The Dock and Lock Method: A Novel Platform Technology for Building Multivalent, Multifunctional Structures of Defined Composition with Retained Bioactivity

Chien-Hsing Chang,1,2 Edmund A. Rossi,2 and David M. Goldenberg1,2,3

Abstract

The idea, approach, and proof-of-concept of the dock and lock (DNL) method, which has the potential for making a large number of bioactive molecules with multivalency and multifunctionality, are reviewed. The key to the DNL method seems to be the judicious application of a pair of distinct protein domains that are involved in the natural association between protein kinase A (PKA; cyclic AMP–dependent protein kinase) and A-kinase anchoring proteins. In essence, the dimerization and docking domain found in the regulatory subunit of PKA and the anchoring domain of an interactive A-kinase anchoring protein are each attached to a biological entity, and the resulting derivatives, when combined, readily form a stably tethered complex of a defined composition that fully retains the functions of individual constituents. Initial validation of the DNL method was provided by the successful generation of several trivalent bispecific binding proteins, each consisting of two identical Fab fragments linked site-specifically to a different Fab. The integration of genetic engineering and conjugation chemistry achieved with the DNL method may not only enable the creation of novel human therapeutics but could also provide the promise and challenge for the construction of improved recombinant products over those currently commercialized, including cytokines, vaccines, and monoclonal antibodies.

The impetus for developing the dock and lock (DNL) method undoubtedly was due to the limitations of existing technologies for the production of antibody-based agents having multiple functions or binding specificities. For agents generated by recombinant engineering, such limitations could include high manufacturing cost, low expression yields, instability in serum, formation of aggregates or dissociated subunits, undefined batch composition due to the presence of multiple product forms, contaminating side-products, reduced functional activities or binding affinity/avidity attributed to steric factors or altered conformations, etc. For agents generated by various methods of chemical cross-linking, high manufacturing cost and heterogeneity of the purified product are two major concerns.

We, of course, recognize that innovative fusion proteins created by recombinant technologies may be built into more complex structures to gain additional attributes that are highly desirable, yet not technically attainable, in the individual engineered construct. Well-known examples include cytokines modified with polyethylene glycol to increase serum half-lives (1), biotinylated proteins to enable immobilization into microarrays (2), and protein-DNA chimeras to quantify specific molecules to which the protein binds (3). To date, these goals are commonly achieved with varied success by judicious application of conjugation chemistries. New strategies that are based on the binding of enzyme to substrate (4) or inhibitor (5), or the high-affinity interaction between two fragments of human RNase I (6, 7), to tether two or more moieties of distinct functions into covalent or quasi-covalent assemblies have been reported; however, these methods are cumbersome and therefore may limit their widespread use.

Protein Kinase A and A-Kinase Anchoring Proteins

The key to the DNL method is the exploitation of the specific protein/protein interactions occurring in nature between the regulatory (R) subunits of protein kinase A (PKA) and the anchoring domain (AD) of A-kinase anchoring proteins (AKAP; refs. 8, 9). PKA, which plays a central role in one of the best studied signal transduction pathway triggered by the binding of the second messenger cyclic AMP to the R subunits, was first isolated from rabbit skeletal muscle in 1968 (10). The structure of the holoenzyme consists of two catalytic subunits held in an inactive form by the R subunits (11). Isozymes of PKA are found with two types of R subunits (RI and RII), and each type has α and β isoforms (12). The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 NH2-terminal residues (13).

Binding of cyclic AMP to the R subunits leads to the release of active catalytic subunits held in an inactive form by the R subunits (11). Isozymes of PKA are found with two types of R subunits (RI and RII), and each type has α and β isoforms (12). The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 NH2-terminal residues (13).

Binding of cyclic AMP to the R subunits leads to the release of active catalytic subunits held in an inactive form by the R subunits (11). Isozymes of PKA are found with two types of R subunits (RI and RII), and each type has α and β isoforms (12). The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 NH2-terminal residues (13). Binding of cyclic AMP to the R subunits leads to the release of active catalytic subunits held in an inactive form by the R subunits (11). Isozymes of PKA are found with two types of R subunits (RI and RII), and each type has α and β isoforms (12). The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 NH2-terminal residues (13). Binding of cyclic AMP to the R subunits leads to the release of active catalytic subunits held in an inactive form by the R subunits (11). Isozymes of PKA are found with two types of R subunits (RI and RII), and each type has α and β isoforms (12). The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 NH2-terminal residues (13). Binding of cyclic AMP to the R subunits leads to the release of active catalytic subunits held in an inactive form by the R subunits (11). Isozymes of PKA are found with two types of R subunits (RI and RII), and each type has α and β isoforms (12). The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 NH2-terminal residues (13).
Since the first AKAP, microtubule-associated protein 2, was characterized in 1984 (15), more than 50 AKAPs that localize to various subcellular sites, including plasma membrane, actin cytoskeleton, nucleus, mitochondria, and endoplasmic reticulum, have been identified with diverse structures in species ranging from yeast to humans (9). The AD of AKAPs for PKA is an amphipathic helix of 14 to 18 residues (16). The amino acid sequences of the AD are quite varied among individual AKAPs, with the binding affinities reported for RI dimers ranging from 2 to 90 nmol/L (17). Interestingly, AKAPs will only bind to dimeric R subunits. For human RIα, the AD binds to a hydrophobic surface formed by the 23 NH₂-terminal residues (18). Thus, the dimerization domain and AKAP binding domain of human RIα are both located within the same NH₂-terminal 44-amino-acid sequence (13, 19), which is termed the dimerization and docking domain (DDD) herein.

**DDD of Human RIα and AD of AKAPs as Linker Modules**

We envisioned a platform technology to exploit the DDD of human RIα and the AD of a certain amino acid sequence as an excellent pair of linker modules for docking any two entities, referred to hereafter as A and B, into a noncovalent complex, which could be further locked into a stably tethered structure through the introduction of cysteine residues into both the DDD and AD at strategic positions to facilitate the formation of disulfide bonds, as illustrated in Fig. 1. The general methods of our DNL approach would be as follows. Entity A would be constructed by linking a DDD sequence to a precursor of A, resulting in a first component hereafter referred to as a. Because the DDD sequence would effect the spontaneous formation of a dimer, A would thus be composed of a₂. Entity B would be constructed by linking an AD sequence to a precursor of B, resulting in a second component hereafter referred to as b. The dimeric motif of DDD contained in a₂ should create a docking site for binding to the AD sequence contained in b, thus facilitating a ready association of a₂ and b to form a binary, trimeric complex composed of a₂b. This binding event could be made irreversible with a subsequent reaction to covalently secure the two entities via disulfide bridges, which might occur very efficiently based on the principle of effective local concentration because the initial binding interactions would bring the reactive thiol groups placed onto both the DDD and AD into proximity (20) to ligate site-specifically. By attaching the DDD and AD away from the functional groups of the two precursors, such site-specific ligations would also be expected to preserve the original activities of the two precursors. This approach would be modular in nature and potentially could be applied to link, site-specifically and covalently, a wide range of substances including peptides, proteins, and nucleic acids.

**Bispecific Trivalent Structures Composed of Three Stably Linked Fab Fragments**

For proof-of-concept, we used the DNL method to assemble bispecific a₂b complexes comprising three Fab fragments from a panel of five fusion proteins, each containing either a DDD or an AD of the sequence shown in Fig. 2A (21). The three DDD-containing A entities were made recombinantly using as a precursor the Fab fragment of the humanized monoclonal antibody hMN-14 (22), which has binding specificity for human carcinoembryonic antigen (CEACAM5). The first A, C-DDD1-Fab-hMN-14, was generated by linking the DDD1 peptide sequence, which is composed of amino acids 1 to 44 of human RIα, to the COOH-terminal end of the Fab chain via a 14-residue flexible peptide linker (Fig. 2B). This construct was modified by incorporation of a cysteine residue adjacent to the NH₂-terminal end of DDD1 to create C-DDD2-Fab-hMN-14 (Fig. 2C). The DDD2 sequence was moved to the NH₂-terminal end of theFd to generate N-DDD2-Fab-hMN-14 (Fig. 2D). Both AD-containing B entities were generated recombinantly using as a precursor the Fab fragment of the humanized monoclonal antibody h679 (23), which has binding specificity for histamine-succinyl-glycine. The first B, C-AD1-Fab-h679, was generated by linking the 17-residue amino acid sequence derived from AKAP-85, a synthetic peptide optimized for RII-selective binding with a reported dissociation constant (Kₐ) of 0.4 nmol/L (17), to the COOH-terminal end of the Fd chain via a 15-residue flexible peptide linker (Fig. 2E). The other B, C-AD2-Fab-h679, was generated in the same fashion as C-AD1-Fab-h679, except with the addition of cysteine residues to both the NH₂ and COOH-terminal ends of AD1 (Fig. 2F).

As expected, C-DDD1-Fab-hMN-14 and C-AD1-Fab-h679 were purified from culture media exclusively as a homodimer (the a₂ structure) and a monomer (the b structure) of Fab, respectively. When C-DDD1-Fab-hMN-14 was combined with C-AD1-Fab-h679, the formation of an a₂b complex was readily shown by size-exclusion high-performance liquid chromatography, with the Kₐ determined by equilibrium gel filtration analysis (24) to be ~8 nmol/L, which is presumably too weak of an affinity to keep the a₂b complex intact at concentrations typical (~1 μg/mL) for in vivo applications. To prevent the dissociation of the noncovalent complex formed from C-DDD1-Fab-hMN-14 and C-AD1-Fab-h679 at lower concentrations, thus allowing in vivo applications, cysteine residues were added into the DDD and AD sequences of the A (N-DDD2-Fab-hMN-14 and C-DDD2-Fab-hMN-14) and B (C-AD2-Fab-h679) entities, respectively. We anticipated that on mixing of the cysteine-modified entities, an a₂b complex could be made irreversible with a subsequent reaction to covalently secure the two entities via disulfide bridges, which might occur very efficiently based on the principle of effective local concentration because the initial binding interactions would bring the reactive thiol groups placed onto both the DDD and AD into proximity (20) to ligate site-specifically. By attaching the DDD and AD away from the functional groups of the two precursors, such site-specific ligations would also be expected to preserve the original activities of the two precursors. This approach would be modular in nature and potentially could be applied to link, site-specifically and covalently, a wide range of substances including peptides, proteins, and nucleic acids.

![Fig. 1. Illustration of a stably tethered structure made by the DNL method.](image-url)
complex would promptly form, which could be further stabilized by the formation of disulfide bridges. Two stably tethered trivalent bispecific structures, referred to as TF1 for the conjugate of N-DDD2-Fab-hMN-14 and C-AD2-Fab-h679 and TF2 for the conjugate of C-DDD2-Fab-hMN-14 and C-AD2-Fab-h679, were obtained in nearly quantitative yields following Tris (2-carboxy-ethyl)-phosphine-HCl reduction, DMSO oxidation, and affinity purification. Similar results were achieved by substituting Tris (2-carboxy-ethyl)-phosphine-HCl and DMSO with reduced and oxidized glutathione, respectively. TF1 and TF2 were each shown by size-exclusion high-performance liquid chromatography to be a single peak of the expected molecular size (150 kDa), by BIAcore to be bispecific for both histamine-succinyl-glycine and a rat anti-idiotype monoclonal antibody to hMN-14 (25), and by competition ELISA to be equivalent to hMN-14 immunoglobulin G (IgG) and h679 Fab, reflecting the full retention of valency and binding affinity. Furthermore, TF1 and TF2 were found to be stable for at least 7 days when incubated at 37°C in human or mouse serum, and the superiority of TF2 as a pretargeting agent for diagnostic imaging has been shown in nude mice bearing carcinoembryonic antigen–expressing human colon cancer xenografts (21).

Multivalent Binding Complexes Composed of Six Fab Fragments

Since the generation of TF1 and TF2, several additional trivalent bispecific Fab-based complexes (the TF series) have been produced. We have also applied the DNL method to successfully generate hexavalent, antibody-based complexes (the DNL series) that are either monospecific (Fig. 3A) or bispecific (Fig. 3B and C). The complexes in the DNL series, all of which comprise an IgG-(AD2)2 module coupled to two Fab-DDD2 modules, were made using the C-DDD2-Fab derived from hLL2 (epratuzumab, anti-CD22 IgG; ref. 26), hA20 (humanized anti-CD20 IgG; ref. 27), or hMN-14, and the IgG-(AD2)2 modules derived from hLL2 or hA20. To evaluate
whether the four extra Fab moieties would affect the functions of the Fc, we produced two types of IgG-(AD2)2 modules, with AD2 fused to each COOH terminus of the heavy chain (C-AD2-IgG) or to each NH2 terminus of the light chain (κ-AD2-IgG). Preliminary results indicate that a hexavalent complex with AD2 placed at the NH2 termini seems to have a functional Fc but may selectively lose the binding activity of the IgG-AD2 module, depending on the epitope. In contrast, a hexavalent complex with AD2 placed at the COOH termini may not have a functional Fc but preserves the binding activity of the IgG-AD2 module. In both types of hexameric complexes, each of the four Fab moieties contributed from the Fab-DDD2 module remains active, however. Additional findings are that the monospecific hexameric complex, designated Hex-hA20 (composed of four hA20 Fab tethered to the COOH termini of hA20 IgG), and the two bispecific hexameric complexes, designated DNL1 and DNL2 (composed of four hA20 Fab tethered to hLL2 IgG and four hLL2 Fab tethered to hA20 IgG, respectively), show much greater potency in inhibiting growth of human Burkitt lymphoma cell lines in vitro than the parental monoclonal antibodies alone or combined (28). The bispecific DNL2 and Hex-hA20 showed >100-fold and >10,000-fold more potent antiproliferative activity, respectively, than hA20 IgG on Daudi cells (Fig. 4A). For Raji cells, Hex-hA20 displayed potent antiproliferative activity, whereas DNL2 showed only minimal activity (Fig. 4B). For Ramos cells, both DNL2 and Hex-hA20 showed potent antiproliferative activity (Fig. 4C). In contrast, Hex-hLL2 (composed of four hLL2 Fab tethered to the COOH termini of hLL2 IgG) and the two bispecific controls, DNL1-c (substituting hA20 Fab in DNL1 for hMN-14 Fab) and DNL2-c (substituting hLL2 Fab in DNL2 for hMN-14 Fab), had little antiproliferative activity under the conditions used (data not shown).

The Advantages of DNL

Based on our experience with the tri-Fab and hexa-Fab constructs, the advantages of the DNL method that distinguish it from other site-specific conjugation methods (1–7) are briefly summarized as follows.

DNL is modular. Each DDD- or AD-containing entity is a module and any DDD module can be paired with any AD module. Such modules can be produced independently, stored separately “on shelf”, and combined “on demand,” without

![Fig. 3. Schematics of IgG-based hexavalent DNL structures. A, monospecific derived from C-AD2-IgG and C-Fab-DDD2; B, bispecific derived from C-AD2-IgG and C-DDD2-Fab; C, alternative bispecific derived from κ-AD2-IgG and C-DDD2-Fab.](image)
requiring purification before mixing. There is essentially no limit on the types of precursors that can be derived into a DDD or AD module, so long as the resulting modules do not interfere with the dimerization of DDD or the binding of DDD to AD. In addition to the DDD sequence of human RIIα, other DDD sequences may be selected from human RIA, human RIβ, or human RIIβ. The DDD sequence of choice will be matched with a highly interactive AD sequence, which can be deduced from the literature (29) or determined experimentally.

**DNL is versatile.** Modules may be made recombinantly or chemically. Recombinant modules, which may be produced in mammalian or microbial systems, may include derivatives of antibodies or antibody fragments, cytokines, enzymes, natural carrier proteins (such as human serum albumin and human transferrin), or a variety of natural or artificial non-antibody binding or scaffold proteins (30–33). Although each recombinant module would usually be produced in a separate expression system, certain pairs of DDD and AD modules may be coexpressed in the same host cell without affecting the formation of the DNL conjugates. Furthermore, DDD or AD can be coupled to the NH2-terminal or COOH-terminal end or positioned internally within the fusion protein, preferably with a spacer containing an appropriate length and composition of amino acid residues, provided that the binding activity of the DDD or AD and the desired activity of the polypeptide fusion partners are not compromised.

Modules may also be made synthetically to contain peptides, peptide mimetics, oligonucleotides or polynucleotides, small interfering RNA, polyethylene glycol, chelators with or without radioactive or nonradioactive metals, drugs, dyes, oligosaccharides, natural or synthetic polymeric substances, nanoparticles, fluorescent molecules, or quantum dots, depending on the intended applications.

**DNL manufacture is easy.** The DNL method is basically a one-pot preparation and requires three simple steps to recover the product from the starting materials: (a) combine DDD and AD modules in stoichiometric amounts; (b) add redox agents to facilitate the self-assembly of the DNL conjugate; and (c) purify by an appropriate affinity chromatography.

**DNL results in quantitative yields of a homogeneous product with a defined composition and in vivo stability.** The facile binding between the DDD and AD modules effects nearly 100% conversion of each into the desired DNL product. The site-specific conjugation also ensures that the full activity of each module is preserved, the molecular size is homogeneous, the composition is defined, and in vivo stability is sustained.

**Potential Limitations**

The yield of the precursor protein may predetermine that of its recombinant DDD or AD module. We have found that modules based on IgG or Fab fragments are expressed at high levels similar to the precursor monoclonal antibody, whereas recombinant proteins expressed at low levels result in DNL modules also expressed at comparably low levels. Thus, the optimal expression system for high-level production of a DDD or AD module should be based on what would be best suited for production of the precursor.

Although all of the DNL complexes that have been generated to date are highly stable, it is possible that some other DNL structures may be susceptible to proteolytic degradation in vivo, and therefore, the serum stability of each new DNL complex will need to be evaluated.

In an effort to minimize immunogenicity, the DDD and AD used for the DNL method consist of the smallest functional peptides derived from human protein sequences. However, the DDD2 and AD2 peptides possess additional cysteine residues, which are not part of the natural domains, and both are fused to precursor proteins via flexible Gly-Ser linker peptides, which are foreign but generally considered to be largely nonimmunogenic. Whether the DNL constructs would be immunogenic in humans can only be assessed in future clinical trials.

**Conclusions**

We believe that the superior preclinical results obtained, to date, with the trivalent bispecific Fab-based complexes as a
pretargeted imaging agent will likely be translated successfully into clinical trials. Present challenges lie in showing that the DNL method is also useful as a tool for creating a new class of cytokines with improved pharmacokinetic properties and enhanced in vivo potency, as well as applicable to developing a new paradigm for more effective vaccines composed of multiple subunits, each being well defined. When such challenges are met, the prospects of the DNL method may indeed be endless and limited only by our imagination.

References


The Dock and Lock Method: A Novel Platform Technology for Building Multivalent, Multifunctional Structures of Defined Composition with Retained Bioactivity

Chien-Hsing Chang, Edmund A. Rossi and David M. Goldenberg


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/13/18/5586s

Cited articles
This article cites 33 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/13/18/5586s.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/13/18/5586s.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.