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Engineering cell signaling modulators from native protein–protein interactions

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Recent studies on genome sequencing and genetic screens with RNAi and CRISPR technology have revolutionized our understanding of aberrant signaling networks in human diseases. A strategy combining both genetic and protein-based technologies should be at the heart of modern drug-development efforts, particularly in the era of precision medicine. Thus, there is an urgent need for efficient platforms to develop probes that can modulate protein function in cells to validate drug targets and to develop therapeutic leads. Advanced protein engineering has enabled the rapid production of monoclonal antibodies and small protein scaffold affinity reagents for diverse protein targets. Here, we review the most recent progress on engineering natural protein–protein interactions for modulation of cell signaling.

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Introduction

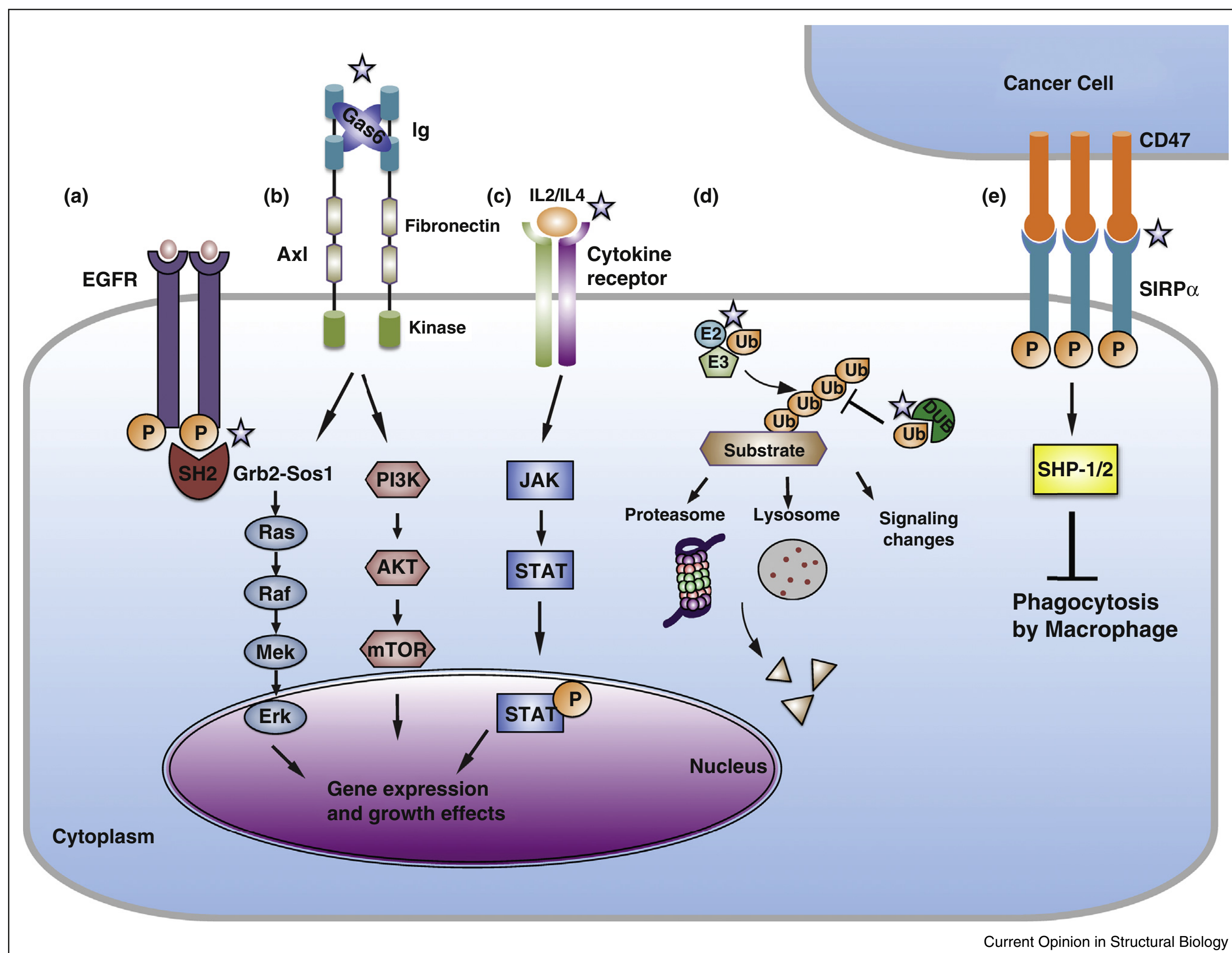
The past decade has witnessed remarkable advances in understanding of the signaling networks that drive disease progression, enabled by genetic approaches such as RNAi (RNA interference) and CRISPR (clustered regularly interspaced short palindromic repeats) [1,2]. While these studies point the way toward new therapies, effective drug discovery requires a toolkit for systematic modulation of the proteins encoded by disease-associated genes to validate drug targets and to develop therapeutic molecules. In light of this, significant efforts have been devoted to generate monoclonal antibodies (MAbs), nature-inspired reagents for research and clinical usage [3,4]. Unfortunately, MAb applications are limited, due to the large size, structural complexity, and poor intracellular activity of the MAb molecule [5].

To overcome these limitations, approximately 50 alternative protein scaffolds have been engineered to develop molecules that can bind clinically relevant targets with high affinity and specificity [6–9]. For example, Affibodies, Affilins, Atrimers, DARPins (Designed Ankyrin Repeat Proteins), Fynomers, and Kunitz domains were developed and actively used in diagnostics, imaging and potential therapeutics [6,8]. Employing combinatorial engineering methods, such as site-directed combinatorial mutagenesis coupled with phage display and other selection techniques [10,11], these protein structures can be equipped with new binding capacities [7,9]. It is noteworthy that scaffold diversification design has been greatly facilitated by studies of natural protein–protein interactions (PPIs), which are mediated by modular domains to ensure regulated and coordinated signal transduction [12]. This has stimulated the usage of short peptides (natural, synthetic or engineered protein fragments) as protein inhibitors [13,14]. Tremendous progress has been made in this field but the small size and conformational flexibility of peptides impose several drawbacks, including proteolytic instability, generally low affinities, and unwanted and unpredictable cross-reactivity in cells [15]. While in principle the optimization of natural interactions could provide potent and specific inhibitors, the use of protein scaffolds to exploit existing natural interactions has been relatively uncommon [8]. In this review, we present recent work on protein engineering of natural PPIs for cell signaling modulators (Figure 1).

Engineering phosphorylation recognition sites

Protein phosphorylation is a post-translational modification that eukaryotic cells commonly employ for signal transduction [16]. The dynamic phospho-epitope recognition is mediated by conserved modular domains, including SH2 (Src homology 2), WW, 14-3-3, FHA (Forkhead-associated), and BRCT (BRCA1 C-terminal) [12,16]. These protein modules can retain their structure and biochemical properties when expressed alone, and are frequently employed by multi-cellular organisms to assemble multi-protein complexes to mediate signaling cascades [17]. Although various protein domains (*e.g.* SH2, SH3, PDZ, WW) have been engineered to create new peptide–ligand interactions [8], not much work was done to modulate natural existing phosphor-dependent PPIs in order to dissect and characterize cellular responses with

Figure 1



Protein–protein interactions that have been engineered to modulate cellular processes (denoted by *stars*). **(a)** Phosphor-epitope recognition mediated by SH2 domains is important for phosphorylation dependent signal transduction [12,20]. For example, the SH2 domain of Grb2 specifically binds pTyr residues in activated EGFR to activate the Ras signaling cascade by recruitment of the guanine nucleotide exchange factor Sos1. **(b)** The Axl-Gas6 interaction is critical for activation of Axl signaling and can be engineered for therapeutic benefit. Axl contains two immunoglobulin-like (Ig) domains, two fibronectin type III repeat (FNIII) domains and a kinase domain. Gas6 dependent dimerization activates Axl and downstream signaling pathways that are important for cell proliferation and survival. **(c)** Cytokines, such as IL2 and IL4, assemble their cognate receptor complexes and trigger downstream signaling through the JAK-STAT pathway. Low binding affinity between cytokines and their secondary receptor subunits can be improved through protein engineering, and the resulting superkines have potential applications for immunotherapy [55]. **(d)** Ubiquitination can lead to changes in cellular signaling or it can direct proteins to the proteasome or lysosomes for degradation [16]. E3 ligases are responsible for the transfer of Ub from E2 conjugating enzymes to substrate proteins, while DUBs cleave Ub moieties from substrate proteins. The E3-Ub and DUB-Ub interaction sites can be targeted with engineered Ub variants. **(e)** CD47 on cancer cells can activate SIRPα on macrophages, which results in recruitment of SHP-1/2 phosphatases that elicit a ‘don’t eat me’ signal and prevent phagocytosis. Blockade of CD47-SIRPα interactions by engineered CD47 and SIRPα variants can potentially enhance macrophage phagocytosis of cancer cells [50,51••].

hyper/hypo phosphopeptide-ligand interactions. Below, we provide an example of generating SH2 variants as superbinders of pTyr peptides to modulate cell signaling [18•,19•].

Strengthening SH2-pTyr interactions

The SH2 domain fold contains ~100 amino acids, was first discovered in the viral oncoprotein v-Src, and is now known to occur in 111 human proteins, including signaling adaptors, kinases and phosphatases [20]. SH2 domains specifically bind to targets containing pTyr peptide motifs (Figure 1a). Structural analysis of various SH2 domains in complex with phospho-peptides revealed that the SH2 domain fold is comprised of a central anti-parallel β-sheet surrounded by two α-helices [20,21]. It provides a positively charged pocket with conserved residues on one

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