Scratching the (cell) surface: cytokine engineering for improved ligand/receptor trafficking dynamics

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Cytokines can be engineered for greater potency in stimulating cellular functions. An obvious test criterion for an improved cytokine is receptor-binding affinity, but this does not always correlate with improved biological response. By combining protein-engineering techniques with studies of receptor trafficking and signaling, it might be possible to identify the ligand receptor-binding properties that should be sought.

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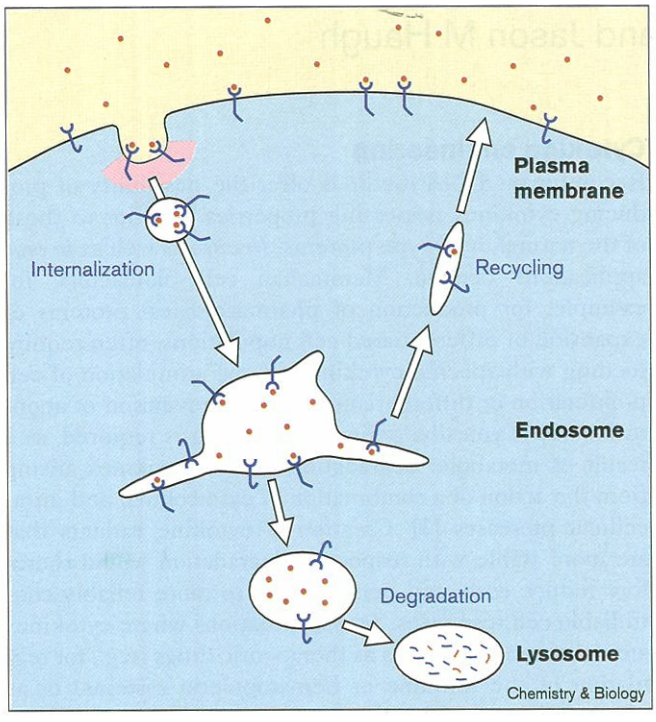
Cytokine engineering

Recombinant DNA methods offer the possibility of producing cytokines possessing properties superior to those of the natural, wild-type proteins. *In vitro* as well as *in vivo* applications beckon. Mammalian cell bioreactors, for example, for production of pharmacological proteins or expansion of differentiated cell populations, often require feeding with specific cytokines for the stimulation of cell proliferation or differentiation, or the prevention of apoptosis [1,2]. Typically, periodic re-feeding is required, as a result of metabolic degradation of the cytokines arising from the action of a combination of extracellular and intracellular proteases [3]. Creation of cytokine variants that are more stable with respect to degradation would therefore reduce costs and perhaps lead to more reliably controllable cell responses. *In vivo* situations where cytokines are delivered to patients as therapeutic drugs (e.g., for regulation of the immune or hematopoietic systems) or as effectors in wound healing or tissue regeneration are frequently limited by physiological processes that lead to the loss of the protein [4]. Some of these processes are physical, such as transport from the bloodstream into renal or hepatic compartments, whereas others arise from extracellular and intracellular proteolysis, similarly to the situations *in vitro*. The use of variants that minimize any of these loss mechanisms could widen the window of therapeutic efficacy, diminishing toxic side effects by reducing the unphysiologically high concentrations of cytokines currently needed [5–7].

One strategy for cytokine engineering is the alteration of protease recognition sites to protect against both extracellular and intracellular proteases. An alternative technique for accomplishing the same goal is the conjugation of the protein with polyethylene glycol, which shields the degradation sites from protease action [8]. This latter technique might also provide benefits against physical transport-loss mechanisms, by increasing the effective size of the protein [9].

More recently, a new potential methodology for the advantageous manipulation of protein structure has arisen, motivated by cell biology studies on endocytic receptor/ligand trafficking. Endocytic uptake of cytokine ligands generally follows binding to their target receptors, potentiating intracellular proteolytic cytokine degradation in endosomal and/or lysosomal compartments [10,11] (Figure 1). Endocytic degradation might have, in fact, more severe consequences than degradation mediated by extracellular proteases, because the internalized cytokine receptors can

Figure 1



Endocytic trafficking. The binding of cytokines to cognate receptors is often concomitant with the specific entrapment of the receptor in specialized clathrin-coated pits in the plasma membrane. This entrapment is mediated by adaptor proteins, which link endocytic motifs exposed in the cytoplasmic domain of a ligated receptor to the clathrin cage. Upon invagination the coated pit pinches off, and the resulting endocytic vesicle fuses with an early endosome, delivering receptor–ligand complexes and other components of the plasma membrane. It is here that receptors and ligands are sorted for either recycling back to the cell surface or degradation. Endosomal tubules collect molecules for return to the surface, whereas molecules that remain in the vesicular portion of the endosome are routed for degradation in lysosomes, which employ proteolytic enzymes to break down ligands and receptors [10,11].

be destroyed at the same time. This additional effect results in receptor downregulation, thereby further diminishing the efficacy of cytokines for stimulating cell responses. Thus, endocytic trafficking-mediated degradation of both ligand and receptor can substantially limit cytokine potency, even when extracellular loss mechanisms are circumvented. Significant gain, therefore, is promised by altering cytokine structure in a manner that retains proper receptor activation for signal transduction but diminishes endocytic internalization and/or enhances endosomal sorting to recycling rather than degradation.

For *in vivo* applications, the alteration of protein primary structure holds the possibility of introducing immune recognition and subsequent adverse pathological reactions. This potential difficulty, of course, is not unique to our present topic but is generic to the broad area of protein therapeutics. Because multiple sequence alterations can be explored for their effects on both trafficking dynamics and immune recognition, it is anticipated that this problem can be overcome.

Cytokine/receptor trafficking and cell biological response

For two well-known proteins, epidermal growth factor (EGF) and interleukin-2 (IL-2), some recent studies have generated findings that are, perhaps, counterintuitive in the field of cytokine biochemistry: at least some cell biological responses can be heavily influenced by cytokine/receptor trafficking properties, to the extent that they are not simply predictable from cytokine–receptor binding affinity at the cell surface. An understanding of how receptor-binding properties of a cytokine affect trafficking dynamics, and, in turn, how trafficking dynamics impact cell biological responses, is required to enable the particular cytokine to be optimally re-engineered. Cytokine variants, generated by rational or random approaches, can then be screened using the most relevant criteria.

Epidermal growth factor

EGF is a 53 amino-acid peptide cytokine capable of stimulating a spectrum of responses, including proliferation and migration, in a variety of connective tissue cell types. Roles for EGF, a cytokine discovered more than 30 years ago, have been identified in tissue organization during development and wound healing. EGF is believed to bind with 1:1 stoichiometry to the EGF receptor (EGFR), a member of the erbB receptor family. Ligand binding concomitantly stimulates receptor tyrosine kinase activity, homo- and hetero-dimerization, and endocytic internalizaton, resulting in multiple signaling pathways regulating the cell behavioral response [12–14].

Tyr13 (Y13) is a residue in human EGF near its binding site for the EGF receptor (EGFR) that has a hydrophobic character shown to be important for high-affinity binding [15]. When this residue is mutated to less hydrophobic amino acids, the K_D value of EGF–EGFR binding increases compared to that of wild-type EGF. This reduction in affinity is ~50-fold at pH 7.4 for the Y13G variant in which Tyr13 is substituted by glycine (Table 1). Surprisingly, in an assay for proliferation of a fibroblast cell line in culture, Y13G was found to be more potent for increasing cell number over three days than was the wild type at all concentrations tested [16]. The underlying explanation appeared to be that ligand depletion from the culture medium as well as receptor downregulation from the cell surface were less dramatic for the Y13G mutant than for the wild type. Cell proliferation assays conducted under conditions minimizing ligand depletion showed that Y13G was, as expected, less potent than the wild type [16].

Dedicated trafficking experiments have found that, when Y13G is internalized as part of a complex with EGFR, the

Table 1

Receptor-binding affinities and off rates for human EGF and related ligands.

	K_D (nM) pH 7.4	K_D (nM) pH 6.0	k_{off} (min^{-1}) pH 7.4	k_{off} (min^{-1}) pH 6.0
EGF	2.5	78	0.16	0.66
TGF-α	6.3	400	0.27	~2
Y13G	130	160	1.2	1.4

K_D refers here to the equilibrium dissociation constant (reciprocal to affinity) describing the interaction of each ligand with human EGFR at equilibrium and k_{off} is the dissociation rate constant of the interaction. pH 7.4 is representative of cell surface conditions, whereas pH 6.0 is representative of the endosomal lumen. Data adapted from [17].

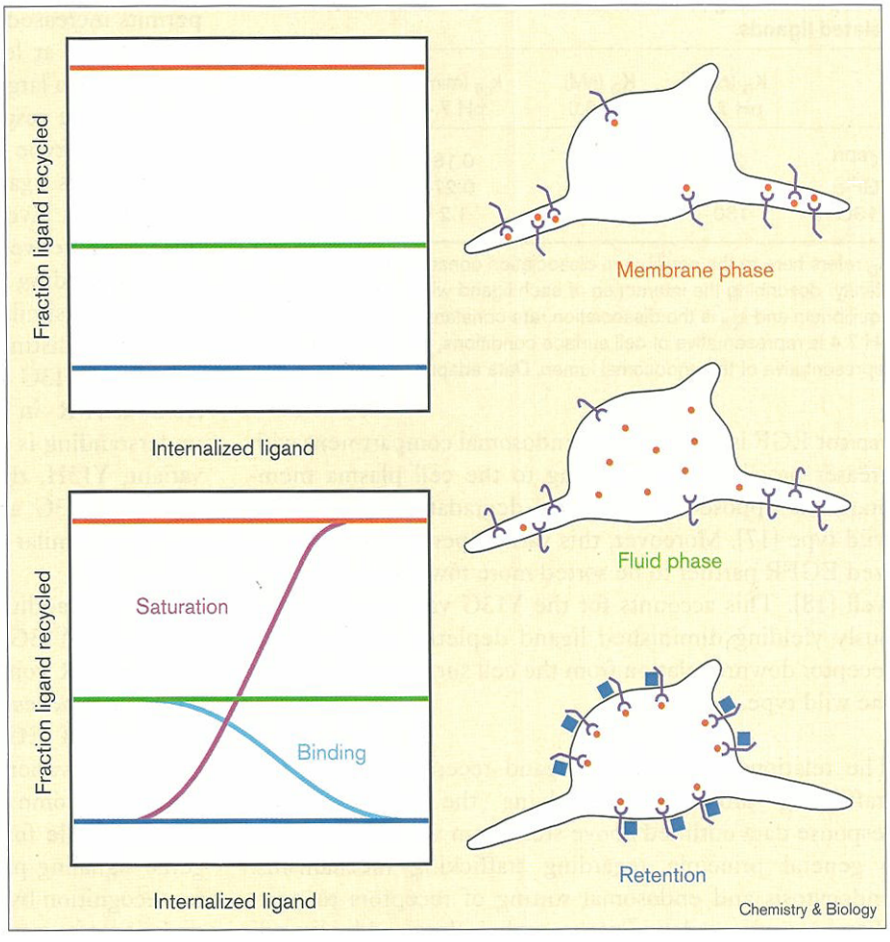
variant EGF is sorted in the endosomal compartment with greater proclivity for recycling to the cell plasma membrane (as opposed to lysosomal degradation) than is the wild type [17]. Moreover, this variant permits its internalized EGFR partner to be sorted more towards recycling as well [18]. This accounts for the Y13G variant simultaneously yielding diminished ligand depletion and reduced receptor downregulation from the cell surface compared to the wild type.

The relationships between ligand–receptor binding and trafficking properties underlying the cell biological response data outlined above stem from what seems to be a general principle regarding trafficking mechanisms: endocytosis and endosomal sorting of receptors to recycling versus degradation are strongly influenced by ligand-dependent interactions of the receptor with cytoplasmic regulatory molecules. The cell's ability to downregulate its ligated receptors is 'saturable' in cell lines overexpressing EGFR, presumably because the accessory proteins that regulate the underlying trafficking processes become stoichiometrically limiting [12,19–21]. For the Y13G EGF variant, the finding of enhanced endosomal sorting towards recycling rather than lysosomal degradation needs to be accounted for in terms of the altered receptor-binding properties of the variant compared to the wild type. Targeting of the ligand and receptor to lysosomes for degradation requires a ligand occupancy-induced EGFR recognition signal for interaction with an endosomal retention element, sorting nexin 1 (SNX-1) [22]. Dissociation of EGF from EGFR in the endosomal compartment relieves this interaction, permitting receptor and ligand to escape this specific targeting mechanism (Figure 2). Thus, a key binding property here should be the dissociation rate constant at endosomal pH (~6); this has been measured to be at least twofold faster for Y13G than for wild-type EGF [17]. The explanation cannot be entirely this simple, however. A natural member of the EGF family, transforming growth factor alpha (TGF-α), has a fast dissociation rate constant at pH 6, similar to that of Y13G EGF, and internalized EGFR is occupied to a lesser extent by TGF-α than by wild-type EGF [23]. But, although binding parameters at endosomal pH suggest that TGF-α permits increased EGFR recycling in a similar manner to Y13G EGF, at least in some cell types TGF-α itself is degraded to a larger degree than wild-type EGF and Y13G [17,24]. As a result, the comparative potency of TGF-α versus wild-type EGF for cell proliferation depends on whether it is ligand or receptor that is stoichiometrically limiting in a given experimental condition [24]. A major difference between TGF-α and Y13G EGF is that the receptor-binding affinity of TGF-α at cell surface pH is much more similar to that of wild-type EGF [17,23]. So, the crucial distinction could reside in the lower binding affinity of Y13G at cell surface pH, allowing dissociation from EGFR in nonacidified endocytic vesicles. This understanding is consistent with studies on another EGF variant, Y13H, that has binding properties intermediate between Y13G and TGF-α and elicits cell-proliferation responses similar to wild-type EGF [25].

Some data additionally suggest that the lower binding affinity of Y13G at the cell surface also reduces the EGF–EGFR complex internalization rate constant [18]. This is not necessarily surprising because endocytic internalization of EGFR is enhanced by ligand occupancy through activation of a recognition signal for EGFR interaction with some component of clathrin-coated pits [26]. If the time-scale for an occupied EGFR to stimulate mitogenic signaling pathways, for instance, is faster than that for recognition by the endocytic machinery, the faster dissociation rate constant of Y13G from the receptor could generate more predominant mitogenic signals. Possibly consistent with this concept is the recent identification of another EGF variant, E4T (actually, a chimera of EGF and TGF-α), that has a similar equilibrium receptor-binding affinity at cell surface pH but a faster dissociation rate constant. E4T depletion from the culture medium was found to take place more slowly than for wild-type EGF or TGF-α, and the underlying change in trafficking dynamics was concluded to be a reduced internalization rate constant [27].

Yet another hypothesis could be that quantitative characteristics of EGFR-mediated signal transduction are influenced by ligand/receptor trafficking. Although ligated EGFRs are sorted in endosomes to affect the long-term levels of receptor and extracellular ligand, it is unclear to what extent these internal complexes should be included with complexes at the cell surface as signaling-competent. Recent work indicates that internalized cytokine–receptor complexes can alter comparative levels of signaling pathway intermediates, and thus, possibly, cell biological responses ([28] and J.M.H., A. Wells, H.S. Wiley and D.A.L., unpublished observations). Another intriguing possibility is that the specificity of signaling can be altered in the endosomal environment, where EGFR might have

Figure 2



Mechanistic analysis of endosomal sorting. Both the geometry of endosomes and specific interactions with endosomal proteins are believed to have a strong influence on the sorting of ligands and receptors. Lipids and other 'membrane-phase' components are predominantly routed into recycling tubules, whereas soluble 'fluid-phase' components in the endosomal lumen are predominantly retained in the vesicular portion of an endosome and routed for degradation. For example, transferrin remains tightly complexed with its receptor in endosomes, and this ligand is constitutively recycled along with its receptor. In contrast, low-density lipoprotein dissociates from its receptor at endosomal pH and this ligand is degraded, whereas its receptor is free to return to the cell surface [11]. The relative fluxes of ligand to recycling and degradative fates can be assessed experimentally by measuring the ligand recycling fraction (the fraction of radiolabeled ligand exocytosed by the cell that is intact) under steady-state conditions. Nondissociative cytokines can achieve recycling fractions even lower than fluid- or membrane-phase ligands if the ligated receptor is recognized by accessory proteins in the vesicular portion of an endosome, yielding endosomal retention (top graph). In this case, the receptor is also routed for degradation, yielding downregulation of total receptor number. For example, EGF is retained in endosomes in an occupancy-dependent manner [20,21], and this process has been linked to the interaction of ligated EGFR with an accessory sorting nexin [22]. Intermediate behaviors have been observed for EGF and TGF-α in B82 fibroblasts; when the ligand recycling fraction is plotted against the level of total intracellular ligand, the TGF-α curve exhibits a slightly negative slope, whereas the EGF curve clearly exhibits a positive slope. Counterintuitively, TGF-α exhibits a higher recycling fraction at low levels of internal ligand, whereas EGF exhibits a higher recycling fraction at high levels of internal ligand [17]. The current model adequately explains these subtle aspects of sorting: as the level of internalized ligand increases, a dissociative ligand such as TGF-α will bind some receptors (leading to retention of ligand and receptor), and a nondissociative ligand such as EGF will occupy enough receptors to saturate the limited number of accessory retention proteins (bottom graph) [30].

access to distinct membrane-associated substrates. Such effects could involve EGFR interactions with other members of the erbB receptor family, which are indicated to occur to different extents in various cell compartments [29]. This issue deserves very careful examination using rigorous quantitative methodologies.

The data outlined above suggest that an EGF variant re-engineered for more potent cell-proliferation responses could be generated by requiring that binding to EGFR has sufficiently high dissociation rate constants, at both cell surface and endosomal pH, to yield increased recycling of both ligand and receptor. At the same time, cell-surface binding should be of sufficiently high affinity that effective levels of cytokine–receptor signaling complexes are formed at practical cytokine concentrations. Thus, using either a rational design or directed evolution approach, an optimal combination of at least two EGFR-binding affinities — or, more precisely, dissociation and association rate constants — at the different trafficking-related pH values, is what should be sought. Although this combination is certainly more complicated than merely seeking the highest possible receptor-binding affinity at cell surface conditions, it might be more likely to lead to useful variants. A mathematical model for 'cell-level pharmacokinetics', permitting prediction of how receptor-binding properties influence the spectrum of trafficking and signaling processes that govern the desired biological response [30–32], will probably be a valuable tool for indicating optimal cytokine characteristics.

It should be noted that for some *in vivo* applications, such as wound healing, cell migration might be at least as important a biological response as proliferation. Almost certainly

the trafficking dynamics — hence receptor-binding properties — yielding maximal migration responses will not be the same as those yielding maximal proliferation responses, because the signaling pathways underlying these responses are divergent [33,34]. Indeed, this situation would offer the potential for designing alternative variants of a single cytokine that are more effective for specific applications.

Interleukin-2

IL-2 is a 133 amino-acid protein whose four-α-helix bundle structure is related to those of leptin, erythropoietin, granulocyte–colony-stimulating factor (G-CSF) and human growth hormone, among others [35,36]. IL-2 was originally identified as T-cell growth factor (TCGF), a soluble mediator of activated T-cell proliferation, and its discovery led to a breakthrough in understanding the mechanism of T-cell-based immunity [37,38]. IL-2 also induces the proliferation of, and the release of, cytokines by natural killer (NK) cells [39,40]. The effects of IL-2 on activated B cells include differentiation, proliferation and stimulation of IgG and IgM release [41,42].

IL-2 presents a more complicated situation than that of EGF, because of the heterotrimeric nature of the IL-2 receptor (IL-2R). IL-2 binds with different affinities to various permutations of IL-2R subunit (α, β and γ) combinations: with K_D values of ~10 pM for αβγ, 10 nM for α, and 1 nM for βγ [43]. The pH dependence of these values has not been reported. The β and γ subunits elicit both activation of signal-transduction pathways, including those leading to cell proliferation, and endocytic internalization [44–48]; IL-2 bound to the α subunit is internalized with a low constitutive rate constant [49,50], whereas that bound to βγ or αβγ is internalized with a higher rate constant [51–53]. The IL-2R subunits similarly appear to be sorted differently in the endosomal compartment; the α subunit largely recycled, whereas the βγ subunit is predominantly targeted for lysosomal degradation [54,55]. Hence, trafficking dynamics of the ligand should depend heavily on the ligand's relative binding properties among the receptor subunits.

As with EGF, an IL-2 variant has been generated that is more potent for lymphocyte proliferation than is wild-type IL-2. Termed 2D1, it is a double substitution in which leucine residues at positions 18 and 19 are replaced by methionine and serine, respectively [56]. 2D1 has an overall binding affinity for the full αβγ IL-2R roughly equal to that of the wild-type ligand (Table 2). For the α subunit, the affinity of the 2D1 variant is approximately twofold greater than that of wild type, whereas for the βγ subunit the affinity of the variant is approximately twofold less. Cell numbers have been found to increase more rapidly under stimulation by the 2D1 variant than by wild-type IL-2 ([56] and E.M.F., K. Lee, T.L. Ciardelli and D.A.L., unpublished observations). The explanation appears to come from reduced depletion of 2D1 ligand from the culture medium compared to wild-type ligand, mediated by IL-2R endocytic trafficking processes. The underlying mechanism has been found to be an increase in fractional endosomal sorting to recycling for 2D1–IL-2R complexes relative to wild-type–IL-2R complexes (E.M.F., K. Lee, T.L. Ciardelli and D.A.L., unpublished observations).

Table 2

Receptor-binding affinities for interleukin-2 and variants.

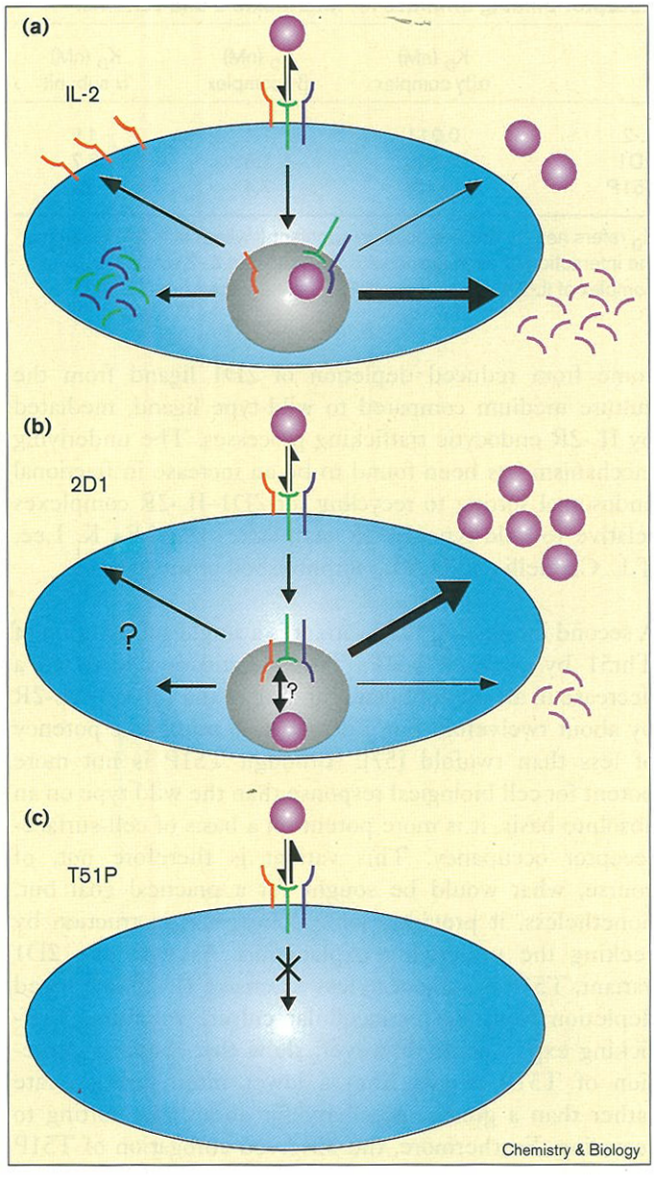
	K_D (nM) αβγ complex	K_D (nM) βγ complex	K_D (nM) α subunit
IL-2	0.011	1.1	11
2D1	0.008	1.8	6.7
T51P	0.13	7.4	35

K_D refers here to the dissociation constant (inverse affinity) describing the interaction of each ligand with the indicated IL-2 subunit or complex of IL-2R subunits at pH 7.4. Data adapted from [56,57].

A second interesting IL-2 variant is a single substitution of Thr51 by proline, T51P. This substitution leads to a decrease in affinity of ligand binding to the full αβγ IL-2R by about twelvefold but a decrease in mitogenic potency of less than twofold [57]. Although T51P is not more potent for cell biological response than the wild type on an absolute basis, it is more potent on a basis of cell-surface-receptor occupancy. This variant is therefore not, of course, what would be sought for a practical goal but, nonetheless, it provides some conceptual instruction by seeking the underlying explanation. As with the 2D1 variant, T51P is subject to less extensive IL-2R-mediated depletion from the extracellular culture medium. Trafficking experiments, however, show that reduced depletion of T51P results from a lower internalization rate rather than a greater proclivity for endosomal sorting to recycling. Furthermore, the observed abrogation of T51P endocytosis cannot be attributed to reduced ligand occupancy of receptors, suggesting a more complicated effect of the mutation on interactions with IL-2R [58].

For IL-2, then, two variants have been identified that possess unique endocytic trafficking properties relative to the wild type (Figure 3), and, in both cases, biological potency does not correlate with cell-surface-binding affinity. Further re-engineering of IL-2 for enhanced efficacy could be realized by optimizing an appropriate combination of binding affinities for the α subunit and βγ complex at both the cell surface pH and endosomal pH. An ideal variant would induce normal activation of IL-2R-mediated signaling pathways while reducing the internalization rate constant and/or increasing endosomal sorting to recycling. Here, a mathematical model for the full set of processes involved in cell-level pharmacokinetics will probably be helpful in identifying an optimal four-way matrix assay for

Figure 3



Trafficking fates of IL-2 and IL-2 variants. **(a)** Wild-type IL-2 binds the high-affinity receptor, consisting of IL-2Rα (red), β (green), and γ (blue), and the entire complex undergoes endocytosis. Upon intracellular sorting, IL-2Rα is recycled to the cell surface, whereas the β and γ subunits are routed to lysosomes for degradation along with IL-2 [51,55]. **(b)** The 2D1 mutein binds the IL-2R trimeric complex with wild-type affinity at the cell surface, and undergoes receptor-mediated endocytosis at the same rate as IL-2 ([56] and E.M.F., K. Lee, T.L. Ciardelli and D.A.L., unpublished observations). Altered sorting of 2D1/IL-2R provides an increase in ligand recycling of approximately twofold when compared to IL-2 (E.M.F., K. Lee, T.L. Ciardelli and D.A.L., unpublished observations). The intracellular mechanism of this enhanced recycling, including the relative binding affinities of 2D1 to the various IL-2R subunits at endosomal pH, is unknown. **(c)** The binding affinity of T51P to the IL-2 receptor is eightfold lower than that of the wild type, and internalization of T51P/IL-2R can not be detected [57,58].

properties of binding to the two receptor subunit classes at the two different pH values.

Conclusions and prospects

What we believe the two examples of EGF and IL-2 suggest is that cytokine variants most potent for cell biological responses will, in general, not be found — whether through rational design or directed evolution methodologies — by using a biochemical criterion of merely maximizing receptor-binding affinity under cell-surface conditions. Instead, what could more productively be used as biochemical screening criteria are combinations of receptor-binding properties that lead to improved endocytic trafficking dynamics. By improved trafficking dynamics we operationally mean those that result in ligand depletion, receptor downregulation and ligand–receptor compartmentation properties that give the optimal cell response. Mathematical models incorporating all the various binding, trafficking and signaling processes will be helpful in identifying the most effective binding parameters. Regardless of specific idiosyncrasies of particular cytokine/cell systems, we expect that a matrix criterion can be identified, depending on the number of receptor subunits involved and the number of relevant trafficking compartment conditions.

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