptide segments, in forming a thiaproline (SPro) and an oxaproline (*O*Pro) bond, respectively.[15,20] The second scheme is the tandem SPro/Cys ligation. It exploits the semi-orthogonality of an N-terminal Cys to two different C-terminal electrophiles, an ester and a thioester, in forming an SPro and a Cys bond, respectively.[20] In this paper, we describe a third scheme that combines the features of the first two schemes to enable a tandem Cys/*O*Pro ligation of three or four segments to form the single-chain and branched-chain peptides. This scheme exploits not only the orthogonalities of two N-terminal specific *O*Pro and Cys ligation, but also a ψGly ligation that mimics a Gly−Gly dipeptide between the chloroacetyl amino group and the newly generated thiol group from the Cys ligation (Figure 3). Cysteine and pseudoglycine ligation can be carried out chemoselectively in aqueous buffers at pH 7 to 8, whereas oxaproline ligation can be achieved in pyridine or methylimidazole−acetic acid solutions. Using these tandem ligation reactions, different architectural peptides with 52 to 75 amino acid residues containing a transportant peptide were demonstrated.

Results and Discussion

Transportants and T-cell Epitopes. Transportant peptides, **3a** and **3d**, representing the truncated N-terminal 24-residue peptide RG24 and a shortened peptide RL17 of the RP-rich peptides from Bac7 were used for the synthesis of multipartite transportant peptides consisting of one-, two-, and three-chain architectures. Because our multipartite peptides were intended for developing synthetic vaccines, they contained both B- and T-cell epitopes chosen from proteins such as NK-Lysin and melittin and the coat protein gp120 of human immunodeficiency virus (HIV-1). To provide support that RG24 and RL17 could act as transportants to translocate the multipartite peptides into cells, they were fluoresceinated using succinimidyl ester of carboxyfluorescein (FAM) to afford FAM-RG24 and FAM-RL17 for analysis by confocal microscopy.

Synthesis of Peptide Segments. Because we used different N-terminal-specific ligation methods of unprotected segments for the synthesizing three architectural forms of peptides, five

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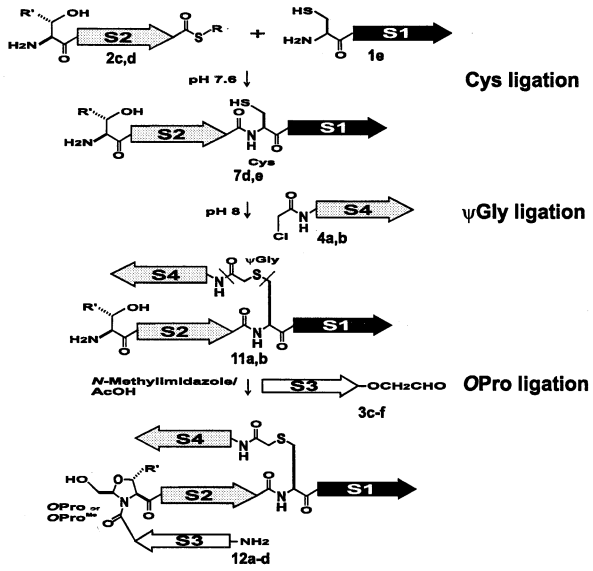

S1-segment	S2-segment	S3-segment	S4-segment
1b. CW14	2c. TA22 (TIQKLEDMVGP-QPNEDTVTQAA)	3c. PG18 (PPPPNPNDPPP-PNPNDLG)	4a. RK15 (RIQRGPGRAFV-TIGK)
	2d. SA15 (SVGPQPNEDTV-TQAA)	3d. RL17 (RRIRPRPPRLPR-PRPPL)	4b. GK9 (GRAFVTIGK)
		3e. RL16 (RRIRPRPPRLPR-PRPL)	
		3f. FAM-RL17	

Figure 3. Tandem ligation scheme for assembly of a two-chain peptide from four different peptide segments using Cys, ψGly, and *O*Pro ligation in tandem.

general types of unprotected peptides designated as S1−S5 were used for the tandem ligation (Figure 1). These peptide segments have a specific N-terminal amino acid (S1, S4, and S5), C-terminal ester (S3), or N-terminal Ser/Thr and C-terminal thioester (S2). They were prepared by a stepwise solid-phase method and purified by RP-HPLC. Their compositions were confirmed by MALDI-MS and amino acid analysis. Segments S1 to S3 were used to form the one-chain peptides. Additional S4- and S5-segments were used to assemble the branched two- and three-chain peptides.

The S1-segments **1a**,**b** were Cys-containing segments with an N-terminal Cys necessary for the Cys ligation. They contained a free carboxylic acid or carboxamide at their carboxyl terminus and were synthesized by Boc chemistry on a commercially available resin-support. HF cleavage afforded the completely free peptides **1a**,**b** in 70−80% yields based on the loading of the first amino acid on the resin.

All S2- and S3-segments contained an ester at their C-termini. S2-segments **2a**−**e** were thioester-containing segments with a CT-thioester for Cys ligation and an N-terminal Ser or N-terminal Thr required for the *O*Pro ligation. The S2 thioester-segments were synthesized directly from a previously developed thiol resin using Boc chemistry.[30] Although there are several methods available to produce a peptide thioester by chemical means,[31] this direct one-step method is straightforward and avoids the need for converting a sensitive thio acid moiety into a thioester as indicated in the original method.[32] HF cleavage provided unprotected peptide thioesters in 50−70% yields.

The S3-segments **3a**−**f** were ester-containing segments necessary for pseudoproline ligation. They must carry a glycoaldehyde ester at the C-terminus, but their N-terminus can be any amino acid except Ser, Thr, and Cys. They were synthesized by Fmoc chemistry on an acetal resin.[33] After the completion of the stepwise synthesis, a TFA-mediated cleavage released the 1,2-diol moiety of the unprotected peptide glycerol ester from the resin. The 1,2-diol moiety of the glycerol ester was then transformed into aldehyde by periodate oxidation under aqueous conditions at pH 4 to 7. The aldehyde moiety was stable in the purification step using a buffer solution with 0.05% TFA. This method has the advantage of avoiding exposure of the sensitive aldehyde moiety to strong acid cleavage.

The S4-segments **4a**,**b** containing an N-terminal chloroacetylamine and a C-terminal COOH were used for the synthesis of the two-chain peptide with a branching architecture. They were synthesized by using Fmoc chemistry on a commercially available Wang resin. The N-terminal chloroacetyl amino peptide was then used for the chemoselective ψGly ligation with the cysteinyl thiol group generated from *S*,*N*-acyl transfer of Cys ligation.

The S5-segment was a branched tetrapeptide **5** that contained two α-amines, an N-terminal Cys and N-terminal Ser linked by a Lys as Cys-βAla-Lys(Ser). These two N-termini were used for ligating S2- and S3-segments to form a branched peptide. The S5-segment was synthesized on a benzyl ester resin starting with an orthogonally protected Lys in which the α-amine was protected by Boc and the ϵ-amine by Fmoc group. Elongation of two amino acids from the α-amine was accomplished by Boc chemistry. Fmoc-deprotection by 20% piperidine/DMF released

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Table 1. Summary of Yields and Mass Spectrometric Data of Tandem Ligation Products inSingle-chain Peptide

cys ligation						*O* proligation		
S1	S2	S1/S2	yield %	Mw[a]	**S3**	**S1/S2/S3**	yield (%)	Mw
CP35(**1a**)	SA16(**2a**)	SP51(**7a**)	86	5606.1	RG24(**3a**)	RP75(**10a**)	69	8580.1
CP35(**1a**)	TA16(**2b**)	TP51(**7b**)	84	5619.9	RG24(**3a**)	RP75[†](**10b**)	71	8590.9
CW14(**1b**)	TA16(**2b**)	TW30(**7c**)	83	3126.8	F[‡]-RG24(**3b**)	F−RW54(**10c**)	52	6451.5

[a] Mw is the observed molecular weight MH^+ and that agrees with the calculated Mw. [b] See Figure 1 for sequence. RP75; amino acid in one-letter code indicating the N-terminus (Arg) and C-terminus (Pro) of a peptide as well as the number the length (75 amino acids). [c] F is carboxyfluorescein.

the ϵ-amine, which was subsequently coupled with the protected Boc-Ser. HF-mediated cleavage gave the desired tetrapeptide S5-segment.

Three-Segment Tandem Ligation to Form Single-Chain Peptides with Normal Architectures. The tandem Cys/*O*Pro ligation is orthogonal and can be performed in the N-to-C or C-to-N direction. For convenience, we chose the C-to-N direction in the order of S3←S2←S1 using first the Cys ligation to couple the S1- and S2-segments, and then *O*Pro ligation to incorporate the RP-rich transportant S3-segment RG24 at the N-terminus to produce three peptides **10a**−**c** containing 54 to75 residues (Figure 2).

a. Cysteine Ligation. The Cys ligation is a two-step reaction between S1 and S2 segments to effect an Xaa-Cys at the ligation site (Figure 2). First, the thiol nucleophile of the N-terminal Cys of S1-segments **1a**,**b** undergoes a thiol-thioester exchange with the C-terminal thioester present on the S2-segments **2a**,**b** to form branched thioester intermediates **6a**−**c**. Second, the thioester intermediates **6a**−**c** spontaneously undergo an intramolecular *S*,*N*-acyl transfer to form the ligated products **7a**−**c** with an amide bond. The cysteine ligation was performed in an aqueous solution at pH 7.6 under reducing conditions.[20] To inhibit disulfide bond formation between the S1-segments and the ligated products, a reducing agent composed of 10 to 20 equivs of 2-mercaptoethanesulfonate was used.[31] Under this condition, the cysteine ligations afforded a steady yield ranging from 83 to 86% (Table 1).

b. Oxaproline Ligation. The second coupling step for forming an amide bond in our scheme was the oxaproline ligation between the ligated products **7a**−**c** and the S3-segments. It involves the S3-segments **3a**,**b** containing a CT-glycoaldehyde ester and the ligated segments **7a**−**c** containing an NT-Ser or NT-Thr to form the three-segment ligated products **10a**−**c**. The oxaproline ligation proceeds through an imine capture, oxazolidine ring formation, and *O*,*N*-acyl transfer (Figure 2B).[22] Because no enthalpic activating agent is involved in the *O*Pro ligation that is mediated through an intramolecular acyl transfer reaction, no epimerization at the α-carbon of Ser or Thr has been known to occur. A new stereocenter is generated at the C2 position as a result of the imine capture step forming the 1,3-oxazolidine. The stereochemistry of the stereocenters 1,3-oxazolidines as well as the related five- or six-ring heterocyclic compounds have been extensively studied.[34] These reactions were surprisingly stereoselective when the syntheses were performed on entantiomerically pure α- or β-amino acids and when an exocyclic N-acyl group was a part of the structural element. Under these conditions, the stereochemical courses of reactions produced exclusively *cis*-substituted heterocycles due to the $A^{1,3}$ effect exerted by an amide group. Previously, we have found that *O*Pro ligation is stereoselective and the stereochemistry of the C2 position is determined by the chirality of α-carbon of Ser or Thr on the basis of NMR data.[15] Our results are consistent with the extensive work by Seebach's group and others who find that the stereochemistry of the C2 is cis to the chiral α-carbon.

The *O*Pro ligation provides a proline mimetic that retains the amide backbone structure of an α-peptide. In contrast to thiaproline ligation[15] which is usually performed in aqueous buffers at pH 4 to 7, *O*Pro ligation requires nearly nonaqueous conditions.[34] Thus, the *O*Pro ligations of the S3-segments **3a**,**b** with the S1/S2-ligated segments **7a**−**c** were performed in a pyridine- or methylimidazole-acetic acid mixture (1:1, mol/mol) to afford the three-segment product **10a**−**c** in yields of 52−71% (Table 1). It should be noted that under such conditions, ligation also occurs with segments containing N-terminal Cys, Trp, or His.[22]

Peptides with Branched Architectures. To expand the N-terminal specific tandem ligation strategy, we synthesized two-chain and three-chain branched peptides using four different segments. Similar to the synthetic scheme of the one-chain peptides, the direction of tandem ligation was C-to-N in the order of S3←S4)←S2←S1, generating three different ligation sites: Xaa-Cys, Xaa-ψGly, and Xaa-*O*Pro (Figure 3).

a. Two-Chain Peptides. Three two-chain peptides consisting of 54, 55, and 69 residues were prepared using four peptide segments with S3-segments containing various lengths of the RP-rich transportant **3c**−**e**. The four-segment tandem ligation produced branched peptides with the main chain consisting of three segments similar to the single-chain peptides produced by the tandem *O*Pro/Cys ligation. The branched chain was the S4-segment containing a peptide epitope **4a** or **4b** that attached at its amino end in a C-to-N direction through a Gly−Gly dipeptide mimetic to the thiol moiety of the S1-segment by the S4-chloroacetylated segment **4a** or **4b**. Thus, the requirements for three of the four starting materials for these syntheses were identical to the tandem ligation of the single-chain peptides that included the S1-cysteine segment **1b**, the S2-thioester segments **2b** or **2a**, and S3-segments **3c**, **3d**, or **3e**.

The syntheses of the two-chain peptides **12a** and **12b** containing 69 and 55 residues, respectively, began first with a Cys ligation. For **12a**, Cys ligation between segments was S1-cysteine segment **1b** and the S2-thioester segment **2c** at pH 7.6 phosphate buffer with TCEP to afford a 36-residue peptide **7d** in 80−85% yield (Figure 4A). For **12b**, Cys ligation between **1b** and another S2-thioester segment **2d** with an N-terminal Ser

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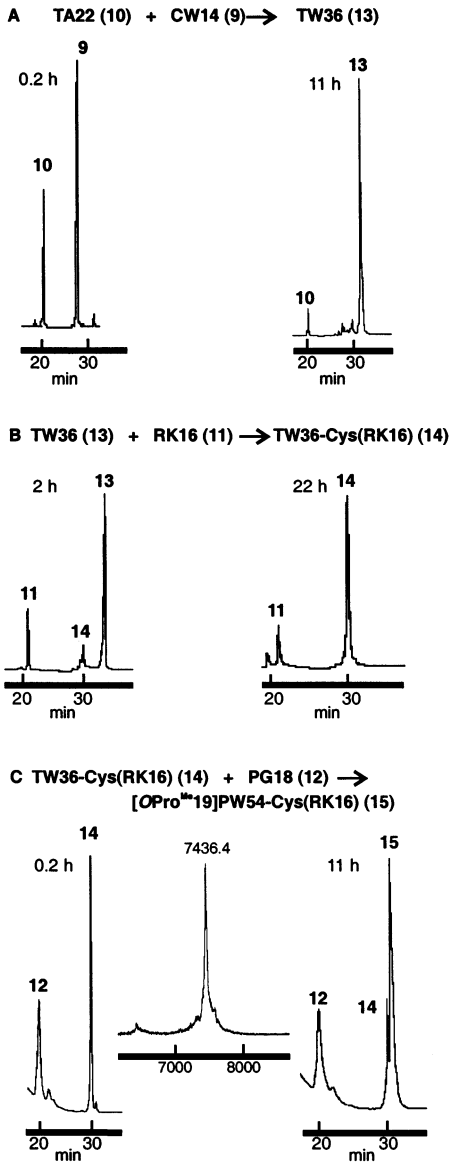

Figure 4. HPLC profiles monitoring the assembly of a two-chain peptide from four peptide segments as shown in Figure 5. (A) Cysteine ligation between peptide thioester TA22**2c** and segment CW14 **1b** producing the 36-residue TW36 **7d**. (B) ψGly ligation between segments RK15 **4a** and **7d** obtained to afford the 51-residue branch peptide TW51 **11a**. (C) Oxaproline ligation between **11a** and segment PG18 **3c**. The reaction was complete after 11 h producing the two-chain 69-residue product PW69 **12a** with the expected mass of *m*/*z* 7436.4.

gave a 29-residue peptide **7e**. The newly generated cysteinyl thiol group of **7d** was then used for the ψGly ligation with the S4-chloroacetylated segment **4a** to form the 51-residue branch peptide **11a** containing a thioether bond as a Gly-Gly mimetic. This ligation was performed at pH 8 in 80−85% yield (Figure 4B). Similarly, ψGly ligation of **7e** and the S4-chloroacetylated segment **4b** under the same pH condition provided 85−95% yield. To complete the synthesis of **12a**, the third ligation between the proline-rich S3-segment containing a glycoaldehyde ester **3c** and the N-terminal Thr of **11a** was obtained through an oxaproline ligation that afforded the final two-chain, 69-residue product **12a** in 55% yield (Figure 4C). Similarly, **12b** was obtained in 50% yield through an oxaproline ligation of **11b** with the S3-segment **3d**. Other four-segment peptides **12c**−**d** containing 54 to 55 amino acid residues from the two different RP-rich transportant S3-segments were obtained in about 50% yield.

b. Three-Chain Peptide. The four-segment tandem ligation was also used to prepare a dendrimer, 52-residue dendritic peptide **15a** containing three chains, **2e**, **4b** and **5c** derived from melittin, gp120 of HIV-1 and Bac7, respectively. These different peptide segments, which constituted two T-cell epitopes and an RP-rich transportant (RG24) to elicit a cytotoxic T-cell immune response, were designed for a synthetic vaccine against HIV-1.

The synthesis of a three-chain peptide employed a symmetrical dendritic core S5-segment **5**, Cys-βAla-Lys(Ser) in which the N^{α}- and N^{ϵ}-amines were equally spaced from the C^{α}-carbon of Lys. The N^{α}-amine and the thiol side chain of Cys as well as the N^{α}-Ser of lysine were then used to couple three different peptide segments by a tandem ligation through Cys, ψGly, and oxaproline methods (Figure 5). Unlike the two previous architectural forms, this sequence of tandem ligation produces a three-chain architecture without a main chain. Instead, it contains the RP-rich transportant S3-segment **3a** and the peptide epitope **2e** attached at their C-termini to the dendritic core **5**, whereas the peptide epitope **4b** is attached at its amino terminus through thioalkylation.

The first ligation through Cys ligation of N-terminal Cys of S5-segment with the S2-thioester segment **2e** produced the **13** in 93% yield (Figure 6A). The second ligation by ψGly ligation at pH 8 formed the thioether linkage between the thiol group of the newly generated cysteine and the N-terminal chloroacetyl moiety of the S4-segment **4b** that permitted a thioalkylation to produce **14** in 83% yield (Figure 6B). The N^{α}-Ser of the ligated product **14** was then employed for the third ligation by an oxaproline ligation with the C-terminal glycoaldehyde ester **3a** to incorporate the RP-rich transportant S3-segment **3a** to complete the synthesis (Figure 6C). The final three-chain product, **15a** was obtained in 67% yield.

Biological Activity. To show that the RP-rich segments RG24 and RL17 representing the N-terminal 1−24 and 1−17 of Bac 7, respectively, were cell permeable, they were fluoresceinated using succinimidyl ester of FAM to obtain FAM-RG24 and FAM-RL17 for analysis by confocal microscopy that could reveal the intracellular localization and distribution of fluoresceinated peptides. Indeed, FAM-RG24 and FAM-RL-17 were found to distribute in the cytoplasm and nucleus (Figure 7). For comparison, cells were also incubated with a known cationic transportant peptide labeled with FAM at its N-terminus, FAM-Tat (RKKRRQRRR) which also showed cytoplasmic and nuclear localization similar to RG24 and RL17 (B,C in Figure 7). In contrast, cells incubated in peptides without the RP-rich transportant sequences such as FAM-**7e** and FAM-**11b** did not show nuclear delivery (data not shown).

To show that RG24 and its truncated peptide RL17 could act as transportants to translocate the multipartite peptides with peptide cargoes into cells, we prepared three fluoresceinated multipartite peptides **10c**, **12d**, and **15b** in different architectures with RG24 or RL17. In these preparations of labeled peptides, the chemistry and starting materials were similar to those described for the unlabeled peptides, but we avoided the use of the highly basic segment **4a** that could serve as a transportant. The single-chain FAM-peptide **10c** was obtained by an *O*Pro ligation of FAM-RG24 to **7c** consisting of two ligated segments,

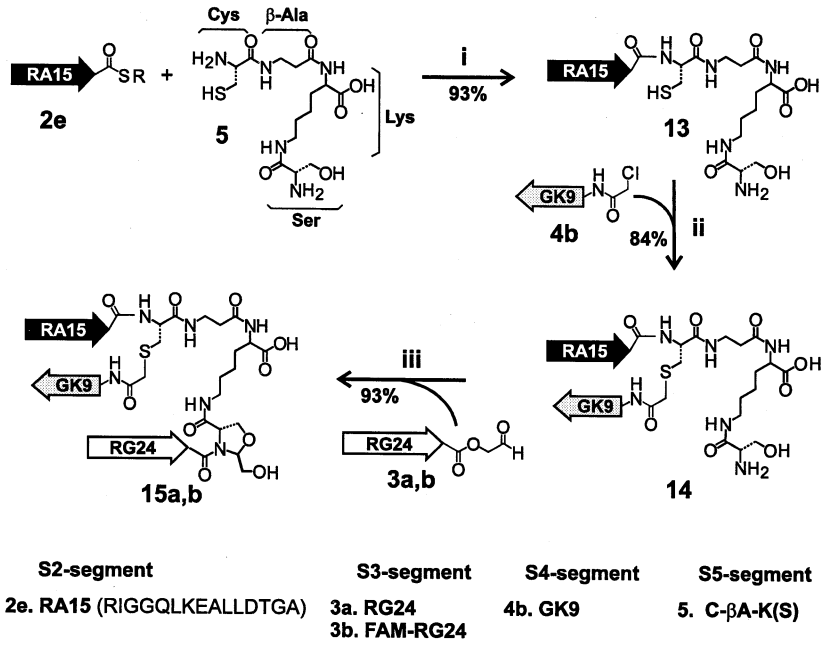

Figure 5. Tandem ligation scheme for the assembly of a three-chain peptide from four segments using (i) Cys, (ii) ψGly, and (iii) *O*Pro ligation methods.

1b and **2b** (Table 1). The two-chain FAM-peptide **12d** was prepared similarly to the unlabeled peptide **12b** using the labeled S3-segment FAM-RL17 (see Figure 3). Finally, a FAM-labeled dendritic peptide **15b** was also prepared using the same strategy and intermediates as **15a** except for the S3-segment which was substituted by **5d**. These labeled peptides contained 52 to 54 amino acids with a molecular mass of about 6500 Da.

The ability of three fluorophor-labeled peptides to translocate across the plasma membrane of HeLa cells was assayed by confocal microscopy (Figure 7). The results revealed a fluorescent pattern consistent with both nuclear and cytoplasmic distribution, confirming that these peptides were translocated into the cells. The one- and three-chain peptides **10c** and **15b** gave stronger fluorescent signals than the two-chain peptide **12d (**compare panels E, H, and K in Figure 7), suggesting that the use of the full-length RP-transportant RG24 in **10c** and **15b** was more effective than the truncated RP-transportant RL17. The translocation was also time- and dose-dependent. Preferential accumulation in the nucleus was found when the cells were incubated with fluorescent-labeled peptides at 37 °C for in 30 min. At low concentration of 0.1 μM, the signal was mostly distributed in cytoplasm. At high concentration of 10 μM, an intense signal was observed at the nucleus, suggesting most of the multipartite peptides were concentrated in the nucleus. There were also subtle differences observed in the fluorescent signals among the three FAM-peptides. For example, in the one-chain peptide **10c**, we found the intense signals surrounding membranes when the cells were incubated with 0.1 and 1.0 μM of **10c**. However, the signals surrounding membranes were scattered or opaque when cells were incubated with a low concentration of two-chain peptide **12d** and three-chain peptide **15b**.

These subtle differences could be resulted in a proteolytic cleavage of our fluorescent-labeled peptides during the translocation experiments. To examine whether there was proteolytic cleavage, **12d** was incubated in the buffered solution containing 2% bovine serum. HPLC monitoring of aliquots withdrawn at 2 h intervals and followed by mass-spectrometric analysis showed no significant degradation in 12 h. Thus, these subtle difference may be attributed to the multipartite peptides carrying different peptide cargoes.

Taken together, RG24 and RL17 derived from the N-terminal fragments of Bac 7 are transportants even when ligated to peptide cargoes. These cell-permeable peptides are capable of translocating the ligated cargoes that contain fragments of melittin and V3 loop of HIV to cross the plasma membrane of HeLa cells. The RP-rich transportant is effective in the multipartite peptides with normal or branched architectures.

Conclusions

Three aspects of our results are worth noting. First, we show a tandem ligation scheme employing three N-terminal specific ligation methods for direct coupling of four different segments to afford a Cys, ψGly, and *O*Pro bond at the ligation sites without a deprotection step in the synthetic scheme. This strategy is flexible for the syntheses of peptides containing one-chain, two-chain, and three-chain architectures with high yield. Such a tandem ligation strategy can be readily utilized for semisynthesis of proteins using building blocks from biosynthetic sources. Potentially, it can also be exploited for combinatorial segment synthesis.

Second, we confirm that the RP-rich peptides RG-24 and RL17 representing the amino terminal segments of Bac 7 can be used as a transportant for intracellular delivery. In peptide-based vaccines, a transportant delivers cytotoxic T-cell epitopes necessary for eliciting cellular immune responses. Furthermore, the branched multipartite design is as effective as the conventional single-chain construct. Transportants provide a noninvasive means of intracellular delivery of functionally active peptides or cargoes to target intracellular protein−protein interactions and signal transduction mechanisms. With the rapid advances in genomics and proteomics, intracellular delivery of peptides, proteins, or DNA provides a useful method to validate molecular targets.

Third, our results suggest that functional peptides can be exploited as heteromeric dendritic peptides in designing drugs

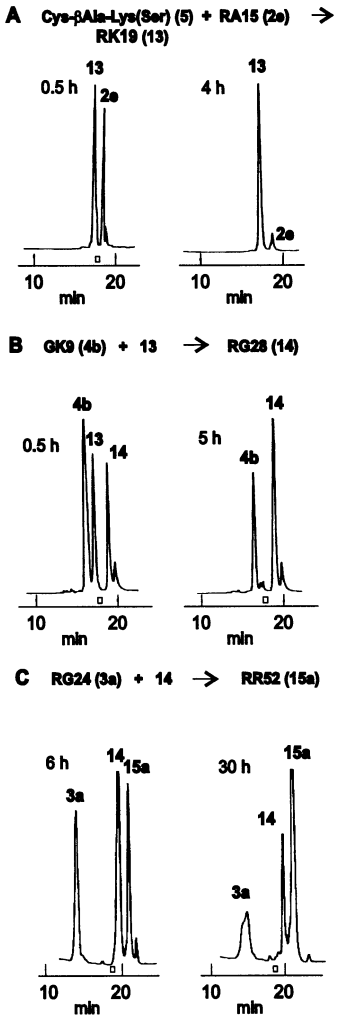

Figure 6. HPLC profiles monitoring the assembly of a three-chain peptide using the scheme shown in Figure 6. (A) Cysteine ligation between the tetrapeptide core **5** and peptide thioester RA15 **2e** to produce RK19 **13** after 0.5 and 4 h. (B) ψGly ligation between segments GK9 **4b** and **13** to produce RG28 **14**. (C) Oxaproline ligation between segments RG24 **3a** and **14** to produce the three-chain 52-residue produce RR52 **15a**. *, impurity from pyridine.

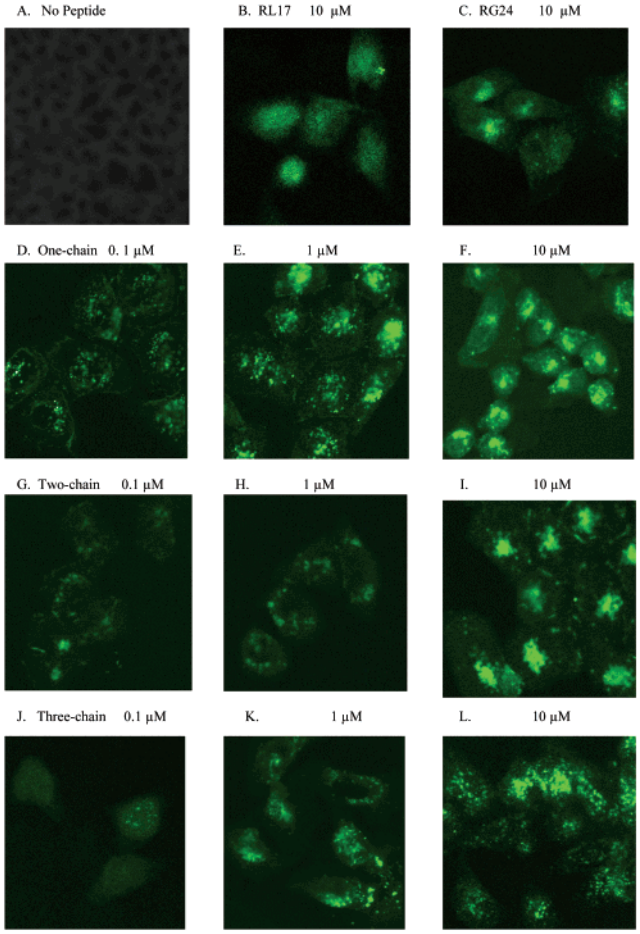

Figure 7. Confocal microscopy analysis on the intracellular uptake of three fluorescein-labeled peptides **10c**, **12d**, and **15b** by HeLa cells in three different concentrations. Cells were incubated with no peptide (A); 10 μM of **3b** (B); 10 μM of **3f** (C); one-chain peptide **10c** at 0.1 (D), 1.0 (E), 10 μM (F); two-chain peptide **12d** at 0.1 (G), 1.0 (H), 10 μM (I); three-chain peptide **15b** at 0.1 (J), 1.0 (K), 10 μM (L) concentrations. Cells were incubated with peptides for 30 min at 37 °C.

and synthetic biologicals to enhance binding avidity and to probe multiple molecular interactions frequently found in nature.[35] Recently, homomeric branched peptides and dendrimers have found applications to increase the specificity and potency of ligands for receptor interactions. The development of multiple antigen peptide(s) (MAP) for diagnostics and synthetic vaccines is an example of increasing binding efficacy through multivalency.[36] Most MAPs in use today are homomeric consisting of multiple copies of same peptides in dendritic forms. However, there is also a need for heteromeric constructs that contain two or more different peptides to produce a molecule capable of interacting with different functional or binding sites. In a recent example, a structurally homologous smaller protein mimetic has been successfully engineered by ligating of a chimeric four-helix bundle onto a template-assembled scaffold.[37] Even more recently, a multipartite construct with peptides, peptide mimetics and lipids that is similar in design to our three-chain peptides has been developed successfully for gene delivery to target vasculatures.[38] The ability to ligate multipartite heteromeric constructs through tandem ligation will likely be useful for designing artificial proteins and novel carriers to inhibit intracellular functions.

Experimental Section

Abbreviations. Standard abbreviations are used for the amino acids and protecting groups [IUPAC−IUB Commission for Biochemical Nomenclature (1985) J. Biol. Chem. 260, 14]. Other abbreviations are as follows: *O*Pro (*OP*), 2-hydroxymethyloxazolidine; *O*Pro^{Me} (*OP*^{Me}), 2-hydoxymethyl-5-methyloxazolidine; Boc, *t*-butoxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; RP-HPLC, reverse phase-high performance liquid chromatography; ESI−MS, electron spray ionization mass spectrometry; FAM, carboxyfluorescein; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; TFA, trifluroacetic acid; HF, hydrofluoride; MBHA, methylbenzhydrylamine; TCEP, tris-

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(carboxyethyl)phosphine; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium-hexafluorophosphate; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate; DCM, dichloromethane; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIC, *N*,*N*-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; HOBt, *N*-hydroxybenzotriazole

General. Analytical HPLC was performed using a Shimadzu 10A system with a Vydac C18 column (4.6 × 250 mm, 5 μm) run at a flow rate of 1.0 mL/min, eluent was monitored at 225 nm. Preparative HPLC was performed on Waters 600 equipment with a Vydac C18 column (22 × 250 mm). All HPLC was carried out with a reversed phase linear gradient of buffer A (0.05% TFA in H_2O) and buffer B (60% CH_3CN in H_2O with 0.04% TFA). Mass spectra were obtained by the MALDI-TOF method using a PerSeptive Biosystems Voyager Elite 2 instrument.

Peptide Segments Syntheses. a. S1-Segments 1a,**b.** The S1-segments **1a**,**b** were synthesized on PAM resin using a Boc/Bzl and HBTU/HOBt strategy. The formyl group of Trp was removed prior to HF cleavage by using 10% piperidine-DMF solution at 0 °C for 2 h. These peptides were cleaved from the resin by anhydrous HF-anisole (95:5, v/v), and then purified by HPLC. Peptide composition was confirmed by amino acid analysis and mass spectroscopy. **1a**, CRPIPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRP−OH, t_R = 22.9 min (20−50 B%), LRMS *m*/*z* 3995.27 (M^+, 3995.8 cald for $C_{188}H_{301}N_{59}O_{36}S$); **1b**, CKVLTTGLPALISW−OH, t_R = 29.3 min (0−100 B%, 40 min) MALDI-MS *m*/*z* 1502.6 (M^+, 1501.8 cald for $C_{70}H_{116}N_{16}O_{18}S$).

b. S2-Segments 2a−e. S2-segments **2a**,**b** were synthesized on a thiol resin.[30] Attachment of the first amino acid to the thiol resin was achieved by the HBTU/HOBt method,[39] and monitored using the Ellman's reagent.[40] In each cycle of the stepwise synthesis, neutralization was accomplished by mixing 5% DIEA in DMF for 1 min, then coupling with 4 equiv of Boc-AA-OH/DIC/HOBt activated for 40 min before use. During synthesis the terminal Boc protecting groups were removed by treatment wit*h*100% TFA for 5 min. The peptides were cleaved from the resin by anhydrous HF/*m*-cresol/*p*-thiocresol (93:5:2, v/v) at 0 °C for 1.5 h, and then purified by HPLC. The amino acid analysis and MS gave the desired results. **2a**, SRIGGQLKEALLDTGA-$SCH_2CH_2CONH_2$, t_R = 22.5 min (20−50 B%), MALDI-MS *m*/*z* 1717.5 (M + H^+, 1717.0 cald for $C_{72}H_{127}N_{22}O_{24}S$); **2b**, TRIGGQLKEALLDTGA-$SCH_2CH_2CONH_2$, t_R = 23.3 min (20−50 B%), MALDI-MS *m*/*z* 1731.8 (M + H^+, 1731.0 cald for $C_{73}H_{129}N_{22}O_{24}S$). **2c**, TIQKLEDMVGPQP-NEDTVTQAA-$SCH_2CH_2CONH_2$, 62% yield, t_R = 21.9 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 2473.3 (M + H^+, 2472.7 cald for $C_{103}H_{171}N_{28}O_{38}S_2$); **2d**, SVGPQPNEDTVTQAA-$SCH_2CH_2CONH_2$, 70% yield, t_R = 17.3 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 1601.2 (M^+, 1600.7 cald for $C_{65}H_{105}N_{19}O_{26}S$); **2e**, RIGGQLKEALLDTGA-$SCH_2CH_2CONH_2$, t_R = 18.0 min (30−70 B%, 30 min), MALDI-MS *m*/*z* 1630.1 (M + H^+, 1629.9 cald for $C_{69}H_{122}N_{21}O_{22}S$).

c. S3-Segments 3a−f. Peptide chain assembly was accomplished on an acetal resin using an Fmoc/tBu strategy.[22,33] Attachment of the first amino acid to the acetal resin was achieved using 4 equiv $(Fmoc\text{-}Gly)_2O$ and a catalytic amount of DMAP and HOBt in anhydrous DMF at 20 °C for 12 h. With HBTU/HOBt as coupling agents and 20% piperidine in DMF as the deprotecting agent, 2.5 equiv of amino acid were used in each cycle of the stepwise synthesis. Capping was mediated through 5 equiv succinimidyl ester of carboxyfluorescein under a basic condition for 3 to 8 h. Final cleavage of peptides from the resin was performed with TFA-glycerol-anisole-thioanisole (90:4:3:3, 40 mL/g resin) for 3 h. Preparative HPLC gave the purified peptide glycerol esters (60−75% yields based on the substitution of resin), which were completely converted to S3-segment **3a**,**b** by 5 equiv $NaIO_4$ under aqueous conditions for 5−10 min at pH 5. Some of S3-segments were used without mass analysis after purification. The analysis data for these segments are shown below. **3a**, RRIRPRPPRLPRPRPRPLPF-PRPG-OCH_2CHO, 74% yield, t_R = 17.4 min (20−50 B%), MALDI-MS *m*/*z* 2980.2 (M + H^+, 2980.6 cald for $C_{135}H_{227}N_{51}O_{26}$); **3b**, $C_{21}H_{11}O_6$−RRIRPRPPRLPRPRPRPLPFPRPG-$OCH_2CH(OH)CH_2$-OH, 69% yield, t_R = 23.9 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 3370.9 (M^+, 3371.0 cald for $C_{157}H_{241}N_{51}O_{33}$); **3c**, PPPPNPNDPPPP-NPNDLG-O−$CH_2CH(OH)CH_2OH$, 71% yield, t_R = 17.5 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 1920.9 (M^+, 1920.0 cald for $C_{85}H_{126}N_{22}O_{29}$); **3d**, RRIRPRPPRLPRPRPPL-O−$CH_2CH(OH)CH_2OH$, 76% yield, t_R = 19.9 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 2205.5 (M^+, 2204.7 cald for $C_{98}H_{174}N_{38}O_{20}$); **3e**, RRIRPRPPRLPRPRPL-O−$CH_2CH(OH)CH_2OH$, 76% yield, t_R = 19.9 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 2108.4 (M^+, 2107.6 cald for $C_{93}H_{167}N_{37}O_{19}$); **3f**, $C_{21}H_{11}O_6$−RRIRPRPPRLPRPRPPL-O−$CH_2CH(OH)CH_2OH$, 76% yield, t_R = 23.5 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 2564.3 (M + H^+, 2564.0 cald for $C_{119}H_{185}N_{38}O_{26}$).

d. S4-Segments 4a,**b.** The S4-segments **4a**,**b** were synthesized on Wang resin using an Fmoc/tBu strategy. The S4-segments bearing the N-terminal chloroacetyl group were prepared by a routine coupling of chloroacetic acid. Final cleavage of peptides from the resin was performed with the routine TFA-mediated cleavage methods for 2−3 h. **4a**, $ClCH_2CO$−RIQRGPGRAFVTIGK−OH, 85% yield, t_R = 21.3 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 1733.1 (M^+, 1732.4 cald for $C_{75}H_{127}N_{26}O_{19}Cl$); **4b**, $ClCH_2CO$-GRAFVTIGK−OH, 74% yield, t_R = 21.9 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 1024.7 (M^+, 1024.6 cald for $C_{45}H_{74}N_{13}O_{12}Cl$).

e. S5-Segment 5. The S5-segment was synthesized on a benzyl ester resin starting with an orthogonally protected Lys in which the α-amine was protected by Boc and the ϵ-amine by Fmoc group. Elongation of two amino acids from the α-amine was accomplished by Boc chemistry. Fmoc-deprotection by 20% piperidine/DMF released the ϵ-amine, which was subsequently coupled with the protected Boc-Ser. HF-mediated cleavage gave the desired tetrapeptide S5-segment. S5 **5**, CβAK(S)-OH, 74% yield, MALDI-MS *m*/*z* 408.2 (M + H^+, 408.5 cald for $C_{15}H_{30}N_5O_6S$).

Cysteine Ligation of S2 and S3 Segments. A typical cysteine ligation between an S1 segment (1.0 mmol) and an S2 segment (1.1 mmol) was carried out in 0.4 to 0.6 mL aqueous buffer at pH 7.6 containing 6 M guanidine HCl and 10 equiv of 2-mercaptoethanesulfonic acid sodium salt. The reaction was monitored by HPLC. The ligation product was identified by MALDI-TOF MS and formation of the amide bond was confirmed by treatment with 1 M H_2NOH at pH 9.0 or by the Ellman test at pH 8. **7a**, SRIGGQLKEALLDT-GA**C**RPIPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRP−OH, t_R = 23.1 min (20−50 B%), MALDI-MS *m*/*z* 5606.1 (M^+, 5606.7 cald for $C_{258}H_{421}N_{80}O_{59}S$); **7b**, TRIGGQLEALLDTGA**C**RPIPRPLPFPRPG-PRPIPRPLPFPRPGPRPIPRP−OH, t_R = 23.3 min (20−50 B%), MALDI-MS *m*/*z* 5619.9 (M^+, 5620.7 cald for $C_{258}H_{422}N_{80}O_{59}S$); **7c**, TRIGGQLKEALLDTGA**C**KVLTTGLPALISW−OH, t_R = 32.0 min (0−100 B%, 40 min), MALDI-MS *m*/*z* 3127.3 (M^+, 3126.7 cald for $C_{140}H_{237}N_{37}O_{41}S$).

Oxaproline Ligation of S3 and Segments 7a−c. The oxaproline ligations between S3 segments **3a**,**b** (2.0 mmol) and segments **7a−c** (1.0 mmol) (1.0−1.5 mmol) were typically performed in 0.4 mL pyridine-acetic acid mixture (1:1, mol/mol) for 25 h at 20 °C, giving the final products **10a−c** at 52−71% yield (Table 1). The oxaproline ligations were monitored by HPLC and the products were confirmed by chemical analysis and MALDI-MS. **10a**, RRIRPRPPRLPRPRP-RPLPFPRPG***O*****P**RIGGQLKEALLDTGACRPIPRPLPFPRPGPRP-IPRPLPFPRPGPRPIPRP−OH, t_R = 25.1 min (20−50 B%), MALDI-MS *m*/*z* 8580.1 (M^+, 8580.3 cald for $C_{393}H_{644}N_{131}O_{84}S$); **10b**, RRIRPRPPRLPRPRPRPLPFPRPG***O*****P**$^{\mathbf{Me}}$RIGGQLKEALL-DTGACRPIPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRP−OH, t_R = 25.2 min (20−50 B%), MALDI-MS *m*/*z* 8590.9 (M^+, 8594.4 cald for $C_{394}H_{646}N_{131}O_{84}S$); **10c**, $C_{21}H_{11}O_6$−RRIRPRPPRLPRPRPRPLPFPRP-G***O*****P**$^{\mathbf{Me}}$RIGGQLKEALLDTGACKVLTTGLPALISW−OH, t_R = 31.8

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min (0−100 B%, 40 min), MALDI-MS *m/z* 6451.5 (M + H^+, 6448.6 cald for $C_{296}H_{473}N_{88}O_{72}S$).

Synthesis of Two-Chain Peptides. The following peptides were used in Cys/ψGly/*O*Pro tandem ligation; **1b** as an S1-segment, **2c** and **2d** as S2-segments, **4a** and **4b** as S4-segments, and **3c**, **3d**, **3e**, and **3f** as S3-segments. The S1-, S2-, and S3-segments were prepared as described in one-chain peptide. The S4-segments bearing the N-terminal chloroacetyl group were prepared by a routine coupling of chloroacetic acid.

The C-termini of the S3-segment precursors **3c**−**f** were converted to aldehyde moieties in quantitative yield by addition of 5 equiv sodium periodate in phosphate buffer pH5 for 5−10 min at 20 °C.

Cysteine ligation of **2c** (8.2 mg, 3.3 mmol) to **1b** (6.2 mg, 4.1 mmol) with TCEP (5.9 mg, 20.7 mmol) was carried out in 1.2 mL phosphate buffer at pH 7.6, the reaction was left to proceed for 11 h at 20 °C. The pH was maintained by addition of a basic solution and the reaction was monitored by HPLC throughout. The ligated product TW36 **7d**, TIQKLEDMVGPQPNEDTVTQAA**C**KVLTTGLPALISW−OH, was obtained at an 80−85% yield, t_R = 32.8 min (0−100 B%, 40 min), MALDI-MS *m/z* 3873.1 (M + H^+, 3872.4 cald for $C_{170}H_{281}N_{43}O_{55}S_2$). A second cysteine ligation between **2d** (2.3 mg, 1.4 mmol) and **1b** (2.0 mg, 1.4 mmol), gave the ligated product, **7e**, SVGPQP-NEDTVTQAA**C**KVLTTGLPALISW−OH, in 85% yield, t_R = 32.8 min (0−100 B%, 40 min), MALDI-MS *m/z* 2998.3 (M^+, 2997.4 cald for $C_{132}H_{214}N_{34}O_{43}S$).

The chemoselective ψGly ligation of **7d** (2.0 mg, 0.7 μmol) and the N-terminal chloroacetyl **4a** (1.0 mg, 1.0 μmol) was carried out in 0.4 mL aqueous 6M guanidine buffer at pH 8.0 for 22 h at 20 °C, yielding **11a**, TIQKLEDMVGPQPNEDTVTQAAC(**S**CH_2CO−RIQRG-PGRAFVTIGK−OH)KVLTTGLPALISW−OH, with a yield of 85%, t_R = 30.1 min (0−100 B%, 40 min), MALDI-MS *m/z* 5566.6 (M^+, 5567.4 cald for $C_{245}H_{407}N_{69}O_{74}S_2$). Another ψGly ligation between **7e** (1.8 mg, 0.6 mmol) and **4b** (0.8 mg, 0.8 mmol) was performed in similar conditions to produce **11b**, SVGPQPNEDTVTQAAC(**S**CH_2CO-GRAFVTIGK−OH)KVLTTGLPALISW−OH, with a yield of 95%, t_R = 30.3 min (0−100 B%, 40 min), MALDI-MS *m/z* 3984.7 (M^+, 3985.5 cald for $C_{177}H_{287}N_{47}O_{55}S$).

Finally, the oxaproline ligation between **11a** (1.5 mg, 0.3 μmol) and **3c** (1.0 mg, 0.6 μmol) was achieved in 0.1 mL of a *N*-methyl-imidazole-acetic acid mixture at 20 °C for 10 h affording the desired two-chain peptide **12a**, PPPPNPNDPPPPNPNDLG***OP***^{Me}-IQKLEDMVGPQPNEDTVTQAAC(SCH_2CO−RIQRGPGRAFVTIGK−OH)KVLTTGLPALISW−OH, in 55% yield, t_R = 31.3 min (0−100 B%, 40 min), MALDI-MS *m/z* 7436.4 (M^+, 7437.4 cald for $C_{329}H_{527}N_{91}O_{101}S_2$). The other oxaproline ligations of **11b** (1.0 mg, 0.3 mmol) and each of **3d**−**f** (0.6 mmol) carried out under same condition as above gave rise to 50% yield of the two-chain peptide, **12b**, RRIRPRPPRLPRPRPPL***OP***VGPQPNEDTVTQAAC(SCH_2CO-GRAFVTIGK−OH)KVLTTGLPALISW−OH, t_R = 30.7 min (0−100 B%, 40 min), MALDI-MS *m/z* 6139.7 (M^+, 6140.1 cald for $C_{274}H_{455}N_{85}O_{73}S$); **12c**, RRIRPRPPRLPRPRPL***OP***VGPQ0PNEDTV-TQAAC(SCH_2CO-GRAFVTIGK−OH)KVLTTGLPALISW−OH, t_R = 30.7 min (0−100 B%, 40 min), MALDI-MS *m/z* 6042.3 (M^+, 6043.0 cald for $C_{269}H_{448}N_{84}O_{72}S$); **12d**, $C_{21}H_{11}O_6$−RRIRPRPPRLPRPRPL***OP***-VGPQPNEDTVTQAAC(SCH_2CO-GRAFVTIGK−OH)KVLTTGL-PALISW−OH, 50% yield, t_R = 31.0 min (0−100 B%, 40 min), MALDI-MS *m/z* 6494.4 (M^+, 6498.5 cald for $C_{295}H_{465}N_{85}O_{79}S$).

Synthesis of Three-Chain Peptides. The peptide segments **2e**, **4b**, and S5 **5** were synthesized by stepwise solid-phase synthesis as described above.

Cysteine ligation between segments **5 (**2.9 mg, 7 mmol) and **2e** (8.1 mg, 5 μmol) was carried out in 0.5 mL aqueous buffer (pH 7.6) containing 6 M guanidine HCl and 10 equiv of 2-mercaptoethane-sulfonic acid sodium salt at 20 °C for 5 h. The reaction was monitored by HPLC. The ligated **13**, RIGGQLKEALLDTGA**C**βAK(S)-OH, was obtained in 93% yield, t_R = 16.0 min (30−70 B%, 30 min), MALDI-MS *m/z* 1932.5 (M + H^+, 1932.2 cald for $C_{81}H_{144}N_{25}O_{27}S$). The chemoselective ligation of **13** (3.9 mg, 2.0 mmol) and **4b** (3 mg, 3.0 mmol) was carried out in 0.4 mL aqueous buffers at pH 8 for 6 h at 20 °C, yielding **14**, RIGGQLKEALLDTGAC(**S**CH_2CO-GRAFVTIGK) βAK(S)-OH, 94% yield, t_R = 18.5 min (30−70 B%, 30 min), MALDI-MS *m/z* 2920.2 (M + H^+, 2920.4 cald for $C_{126}H_{217}N_{38}O_{39}S$). Finally, the oxaproline ligation between **14** (2.9 mg, 1.0 mmol) and **3a** (6.0 mg, 2.0 mmol) was achieved in 0.3 mL of pyridine-acetic acid mixture at 20 °C for 35 h affording the desired three-chain peptide **15a**, RIGGQLKEALLDTGAC(SCH_2CO-GRAFVTIGK)βAK(***OP***-GPRPF-PLPRPRPRPLRPPRPRIRR), 67% yield, t_R = 21.3 min (30−70 B%, 30 min), MALDI-MS *m/z* 5881.6 (M + H^+, 5881.9 cald for $C_{261}H_{441}N_{89}O_{64}S$). A second oxaproline ligation of **14** (2.9 mg, 1.0 mmol) and **3b** (2.0 mmol) carried out under methylimidazole-acetic acid mixture at 20 °C for 6 h gave 50% yield of the three-chain peptide, **15b**, RIGGQLKEALLDTGAC(**S**CH_2CO-GRAFVTIGK)βAK-(***OP***-GPRPFPLPRPRPRPLRPPRPRIRR−$C_{21}H_{11}O_6$), 50% yield, t_R = 31.0 min (0−100 B%, 40 min), MALDI-MS *m/z* 6242.7 (M + H^+, 6242.3 cald for $C_{282}H_{453}N_{89}O_{70}S$).

Cells and Cultures. HeLa cells, a cervical adenocarcinoma − derived from human malignant tissue, were gift from Dr. Christopher Aiken, Vanderbilt Univesity. Cells were propagated in Dulbecco's Modified Eagle's (DME) medium (Cellgro, VA) containing 10% v/v heat-inactivated fetal bovein serum (Atlanta Biologicals, GA), 2 mM glutamine and 50 U/ml penicillin/50 mg/mL streptomycin. Cells were incubated at 37 °C with 95% humidity and 5% CO_2 and grown in 5% FBS in DME medium prior to use for all the cellular uptake experiments.

Confocal Laser Scanning Microscopy Imaging. Cells at 6−10 × 10^4/ml density were plated on slide flask chambers with 22 × 22 mm flat bottom (Nalge Nunc International, Denmark). The fluorescein-labeled peptides were dissolved in pH 7.4 phosphate-buffered saline (PBS). Each 20 μL aliquote from the stock solutions was added to cultures in a total of 200 μL, and final concentrations of peptides were 0.1, 1 and 10 μM, respectively. The cultures were incubated at 37 °C for 30 min. Cells were washed three times with cold PBS and fixed by 3.7% paraformaldehyde and permeabilized in 0.1% Triton 100 and were analyzed with Zeiss LSM410 confocal microscopy (Zeiss, Germany) equipped with a laser. An excitation wavelength of 488 nm was selected from an argon-ion laser and a 510−550 band-pass filter was used to collect emission wavelengths. Software merging of images was carried out using the COMOS software (Zeiss, Germany). All experiments were performed under dim light to avoid the photobleaching of the fluorescence-labeling peptides.

Proteolytic Stability. Two-chain peptide **12b** with the buffer solution containing 2% serum was incubated at 37 °C. The aliquots withdrawn at 2 h intervals were monitored by HPLC, and confirmed by mass-spectrometry.

Acknowledgment. This work was supported in part by US Public Health Service NIH CA36544.

JA020529R