Analysis of Trafficking Dynamics and Cellular Response in the Interleukin-2 Ligand/Receptor System

by

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ABSTRACT

Interleukin-2 (IL-2) has been widely studied as a protein therapeutic due primarily to its central role in potentiating cell-based immunity. The effects of IL-2 on its cellular targets are mediated solely through interaction with one or more forms of the IL-2 receptor (IL-2R). The overall goal of this thesis is to quantitatively characterize IL-2/IL-2R interactions toward the scientific end of advancing the understanding of the effects of molecular perturbations on cell function, and the technological end of providing insight into the design of improved IL-2 therapies.

An interleukin-2 (IL-2) variant displays binding affinity to the heterotrimeric IL-2 receptor similar to that of wild-type (WT) IL-2, and was previously found to exhibit increased bioactivity in a T cell proliferation assay. Dedicated trafficking studies show that endocytic trafficking of this 2D1 variant might be responsible for this increased potency, as a significantly increased fraction of internalized 2D1 is sorted to recycling instead of to lysosomal degradation. Denaturation experiments indicate that wild-type IL-2 and the 2D1 analog have similar stabilities at neutral pH; however, the analog displays decreased stability of the native and intermediate states under endosomal sorting conditions when compared to wild-type IL-2. This behavior correlates with differential pH-sensitivities of receptor binding affinity measured for IL-2 and the 2D1 variant. Improved sorting leads to an extended half-life of intact 2D1, providing enhanced mitogenesis as compared to IL-2. We further conclude that this IL-2 variant minimizes undesired stimulation of NK cells.

We describe a mathematical model that relates ligand-receptor trafficking properties to T cell proliferation in response to interleukin-2 (IL-2). The steady-state sorting behavior of the 2D1 variant is predictable from the model, as are criteria for designing IL-2 variants with further improvements in bioactivity. The concept of altering trafficking dynamics may offer a generalizable approach to generating improvements in the pharmacological efficacy of therapeutic cytokines.

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Chapter 1: Introduction and Background

Mammalian cell functions are tightly regulated by extracellular cues. The primary means of communication of these cells with their environment is via their membrane receptors. These receptors perform a variety of functions including nutrient uptake, clearance of non-nutrient molecules from the cellular environment, and regulation of cell migration through recognition of extracellular matrix proteins. A large class of regulatory proteins called growth factors modulates cellular functions such as proliferation, differentiation, and chemotaxis by interacting with cognate membrane receptors. An essential growth factor in potentiating cell-mediated immunity is interleukin-2 (IL-2).

Interleukin-2 (IL-2) has been widely studied as a protein therapeutic, with demonstrated applications in the treatment of AIDS and several forms of cancer. IL-2 acts upon both CD4$^+$ and CD8$^+$ T cells, as well as on natural killer (NK) cells, among others. Its role as the growth factor for T lymphocyte proliferation is a key step in cell-based immunity, and IL-2’s effect on its cellular targets is mediated solely through interaction with one or more forms of the IL-2 receptor (IL-2R). The overall goal of this thesis is to quantitatively characterize IL-2/IL-2R interactions toward the scientific end of advancing the understanding of the effects of molecular perturbations on cell function, and the technological end of providing insight into the design of improved IL-2 therapies.

1.1 Interleukin-2

Interleukin-2 (IL-2) was originally identified as T-cell growth factor (TCGF), a soluble mediator of activated T-cell proliferation (Gillis et al., 1978). An IL-2 dependent mouse cytotoxic T-cell line (CTLL) was established. This provided accurate
measurement of IL-2 concentration based on the dose-dependent proliferation of CTLL. This CTLL bioassay is still of great utility in defining effective concentrations of IL-2 and its variants. IL-2 was isolated from a leukemic T-cell line (JURKAT) and later shown to be a variably glycosylated protein of molecular weight 15 kD (Robb and Smith, 1981). IL-2 was cloned and later produced recombinantly in E. coli with no loss of activity vs. the native form (Taniguchi et al., 1983; Kato et al., 1985; Rosenberg et al., 1984; Williams et al., 1988). Though monomeric, IL-2 was shown to self-associate with a $K_d$ of 0.6 μM, a physiologically irrelevant concentration (Fleischmann et al., 1988).

The original crystal structure of IL-2 was later revised and shown to be similar to that of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, two other cytokines (Bazan, 1992; Brandhuber et al., 1987; Brandhuber et al., 1987). The four α-helix structure is shown in Figure 1.1. IL-2 contains one essential disulfide bond between Cys58 and Cys105 (Ju et al., 1987; Arakawa et al., 1986).

The discovery of IL-2 led to a breakthrough in understanding the mechanism of T-cell based immunity (Smith, 1988). T cells are activated in vivo by antigen presenting cells (APC) via the MHC-TCR interaction and costimulatory signals, such as B7 binding to CD28. This activation leads to autocrine secretion of IL-2, as well as expression of IL-2R. Subsequent IL-2 interaction with its high affinity receptor then initiates “progression” by mediating T-cell proliferation, wherein the progeny are activated as well (Abbas et al., 1994; Herzberg and Smith, 1987). Thus, IL-2 induces in vivo proliferation only of activated T-cells, and is illustrative of the essential role of cytokines as mediators of immune response (Gaulton and Williamson, 1994).
The effects of IL-2 are pleiotropic. It induces the proliferation of, and the release of cytokines by natural killer (NK) cells (Henny et al., 1981; Lee and Ciardelli, 1992). Interestingly, NK cells respond to IL-2 despite the fact that the majority of these cells express only an intermediate form of the receptor, implying that this receptor is competent for signaling (Caligiuri et al., 1990). Effects of IL-2 on activated B cells include differentiation, proliferation, and stimulation of IgG and IgM release (Jung et al., 1984; Waldmann et al., 1984). IL-2 has also been shown to mediate growth signals for some populations of nerve cells (Benvenisto and Merrill, 1986; Hanisch and Quirion, 1996).

1.2 The Interleukin-2 Receptor

The IL-2 receptor (IL-2R) contains three distinct, single-transmembrane spanning subunits designated α (55 kD), β (75 kD), and γ (65 kD). The α chain, originally called Tac, was first identified through the use of anti-Tac antibodies that specifically blocked IL-2 binding and proliferation of a human T cell line (Uchiyama et al., 1981; Uchiyama et al., 1981; Leonard et al., 1983; Leonard et al., 1982; Robb and Greene, 1983). At the same time, a direct quantitative correlation between biological response (proliferation) of CTLLs and receptor occupancy was demonstrated (Robb, 1982; Robb et al., 1981). The alpha chain was cloned and later shown to be insufficient in mediating a growth response (Cosman et al., 1984; Nikaido et al., 1984; Greene and Leonard, 1986; Wang and Smith, 1987). This was not surprising since the cytoplasmic domain of the α subunit possesses only 13 residues (Nikaido, et al., 1984). The extracellular domain of the α chain possesses ten structurally essential Cys residues, and exhibits no homology to other known receptors (Rusk et al., 1988).
Upon cloning of the alpha chain, experiments showed that higher affinity binding sites existed on activated T cell lines that were distinct from those on cells transfected with α cDNA (Hatakeyama et al., 1985; Robb et al., 1984; Sharon et al., 1986). Thus, a distinct receptor with higher IL-2 affinity and growth signaling capability was identified as IL-2Rβ (Smith and Cantrell, 1985; Teshigawara et al., 1987; Bich-Thuy et al., 1987). The β chain was found to form heterodimers with α (Minamoto et al., 1990). It possesses a large cytoplasmic domain of 286 residues and exhibits homology with several other receptors of the hematopoietic receptor superfamily (Bazan, 1990; Cosman et al., 1990; Nicola and Metcalf, 1991). The β subunit is shared by the recently identified IL-15 ligand (Carson et al., 1994; Grabstein et al., 1994).

The existence of the γ chain was finally postulated from binding and co-immunoprecipitation studies (Takeshita et al., 1992; Takeshita et al., 1990). The gene encoding γ was cloned (Takeshita et al., 1992), and it was shown to possess 86 cytoplasmic residues. The γ chain has also been called the γc receptor (γ-common), as IL-4, IL-7, IL-9, and IL-15 functionally bind to the native form (Kondo et al., 1993; Russell et al., 1993; Kondo et al., 1994; Noguchi et al., 1993; Kimura et al., 1995; Giri et al., 1994). Mutations in the γ gene were found in patients with X-linked severe combined immunodeficiency syndrome (X-SCID) (Noguchi et al., 1993; Russell et al., 1994). This implied key roles for the γ chain in the IL-2 mediated immune response (DiSanto et al., 1994; DiSanto et al., 1994).

Fully activated peripheral blood lymphocytes (PBL) express IL-2 and IL-2Rα, β, and γ, though expression levels may not be temporally or quantitatively coordinated (Cantrell et al., 1988). Induction of IL-2R subunit expression occurs only upon in vivo
activation or through exogenous antigen presentation in culture, though resting T cells
have been shown to express low levels of γ (Nakarai et al., 1994). Subpopulations of NK
cells have been shown to constitutively express β (Caligiuri, et al., 1990). IL-2 itself can
regulate the level of expression of various subunits upon binding (Bismuth et al., 1985;
Duprez et al., 1988; Smith and Cantrell, 1985; Waldmann, 1989). Other factors,
including interferon-γ (IFN-γ) and cytotoxic T-cells can also influence IL-2R expression
levels on some lymphocytes (Kaempfer, 1994; Rodriguez et al., 1990).

1.3 IL-2/IL-2R Subunit Interactions

As shown in Table 1.1, each IL-2R subunit combination displays a distinct IL-2
binding affinity. This has allowed dissection of the site specificity of receptor-ligand
interactions through binding assays with IL-2 mutants (Cohen et al., 1986; Liang et al.,
1986). Fortunately, the structural integrity of IL-2 has been quite resilient to site-directed
mutation, and many partially active mutants have been successfully expressed (Berndt et
al., 1994; Collins et al., 1988; Ernst and Richman, 1989; Weir et al., 1988). Studies have
shown that the N-terminal helix, specifically Asp20, interacts with the β subunit (Collins,
et al., 1988). Sites 38-43, especially Thr41 and Phe42 in a trans-helix loop interact with
the α chain (Kuziel et al., 1993; Grant et al., 1992; Suave et al., 1991). The C-terminal
helix, specifically Gln126, is requisite in IL-2/IL-γ interaction (Buchli and Ciardelli,
1993; Kuziel, et al., 1993). Glu62 affects α interaction directly, but also perturbs binding
affinity to the αβγ complex (Wang et al., 1995). Thus, IL-2 interacts directly with each
subunit in the high affinity (αβγ) complex.

These results, and the data in Table 1.1, implicate the modular yet cooperative
interactions among α, β, and γ in IL-2 binding. Interactions among the various subunits
have been identified, independent of those with IL-2 (Gutgsell and Malek, 1994; Roessler et al., 1994; Voss et al., 1992). Since activated T-cells express $10^3$-$10^4$-fold more α receptors than β or γ, it was originally thought that IL-2 binding was sequential (Lowenthal and Greene, 1987). The “affinity conversion” model stated that IL-2 first bound to α with a fast on-rate, then IL-2•α bound to β with IL-2 acting as the converter of α to the higher affinity αβ complex (Kamio et al., 1990; Saito et al., 1988). The β subunit contributes to this higher αβ affinity with a slow off-rate (Goldstein et al., 1992). Later work established the “preformed heterodimer” model, wherein αβ exists without IL-2 and can bind IL-2 directly (Saragori and Malek, 1988; Grant, et al., 1992; Landgraf et al., 1992). Theoretical analysis of previous binding-study data validated this model (Goldstein, et al., 1992). The excess α receptors likely maintain a role as ligand carriers, promoting the formation of functional, high-affinity (αβγ) complexes on autocrine T-cells (Forsten and Lauffenburger, 1994; Smith, 1989).

The role of γ in forming high-affinity complexes is less clear, though it likely contributes by slowing the off-rate in a similar fashion to β via avidity effects (Lauffenburger and Linderman, 1993; Matsuoka et al., 1993). Binding to γ alone has not been observed on cells, thus a conformational change during complex formation or avidity effect likely accounts for the observed IL-2•γ interaction in high-affinity complexes (Voss et al., 1993; Nakarai, et al., 1994). Direct interaction of β and γ in the absence of IL-2 has not been observed (Kuziel, et al., 1993; Voss, et al., 1992).

Functional homodimerization has not been observed for any of the three IL-2R subunits.
1.4 Signaling Through the IL-2 Receptor

The complete signaling events leading to IL-2 induced T-cell proliferation are as yet unclear. Much is known, however, about the contribution of each subunit to functional signaling, and several downstream mediators have been identified (Gomez et al., 1996; Flores et al., 1996; Beadling et al., 1994; Cano et al., 1992). This work has also illustrated the importance of using lymphocyte systems in addressing IL-2/IL-2R binding and signaling, as anomalous results in fibroblastoid transfectants have been observed (Mills et al., 1991; Minami et al., 1994; Minamoto, et al., 1990).

A diagram of the IL-2R in Figure 1.2 shows the interaction with various signaling molecules. The α chain does not contribute to signaling, though co-expression of α increases the “sensitivity” of cells to IL-2, likely through binding events (Morella et al., 1995; Bich-Thuy, et al., 1987; Wang and Smith, 1987). The cytoplasmic domains of β and γ have been shown to mediate at least three and two distinct signaling pathways, respectively (Nakamura et al., 1994; Nelson et al., 1994; Asao et al., 1993; Miyazaki et al., 1995). Neither subunit possesses intrinsic tyrosine kinase activity, yet activation of intracellular PTKs is dependent on the tyrosine-phosphorylation state of the subunits (Shackelford and Trowbridge, 1991; Asao et al., 1990; Fung et al., 1991; Goldsmith et al., 1995; Mills et al., 1990). Subunit phosphorylation states are influenced by JAK1, p56\(^{lck}\), and syc, which interact with β, and JAK3 that couples to γ (Friedman et al., 1996; Einspahr et al., 1992; Hatakeyama et al., 1991; Minami et al., 1993; Minami and Taniguchi, 1995; Johnston et al., 1994; Nelson et al., 1996).

Increased expression of the proto-oncogenes c-fos/c-jun and c-myc has been localized to two distinct regions of the β subunit (Hatakeyama et al., 1992; Asao et al., 1994; Shibuya et al., 1992; Goldsmith et al., 1994; Satoh et al., 1992). IL-2 induced
expression of \( bcl-2 \), an anti-apoptosis gene, via the \( \beta \) subunit has recently been observed (Mor and Cohen, 1996). A conserved WSXWS motif in the extracellular region of \( \beta \) is crucial in both ligand binding and proliferation, by analogy with other hematopoietic superfamily receptors (Miyazaki \textit{et al.}, 1991).

Though this thesis does not address signaling mechanisms \textit{per se}, an understanding of the functional distinctions between proliferative response and receptor-mediated ligand processing is essential. For instance, signaling-impaired mutants of \( \gamma \) can still contribute to receptor-mediated internalization, indicating that \( \gamma \) is essential for both phenomena (Asao \textit{et al.}, 1993; Mills, \textit{et al.}, 1994). Cell lines expressing \( \beta \) mutants unable to interact with p56\(^{lck}\) could still respond to IL-2, invoking an active signaling pathway distinct from that associated with p56\(^{lck}\) (Howard \textit{et al.}, 1995; Liu \textit{et al.}, 1995; Taniguchi \textit{et al.}, 1995). A \( \beta \) truncation mutant possessing only its 27 membrane proximal residues could internalize IL-2 at a wild-type rate, though it was signaling incompetent (Hatekeyama \textit{et al.}, 1989). Thus, the modularity of the IL-2R system is relevant not only in IL-2 binding, but also in intra-subunit signaling and trafficking events (Imler, 1992; Kumar \textit{et al.}, 1987; Ortaldo \textit{et al.}, 1991). Mutant ligands might therefore specifically affect one of these phenomena (Chang \textit{et al.}, 1996; Weigel \textit{et al.}, 1989).

1.5 IL-2 Receptor-Mediated Trafficking

In contrast to signaling, which has only recently been approached from an engineering perspective, binding and trafficking events have lent themselves well to quantitation (Haugh and Lauffenburger, 1998; Lauffenburger \textit{et al.}, 1996; Lauffenburger and Linderman, 1993). Whole-cell models of IL-2 effects on T-cell growth implicate
three essential factors: IL-2 concentration, IL-2R surface density, and the duration of the ligand-receptor interaction (Cantrell and Smith, 1984). Any abrogation of one of these three parameters will diminish T-cell responsiveness (Smith, 1995). Clearly, IL-2/IL-2R internalization and down-regulation impacts all three. IL-2 “consumption” by T-cells led to its identification, and receptor-mediated internalization and degradation by T-cells have since been quantified (Gillis, et al., 1978; Fujii et al., 1986; Gullberg, 1987; Robb and Greene, 1987). T-cell lines degrade exogenous IL-2 with a $t_{1/2}$ of 60-80 minutes following addition of saturating amounts of ligand. Degradation of IL-2 by autocrine T cells can reduce the amount of active ligand released into the medium 20-fold vs. when ligand-binding is blocked with anti-receptor antibodies (Duprez et al., 1985; Claret et al., 1992).

The role of each IL-2R subunit in mediating IL-2 internalization has been addressed using $^{125}$I-IL-2 and IL-2•toxin chimeras in various T-cell lines (Furse and Malek, 1993; Lorberboum-Galski et al., 1988; Re et al., 1996). The α subunit alone cannot induce internalization, but the αβ complex and αγ form can both internalize (Tanaka et al., 1988; Weissman et al., 1986; Fung et al., 1988; Plaisance et al., 1993; Tsudo et al., 1990; Lorberboum-Galski, et al., 1988). The β chain has been shown to internalize in the absence of ligand with a $t_{1/2}$ of roughly 120 minutes (Hemar et al., 1994). It is not known whether IL-2 can mediate internalization of β or γ alone, though the IL-2•βγ complex is endocytosed with a $t_{1/2}$ of 10-15 minutes (Robb and Greene, 1987). This rate is similar to that seen with the IL-2•αβγ holocomplex (Chang, et al., 1996; Gullberg, 1987; Hemar et al., 1995). Though a correlation has been shown
between internalization and signaling of the high affinity complex, a causative relationship has not been proven (Duprez et al., 1991).

The intracellular trafficking events following IL-2 binding are illustrated in Figure 1.3. Within ten minutes of internalization through clathrin-coated or uncoated pits IL-2 remains bound to α, β, and γ in early endosomes (Peters and Norback, 1990; Subtil et al., 1994; Duprez et al., 1992; Ferrer et al., 1993). The ligand itself has been localized to lysosomes by this time, and in late lysosomes after 30 minutes (Duprez et al., 1994; Lowenthal et al., 1986). After 100 minutes, α appears to recycle to the cell surface while IL-2 and βγ are sorted to the degradative pathway (Hemar, et al., 1995). The basis of this sorting is unknown, though it is known that IL-2 dissociates from its receptor in the acidic endosome (Ciardelli and Smith, 1989; Hemar, et al., 1995; Tyco and Maxfield, 1982). It has been proposed that an endosomal retention signal is present on one or more of the receptor subunits, which may promote interaction with a sorting determinant as seen in the EGFR system (French and Lauffenburger, 1996; Kurten et al., 1996).

The end result of IL-2/IL-2R trafficking is degradation of the ligand and the β & γ subunits, as well as down-regulation of surface high-affinity receptors (Duprez, et al., 1988; Lee and Mookerjee, 1989: Morelon et al., 1995). In the absence of receptor synthesis, the half-life of β and γ appears to be only one hour, while that of α is 48 hours (Duprez and Dautry-Varsat, 1986; Hemar, et al., 1995). The level of receptor down-regulation is dependent on receptor occupancy, and degradation of IL-2 abrogates this effect (Duprez, et al., 1988). Similar effects have been shown in the TGFα/EGFR system (Reddy et al., 1996). The t_{1/2} of IL-2 degradation on various lymphocyte cell lines expressing α, β, and γ ranges from 70-90 minutes, as measured by TCA
precipitability of released ligand (Fujii, et al., 1986; Furse and Malek, 1993; Robb and Greene, 1987). All studies of receptor-mediated IL-2 degradation have been performed using a single, saturating ligand concentration, which might mask any effects of sorting pathway saturation (French et al., 1994; French et al., 1995). Also, much of this work has been performed using an autocrine-secreting IL-2 dependent T-cell line; this may limit the quantitative validity of receptor occupancy data (Claret, et al., 1992; Duprez and Dautry-Varsat, 1986).

1.6 Previous Studies in Other Ligand-Receptor Systems

Ligand-receptor binding is only one aspect of cytokine-mediated cell responses. The functional IL-6 receptor consists of IL-6R and gp130, the latter being exclusively responsible for signaling (Taga et al., 1989). Thus, like IL-2, binding and signaling are separable at the receptor level (Grube and Cochrane, 1994). Dimeric IL-6 binds with higher affinity to its receptor complex, while the monomer is a more potent agonist (Ward et al., 1996). Though some IL-6 variants express enhanced binding and bioactivity, results were obtained using a soluble IL-6R in conjunction with the gp130 protein (Toniatti et al., 1996). Hence, this experimental system did not account for down-regulation due to endocytosis.

A mutant of GM-CSF was shown to be unaffected in low-affinity binding, but displayed decreased bioactivity and high-affinity binding (Lopez et al., 1992). This result implicated two distinct receptor types in the GM-CSFR system (Lock et al., 1994). The distinction between binding and agonism was crucial in isolating the murine IL-2Rγ chain (Zurawski et al., 1990). The naturally occurring IL-1 antagonist ligand was converted into a partial agonist via a point mutation, indicating the subtle relationship
between binding and signaling (Ju, 1991). Conversely, some IL-3 mutants with higher binding affinity to the IL-3R also show greater bioactivity as compared to WT (Lopez et al., 1992). Binding and efficacy have also been decoupled using IL-4 mutants (Kruse et al., 1992). In another example, though heparan sulfate was thought to be required for basic fibroblast growth factor (bFGF) signaling in fibroblasts, it has been shown that lack of heparan sulfate contributes only a binding effect (Fannon and Nugent, 1996). Response vs. bFGF receptor occupancy was thus unchanged in the presence or absence of heparan sulfate.

Distinctions between binding and post-binding events have been made most clearly with the EGFR system. Internalization and mitogenic signaling are separable at the level of receptor structure (Reddy, 1994; Wells et al., 1990). Though EGF and TGFα have similar binding affinities to EGFR at physiological pH, their mitogenic potencies vary due to differential ligand depletion (Reddy et al., 1994). This effect was produced by endosomal sorting events via distinct EGFR binding affinities for the two ligands at endosomal pH (French and Lauffenburger, 1996; French, et al., 1994; French, et al., 1995). Both ligand and receptor mutants have provided the tools for dissecting binding, signaling, and trafficking in the EGFR and growth hormone (GH) systems (Wells, et al., 1990; King et al., 1996; Lowman et al., 1991). The same approach should be instructive in deconvoluting the IL-2R system by exploiting novel IL-2 variants (Berndt, et al., 1994; Chang et al., 1995; Chang, et al., 1996).

1.7 Significance
The IL-2/IL-2 receptor system has been cited as an essential element in controlling immune response (Waldmann, 1993; Waldmann et al., 1993). IL-2 has been
FDA-approved for treatment of melanoma and renal cell carcinoma (Abbas, et al., 1994). IL-2 has been used clinically in numerous immunotherapy applications, either alone or in conjunction with lymphokine-activated killer (LAK) cells (Rosenberg and Lotze, 1986; Rosenberg et al., 1986). IL-2/ LAK immunotherapy has been applied to cancers of the lung, brain, pancreas, spleen, and liver (Burger et al., 1995; Ratto et al., 1995; Sankhla et al., 1995; Marincola et al., 1990; Dubinett et al., 1993; Liu et al., 1994). Often this therapy involves removal of tumor-infiltrating lymphocytes (TIL), in vitro expansion of TIL numbers with IL-2, and re-injection into the patient (Chin et al., 1992; Freedman et al., 1994). The expansion is often performed in bioreactors wherein rIL-2 is added exogenously (Hillman et al., 1994). An IL-2 variant with abrogated cell-based depletion would lessen the material costs of this process.

In vivo injection of IL-2 into patients with HIV has shown moderate promise in potentiating immune response by increasing CD4+ T-cell numbers (Adachi et al., 1996; Johnston and Hoth, 1993). Most therapy protocols have been performed with high-dose IL-2 injection near the maximal tolerance limit (Lotze et al., 1986). This results in side effects including capillary leak, fluid retention and fever, and even increases in plasma HIV levels in AIDS patients (Rosenstein et al., 1986; Lotze et al., 1985; Kovacs et al., 1995). These doses are well above the saturating concentrations of high affinity IL-2R’s on T-cells. In fact, the undesired stimulation of NK cells which express intermediate-affinity IL-2R leads to secretion by these cells of IFN-γ, TNFα, and GM-CSF (Smith, 1993). These “secondary” cytokines are responsible for most of the toxic side effects. A lower-affinity, equipotent IL-2 variant displaying reduced interaction with NK cells might therefore eliminate these pathologies. Recently, low-dose IL-2 therapy with HIV
patients resulted in an increase in CD4+ T-cell numbers without toxicity or elevation of HIV levels (Jacobson et al., 1996). Selective stimulation of high-affinity receptors was essential, illustrating the need to combine an understanding of IL-2/IL-2R cell biology with rational immunotherapy.

The IL-2 receptor has been exploited in mediating the destruction of transformed T-cells or in modulating immune response in transplant patients. Antibodies to IL-2Rα have been shown to inhibit intracellular processing of this subunit (Richardson et al., 1995). IL-2–cell toxin chimeras mediate tumor cell destruction in neuroblastoma metastases and Hodgkin’s disease patients (Sabzevari et al., 1994; Tepler et al., 1994). Toxin chimeras have also been shown to induce immune response and increase the half-life of IL-2 in the bloodstream (Becker et al., 1996; Harvill et al., 1996).

An increased understanding of the trafficking phenomena of IL-2 and the IL-2R can improve the potency of IL-2-based therapies. IL-2 antagonists that suppress immunity might be useful in self-tolerance pathologies such as arthritis, or in reducing transplant rejections. IL-2 agonists could potentiate immunity in HIV or cancer treatment, while maintaining long-term efficacy through abrogated depletion. A quantitative study of IL-2 binding and trafficking will prove invaluable in both these applications.

1.8 Thesis Overview

The overall aim of this thesis research is to characterize the impact of IL-2 receptor-ligand trafficking on T-lymphocyte mitogenesis, and identify key molecular parameters that should be addressed in designing IL-2 variants with improved agonist properties. The thesis itself is divided into three chapters. Chapter 2 deals with
experiments aimed at correlating receptor/ligand trafficking and cellular proliferation within a single system. The central tool in this work is the KIT-225 human T-lymphocyte cell line. This cell line expresses all three subunits (α, β, and γ) of the IL-2 receptor, and is IL-2 dependent. A double mutant of IL-2, termed 2D1, is utilized to perturb the native trafficking and proliferation dynamics of the cellular system on the molecular level. Novel cell-based trafficking assays are developed to compare the native and mutant cytokines in terms of their molecular fates following surface receptor binding and intracellular sorting. These assays are carried out in parallel with the quantitation of KIT cell growth response, allowing the extension of a quantitative, system-integrative approach to the effects IL-2R-mediated ligand processing on T cell function. The binding affinities of IL-2 and 2D1 to the α and β subunits of the IL-2 receptor are measured at both cell surface and endosomal pH. These results provide mechanistic insight into the sorting phenomena that drive the observed trafficking differences between IL-2 and 2D1. The hypothesized concept of an expanded “therapeutic window” for 2D1 is tested using the 2C2 natural killer cell line. Quantification of interferon-γ secretion tests the relative stimulation of these cells by IL-2 and 2D1. In chapter 3, spectroscopic characterization of IL-2 and the 2D1 mutant is undertaken using circular dichroism (CD) and intrinsic fluorescence techniques. This is done to understand the effects of intracellular vesicle acidification on the structure of these proteins, and how these structural changes might affect the relative sorting of these ligands toward recycling or lysosomal degradation. Chapter 4 draws upon the insights gained from experimental results, and details the development of a mathematical model relating trafficking, ligand depletion, and IL-2 potency. The final chapter summarizes the
conclusions and implications of this study for future work.
Table 1.1  Binding affinities and signaling function of IL-2R subunit combinations.
(adapted from Gaulton and Williamson 1994).

<table>
<thead>
<tr>
<th>IL-2R</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\gamma$</th>
<th>$\alpha\beta$</th>
<th>$\beta\gamma$</th>
<th>$\alpha\beta\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding Affinity (M)</td>
<td>$10^{-8}$</td>
<td>$10^{-7}$</td>
<td>$10^{-6}$</td>
<td>$10^{-10}$</td>
<td>$10^{-9}$</td>
<td>$10^{-11}$</td>
</tr>
<tr>
<td>Signaling Functions</td>
<td>No</td>
<td>?</td>
<td>No</td>
<td>?</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure 1.1 Secondary structure of IL-2.

The mutation sites in 2D1 (Leu18 and Leu19) are indicated. (adapted from the Brookhaven Protein Data Base)
Figure 1.2 Interleukin-2 receptor signaling.

The serine-rich (S), acidic (A), and proline-rich (H) regions of the β-subunit are shown. The latter is required for Stat5 activation. Distinct regions of the IL-2R potentiate signaling through various pathways. (adapted from Miyazaki et al. 1995)
Figure 1.3 IL-2/IL-2R trafficking.

Following internalization and early endosomal sorting, α subunits are recycled. The IL-2/β/γ complex is routed to late endosomes and eventually to lysosomal degradation.
Chapter 2: Experimental Trafficking and Potency Studies

An interleukin-2 (IL-2) variant containing adjacent point mutations (Leu18Met, Leu19Ser, termed 2D1) displaying binding affinity to the heterotrimeric IL-2 receptor similar to that of wild-type (WT) IL-2 had been previously found to surprisingly exhibit increased bioactivity in a peripheral blood lymphocyte proliferation assay. In order to provide an explanatory mechanism for this unexpected potency enhancement, we hypothesize that altered endocytic trafficking of the 2D1 variant might be responsible by increasing the number of ligand/receptor complexes. We demonstrate here that the internalization kinetics of 2D1 via the high-affinity IL-2 receptor are equivalent to those of WT IL-2, but that a significantly increased fraction of internalized 2D1 is sorted to recycling instead of to lysosomal degradation. We further find a reduced pH sensitivity of binding to IL-2Rα relative to IL-2Rβ compared to WT IL-2, which could be responsible for the altered sorting behavior of 2D1 in the acidic endosomal compartment. Accordingly, the 2D1 variant displays a half-life 36 hours longer than that of IL-2 in T-lymphocyte culture at concentrations equal to the $K_D$ of the IL-2 receptor. The extended half-life of intact 2D1 provides enhanced mitogenesis as compared to IL-2. In addition, 2D1 stimulates natural killer (NK) cells to a lesser degree than IL-2 at equal concentrations. We conclude that this IL-2 variant provides increased mitogenic stimulation that could not be easily predicted from its cell-surface receptor binding affinity, while minimizing undesired stimulation of NK cells. This concept of altering trafficking dynamics may offer a generalizable approach to generating improvements in the pharmacological efficacy of therapeutic cytokines.
2.1 Introduction

The diverse cell-mediated events of immune and inflammatory responses are tightly regulated by cytokines, and these proteins have been extensively studied as targets for therapeutic intervention (Nicola, 1994). Cytokines direct communication among cells of the hematopoietic system by binding to specific cell-surface receptors (Paul and Seder, 1994). These receptors generally bind their soluble ligands with very high affinity; thus optimum response requires delivery of cytokines to target cells at very low concentrations (~$10^{-10}$-$10^{-12}$ M). These low levels are difficult to sustain due to rapid systemic clearance. Endocytosis of cytokines via their cognate receptors provides cells with an efficient means of modulating their response to ligand-receptor binding events (Trowbridge et al., 1993). This down-regulatory mechanism can cause significant depletion of therapeutic cytokines, however, creating the need to deliver unphysiologically high doses in order to maintain reasonable in vivo concentrations (Metcalf and Nicola, 1995; Ono, 1994). High bolus concentrations often produce toxic, sometimes lethal, side effects (Vial and Descotes, 1995; Kung et al., 1993).

Interleukin-2 (IL-2) was initially isolated as a T-cell growth factor, and has since been shown to direct the expansion and differentiation of several hematopoietic cell types (Theze et al., 1996; Smith, 1988). Clinical studies using IL-2 in the treatment of AIDS have been encouraging, due to its critical role as a proliferative signal for activated T-lymphocytes (Kennedy and Park, 1996; Ghezzi et al., 1997; Kinter and Fauci, 1996; Kovacs et al., 1996; Jacobson, et al., 1996). IL-2 has also undergone trials in the treatment of several types of cancer, based on its stimulation of cytotoxic, antitumor cells (Noble and Goa, 1997; Jeal and Goa, 1997; Vlasveld and Rankin, 1994; Whittington and Faulds, 1993). Toxicity has severely limited the effectiveness of IL-2 treatments,
however, making cell receptor-level pharmacodynamic issues crucial in expanding its therapeutic applicability (Anderson and Sorenson, 1994; Smith, 1993). Pharmacokinetics can be addressed on both a systemic level and at the site of IL-2 activity, the IL-2 receptor (IL-2R) expressed in various forms on hematopoietic cells (Smith, 1997). Cell-level pharmacokinetics involves consideration of receptor-mediated endocytic turnover and intracellular degradation of internalized cytokine molecules that, over time, leads to depletion of ligand from the extracellular medium (Lauffenburger et al., 1998).

The IL-2 receptor on activated T-lymphocytes consists of α, β, and γ subunits, the latter two of which have been shown to mediate IL-2-induced proliferative signaling in these cells (Nakamura, et al., 1994; Nelson, et al., 1994). Expression of the α subunit is upregulated by T cells upon antigen activation, and is thus found in ~100-fold excess on the surface of activated T cell populations (Theze, et al., 1996). The α subunit serves solely to enhance the affinity of the IL-2R complex for soluble IL-2 (Gutgsell and Malek, 1994; Forsten and Lauffenburger, 1994), and is not involved in signaling as it possesses only 13 residues in its cytoplasmic domain (Nikaido, et al., 1984). This heterotrimeric receptor binds with high affinity to IL-2 (K_\text{D}=10 \text{ pM}), followed by rapid internalization and specific degradation of both IL-2 and the β and γ subunits (Subtil and Dautry-Varsat, 1998; Morelon and Dautry-Varsat, 1998; Subtil et al., 1997; Hemar, et al., 1995).

Consistent with its role as a signaling-deficient enhancer of cell-surface ligand binding affinity, the α subunit is constitutively recycled following postendocytic sorting (Hemar, et al., 1995). Therefore, receptor/ligand trafficking dynamics should influence the potency of IL-2 to at least as significant a degree as receptor binding affinity, as has been illustrated in the EGF ligand-receptor system (Reddy, et al., 1996; Reddy et al., 1996).
The functional β and γ IL-2 receptor subunits are also constitutively expressed on natural killer (NK) cells, thus allowing this cell type to respond to IL-2 through upregulation of cytokine secretion and enhancement of cytotoxicity (Kornbluth and Hoover, 1988; Handa et al., 1983). IL-2 therapies have been hampered by a therapeutic "window" in concentration, defined at the upper end by undesired stimulation of intermediate-affinity (K₈D=0.1-10 nM) βγ IL-2 receptors on natural killer NK cells, and at the lower end by desired stimulation of high-affinity (K₈D=1-100 pM) αβγ IL-2 receptors on antigen-activated T and B cells (Jacobson, et al., 1996; Smith, 1993). Dangerous inflammatory responses are activated at the upper end of IL-2 concentration, while therapeutic effects are not observed below the lower end.

We demonstrate that a double mutant of IL-2, Leu18Met/Leu19Ser (termed 2D1), displays reduced endocytic degradation when compared to wild-type IL-2 (WT), and that this effect is due to enhanced ligand recycling. Analysis of ligand binding to the IL-2Rα and β subunits at cell-surface and endosomal pH indicates that the affinity of 2D1 for IL-2Rα decreases to a lesser degree than that of WT as ligand-receptor complexes undergo sorting. These intracellular trafficking differences permit greater sustenance of 2D1 concentrations in cell culture, and increased T-lymphocyte proliferation. The reduced binding affinity of 2D1 to the βγ IL-2R on NK cells also provides reduced stimulation of these cells, a desired outcome in an in vivo setting. This suggests that IL-2 mutants engineered for improved cellular pharmacokinetics and optimal binding affinities to cell type-specific IL-2 receptor isoforms can provide enhanced mitogenic response of T-lymphocytes while minimizing potential in vivo toxicity.
2.2 Experimental Procedures

2.2.1 Materials

RPMI 1640 medium, L-glutamine, and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from Intergen (Purchase, NY). Solution for the Coulter counter (ISOTON II, Coulter Diagnostics, Hialeah, FL) was acquired from Curtin Matheson Scientific Inc. (Houston, TX). $^{125}\text{I}$ was obtained from Dupont NEN Research Products (Boston, MA). Wild-type IL-2 and 2D1 (L18M/L19S) were previously prepared via cassette mutagenesis on a synthetic IL-2 gene (Williams, et al., 1988; Berndt, et al., 1994). Proteins were expressed in E. coli, refolded, and purified as previously described (Landgraf et al., 1991). Concentrations of purified proteins were determined by UV absorption ($\varepsilon_{280} = 9.53 \times 10^3 \text{ M}^{-1}$) (Johnson, 1990). Radioiodinated IL-2 and 2D1 were prepared using the indirect IODO-GEN method according to the manufacturer's protocol (Pierce Chemical Company, Rockford, IL). Specific activities were determined by CTLL bioassay (Gillis, et al., 1978) and ranged from 2-4 Curies/µmole.

2.2.2 Cell culture

The KIT-225 human IL-2-dependent cell line, originally derived from a T-lymphocytic leukemia patient, expresses roughly 3,000 high-affinity IL-2 receptors (IL-2Rαβγ, $K_D=10 \text{ pM}$) and 300,000 low affinity sites (IL-2Rα, $K_D=10 \text{ nM}$) (Hori et al., 1987; Arima et al., 1992). The YT-2C2 human leukemic NK cell line expresses approximately 20,000 intermediate-affinity IL-2 receptors (IL-2Rβγ, $K_D=1 \text{ nM}$) (Teshigawara, et al., 1987). KIT-225 cells were routinely cultured in Corning 75-cm$^2$ tissue culture flasks in RPMI 1640 medium supplemented with 1 nM IL-2, 200 mM L-glutamine, 50 µg/ml gentamycin, 50 units/ml penicillin, and 10% heat-inactivated FBS in
a humidified atmosphere with 5% CO₂. YT-2C2 cells were cultured in the same medium without IL-2. All cells were passaged to $10^5$ cells/ml every 2-3 days.

### 2.2.3 Internalization of $^{125}$I-labeled IL-2 and 2D1

Internalization experiments were performed in a 96-well format using the MultiScreen Assay System (Millipore, Bedford, MA). The bottom of each well in a MultiScreen microtiter plate consists of a low-protein binding, semi-permeable (0.65 μm pore size) membrane that provides separation of cell-associated ligand and free protein upon vacuum filtration of the plate base. KIT-225 cells were washed three times with supplemented RPMI 1640 to remove exogenous IL-2, and were resuspended at $4 \times 10^6$ cells/ml. 100 μl of the cell suspension was loaded into each well, and cells were exposed to a range of labeled ligand concentrations (1-200 pM) for 0-30 minutes at 37°C to allow receptor-mediated endocytosis to occur. Following ligand exposure, free protein was suctioned through the base of the plate, and wells were washed six times with 250 μl of ice-cold PBS. Half the plate was then exposed to three 250-μl rinses of acid strip containing urea (10 mM citrate, 0.14 M NaCl, 50 μg/ml BSA, and 2M urea, pH 2.8) for three minutes each to remove cell surface-bound ligand. Strip efficiency was determined to be ~90% in control experiments. The remaining half of the plate was simultaneously rinsed with PBS, and membranes were then counted individually by solid scintillation.

Stripped wells were considered as internalized counts ($C_i$) and PBS-rinsed wells as total counts ($C_T$), the difference being cell surface-associated ($C_s$). Each data point was measured in triplicate, subtracting membrane-bound ligand in the absence of cells. An endocytic rate constant ($k_e$) was calculated for each ligand concentration as the slope of a straight-line fit to a plot of $C_i$ vs. $\int C_s \Delta t$, as described (Wiley and Cunningham, 1982).
2.2.4 Steady-state sorting experiments

KIT-225 cells were washed three times with supplemented RPMI 1640, and resuspended in the same media including various concentrations of $^{125}\text{I}-\text{IL-2}$ and $^{125}\text{I}-\text{2D1}$ (10-100 pM) at $5 \times 10^6$ cells/ml. Cells were incubated for 3 hours at 37°C to allow intracellular sorting processes to reach steady-state, as previously described (French, et al., 1995; French, et al., 1994). Samples were cooled to 4°C and washed twice with ice-cold PBS, followed by two washes with ice-cold acid strip (10 mM citrate, 0.14 M NaCl, 50 μg/ml BSA, pH 2.8) to remove surface bound $^{125}\text{I}$-ligand. This strip protocol removed all the cell surface-associated label, as indicated by control experiments in which cells were incubated with radiolabeled ligands for 3 hours at 4°C prior to washing. After an additional wash with ice-cold PBS, cells were resuspended at 37°C in media containing 200 nM unlabelled IL-2 to prevent rebinding of released, labeled ligands to cell-surface receptors. Aliquots containing $5 \times 10^6$ cells were removed to Eppendorf tubes at 0, 5, 10, and 15 minutes and pelleted. The supernatant was collected, and the cell pellet was rinsed in ice-cold media and pelleted again. Cell pellets were resuspended in 1M NaOH and counted by solid scintillation to calculate intracellular ligand concentrations.

Supernatants were combined and counted to determine the amount of radioactivity released by the cells. Parallel samples were centrifuged through 10 kD molecular weight cut-off filtration units to separate intact from degraded $^{125}\text{I}$-ligand (Ultrafree-MC, Millipore, Bedford, MA). Parallel samples passed through Sephadex G-10 columns provided equally reliable results.

2.2.5 Surface plasmon resonance analysis of ligand binding to IL-2R complexes

Surface plasmon resonance (SPR) analysis of WT and 2D1 binding to immobilized IL-2Rα- and IL-2Rβ-ectodomains was performed as described (Wu et al.,
1995). Sensor chip surfaces with oriented IL-2Rα or IL-2Rβ subunits were prepared via thioether linkages at single cysteine residues present in the native extracellular domain (IL-2Rα) or introduced by mutagenesis (IL-2Rβ) (Sana et al., 1994) as previously described (Myszka et al., 1996). SPR instrumentation (BIAcore™), CM5 sensor chips, and amine-coupling reagents containing N-hydroxysuccinimide, N-ethyl-N′-(3-diethylaminopropyl)carbodiimide, and ethanolamine HCl were obtained from Pharmacia Biotech Inc. Thiol conjugation was achieved using m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce). Purified IL-2Rα or IL-2Rβ complex was diluted into NaOAc buffer (10 mM), pH 5, and coupled to the dextran-modified gold surface of a CM5 sensor chip using the manufacturer’s coupling chemistry as described in the BIAcore™ systems manual. Surface densities of 800-3500 resonance units of IL-2R complex were used. Prior to analysis, stock solutions of WT and 2D1 were dialyzed against phosphate buffer (10 mM sodium phosphate, pH 7.2 or pH 6, 150 mM NaCl), and protein concentrations were determined from A280 values (Berndt, et al., 1994). Samples were then diluted to desired concentrations in analysis buffer (10 mM sodium phosphate, pH 7.2 or pH 6, 150 mM NaCl, 0.005% surfactant P-20) containing 100 µg/ml bovine serum albumin. Six to eight serial dilutions of each protein were injected over the respective surfaces at a flow rate of 10 µl/min. Sensorgrams were recorded and normalized to a baseline value of 0 RU. Equivalent samples were injected over BSA surfaces for simultaneous subtraction of non-specific binding and bulk refractive index background. Each determination was performed in triplicate, and the same surface was used for each set of data collected at pH 7.2 and pH 6.
Since binding of WT and 2D1 reached a constant equilibrium value during injection on both IL-2R complex surfaces at pH 7.2 and pH 6, this equilibrium value (RU_{eq}) was used to fit the data to a single-site binding isotherm (A+B=AB) at each condition. After subtraction of background and nonspecific RU (determined by injection over a BSA surface), RU_{eq} was plotted vs. C, the concentration of ligand injected over the sensor chip surface. The dissociation constants were estimated by weighted, nonlinear least squares curve fitting using KaleidaGraph^TM (version 3.09, Synergy Software).

2.2.6 Growth experiments and cell-surface receptor quantification

KIT-225 cells were passaged into supplemented RPMI 1640 (without IL-2) at 2x10^5 cells/ml six days prior to the addition of exogenous IL-2 analogs. Cells were switched into fresh IL-2-free media on day three of quiescence. Growth experiments were initiated by switching cells into media containing a range of ligand concentrations (1, 10, and 100 pM). Cells were counted in a Coulter counter to monitor growth. Cell aliquots from equivalent populations were washed in ice-cold media, and resuspended in media containing 500 pM ^{125}I-IL-2 at approximately 2x10^6 cells/ml. Cell suspensions were transferred to 0.65 μm MultiScreen microtiter plates and incubated for three hours at 4°C. Following incubation, free protein was suctioned through the base of the plate, and wells were washed six times with 250 μl of ice-cold PBS. Membranes were then counted individually by solid scintillation to determine cell-surface associated radioactivity. Each data point was measured in triplicate, subtracting nonspecific binding as determined by addition of 200-fold molar excess of unlabelled wild-type IL-2.
2.2.7 Intact ligand quantification
Quiescent KIT-225 cells or YT-2C2 cells were incubated in media containing 125I-labeled ligands. Controls for nonspecific degradation included incubation with 200-fold molar excess of unlabelled IL-2. Cell aliquots were removed and pelleted every twelve hours, and supernatants were centrifuged through 10 kD molecular weight cut-off filtration units to separate intact from degraded 125I-ligand. This method proved to be equivalent to gel filtration in quantifying relative amounts of degraded protein.

2.2.8 Determination of IL-2-induced IFN-γ secretion
YT-2C2 cells were incubated for 48 hours in media containing 1nM IL-2 or 2D1. At that time, the concentration of IFN-γ was determined using an ELISA kit obtained from Intergen (Purchase, NY). No cross-reactivity of the IFN-γ antibody was observed with either WT or 2D1 under experimental conditions (data not shown). Medium samples were centrifuged to remove cellular debris prior to analysis.

2.3 Results

2.3.1 Selection of IL-2 variant L18M/L19S (2D1)
A double mutant of IL-2, L18M/L19S (2D1), was shown to yield an equivalent biological response to wild-type at a two-fold lower concentration (Table 2.1) (Berndt, et al., 1994). One possible explanation for this enhanced potency might be increased binding to the high-affinity IL-2 receptor (αβγ complex) expressed on activated T-lymphocytes. This would be expected to generate more ligand-receptor complexes at a given cytokine concentration, resulting in up-regulated cell proliferation (Smith, 1995). The binding affinities of WT and 2D1 to the αβγ complex are roughly equivalent, however, requiring an alternative explanation for 2D1’s superagonism (Table 2.2)
(Berndt, et al., 1994). We sought to characterize the trafficking behavior of WT and 2D1 as a potential source of their differences in observed mitogenic potency.

2.3.2 Receptor-mediated endocytosis of WT and 2D1

A different IL-2 variant, T51P, had been previously shown to be deficient in receptor-mediated endocytic uptake, resulting in negligible cellular depletion of ligand from the culture medium. Therefore, despite a reduced receptor binding affinity, T51P generated a proliferative response equivalent to that of WT due to abrogated down-regulation of ligand-receptor complexes from the cell surface (Chang, et al., 1996). To test whether this mechanism was responsible for the enhanced mitogenic potency of 2D1, we measured the internalization rates of WT and 2D1 on T-lymphocytes. The KIT-225 human cell line was chosen because it is IL-2 dependent, providing the opportunity to correlate receptor-mediated trafficking events and mitogenesis within a single system. Cells were incubated with ligand concentrations both below and above the $K_D$ of the high-affinity IL-2 receptor (10 pM) to investigate endocytic behavior at potentially sub-saturating and saturating conditions (Lund et al., 1990). Radiolabeled WT and 2D1 were internalized with comparable rates at both low and high surface receptor occupancy (Figure 2.1). Over most of the occupancy range, the internalization rate constants fell within the interval of 0.03-0.04 min$^{-1}$, which corresponds to the internalization half-time of 17-23 minutes observed in activated peripheral blood lymphocytes (Chang, et al., 1996). Surface binding and receptor-mediated endocytosis of 2D1 are thus indistinguishable from WT. The increase in the value of the internalization rate constant with increasing receptor occupancy at low concentrations is intriguing, and may indicate
an interesting regulation point; this phenomenon, however, is beyond the scope of the present work.

2.3.3 Steady-state sorting of WT and 2D1

We therefore hypothesized that altered intracellular sorting might be the trafficking process responsible for the differential bioactivity. To measure trafficking events downstream of internalization, cells were allowed to process radiolabeled WT or 2D1 until the sorting process reached steady-state (French, et al., 1994). Exogenous and surface-bound proteins were removed at 4°C (to inhibit endocytosis), and the initial rate of ligand release was measured by collecting media up to fifteen minutes after returning the cells to 37°C. Sorting fractions were measured by filtering samples through 10 kD molecular weight cutoff membranes and counting the radioactivity in the filtrate and retentate. Over the range of extracellular ligand concentrations tested (10-100 pM), the 2D1 variant was recycled to a significantly greater extent than WT (Figure 2.2). The fraction of 2D1 recycled was nearly 50%, while only 20-35% of intracellular WT was released intact. These sorting results further confirm the equivalence of binding and internalization events for WT and 2D1, since the number of intracellular complexes was roughly equivalent.

2.3.4 SPR analysis of ligand binding to IL-2Rα and β subunits

The pH sensitivity of ligand-receptor interactions has been shown to be a crucial factor in affecting endosomal sorting outcomes (French, et al., 1995). In the IL-2R system, the β and γ subunits are preferentially degraded, while the α subunit recycles following IL-2 binding and internalization (Hemar, et al., 1995). Thus, it follows that the relative affinity of an IL-2 analog for these individual receptor subunits as pH is
decreased from ~7.2 at the cell surface to ~6 in endosomes should directly impact the ligand's sorting fate. We chose to examine the affinity of WT and 2D1 to the α and β subunits, individually, at both pH 7.2 and pH 6 using SPR (BIAcore™) analysis. Because the γ subunit displays equivalent sorting behavior to IL-2Rβ, exhibits extremely low binding affinity to IL-2 (K_D > 50 μM) (Johnson et al., 1994), and is unstable to the low pH conditions required to prepare and regenerate biosensor surfaces, binding to IL-2Rγ was not examined. Experiments were performed at pH 7.2 and pH 6, and equilibrium RU values were plotted versus the concentration of ligand for IL-2Rα and IL-2Rβ surfaces as shown in Figure 2.3 (A and B, respectively). The binding of IL-2 to these biosensor surfaces has been previously characterized, and the kinetic and equilibrium binding constants were similar to those obtained in cell-based assays (Wu, et al., 1995; Myszka, et al., 1996). The data were fit to a single-site binding model under each experimental condition, and resulting dissociation constants are listed in Table 2.3. An equivalent 10-fold decrease in affinity to IL-2Rβ was observed for both WT and 2D1 as pH was reduced from 7.2 to 6. The affinity of WT for IL-2Rα is reduced 8-fold, while that of 2D1 is reduced only 5-fold as pH decreases from 7.2 to 6.

The potential effect of these affinity changes on ligand sorting behavior can be visualized by plotting the ratio of dissociation constants for the alpha versus beta subunits at pH 6 relative to pH 7.2; i.e., [K_D(β)/K_D(α) at pH 6]/[K_D(β)/K_D(α) at pH 7.2] for both WT and 2D1 IL-2 (Figure 2.4). This ratio represents the change in relative preference for ligand binding to IL-2Rα versus IL-2Rβ as the complex moves from the cell surface to the endosomal sorting compartment. The greater the value of this ratio, the greater the proclivity the ligand should have for endosomal sorting toward recycling along with IL-
2Rα than toward lysosomal degradation along with IL-2Rβ. Figure 2.4 shows that this ratio is clearly greater for 2D1 than for WT, consistent with our findings shown in Figure 2.2.

### 2.3.5 Ligand depletion by T cells in culture

Proliferating T cells are known to deplete IL-2 as a means of down-regulating their mitogenic stimulus (Fujii, et al., 1986). We sought to determine if the altered intracellular sorting of 2D1 provided this variant with improved pharmacokinetics in culture. KIT-225 cells were starved of IL-2 as described in “Experimental procedures”, and incubated with radiolabeled WT or 2D1 at concentrations of 10 pM or 100 pM. These concentrations were chosen to approximate 50% and 100% occupancy of high-affinity IL-2 receptors, respectively. Intact ligand remaining in five-day cultures was quantified every twelve hours by passing samples of the cell supernatant through 10 kD molecular weight cutoff membranes. Media containing 10 pM radiolabeled ligand was depleted more rapidly by the cells than media containing 100 pM ligand for both WT and 2D1. This result is consistent with a depletion mechanism wherein a fixed number of molecules is processed by a constant number of cells in a given time. Thus, the larger pool of labeled ligands (100 pM) is depleted less rapidly than the smaller population (10 pM). The degradation of both proteins was abrogated by co-incubating cells with a 200-fold excess of unlabelled WT, indicating that ligand consumption was mediated specifically by the IL-2 receptor (Figure 2.5). Wild-type IL-2 was degraded at a significantly faster rate than 2D1 for both conditions, though the difference in depletion rate for 100 pM starting concentrations was not apparent until day three due to the larger pool of extracellular ligand molecules (Figure 2.5). The viable concentration of 10 pM
WT in the media was halved within 48 hours, while the half-life of 10 pM 2D1 was 84 hours. Therefore, at cytokine concentrations near the $K_D$ of the high-affinity IL-2 receptor (10 pM), the 2D1 mutant should retain its efficacy longer than IL-2 due to its enhanced stability to receptor-mediated degradation.

2.3.6 Cell-surface receptor-ligand complex numbers

Cells receive their mitogenic stimulus from IL-2/IL-2R complexes on the cell surface. Thus, quantification of IL-2 receptor numbers was necessary to fully determine the effects of ligand depletion on cell proliferation. Quiescent KIT-225 cells were incubated with WT or 2D1 at concentrations of 10 pM or 100 pM. Cell aliquots were removed every 24 hours, and radioreceptor binding assays were performed at 4°C using 500 pM $^{125}$I-WT to saturate high-affinity receptors. Receptor levels were depressed on day one following quiescence. Upon exposure to either WT or 2D1, receptor levels increased throughout the five-day culture, presumably due to enhanced IL-2Rα expression (Figure 2.6). This effect was likely due to up-regulation of IL-2Rα upon introduction of IL-2, which has been described to occur both in activated PBLs and in another human leukemic T cell line (Hemar, et al., 1995; Smith and Cantrell, 1985; Deppen et al., 1985; Smith, 1989; Duprez, et al., 1988). Receptor numbers increased at equivalent rates both ligands at 10 pM starting concentrations then displayed relatively constant levels beginning at day three. Cell-surface receptor levels increased throughout the five-day culture for both WT and 2D1 at 100 pM, with a significantly greater increase observed in the presence of 2D1 at later times.

Receptor data were combined with results of the ligand depletion studies to estimate cell-surface ligand-receptor complex numbers at each time using the following
relation: \( C_s = \frac{R \cdot L}{[K_D + L]} \); where, \( R \) is the number of receptors per cell, \( L \) is the intact ligand concentration as shown in Figure 2.5, and \( K_D \) is the respective equilibrium dissociation constant describing binding of WT or 2D1 to the high-affinity IL-2R (see Table 2.2). Although the receptor levels seen in the presence of 10 pM WT and 10 pM 2D1 are essentially equal, the reduced depletion of 2D1 leads to greater predicted ligand-receptor complex numbers up to day 3, followed by a decrease upon eventual depletion of this ligand (Figure 2.7). Depletion is less significant at 100 pM ligand concentrations, so the predicted complex numbers track in accord with the receptor numbers shown in Figure 2.6.

2.3.7 T-cell proliferation in response to WT and 2D1

Bio-activity data were previously obtained on activated PBLs, and 2D1 was shown to induce a mitogenic response equivalent to WT at a two-fold lower concentration in 48-hour assays (Table 2.1). Proliferation of KIT-225 cells was monitored to determine the influence of ligand depletion on relative potencies of WT and 2D1 within one cell line. Cell growth was monitored over several days following incubation with various concentrations of WT or 2D1 (0-100 pM). Cell numbers were essentially constant during the first 48 hours of incubation, although cells were actively processing ligand (see Figure 2.5). Proliferation between 48 and 72 hours was equal to the ligand-free control at very low (1 pM) concentrations for both ligands. Both WT and 2D1 had equivalent potencies during this time at concentrations that saturate the high affinity IL-2 receptor (100 pM), suggesting that 2D1 does not generate superior signal transduction upon receptor binding. However, the growth rate of KIT-225 cells initially exposed to 10 pM 2D1 was roughly three times that of cells exposed to 10 pM WT from
48 to 72 hours, and reached essentially the maximum not ordinarily seen for WT until 100 pM (Figure 2.8). This time period corresponded to that in which significantly increased numbers of cell-surface ligand-receptor complexes were predicted for 2D1 relative to WT (see Figure 2.7). Depletion effects have been shown to impact cell response under conditions wherein the number of ligand/receptor complexes is highly dependent on the concentration of active cytokine in the media (Reddy, et al., 1996). This occurs at concentrations near or below the $K_D$ (~10 pM for the high affinity IL-2 receptor). The altered intracellular trafficking fate of 2D1 directly heightens its mitogenic potency over WT in this concentration range.

### 2.3.8 Functional response of NK cells to IL-2 and 2D1

The pleiotropic immunologic effects of IL-2 include stimulation of NK cells through the intermediate affinity (IL-2Rβγ, $K_D=1$ nM) IL-2 receptor. Since the 2D1 variant is known to bind this receptor with a lower affinity than wild-type IL-2 (see Table 2.2), we sought to quantify the relative response of NK cells to the two ligands. One indicator of NK cell activation in the presence of IL-2 is secretion of IFN-γ (Handa, et al., 1983; DeSanctis et al., 1997; Carson et al., 1997; Chan et al., 1992; Chan et al., 1991). To this end, YT-2C2 NK cells were incubated for 48 hours with initial concentrations of 1 nM WT or 2D1 in the culture medium. This concentration is equivalent to the $K_D$ describing the binding of IL-2 with the IL-2 receptor expressed on these cells, and negligible binding to either of the individual β or γ subunits occurs at this concentration. These cells were capable of specifically internalizing, and hence down-regulating their response to IL-2 analogs as evidenced by observable ligand depletion over the course of the experiment (Figure 2.9). Wild-type IL-2 was degraded to a greater extent than 2D1
via the intermediate-affinity IL-2 receptor. This result is consistent with the relative endocytic depletion seen in T-cell culture via the high-affinity IL-2R, and highlights the functional significance of the βγ complex in effecting cellular response. An ELISA was used to quantify the amount of IFN-γ present in the NK cell culture medium, and results indicated that these cells secreted a significantly greater amount of IFN-γ in the presence of WT than was observed in response to 2D1 on a per-cell basis (Figure 2.10). This difference is qualitatively consistent with the reduced binding affinity of 2D1 to the βγ IL-2 receptor, and illustrates the diminished sensitivity of NK cells to an equivalent bolus of 2D1 relative to WT IL-2.

2.4 Discussion

Our experimental data detailed here show that consideration of receptor-mediated trafficking phenomena can lead to engineered ligand variants with improved functional properties. This concept was previously suggested by studies with EGF (Reddy, et al., 1994), and we now prove that it is generalizable to an immune system cytokine. The improved potency of 2D1 would not have been predicted from its binding affinity to the IL-2 receptor (αβγ) at the cell surface, yet it can be explained by modified endosomal sorting leading to abrogated receptor-mediated depletion.

Following internalization of the IL-2/IL-2R complex into the endosomal compartment, wild-type IL-2 is typically sorted together with the β and γ subunits of the IL-2 receptor toward lysosomal degradation while the α subunit is released to recycle back to the cell surface (Hemar, et al., 1995). The enhanced recycling of 2D1 relative to WT correlates qualitatively with the mutant’s heightened proclivity to bind IL-2Rα versus IL-2Rβ as pH is decreased. In Chapter 3 we show that, although WT and the 2D1
variant display equivalent structural integrity at pH 7, the stability of 2D1 decreases relative to that of WT as pH is decreased to mimic transport through endocytic sorting vesicles. These structural differences might directly impact conformation-dependent binding properties of 2D1 to the differentially-sorted α, β, and γ IL-2R subunits at endosomal pH. Although the absolute difference in IL-2Rα affinity between IL-2 and 2D1 at pH 6 does not point directly to a difference in recycling, the sensitivity of the ligand-receptor interaction to a pH change upon endocytosis is likely the relevant parameter in considering relative sorting outcomes as in the EGF system (French, et al., 1995). Mutations that perturb ligand-receptor interactions in acidic endosomes and subsequently alter the intracellular trafficking of ligand-receptor complexes have also been identified in both the LDL and insulin receptor systems (Kadowaki et al., 1990; Miyake et al., 1989; Davis et al., 1987). The high affinity IL-2R system adds an additional level of complexity, and potentially an added route of intervention, based on the differential sorting of its distinct receptor subunits. Since IL-2 constantly undergoes endocytic turnover, mutations that cause slight differences in recycling efficiency can have profound effects on the effective ligand concentration in culture as illustrated in the present work.

From comparison of Figure 2.2 and Figure 2.4, it can be seen that a roughly 50%-fold increase in ligand binding preference for the recycling receptor subunit relative to the degrading receptor subunit in this case yields an approximately 2-fold increase in the fraction of ligand sorted to recycling, from about 25% to about 50%. This manner of relationship between ligand/receptor binding and trafficking properties can be analyzed using a mathematical model previously described for the endosomal sorting process.
(French and Lauffenburger, 1996). Further including Figure 2.8 in the comparison demonstrates that this increase in sorting to recycling leads to an essentially 10-fold enhancement in ligand potency for stimulation of T-cell proliferation. We infer that further increases in sorting to recycling, perhaps up to near 90% (which appears to be a constitutive maximum (French, et al., 1994)), may be obtained by rational or combinatorial engineering of IL-2 variants possessing a greater increase in binding preference for IL-2Rα relative to IL-2Rβ -- and that such increases could lead to another 1-2 orders of magnitude enhancement of IL-2 potency.

The reduced binding affinity of 2D1 to the IL-2R βγ complex expressed on NK cells should dampen induced inflammatory activity of these cells, as evidenced by the decrease in IFN-γ secretion by the YT-2C2 cell line (Yoneda et al., 1996). Secretion of IFN-γ by NK cells can be an undesired side effect of IL-2 therapy directed at specific stimulation of the high affinity IL-2 receptor on T cells (Smith, 1993). Thus, the 2D1 variant can be predicted to exhibit an expanded range of therapeutic efficacy: by maximizing potency with respect to activated T cells at low concentrations, and minimizing of harmful side effects resulting from NK cell stimulation at high IL-2 concentrations.

Results gleaned from studies of the 2D1 variant can contribute to the concept of a cell-level dynamic analysis, involving “matrix” criteria related to optimization of multiple ligand/receptor binding properties within diverse cellular trafficking compartments (Lauffenburger, et al., 1998). For example, a more effective IL-2 variant should display increased affinity to both the αβγ IL-2R complex on the surface of T cells and to the constitutively-recycling IL-2Rα in postendocytic sorting vesicles, while maintaining
decreased affinity to the βγ IL-2R both on NK cells and under endosomal conditions wherein this complex is lysosomally-routed. Utilization of the pH changes associated with transport through endocytic vesicles could be employed in a high-throughput combinatorial manner or toward rational design based on the pH sensitivity of ligand-receptor binding interactions. An optimally re-engineered IL-2 variant might affect not only enhanced T-cell mitogenesis via improved postendocytic recycling efficiency, but also reduced stimulation of NK cells upon exogenous delivery in vivo.
Table 2.1 Mitogenic potencies of IL-2 and 2D1 on human PBL’s.

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ (pM)</th>
</tr>
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<tbody>
<tr>
<td>WT IL-2</td>
<td>16.0±2.0</td>
</tr>
<tr>
<td>2D1</td>
<td>7.6±0.7</td>
</tr>
</tbody>
</table>

Concentrations of protein producing 50% of the maximal cell response in 48-hour $^3$H-thymidine uptake bioassays, as described (Landgraf et al., 1989). Data are adapted from Berndt et al. (Berndt, et al., 1994)

Table 2.2 Binding affinities of IL-2 and 2D1 to functional forms of the IL-2 receptor.

<table>
<thead>
<tr>
<th></th>
<th>$K_D$ (pM)</th>
<th>$K_D$ (nM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$\alpha\beta\gamma$ complex</td>
<td>$\beta\gamma$ complex</td>
</tr>
<tr>
<td>WT IL-2</td>
<td>11.1±0.4</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>2D1</td>
<td>8.2±0.2</td>
<td>1.8±0.4</td>
</tr>
</tbody>
</table>

All data were obtained from radioreceptor binding assays performed on antigen-activated PBL’s ($\alpha\beta\gamma$ complex) and YT-2C2 cells ($\beta\gamma$ complex) at pH 7.4, representative of cell surface pH. Data are adapted from Berndt et al. (Berndt, et al., 1994)
Table 2.3  Binding affinities of IL-2 and 2D1 to IL-2R subunits.

<table>
<thead>
<tr>
<th></th>
<th>pH 7.2</th>
<th></th>
<th>pH 6</th>
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<tbody>
<tr>
<td></td>
<td>$K_D$ (nM)</td>
<td></td>
<td>$K_D$ (nM)</td>
</tr>
<tr>
<td>IL-2Rα</td>
<td>20.2±0.4</td>
<td></td>
<td>155±7</td>
</tr>
<tr>
<td>IL-2Rβ</td>
<td>625±1</td>
<td></td>
<td>6110±80</td>
</tr>
<tr>
<td>WT IL-2</td>
<td>43.2±1.3</td>
<td>890±3</td>
<td>227±18</td>
</tr>
<tr>
<td>2D1</td>
<td>8800±190</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dissociation constants were obtained from SPR analysis of ligand binding to immobilized IL-2R ectodomain. Each value was derived from a weighted nonlinear least-squares fit of equilibrium RU data to a single-site binding model.
Figure 2.1 Ligand internalization.

Internalization rate constants of $^{125}$I-WT IL-2 (filled circles) and $^{125}$I-2D1 (open circles) are plotted versus the number of surface complexes, as described (52). Error bars represent the standard error in the slope of a plot of the number of internalized complexes vs. the average number of surface complexes over the measurement interval. Results of four experiments are shown.
Figure 2.2 Ligand recycling.

Fractions of $^{125}$I-WT IL-2 (filled circles) and $^{125}$I-2D1 (open circles) sorted to recycling. KIT-225 cells were incubated for 3 hours at 37°C with concentrations of labeled ligand ranging from 10 pM to 100 pM. Cells were washed with a pH 2.8 acid strip to remove surface-bound moieties, and chased with 200 nM unlabelled wild-type IL-2. Media was collected from the cell suspension at 0, 5, 10, and 15 min. Medium samples were centrifuged through 10 kD-cutoff membrane filtration units to separate degraded and viable (intact) radiolabeled ligands. Sorting fractions for each time point are shown.
Figure 2.3 Surface plasmon resonance analysis of ligand binding.

Equilibrium RU values were obtained following injection of WT IL-2 (open symbols) or 2D1 (filled symbols) in pH 7.2 (circles) or pH 6 (square) buffer over sensor chip surfaces containing covalently linked IL-2Rα (A) and IL-2Rβ (B) ectodomains as described in the Experimental Procedures section. Results shown are the mean +/- S.E.M. of triplicate measurements, and values were corrected for nonspecific binding by injecting parallel samples over BSA surfaces. Each curve represents the result of a weighted nonlinear least squares fit to a single-site binding model for each condition.
Figure 2.4 Relative binding affinities among IL-2Rα and IL-2Rβ between pH 6 and pH 7.2.

Equilibrium dissociation constants tabulated in Table 2.3 were used to calculate the ratio of ligand affinity for IL-2Rα versus IL-2Rβ for WT IL-2 (black bar) relative to 2D1 (gray bar). The magnitude of this ratio represents the enhancement of ligand affinity to IL-2Rα relative to IL-2Rβ as pH is decreased from 7.2 to 6.
Figure 2.5 Degradation of IL-2 and 2D1 in T cell culture.

Quiescent KIT-225 cells were incubated with 10 pM (circles) and 100 pM (squares) $^{125}$I-WT IL-2 (filled symbols), or 10 pM (circles) and 100 pM (squares) $^{125}$I-2D1 (open symbols) beginning at day zero. Media was removed at the indicated times and centrifuged through 10 kD-cutoff membrane filtration units to separate intact and degraded ligand. Negative controls included incubation of 10 pM (triangles) and 100 pM (diamonds) $^{125}$I-WT IL-2 (filled symbols), or 10 pM (triangles) and 100 pM (diamonds) $^{125}$I-2D1 (open symbols) plus a 200-fold molar excess of unlabelled WT IL-2.
Figure 2.6 High-affinity IL-2 receptor numbers.

Quiescent KIT-225 cells were incubated with 10 pM (circles) and 100 pM (squares) WT IL-2 (filled symbols) or 2D1 (open symbols) beginning on day zero. At the indicated times, cells were removed from culture, washed, and resuspended in triplicate at approximately 2x10^6 cells/ml in ice-cold RPMI + 10% FCS containing 500 pM ^125^I-IL-2 to saturate high affinity receptors. Non-specific binding was subtracted from each measurement by incubating cells with 200-fold molar excess of unlabelled wild-type IL-2. Cells were incubated for 3 hours at 4°C, separated from free ligand using the Millipore MultiScreen unit, and then washed six times with ice-cold PBS. Parallel cell samples were counted to determine receptor number per cell, and results were normalized to values on day 1. Results shown are the mean +/- S.E.M. of two independent experiments.
Figure 2.7 Cell-surface ligand-receptor complex numbers.

Free ligand determined from degradation experiments (Figure 2.3) were combined with receptor numbers determined from equilibrium binding studies (Figure 2.4) to calculate expected surface receptor-ligand complex numbers per cell versus time for each initial incubation concentration: 10 pM (circles) and 100 pM (squares) WT IL-2 (filled symbols) or 2D1 (open symbols).
Figure 2.8 Growth rate of KIT-225 cells.

Growth rates are shown as a function of concentrations of WT IL-2 (filled symbols) and 2D1 (open symbols) at time zero. Values are based on the increase in cell density from 48-72 hours. Error bars represent the standard deviation of triplicate measurements made on each of two individual samples.
Figure 2.9 Degradation of IL-2 and 2D1 in NK cell culture.

YT-2C2 cells were incubated at $10^6$ cells/ml with 1 nM (filled circles) $^{125}$I-WT IL-2 or 1 nM (filled squares) $^{125}$I-2D1 beginning at day zero. Media was removed at the indicated times and centrifuged through 10 kD-cutoff membrane filtration units to separate intact and degraded ligand. Negative controls included incubation of 1 nM $^{125}$I-WT IL-2 (open squares) or 1 nM $^{125}$I-2D1 (filled squares) plus a 200-fold molar excess of unlabelled wild-type IL-2. Results shown are the mean +/- S.E. of two independent experiments.
Figure 2.10 Secretion of IFN-γ by NK cells in culture.

YT-2C2 cells were incubated at 10^6 cells/ml with 1 nM WT IL-2 (black bar) or 1 nM 2D1 (gray bar) beginning at day zero. IFN-γ secretion was quantified after 48 hours by ELISA, and secretion by control cultures in the absence of IL-2 was subtracted. Results were normalized to cell number and the average intact IL-2 analog concentration (see Figure 2.7) over the 48-hour time course. Viable cell density was unchanged throughout the experiment (data not shown). Results shown are the mean ± S.E.M. of two independent experiments.
Chapter 3: Structural Studies

The formation of partially unfolded intermediates can occur when an internalized cytokine encounters the acidic environment of the endosomes. In this chapter, the nature of these structural transitions is studied for wild-type IL-2 and the 2D1 analog. A noncoincidence of denaturation transitions in the secondary and tertiary structure for both proteins is observed, suggesting the presence of an intermediate state. Folding intermediates are a common feature of this structural family of four-helical-bundle proteins. Wild-type IL-2 and the 2D1 analog have similar stabilities at neutral pH; however, the analog displays decreased stability of the native and intermediate states under acidic conditions when compared to wild-type IL-2. An understanding of the structural changes accompanying unfolding might lend insight to the conformational behavior of cytokines as they enter acidified endosomes. This behavior could underlie the differential pH-sensitivities of receptor binding affinity for IL-2 and the 2D1 variant that lead to increased ligand recycling, and enhanced therapeutic efficacy for the 2D1 analog.

3.1 Introduction

Interleukin-2 is a member of the four-helix bundle family of proteins, having a structure similar to that of human growth hormone (hGH), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin, leptin, interferon-β, megakaryocyte growth and development factor (MGDF), and other cytokines (Brandhuber, et al., 1987; Hill et al., 1993). For some of these proteins, physical stability is a problem for long-term storage and delivery.
Precipitation and loss of protein during refolding, purification, storage, or delivery has been previously studied for many of these proteins, including IL-2 (Tzannis et al., 1996; Vlasveld et al., 1993; Prestrelski et al., 1995; Marston, 1986; Brems, 1988; Schein, 1990; DeYoung et al., 1993). Conformational studies of G-CSF, hGH, and MGDF as a function of pH and denaturant concentration have shown the presence of folding intermediate states (Narhi et al., 1991; Hamburger et al., 1998). Understanding the mechanism of the structural changes that accompany protein unfolding and precipitation may lead to improved formulations for similar therapeutic proteins.

Several analogs of IL-2 have been designed and characterized for biological activity (Berndt, et al., 1994; Chang, et al., 1995). The IL-2 receptor is composed of α, β, and γ subunits, and some of the IL-2 analogs which display altered biological activity have mutations located at the receptor subunit binding sites (Berndt, et al., 1994; Chang, et al., 1995). One of these analogs, termed “2D1”, has mutations L18M and L19S located in the N-terminal helix near a site known to be essential for binding to the β-subunit of the receptor (Figure 3.1) (Collins, et al., 1988). Cell trafficking studies show that this mutant displays similar binding affinity and internalization rates as the wild-type IL-2. However, the analog displays greater ligand recycling and enhanced mitogenic potency compared to the wild-type IL-2 (see Chapter 2). This mutant may have a greater affinity for the recycled α-subunit of the receptor or a lower affinity for the βγ-receptor complex, which undergoes degradation in the lysosome (Hemar, et al., 1995). The altered ligand trafficking and recycling may be linked to structural or stability differences. This series of experiments addresses the structural basis for the modified trafficking of the IL-2 analog.
3.2 Experimental Procedures

3.2.1 Materials

Recombinant human IL-2 (Cys 125 Ala) was produced in *E. coli* at Amgen Inc. Additional wild-type IL-2 and analog 2D1 (Leu 18 Met, Leu 19 Ser) was produced and purified as previously described (Berndt, et al., 1994). The purified IL-2 was stored in a formulation buffer of 10 mM sodium acetate, pH 4. The buffer for the full CD scans was 1 mM sodium citrate, 1 mM sodium phosphate, 1 mM sodium borate, pH 4 through 7. For the denaturation studies, the buffer was 50 mM Tris, 20 mM sodium acetate, 20 mM MES, pH 4 through 7. Sequenal grade guanidine hydrochloride (GuHCl) was obtained from Pierce.

3.2.2 Circular dichroism

Circular dichroism (CD) was performed using an Aviv instrument with a titrator. The far-UV signal was measured at 222 nm and the full spectrum was recorded between 300 nm and 190 nm. The pH titration was performed by adding 1 uL shots of 1N HCl to a 5 mL stock solution of IL-2 at 0.05 mg/mL in 1 mM sodium citrate, 1 mM sodium phosphate, 1 mM sodium borate, and full spectrum measurements were recorded at pH steps of ΔpH = 0.5 from pH 8.3 to pH 3.4. The equilibrium denaturation studies were performed by preparing stock protein solutions at 0.05 mg/mL in 50 mM Tris, 20 mM sodium acetate, 20 mM MES with 0 M or 6 M GuHCl at each given pH. After each CD measurement, the titration apparatus removed a set volume of sample from the cuvette and injected an equivalent volume of denatured protein. Mixing and equilibration times were incorporated into the sequence between each measurement.
3.2.3 Fluorescence

A PTI fluorometer was used to measure intrinsic fluorescence at 280 nm, and emission scans were performed at wavelengths of 300 nm to 400 nm. A manual pH titration was performed from pH 2 to 7 by removing a given volume of protein solution and mixing in an equivalent volume of pH 7 protein solution. Stock solutions of IL-2 were at a protein concentration of 0.05 mg/mL in 50 mM Tris, 20 mM sodium acetate, 20 mM MES at pH 7 and pH 2. For the equilibrium denaturation studies, separate samples across a range of denaturant concentrations were prepared individually at 0.05 mg/mL protein concentration in 50 mM Tris, 20 mM sodium acetate, 20 mM MES, pH 4.

3.3 Results

Since IL-2 encounters a pH environment ranging from the neutral extracellular space to the acidic endosomal compartments, the structure and stability of IL-2 were examined as a function of pH. Equilibrium denaturation studies were performed to characterize the structure and stability of IL-2. Circular dichroism (CD) and intrinsic fluorescence were monitored as a function of pH and/or denaturant concentration to provide information on changes in structure and/or stability. Comparison of the denaturation transitions as measured by CD and fluorescence may elucidate the nature of these structural changes.

3.3.1 Structural analysis of IL-2 and 2D1 as a function of pH

The secondary structure of wild-type IL-2 was analyzed by CD and compared to that of the 2D1 analog. Far-UV CD data indicated that the two proteins displayed similar helical content (Figure 3.2A). No apparent changes in secondary structure were found as a function of pH within the physiological pH range of cytokine trafficking.
Tertiary structure was also studied as a function of pH using intrinsic fluorescence as a conformational probe (Figure 3.2B). An increase in intrinsic fluorescence occurred at low pH (below pH 4). No shift in the fluorescence emission wavelength maximum was observed, and therefore only the intensity of the fluorescence emission is reported. Additional data show that this transition is accompanied by an increase in hydrophobicity as measured by 1-anilinonaphthalene-8-sulfonyl acid (ANS) fluorescence. The tertiary structural transition is similar for both wild-type and the 2D1 mutant. No observable structural changes occurred for either protein in the pH range of interest between the extracellular pH of 7 and the lysosomal pH of 4.

3.3.2 Secondary structural stabilities of IL-2 and 2D1

The stability of wild-type IL-2 and the 2D1 analog were determined by measuring the secondary structural signal as a function of guanidine hydrochloride (GuHCl) concentration. The far-UV CD signal at 222 nm was followed as a measure of helical structure during titrations of GuHCl. These titrations were performed at pH values between 7 and 4 to mimic the transport of IL-2 from the cell surface to the lysosome. At neutral pH, the secondary structural transition for the wild-type IL-2 was similar to that of the 2D1 analog (Figure 3.3A & C). However, at pH 4, the secondary structural transition midpoint is lower for 2D1 compared to that of the wild-type (Figure 3.3B & C). Therefore, the analog has comparable stability to the wild-type IL-2 at neutral pH, but is less stable than wild-type IL-2 under acidic conditions.

The secondary structural transition for IL-2 is dependent on pH. For wild-type IL-2, the stability increases under acid conditions (Figure 3.3C). Although the 2D1 mutant has a similar pH dependence, the effect is less pronounced. The 2D1 variant has a
lower stability than wild-type IL-2 in acid and displays a minimal pH dependent shift in its secondary structural denaturation transitions.

The mutations in the 2D1 analog do not cause any identifiable structural changes, but rather a decrease in stability compared to wild-type IL-2 in acid. The analog has a lower denaturation midpoint of unfolding in acid, resulting in an altered equilibrium between the folded and unfolded forms of the molecule. The differences in stability between the wild-type and 2D1 analog as a function of pH may be related to the modified trafficking.

3.3.3 Tertiary structural stabilities of IL-2 and 2D1

Tertiary structural transitions were characterized for the wild-type and mutant IL-2 by monitoring intrinsic fluorescence as a function of denaturant concentration. IL-2 displays a linear increase in intrinsic fluorescence at low GuHCl concentrations due to the solvent conditions. At relatively high concentrations of denaturant, the fluorescence emission maximum shifts to a longer wavelength (Figure 3.4A). This red shift in emission maximum is consistent with the solvent exposure of the lone tryptophan residue (Trp 121) from within the buried hydrophobic core. Emission maximum wavelengths were plotted versus GuHCl concentration for wild-type IL-2 and the 2D1 analog. A comparison of these transitions shows that wild-type IL-2 displays an increased tertiary structural stability than the mutant (Figure 3.4B).

3.3.4 Noncoincidence of structural transitions

Comparison of the secondary and tertiary structural transitions reveals a noncoincidence of signals (see Figure 3.4B). For both the wild-type and the 2D1 mutant, loss of helical structure occurs at a lower denaturant concentration than the exposure of
the hydrophobic core. Therefore, under partially denaturing conditions, a folding intermediate is populated having an intact hydrophobic core and disrupted helical structure. This suggests that the unfolding occurs via a multistate transition, which is a pathway more complicated than a two-state (native ↔ denatured) model.

The difference in CD and fluorescence detected transitions for wild-type IL-2 is maintained and shifted to lower GuHCl concentrations for the 2D1 analog. The mutant's structural transitions are not coincident with the wild-type IL-2 CD signal transition. This suggests that the 2D1 analog has decreased stability in both the native and intermediate states.

3.4 Discussion

Differences in intracellular trafficking cause the 2D1 analog to be more potent than wild-type IL-2 in stimulating T lymphocyte proliferation (see Chapter 2). Subtle structural disparities between the wild-type and analog as a function of pH might influence the observed trafficking dynamics. We have shown that stability differences between IL-2 and 2D1 exist under acidic conditions that mimic the endosomes where receptor/ligand sorting occurs (Figure 3.5). The 2D1 analog, having lower stability than the wild-type IL-2, has a greater population of partially unfolded intermediates and unfolded forms in equilibrium with the native state. Due to the location of the mutations, the analog has a lower binding affinity to the βγ-receptor complex destined for degradation in the lysosomes (Berndt, et al., 1994). The analog may preferentially follow the pathway of the α-subunit of the receptor, which is constitutively recycled to the cell surface. Stability differences may play a role in disrupting binding to the βγ-receptor complex and increasing affinity to the α-subunit.
The role of pH in trafficking of proteins and receptors within cells is critical for many systems. The pH dependence on receptor-ligand affinity balances the amount of ligand and receptor that are recycled to the extracellular fluid, degraded in the lysosomes, or transported across the plasma membrane (Maier and Steverding, 1996). The IL-2 analog does not introduce any ionizable residues that would disrupt ligand binding to the receptor subunits when the complex encounters the acidic endosomal compartments. However, the binding affinity of IL-2 analogs to both the α and β receptor subunits decreases considerably as the pH is reduced from 7.2 to 6.0 (see Chapter 2).

In addition to binding differences, structural perturbations at low pH may also increase the susceptibility to proteolysis in the lysosome. Since the IL-2 analog is less stable than wild-type IL-2 at low pH, one would expect the variant to be more susceptible to proteolysis. However, data indicate that the analog undergoes greater recycling of intact ligand compared to wild-type IL-2 (see Chapter 2). The IL-2 analog may completely avoid exposure to the lysosomal proteases by remaining bound to the α-receptor subunit, thereby undergoing recycling to the extracellular space. Another possibility is that the IL-2 analog interacts with vesicular membranes, providing protection from proteolysis. Slight differences in the free energy of unfolding of the IL-2 analog in acid can lead to significant differences in membrane interactions and receptor-ligand binding affinity. Since only stability differences rather than structural changes in acid were observed, it is likely that interactions with either receptor subunits or other cellular components play a role in protecting the analog from degradation.

Structural analysis of related cytokines has frequently identified a molten globule intermediate with partial disruption of tertiary structure and intact native-like secondary
structure (Hamburger, et al., 1998; Ptitsyn et al., 1990; Brems and Havel, 1989; Cleland and Wang, 1991). An apparent difference in the IL-2 folding intermediate is that the secondary structural transition precedes the disruption of tertiary structure, as indicated by the fluorescence shift. In comparing changes in secondary and tertiary structure, the exact location of the tryptophan moieties is critical. The probe for monitoring tertiary structure of IL-2 is a single tryptophan residue in the hydrophobic core. For example, the sole tryptophan residue may reflect the environment of a highly stable core region that is more stable than certain helical segments. In addition, subtle tertiary structural changes may occur at lower denaturant concentrations than the disruption of helices that are undetected by the fluorescence of the sole tryptophan residue.

During the early kinetic stages of refolding of small globular proteins, a hydrophobic collapse occurs as the extended polypeptide chain protects its hydrophobic residues from exposure to the aqueous environment (Kim and Baldwin, 1990). In the equilibrium denaturation of IL-2, the partially unfolded state is similar to this collapsed form with an intact hydrophobic core without defined native-like structure. Thus, the denaturation experiments identify equilibrium conditions that populate species resembling kinetic intermediates in the refolding pathway. The location of the single tryptophan in IL-2 appears to be conveniently located to provide key spectral information regarding the hydrophobic core of an important equilibrium intermediate and possibly a critical early kinetic intermediate.

The dramatic effect of specific amino acid mutations on protein folding and stability has been documented for several proteins (Mitraki et al., 1991; Brems et al., 1988; Wetzel, 1994). Minor changes in the stability and solubility of partially folded
polypeptide chains can have significant effects on the global properties of the protein. The 2D1 analog described in these studies underscores the specificity of mutations in regulating protein stability, receptor-ligand interactions, and trafficking of cytokines. By engineering therapeutic proteins, the resulting analogs may prove to have greater stability, solubility, and/or efficacy.
Figure 3.1 Ribbon diagram of IL-2.

Note the binding site of the α receptor subunit to the first loop region, the β subunit to the N-terminal helix, and the γ subunit to the C-terminal helix. IL-2 analog mutations L18M and L19S, which are found in the β subunit binding site, are also shown.
Figure 3.2 Structural analysis of wild-type IL-2 and 2D1.

A. CD spectrum of wild-type IL-2 and 2D1 at pH 4. The pH titration was performed at 0.05 mg/mL from pH 8.3 to 3.4. For clarity, only data at pH 4 are shown.

B. Intrinsic fluorescence of wild-type IL-2 and 2D1 as a function of pH. Intrinsic fluorescence emission wavelength maxima were recorded at an excitation wavelength of 280 nm, and a pH titration was performed from pH 2 to 7 at a protein concentration of 0.05 mg/mL.
Figure 3.3 Secondary structural stability of wild-type IL-2 and 2D1.

Secondary structural transitions of wild-type IL-2 and the 2D1 analog as determined by equilibrium denaturation, monitoring the far-UV CD signal at 222 nm as a function of GuHCl concentration. IL-2 was at a protein concentration of 0.05 mg/mL at pH 7 (A) through pH 4 (B). The denaturation midpoints for wild-type IL-2 and 2D1 for pH 7 through pH 4 are shown in C. Error bars represent the reproducibility of the denaturation transitions for multiple experiments.
Figure 3.4 Structural transitions of wild-type IL-2 and 2D1.

A. Intrinsic fluorescence emission spectra for wild-type IL-2 at GuHCl concentrations of 4.0 M, 4.5 M, 5.0 M, and 5.5 M. Emission spectra were recorded at an excitation wavelength of 280 nm.

B. Far UV-CD data (circles) and intrinsic fluorescence (squares) of wild-type IL-2 (filled symbols) and IL-2 analog (open symbols) as a function of GuHCl. Far-UV CD data was measured at 222 nm. All samples were at a protein concentration of 0.05 mg/mL in pH 4 buffer.
Figure 3.5 IL-2 trafficking and pH.

The IL-2 ligand binds to the receptor complex at the cell surface (neutral pH), and is internalized within clathrin-coated pits. A branch in the trafficking pathway of IL-2 occurs in the early endosome (pH 6) wherein the ligand may either remain bound to the βγ-receptor complex and enter the late endosomes (pH 5.5) followed by degradation in the lysosome (pH 4), or may follow the route of the α-receptor subunit and get recycled.
Chapter 4: Mathematical Model of Trafficking and Potency

Multi-subunit cytokine receptors are ubiquitous in various hematopoietic cell types. These receptor chains can serve divergent functional roles with respect to both intracellular signaling and post-endocytic trafficking. Differential sorting outcomes can have dramatic effects on both receptor down-regulation and ligand availability, impacting the functional response of cells to exogenous cytokines. In this chapter, we describe a mathematical model that relates ligand-receptor trafficking properties to T cell proliferation in response to interleukin-2 (IL-2). The model comprises kinetic equations describing binding, internalization, and post-endocytic sorting of IL-2 and the IL-2 receptor complex as well as an experimentally derived dependence of cell proliferation rate on these properties. Our results predict reduced ligand depletion and a concomitant increase in T cell mitogenesis for IL-2 mutants displaying either slower binding kinetics than wild-type, or reduced affinity to the βγ IL-2 receptor complex at endosomal pH. The steady-state sorting behavior of an IL-2 mutant previously shown to exhibit enhanced mitogenic potency is accurately predicted from the model, as are criteria for designing IL-2 variants with further improvements in bioactivity. A quantitative understanding of receptor-mediated ligand depletion in multi-subunit receptor systems could provide molecular design criteria for advancing biomedical applications such as in vivo cytokine delivery conditions, and optimization of serum-free media formulations for biotechnological use.
4.1 Introduction

A complex network of molecular and cellular events underlies the diverse responses of the mammalian immune system. Cytokines play an essential role in this system by tightly regulating communication among cells, and have thus been explored as a means of therapeutic intervention (Nicola, 1994). These proteins exhibit their functionality by binding to specific cell-surface receptors with very high affinity (Paul and Seder, 1994). Therefore, optimum response requires delivery of cytokines to target cells at very low concentrations ($\sim 10^{-10}-10^{-12}$ M). These low levels are difficult to sustain due to rapid clearance via physiological mechanisms in the kidney, liver, and/or lung. In addition, negative feedback is often provided via specific endocytosis of ligand upon binding to its cognate receptor (Trowbridge, 1991). This down-regulatory mechanism can be a significant cause of systemic cytokine depletion, as has been demonstrated with GCSF (Cohen, 1993). This creates the need to deliver dangerously high doses in order to maintain reasonable in vivo cytokine concentrations (Metcalf and Nicola, 1995; Ono, 1994). These doses often produce toxic, sometimes lethal, side effects (Vial and Descotes, 1995; Kung, et al., 1993).

Interleukin-2 (IL-2) was one of the first cytokines isolated, and its role is crucial in governing the expansion and differentiation of several hematopoietic cell types (Theze, et al., 1996; Smith, 1988). It was originally termed T cell growth factor (TCGF) based on its critical role as a proliferative signal for CD4$^+$ T-lymphocytes, and has been involved in numerous clinical studies in the treatment of AIDS (Khatri et al., 1998; Ghezzi, et al., 1997; Kinter and Fauci, 1996; Kovacs, et al., 1996; Jacobson, et al., 1996). IL-2 has also been utilized as an adjuvant for cancer therapy based on its stimulation of CD8$^+$ or cytotoxic T cells (Noble and Goa, 1997; Jeal and Goa, 1997; Vlasveld and
Rankin, 1994; Whittington and Faulds, 1993). Unfortunately, systemic toxicity has been a major setback in broadening FDA approval of IL-2. The pleiotropic effects of IL-2 on multiple hematopoietic cell types can create undesired stimulation of cell types other than T cells, since various cells express different isoforms of the IL-2 receptor. Cell receptor-level pharmacodynamic issues are thus essential in understanding the nature of IL-2's effects in vivo, and in expanding its therapeutic applicability (Anderson and Sorenson, 1994; Smith, 1993). In addition, cell-level pharmacokinetics requires consideration of receptor-mediated endocytic turnover cytokine molecules that, over time, can lead to depletion of ligand from the extracellular medium (Lauffenburger, et al., 1998).

The IL-2 receptor on activated T-lymphocytes consists of α, β, and γ subunits, the latter two of which are shared among several other interleukins (Bazan, 1990; Cosman, et al., 1990). The α subunit is exclusive to IL-2, and its expression levels are 10-100 fold greater than those of β or γ on antigen-activated T cells (Theze, et al., 1996). The α subunit enhances the affinity of the IL-2R complex for soluble IL-2 (Gutgsell and Malek, 1994; Forsten and Lauffenburger, 1994; Kuziel, et al., 1993) and is signaling-deficient, as it possesses only 13 residues in its cytoplasmic domain (Nikaido, et al., 1984). The β and γ subunits have both been implicated in specific intracellular signaling pathways that are activated upon IL-2 binding (Sugamura et al., 1996; Friedman, et al., 1996). The heterotrimeric IL-2 receptor binds its ligand with high affinity (K_D=10 pM), and then undergoes rapid internalization and specific degradation of both IL-2 and the β and γ subunits (Subtil and Dautry-Varsat, 1998; Morelon and Dautry-Varsat, 1998; Subtil, et al., 1997). The β and γ subunits undergo specific lysosomal degradation along with bound IL-2, while the α subunit is constitutively recycled following postendocytic sorting
(Hemar, et al., 1995). Therefore, receptor/ligand trafficking dynamics should influence the potency of IL-2 to at least as significant a degree as receptor binding affinity, as has been illustrated in the EGF ligand-receptor system (Reddy, et al., 1996; Reddy, et al., 1996).

The effects of post-endocytic trafficking dynamics on potency can be counter-intuitive with respect to the binding affinity of ligand-receptor interactions. For example, an EGF mutant was shown to be a more potent mitogen than wild-type EGF despite possessing a 50-fold lower binding affinity to the EGF receptor (Reddy, et al., 1996). This result stemmed from a decreased internalization rate and increased recycling rate for both ligand and receptor, opposing the signal-attenuating effects of ligand depletion and receptor downregulation. An IL-2 variant with increased affinity for the α chain but reduced affinity for the βγ-complex demonstrated greater potency based on similar effects in a different system (see Chapter 2). This result is also counter-intuitive because the mutation increased the ligand’s affinity for the subunit not directly involved in signal transduction while decreasing the affinity for the chains that generate a mitogenic signal.

A quantitative relationship between IL-2/IL-2R binding and T cell proliferation was initially proposed by Robb (Robb, 1982). Smith and co-workers later showed that only three elements were crucial for cell cycle progression in T cells: IL-2 concentration, IL-2R density, and the duration of the ligand/receptor interaction (Smith, 1995; Cantrell and Smith, 1984; Herzberg and Smith, 1987). This suggested that the dynamics of the IL-2/IL-2R interaction were crucial determinants of cellular function, and numerous studies have been undertaken to elucidate the nature of binding, internalization, and trafficking in this system (Subtil et al., 1998; Subtil and Dautry-Varsat, 1998; Subtil, et
al., 1997; Morelon and Dautry-Varsat, 1998; Subtil, et al., 1994; Duprez, et al., 1994; Morelon, et al., 1995; Duprez, et al., 1992; Duprez and Dautry-Varsat, 1986). Detailed mathematical models describing alternative mechanisms of IL-2 binding and endocytosis have been proposed, as have separate models for cytokine-induced T cell proliferation (Forsten and Lauffenburger, 1992; Goldstein, et al., 1992; Borisova and Kuznetsov, 1996; Borisova et al., 1998; Burke et al., 1997). Much of the experimental and computational work to date, however, has considered IL-2 trafficking independent from quantitative growth studies.

Our goal is to develop a quantitative framework for directly relating molecular-level binding and trafficking events to cell-level function, rather than considering these as separate entities. This approach has yielded insight into the events underlying mitogenic responses of fibroblasts to the EGF family of ligands, thus aiding in the molecular manipulation of cellular responses (Knauer et al., 1984; Starbuck et al., 1990; Starbuck and Lauffenburger, 1992; Reddy, et al., 1996; Reddy, et al., 1996). We extend similar analyses to the more therapeutically relevant IL-2 system, and include the added level of complexity associated with its multi-subunit receptor. The model follows from experimental work wherein detailed binding, trafficking, and mitogenesis studies were performed within a single cell system. This work demonstrated that a double mutant of IL-2, Leu18Met/Leu19Ser (termed 2D1), displayed reduced endocytic degradation when compared to wild-type IL-2 (WT), and that this effect was due to enhanced ligand recycling. The continuous flow of ligands through intracellular sorting pathways thus led to greater sustenance of 2D1 concentrations in cell culture, and increased T-lymphocyte proliferation (see Chapter 2). The objective of our modeling work is not to “fit” these
experimental results, but to elucidate the essential molecular properties that should be considered in a ligand-based protein engineering approach to altering cell function. The insights derived from this model can aid in interpreting our experimental trafficking and mitogenesis results, while highlighting receptor/ligand-binding parameters that predict enhanced ligand potency in biotechnological applications.

4.2 Mathematical Model

Our ligand depletion model describes the fate of an IL-2 analog upon binding, receptor-mediated internalization, and post-endocytic sorting via the heterotrimeric IL-2 receptor (Figure 4.1). A central feature of the model is its ability to simultaneously account for IL-2-mediated molecular and cellular events that occur on multiple time scales. These include ligand-receptor binding events that transpire on the scale of seconds/minutes, endocytosis and sorting that develops over minutes/hours, and the proliferative response of cells cell-surface IL-2/IL-2R complexes that manifests over hours/days in the presence of extracellular ligand (Smith, 1995). The model therefore illustrates the balance between cell receptor-mediated degradation of extracellular IL-2 and the cell's mitogenic response to intact ligand in the surrounding medium.

This dynamic model simulates the fate of ligands and receptors upon introduction of a bolus concentration of ligand into the extracellular medium at time zero. In the absence of ligand, the number of cell-surface and internal receptors represents a balance between constitutive internalization (k_i) and new receptor synthesis (V_s) (Wiley and Cunningham, 1981). The presence of ligand causes both specific endocytosis of ligand-receptor complexes (k_e) and up-regulation of receptor synthesis via intracellular signaling cascades (k_syn). Both of these processes have been observed and quantified in IL-2/T cell
systems (Chang, et al., 1996; Smith and Cantrell, 1985). The binding of IL-2 to the high-affinity (αβγ) receptor complex has been well characterized, and resultant association (k\text{f}) and dissociation (k\text{r}) rate constants are indicated in Figure 4.1 (Smith, 1989; Wang and Smith, 1987).

We utilize the common sorting behavior of the β and γ subunits by designating receptors (R) as the IL-2Rβγ complex throughout the model. This assumption does not confound the analysis of ligand (L) binding with cell surface receptors to form ligand/receptor complexes (C), despite the fact that IL-2Rα is expressed in excess of the β and γ chains. The α, β, and γ chains have been shown to co-localize even in the absence of IL-2 on the KIT-225 human T cell line (Damjanovich et al., 1997). Although IL-2Rα can bind IL-2 with a K\text{D}=10 \text{ nM}, at the ligand concentrations considered (<200 \text{ pM}) the vast majority of these receptors are unbound. In addition, IL-2Rα is internalized only as part of high-affinity (αβγ) receptors in the presence of IL-2, and cannot internalize IL-2 by itself (Hemar, et al., 1995; Fujii, et al., 1986; Hatakeyama et al., 1989; Nabholz et al., 1987).

Non-specific or fluid-phase uptake of ligand is neglected, as this effect is typically apparent only at ligand concentrations greater than 10 \text{ nM} (Lauffenburger and Linderman, 1993). Thus, internalization of IL-2 occurs solely via receptor-mediated endocytosis (Sorkin and Waters, 1993; Trowbridge, et al., 1993). Although this process is saturable at high ligand concentrations, the low density of high affinity IL-2 receptors on T cells makes saturation an unlikely phenomenon (Lund, et al., 1990). Receptor-mediated endocytosis of IL-2 is thought to transpire both through clathrin-coated pit and non-coated pit mechanisms, yet the model incorporates a lumped internalization rate
constant to account for this process (Borisova and Kuznetsov, 1996; Subtil, et al., 1994; Wiley and Cunningham, 1982).

The heterotrimERIC IL-2 receptor displays unique sorting behavior, and the events that underlie this behavior are central to the model’s structure. As stated in the Introduction, growth factor/receptor complexes are generally targeted for lysosomal degradation in lieu of the default recycling pathway upon undergoing postendocytic sorting. Experimental evidence indicates that the components of the IL-2/IL-2R complex undergo differential sorting, wherein IL-2Rα is recycled to the cell surface while IL-2, IL-2Rβ, and IL-2Rγ all remain associated en route to lysosomes (Hemar, et al., 1995). Specific regions have recently been identified in the cytoplasmic tails of both the IL-2Rβ and γ chains that mediate specific sorting of these subunits to degradation (Subtil and Dautry-Varsat, 1998; Morelon and Dautry-Varsat, 1998; Subtil, et al., 1997). No such signals exist in the short 13-residue intracellular tail of IL-2Rα, allowing it to follow the default-recycling pathway upon dissociation from the IL-2/IL-2R complex (Nikaido, et al., 1984). These results implicate potential accessory proteins that may be involved in endosomal retention and subsequent lysosomal sorting of ligand-bound IL-2Rβγ complexes, as has been postulated in the EGF system (Kurten, et al., 1996; French and Lauffenburger, 1996). Although this EGF pathway has been shown to be saturable at high intracellular ligand concentrations, the relatively low expression levels of IL-2Rβγ on T cells makes it unlikely that the IL-2 system would exhibit such saturation behavior (French, et al., 1994; French, et al., 1995). Interleukin-2 sorting outcomes are thus derived solely from association (k_ε) and dissociation (k_Re) rate constants that depict ligand binding to the IL-2Rβγ complex in the endosome. Consideration of the kinetics of
these interactions is essential since ligand enters endosomes as a component of bound ligand/receptor complexes, but can thus dissociate from and rebind to receptors under endosomal conditions (Schiff et al., 1984). The binding of IL-2 to both the IL-2Rαβγ complex and the IL-2Rβγ heterodimer has been well-characterized at cell-surface pH, as each is expressed on distinct hematopoietic cell types (Smith, 1989; Waldmann, 1989).

Upon localization to endosomes, IL-2Rα is assumed to be dissociated from the IL-2/IL-2Rβγ complex as has been observed experimentally (Hemar, et al., 1995). Free βγ heterodimer (termed Rf) and IL-2 bound to this heterodimer (Cf) are assumed to undergo degradation at a rate described by k_h. The rate of generation of degraded ligand (L_d) is then calculated in order to determine recycling fractions, or the percentage of ligand entering the cell that is released intact following endosomal sorting. Free intracellular IL-2 (L_s) is considered to be subject to the same fate as intracellular IL-2Rα, namely recycling (k_s). Thus, the sorting outcome of an internalized IL-2 molecule is described entirely by the kinetics of binding to IL-2Rβγ and the competing rates of recycling and degradation. Recycling and degradation rate constants represent the experimentally accessible lumped parameters that describe the translocation of molecules from sorting endosomes to late (recycling) endosomes or lysosomal compartments, respectively. The detailed molecular events underlying endosomal sorting have been modeled elsewhere and are not the focus of the present work (French and Lauffenburger, 1997; French and Lauffenburger, 1996). Rather, this model seeks to relate the causal effects of endocytic ligand turnover on the functional response of T cells to physiologic concentrations of IL-2, as these cells are known to rapidly deplete IL-2 via this mechanism (Smith, 1989; Fujii, et al., 1986; Robb, et al., 1981).
Ligand-receptor complexes on the cell surface are assumed to stimulate both new
receptor synthesis \( (k_{syn}) \) and mitogenesis, as both have been quantified experimentally in
IL-2/T cell systems (Robb, et al., 1984; Robb, et al., 1981). Up-regulated expression of
IL-2Rα subunits in response to exogenous IL-2 has been measured in activated peripheral
blood lymphocytes, and the model includes a first-order rate constant to describe this
process (Smith and Cantrell, 1985). The mitogenic response of T cells to IL-2 is more
difficult to capture quantitatively, as cell cycle events occur over much longer time scales
than the ligand-receptor binding interactions that initiate intracellular signaling cascades.
Data obtained using the KIT-225 human leukemic T cell line implicate a mechanism
whereby the growth rate is minimal below a threshold number of IL-2/IL-2R complexes
per cell, then increases rapidly to a relatively constant value above this minimum number
of complexes required to elicit a response (see Figure 4.2). This mechanism was
hypothesized by Cantrell and Smith (Cantrell and Smith, 1984; Smith, 1995), and has
also been observed in studies relating the mitogenic response of fibroblasts to EGF
(Reddy, et al., 1996; Starbuck, et al., 1990). The present model incorporates this
threshold-type mechanism in relating the change in cell density \( (Y) \) to cell-surface
complex number \( (C_s) \). This clearly neglects the complex molecular events underlying
growth factor induced mitogenesis, but the model does provide a means of relating the
amount of active IL-2 to the primary cellular function induced by this ligand.

Corresponding to the concepts outlined above, the following equations govern the
dynamic processes diagrammed in Figure 4.1. All symbols have been defined above,
with the subscripts \( s \) and \( i \) referring to the cell surface and intracellular compartments,
respectively.
\[
\frac{dR}{dt} = -k_f \cdot L[t] \cdot R_s[t] + \left( k_r + k_{syn} \right) \cdot C_s[t] - k_t \cdot R_s[t] + V_s
\]  
(1)

\[
\frac{dC}{dt} = k_f \cdot L[t] \cdot R_s[t] - \left( k_r + k_e \right) \cdot C_s[t]
\]  
(2)

\[
\frac{dR_i}{dt} = -k_{fe} \cdot L_i[t] \cdot R_i[t] + k_{re} \cdot C_i[t] + k_t \cdot R_s[t] - k_h \cdot R_i[t]
\]  
(3)

\[
\frac{dC_i}{dt} = k_{fe} \cdot L_i[t] \cdot R_i[t] - \left( k_{re} + k_h \right) \cdot C_i[t] + k_e \cdot C_s[t]
\]  
(4)

\[
\frac{dL}{dt} = \left( -k_{fe} \cdot L[t] \cdot R_s[t] + k_{re} \cdot C_i[t] \right) / \left( V_e \cdot N_A \right) - k_s \cdot L[t]
\]  
(5)

\[
\frac{dL_i}{dt} = k_h \cdot C_i[t]
\]  
(6)

\[
\frac{dL}{dt} = \left( -k_f \cdot L[t] \cdot R_s[t] + k_r \cdot C_s[t] + k_s \cdot L_i[t] \cdot V_e \cdot N_A \right) \cdot Y[t] / \left( N_A \right)
\]  
(7)

\[
\frac{dY}{dt} = \text{Max}\left\{600 \cdot C_s[t] / (250 + C_s[t]) - 200, 0\right\} \cdot 10^3
\]  
(8)

### 4.3 Parameter Evaluation and Computational Procedure

Base values for the parameters used in the model are listed in Table 4.1. All values are derived from previously reported literature data, or from the experimental work related to the development of this model. The previously described IL-2 analog, termed 2D1, is considered in parallel with wild-type to explore potential effects of ligand-based alterations in receptor binding affinities on post-endocytic trafficking outcomes.

The dissociation constants describing the binding of this variant to the high-affinity (αβγ) IL-2 receptor have been reported (Berndt, et al., 1994). The kinetics of wild-type IL-2/IL-2R binding were measured using radioreceptor assays on cell membrane preparations, and the resultant dissociation rate constant is used as the base value at cell
surface pH (Wang and Smith, 1987). Association rate constants for IL-2 and 2D1 binding to the IL-2R\(\alpha\beta\gamma\) complex are estimated by dividing this base value of the dissociation rate constant by the \(K_D\) of each ligand, respectively. In the analysis that follows, dissociation rate constants are varied from 0.00138 min\(^{-1}\) to 0.138 min\(^{-1}\) independent of the \(K_D\) in order to separate the kinetics of binding from equilibrium affinities.

The pH in sorting endosomes is decreased relative to the cell surface (~6.0 vs. 7.2), and this pH drop was shown to decrease the affinity of IL-2 ligands for both the \(\alpha\) and \(\beta\) IL-2R subunits (see Chapter 2). Receptor-ligand binding kinetics in endosomes are thus estimated by multiplying the dissociation rate constant at the cell surface by a factor greater than one. This factor differed between IL-2 and the 2D1 variant, and values determined from experimental data for each ligand are included in the base model comparisons. The interaction of ligand with the IL-2R\(\alpha\beta\gamma\) complex is most relevant in approximating sorting outcomes, thus the endosomal association rate constant is calculated by dividing the endosomal off-rate by the \(K_D\) describing binding to this intermediate-affinity receptor. These \(K_D\) values, though measured experimentally for IL-2 and 2D1 at cell surface pH, are approximated at endosomal conditions by assuming that the relative affinity between the two ligands is roughly equivalent at pH 6.0 and pH 7.2 (Berndt, et al., 1994).

A dynamic balance between biosynthesis and constitutive endocytosis defines the total number receptor at steady state, even in the absence of ligand. Hemar and co-workers quantified the constitutive internalization rate constant for IL-2 receptors on T cells by Hemar by observing receptor decay from the cell surface while protein synthesis
was chemically inhibited (Hemar, et al., 1994). In similar experiments, the reappearance of receptors was probed with radiolabelled IL-2 immediately following trypsinization of the cell surface (Duprez and Dautry-Varsat, 1986). This reappearance was quantified over time to yield a constitutive receptor biosynthesis rate. In the presence of IL-2, overall receptor turnover is influenced by these constitutive rates and specific events that occur upon IL-2 binding to its receptor. IL-2 has been shown to induce synthesis of IL-2Rα, while the overall rate of IL-2Rαβγ receptor endocytosis is enhanced upon binding. This allows the cell to attenuate its response to the signaling competent IL-2Rβγ complex while retaining the capacity to bind IL-2. The result of these competing effects is the maintenance of a relatively constant population of high-affinity receptors on the cell surface, though the greater number of IL-2Rα subunits creates a net increase in the number of measurable receptors on the cell surface (Depper, et al., 1985; Smith, 1989; Duprez, et al., 1988). Smith and Cantrell simultaneously measured receptor synthesis and endocytosis using receptor subunit specific antibodies, and the net induced receptor synthesis rate is included in the model (Smith and Cantrell, 1985).

The intracellular trafficking parameters (kₓ and kₘ) describe the rate of movement of components from hypothetical post-sorting recycling and degradation compartments, respectively. These transport rates have been shown to be dependent only on the endocytic apparatus, and not on specific ligand-receptor systems (Ward et al., 1989). Thus, the recycling rate constant reported for the constitutively recycled transferrin receptor was used as an estimate of kₓ (Ghosh et al., 1994). Since we are considering kₘ as a lumped parameter that represents the outcome of potentially complex sorting events, the specific degradation rate previously reported for IL-2 receptors in T cells is employed
in the model (Duprez and Dautry-Varsat, 1986). Consideration of the sorting events undergone by soluble ligands in the endosome requires an estimate of the endosomal volume within the cell. French compiled the results of several quantitative morphological studies, and these yielded the estimate of $10^{-14}$ L/cell used in our model (French and Lauffenburger, 1996).

Model equations are solved numerically using Mathematica (version 4.0, Wolfram Research) with nonzero initial conditions including the extracellular ligand concentration and cell density at the start of a hypothetical experiment. The initial cell surface receptor number is \(~1500\) per cell as defined by the estimates of $V_s$ and $k_i$ shown in Table 4.1. This is representative of the number of high affinity IL-2 receptors found on both activated T lymphocytes and on the KIT-225 cell line used in previous experiments. Initial internal receptor numbers is similarly fixed at \(~30\) per cell by the estimates of $V_s$ and $k_h$. Simulations are carried for five days to mimic a ligand depletion/cell proliferation experiment. Intact ligand concentration ($L$), cell density ($Y$), and cell-surface complex number ($C_s$) are calculated throughout the simulation.

Sorting experiments are mimicked by performing simulations over a range of input ligand concentrations for 180 minutes, at which time sorting reaches steady-state following the initial perturbation due to introduction of extracellular ligand (French, et al., 1994; French, et al., 1995). The values of $R_s$, $R_i$, $C_i$, and $L_i$ are recorded and used as inputs into second simulation wherein these values serve as initial conditions along with zero extracellular ligand concentration (see Chapter 2). Degraded and intact ligand concentrations are recorded throughout this fifteen-minute simulation and used to
calculate recycling fractions, or the amount of ligand sorting to recycling vs. the amount sorted to degradation.

4.4 Results

4.4.1 Comparison of base model predictions to experimental results with WT IL-2

The goal of this modeling effort is to accurately link the effects of receptor-mediated ligand degradation to T cell proliferation in a quantitative fashion. We have previously measured these two events simultaneously using the IL-2 dependent KIT-225 human T cell line. These cells rapidly degraded IL-2 via receptor-mediated endocytic depletion, and this effect was observed at both saturating and half-saturating concentrations with respect to the high-affinity IL-2 receptor. The kinetic parameters used in the model that describe binding, trafficking, and signaling events were accumulated from a variety of experimental sources. Therefore, in order to test the ability of our model to accurately predict the effects of molecular parameters on cell-mediated ligand depletion simulations were performed using the trafficking parameters shown in Table 4.1 including those specific for wild-type IL-2. The experimentally observed depletion behavior is accurately predicted at both high and low ligand concentrations (Figure 4.3).

Results of five-day proliferation experiments employing 10 pM and 100 pM IL-2 are shown along with model predictions in Figure 4.4. The model incorporates a threshold type dependence of cell growth rate on cell-surface receptor/ligand complex number (see Figure 4.2). This functionality predicts negligible growth below a fixed value of $C_s$, and a relatively constant increase in cell density as long as $C_s$ remains above this value. This behavior is shown clearly in Figure 4.4, as the higher ligand
concentration serves to maintain $C_s$ at a value above the growth response threshold while little proliferation is observed following exposure to an initial IL-2 concentration of 10 pM. Little cell growth was observed experimentally during the first two days due to the quiescent state of the cells following starvation of IL-2 prior to the start of the experiments. Although this artifact is not accounted for in the model, predicted growth rates are quantitatively similar to those observed experimentally after day two.

**4.4.2 Effects of ligand-receptor binding kinetics**

The model describes a set of inter-related transport processes, beginning with the ligand-receptor binding interaction at the cell surface. Alterations in the kinetics of binding should thus be expected to influence both the post-endocytic ligand turnover rate and the lifetime of cell-surface complexes that signal for both new receptor synthesis and cell proliferation. The effects of altered binding kinetics on the receptor-mediated depletion of ligand at an initial concentration of 10 pM are shown in Figure 4.5A. Roughly half of the available cell surface receptors are initially bound at this concentration, since the $K_D$ of the ligand is fixed at 11.1 pM. A decrease in the dissociation rate (and commensurate decrease in the association rate) leads to enhanced stability to endocytic degradation by cells at an initial density of $2.5 \times 10^8$ per liter. This effect is explained by observing the levels of cell surface ligand-receptor complexes as illustrated in Figure 4.5B. Since ligand can undergo depletion only upon receptor binding, heightened levels of $C_s$ lead to greater degradation. Concomitantly, the value of $C_s$ declines more rapidly for greater initial complex numbers. These effects are apparent, though less pronounced when simulations are performed with an initial ligand concentration of 100 pM. Decreased kinetic rate constants slightly improve ligand
stability in culture (Figure 4.6A). Cell surface complex numbers rapidly reach a steady state under these conditions, and the heightened stability of ligand again correlates with decreased C₅ levels (Figure 4.6B). Available receptors are nearly saturated at this initial concentration, thus the sensitivity of the system relative to small decreases in ligand concentration is diminished.

The predicted growth response of T cells to IL-2 analogs with altered binding kinetics is shown for initial ligand concentrations of 10 pM and 100 pM (Figure 4.7A and 4.7B, respectively). As cells deplete ligand, C₅ can fall below the threshold level required to stimulate cell division. This effect is realized under conditions of low initial ligand concentration, as shown in Figure 4.7A. At an initial IL-2 concentration of 100 pM, C₅ never falls below the minimum value during the five-day simulation, and cell growth is thus maintained at a relatively constant rate (Figure 4.7B). Although depressed binding kinetics lead to enhanced ligand stability, the greater half-life of intact IL-2 does not correspond to increased proliferation in this case. This counterintuitive result illustrates the dual role of cell surface ligand-receptor complexes: as vehicles for the down-regulation of extracellular ligand, and as components that stimulate the cell’s proliferative response.

4.4.3 Effects of endosomal ligand-receptor binding affinity

The characteristics of the heterotrimeric IL-2 receptor are unique in that its subunits are subject to distinct post-endocytic sorting outcomes. The α subunit recycles to the cell surface, while the IL-2/IL-1Rβγ complex is routed to lysosomal degradation (Hemar, et al., 1995). Recycling is the default pathway for membrane-bound components following internalization, implicating a specific endosomal retention mechanism for the
IL-2/IL-Rβγ complex (Hopkins, 1992; French and Lauffenburger, 1996; Subtil, et al., 1998; Subtil, et al., 1997). Therefore, upon internalization of the IL-2/IL-2Rαβγ complex and subsequent dissociation of the α chain, model predictions of ligand sorting outcomes incorporate the binding affinity of IL-2 for the βγ complex at endosomal pH. The $K_D$ for binding to this complex has been measured for both wild-type IL-2 and the 2D1 analog at cell surface conditions, as has the pH-sensitivity of the affinity of these ligands for the α and β chains individually (Berndt, et al., 1994; Fallon et al., 1999). The effects of changes in this binding affinity on receptor-mediated ligand depletion are shown for initial ligand concentrations of 10 pM and 100 pM (Figure 4.8A and 4.8B, respectively). Cell surface receptor-ligand binding affinities are unchanged in these simulations. Decreasing affinity for the lysosomally routed βγ complex in endosomes leads predictably to improved stability of ligand to cell-mediated degradation. The model is insensitive to whether ligand is free in the endosome or bound to IL-2Rα, and assumes both entities are routed solely to recycling.

Cell proliferation is superior in response to ligands exhibiting decreased affinity to the IL-2Rβγ complex in endosomes. The effects of ligand depletion on cell growth are most apparent for initial ligand concentrations of 10 pM (Figure 4.9A). Proliferation in the presence of 100 pM ligand is relatively insensitive to the changes in endosomal binding affinity, indicating that $C_s$ never falls below the threshold level required for growth in this scenario (Figure 4.9B). As observed in the simulations incorporating altered cell surface binding kinetics, cells are most sensitive to changes in the levels of stimulatory ligand when its concentration is near the $K_D$ describing the ligand-receptor interaction.
4.4.4 Ligand recycling

Post-endocytic sorting forms the mechanistic basis for differences in receptor-mediated ligand depletion. The steady state sorting behavior of a given ligand can be described by its recycling fraction. A recycling fraction is defined as the ratio of ligand that undergoes post-endocytic recycling to all the ligand that leaves the sorting endosome and undergoes either degradation or recycling (French, et al., 1994). We have previously estimated endosomal sorting outcomes by directly measuring the ratio of intact labeled ligand in the medium to the total labeled ligand during 15-minute chase periods after the sorting process reaches steady state (see Chapter 2). Recycling fractions previously obtained from steady-state sorting experiments performed on wild-type IL-2 and 2D1 are shown in Figure 4.10A. Data are plotted versus the intracellular ligand concentration (number per cell) rather than the incubation concentration. This allows sorting events to be considered independent of internalization events. Simulated sorting experiments employing the base values for IL-2 and 2D1 (Table 4.1) were performed as described in "Parameter evaluation and computational procedure," and results are shown in Figure 4.10B. For comparison with experimental data, simulated recycling fractions are plotted versus the total intracellular ligand concentration (L_i+C_i, number per cell). Qualitative agreement is observed between model and experiment, though the model underestimates the recycling fraction of the 2D1 analog. The model predicts an increase in recycling as the intracellular ligand concentration increases. This effect has been observed in the EGF system wherein the endosomal retention machinery can become saturated in cells overexpressing the EGF receptor, leading to increased recycling fractions (French and Lauffenburger, 1997; French, et al., 1994).
In order to more directly compare the experimental results with model predictions, recycling fractions are plotted for a fixed intracellular ligand concentration of 200 per cell (Figure 4.11A). This again illustrates the model's underestimation of the recycling fraction seen experimentally for the 2D1 analog. The $K_D$ for binding to the IL-2Rβγ complex at endosomal pH has not been measured directly for either wild-type IL-2 or the 2D1 mutein, and this parameter should have tremendous influence on ligand sorting outcomes. The endosomal $K_D$ was varied over several orders of magnitude, and simulated steady state sorting experiments were performed. Recycling fractions at a fixed intracellular ligand concentration of 200 per cell are shown in Figure 4.11B. These results suggest that an order of magnitude difference in endosomal binding affinity can account for the relative sorting behavior of IL-2 and the 2D1 variant. This is indeed greater than the factor of three difference assumed in the base model. Differences in the pH sensitivity of binding between members of the EGF family of ligands have been shown to directly impact trafficking outcomes in this system (French, et al., 1995).

4.5 Discussion

We have developed a model that predicts the effects of modified receptor-ligand binding parameters on the proliferative response of T cells to IL-2. The model resolves the counterintuitive behavior of a ligand that exhibits heightened potency without displaying increased binding affinity to its signaling receptor, as in the case of the IL-2 analog 2D1 (see Chapter 2). This behavior is explained mechanistically by changes in the ligand's sorting behavior caused by differential binding affinity to receptor subunits that are routed to either lysosomal degradation or recycling. These results also imply that the cellular response to a stimulatory ligand cannot be accurately predicted based solely
on knowledge of the ligand's receptor binding affinity at the cell surface. Ligands with equivalent affinities but dissimilar kinetics of binding can exhibit differences in both potency and stability to post-endocytic degradation according to the model. Furthermore, reduced ligand depletion does not necessarily predict enhanced potency. Increases in cell-surface complex number lead to both greater down-regulation of extracellular ligand and increased proliferation as shown in Figures 4.5 through 4.7. Assuming the growth model in Figure 4.2 holds qualitatively, an optimal ligand would be one that maintains the minimum "threshold" number of signaling complexes required to generate a proliferative response over time. A greater number of complexes than this minimum value leads only to enhanced endocytic down-regulation without heightening the growth response. Since the concentration of active ligand decreases with time, a quantitative understanding of the competing rates of cell growth and post-endocytic depletion can thus aid in predicting optimal ligand dosage rates in an in vivo or ex vivo setting.

The model illustrates the deterministic effect of endocytic sorting on the global depletion of ligand from the extracellular medium. A subtle increase in the recycling fraction of a growth factor mutant can greatly extend the analog's half-life over several days in culture since the ligand is subjected to numerous endocytic cycles during this time (see Chapter 2). Thus, consideration of endosomal sorting behavior can provide an added level of intervention for protein engineering efforts aimed at improving the potency of stimulatory ligands (Lauffenburger, et al., 1996). Although screening for re-engineered cytokine mutants exhibiting altered sorting behavior might appear daunting, the model suggests that "matrix" criteria related to multiple ligand/receptor binding properties within distinct cellular compartments can suffice in fully defining the system
(Lauffenburger, et al., 1998). For instance, the only differences between IL-2 and its 2D1 analog are their relative affinities for IL-2R subunits, and the sensitivity of these affinities to pH (see Table 4.1). These differences cause 2D1 to exhibit a higher recycling fraction than wild-type IL-2, as shown in Figure 4.10. Therefore, one could potentially screen for IL-2 variants with greater binding affinity for IL-2Rα or reduced affinity for IL-2Rβγ at endosomal pH and expect these variants to display increased recycling fractions relative to wild-type IL-2.

Depletion of stimulatory ligands by cells in culture is an important issue both in tissue engineering applications where the organization of multiple cell types is tightly controlled by extracellular cues, and in biotechnological arenas where optimal feeding conditions in mammalian cell bioreactors are desired (Saltzman, 1996; Zandstra et al., 1997). Ligand-receptor binding affinities can be readily addressed experimentally through protein engineering and screening technologies (Dang et al., 1997; Reidhaar-Olson and Sauer, 1988). Thus, the focus of parameter sensitivity analyses in the present work is on these binding interactions both at the cell surface and in sorting endosomes. Although these molecular events can be altered and quantified, it is the translation of these changes into predictable cellular responses that is of greatest interest to the biochemical or tissue engineer.

This goal in developing this model is not to quantitatively “fit” experimental data, but rather to provide a tool with which one can analyze and possibly interpret relationships between molecular and cellular events. These concepts have been addressed previously in studies of fibroblast proliferation in response to the EGF family of ligands (Reddy et al., 1998; Reddy, et al., 1996). The present work focuses not only
on a system of tremendous therapeutic interest, but it also provides the ability to incorporate the added level of complexity associated with a heteromeric receptor. Numerous cytokines utilize heteromeric receptors in transmitting their diverse signals to hematopoietic cells (Moutoussamy et al., 1998; Cosman, et al., 1990; Bazan, 1990). Several of these receptors, including IL-2Rβ and γ, are shared among a “superfamily” of cytokines, immediately extending the potential applicability of the present model to other ligands of therapeutic interest (Sugamura, et al., 1996; Sugamura et al., 1995; Edgington, 1992).
Table 4.1 Model parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Reference</th>
<th>Base Value(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_r$</td>
<td>Dissociation Rate Constant</td>
<td>Wang and Smith, 1987</td>
<td>0.0138 min$^{-1}$</td>
</tr>
<tr>
<td>$k_f$</td>
<td>Association Rate Constant</td>
<td>Berndt et al., 1994</td>
<td>$k_r/11.1$ pM$^{-1}$ min$^{-1}$ (IL-2) $k_r/8.2$ pM$^{-1}$ min$^{-1}$ (2D1)</td>
</tr>
<tr>
<td>$k_{rec}$</td>
<td>Dissociation Rate Constant, Endosome</td>
<td>Fallon et al., 1999</td>
<td>$8 k_r$ min$^{-1}$ (IL-2) $5 k_r$ min$^{-1}$ (2D1)</td>
</tr>
<tr>
<td>$k_{fe}$</td>
<td>Association Rate Constant, Endosome</td>
<td>Fallon et al., 1999</td>
<td>$k_{rv}/1000$ pM$^{-1}$ min$^{-1}$ (IL-2) $k_{rv}/3000$ pM$^{-1}$ min$^{-1}$ (2D1)</td>
</tr>
<tr>
<td>$k_t$</td>
<td>Constitutive Internalization Rate Constant</td>
<td>Hemar et al., 1994</td>
<td>0.007 min$^{-1}$</td>
</tr>
<tr>
<td>$V_s$</td>
<td>Constitutive Receptor Synthesis Rate</td>
<td>Duprez et al., 1986</td>
<td>11 min$^{-1}$</td>
</tr>
<tr>
<td>$k_{syn}$</td>
<td>Induced Receptor Synthesis Rate</td>
<td>Smith et al., 1985</td>
<td>0.0011 min$^{-1}$</td>
</tr>
<tr>
<td>$k_e$</td>
<td>Internalization Rate Constant</td>
<td>Fallon et al., 1999</td>
<td>0.04 min$^{-1}$</td>
</tr>
<tr>
<td>$k_x$</td>
<td>Recycling Rate Constant</td>
<td>Ghosh et al., 1994</td>
<td>0.15 min$^{-1}$</td>
</tr>
<tr>
<td>$k_h$</td>
<td>Degradation Rate Constant</td>
<td>Duprez et al., 1986</td>
<td>0.035 min$^{-1}$</td>
</tr>
<tr>
<td>$V_e$</td>
<td>Total Endosomal Volume</td>
<td>French, 1995</td>
<td>$10^{-14}$ L/cell</td>
</tr>
</tbody>
</table>
Figure 4.1 Model schematic.

This model follows the path of an extracellular ligand, beginning with the kinetics of binding to the IL-2 receptor (kf and kr) and receptor-mediated internalization (ke) into sorting endosomes (volume Ve). The off-rate (kre) increases at this point due to the pH sensitivity of binding, and the on-rate (kfe) is recalculated using the Kd for binding to the relevant IL-2Rβγ complex. Receptor-associated ligand is routed to lysosomal degradation (kh), while free ligand or that bound to IL-2Rα is recycled (kx). Steady state receptor levels in the absence of ligand are described by constitutive internalization (kf) and biosynthesis (Vs) rates. Ligand-receptor complexes stimulate new receptor synthesis (ksyn) and cell proliferation (μ) during their lifetime on the cell surface.
Figure 4.2 Threshold model of cell growth dependence on IL-2/IL-2R complex number.

Growth response to various concentrations of wild-type IL-2 (●) and the 2D1 analog (○) over 24-hour intervals throughout five-day proliferation experiments. Cell-surface complex numbers were quantified by monitoring intact ligand concentration and receptor levels during the growth experiments. A non-linear least squares estimate of Michaelis-Menton behavior reveals the steep, threshold type behavior of the proliferation data. This growth model was used to estimate the growth response of cells as a function of $C_S$ in subsequent simulations (data adapted from the growth experiments detailed in Chapter 2).
Figure 4.3 Ligand depletion- base model prediction and experimental data.

Intact concentrations of wild-type IL-2 in T cell cultures of density 2.5x10^8 cells per liter. Experimental data (●,□) and model predictions (gray curves) using the base values for IL-2 (Table 4.1) are indicated. Simulations and experiments were performed at two initial ligand concentrations, as shown (data adapted from the depletion experiments detailed in Chapter 2).
Figure 4.4 Cell density- base model prediction and experimental data.

T cell densities measured in the presence of 10 pM (●) and 100 pM (■) wild-type IL-2 are shown along with model predictions (gray curves) using the base parameter values shown in Table 4.1 (data adapted from the growth experiments detailed in Chapter 2).
Figure 4.5 Effect of ligand-receptor binding kinetics on ligand depletion at 10 pM ligand concentration.

Intact ligand concentrations (A) and cell-surface complex numbers (B) are shown for hypothetical IL-2 analogs with dissociation rate constants \( (k_e) \) of 0.138, 0.069, 0.0276, 0.0138, 0.0069, 0.00345, and 0.00138 min\(^{-1}\). The \( K_D \) is held constant at the wild-type value of 11.1 pM, and the association rate is adjusted accordingly for each scenario.
Figure 4.6 Effect of ligand-receptor binding kinetics on ligand depletion at 100 pM ligand concentration.

Intact ligand concentrations (A) and cell-surface complex numbers (B) are shown for hypothetical IL-2 analogs with dissociation rate constants ($k_d$) of 0.138, 0.069, 0.0276, 0.0138, 0.0069, 0.00345, and 0.00138 min$^{-1}$. The $K_D$ is held constant at the wild-type value of 11.1 pM, and the association rate is adjusted accordingly for each scenario.
Figure 4.7 Effect of ligand-receptor binding kinetics on cell proliferation.

T cell proliferation in response to hypothetical IL-2 analogs with dissociation rate constants ($k_r$) of 0.138, 0.069, 0.0276, 0.0138, 0.0069, 0.00345, and $0.00138 \text{ min}^{-1}$ is indicated for initial ligand concentrations of 10 pM (A) and 100 pM (B). The $K_D$ is held constant at the wild-type value of 11.1 pM, and the association rate is adjusted accordingly for each scenario.
Figure 4.8 Effect of endosomal binding affinity on ligand depletion.

Intact concentrations of hypothetical IL-2 analogs with endosomal dissociation constants ($=k_{e}/k_{fe}$) of $10, 10^2, 10^3, 3 \times 10^3, 3 \times 10^4, 3 \times 10^5$, and $3 \times 10^6$ pM are shown for initial ligand concentrations of 10 pM (A) and 100 pM (B). Cell surface binding affinities are unaltered in this analysis, and the initial cell density is $2.5 \times 10^8$ per liter.
Figure 4.9 Effect of endosomal binding affinity on cell proliferation.

T cell proliferation in response to hypothetical IL-2 analogs with endosomal dissociation constants \( (=k_{re}/k_{ic}) \) of \( 10, 10^2, 10^3, 3\times10^3, 3\times10^4, 3\times10^5 \), and \( 3\times10^6 \) pM are shown for initial ligand concentrations of 10 pM (A) and 100 pM (B). Cell surface binding affinities are unaltered in this analysis.
Figure 4.10 Comparison of experimental sorting outcomes with modeling results.

A) Steady-state sorting behavior of wild-type IL-2 (●) and the 2D1 analog (○) determined from experiment. Briefly, KIT-225 cells were incubated at 37°C for three hours in various concentrations of 125I-labeled ligands. Cells were then washed with acid-strip (pH 2.8) at 4°C to remove surface-bound ligand, returned to 37°C, and chased with an excess of unlabeled IL-2. Medium was collected at 0, 5, 10, and 15 minutes. Degraded and intact (recycled) radiolabeled ligand in the media was separated using 10kD-cutoff membrane filtration units. B) Sorting results are indicated from simulations performed to mimic experimental conditions using the base values for wild-type IL-2 (●) and 2D1 (○) shown in Table 4.1 (data adapted from the steady-state sorting experiments detailed in Chapter 2).
Figure 4.11 Effect of endosomal $K_D$ on sorting - comparison of modeling results with experimental outcomes at $C_t$=200 per cell.

A) Recycling fractions corresponding to an intracellular ligand concentration (bound+unbound) of 200 per cell are shown for experimental determinations and base case model predictions of the steady-state sorting behavior of wild-type IL-2 and 2D1. B) Results are shown from simulations of steady-state sorting experiments performed using indicated values of the endosomal dissociation constant ($=k_{re}/k_{fc}$). Recycling fractions corresponding to an intracellular ligand concentration (bound+unbound) of 200 per cell are shown.
Chapter 5: Conclusions

The potency of a therapeutic protein, in particular a cytokine or growth factor, is influenced by a number of its molecular properties, including its half-life in the circulation (or more restricted delivery arena) and its local dose-response effect on cell function. Circulatory half-life typically depends mainly on physical clearance mechanisms in the kidney, liver, and/or lung, although for proteins recognized by blood and blood vessel cells biochemically-specific, receptor-mediated cellular uptake can be the predominant clearance mechanism (e.g., GCSF (Cohen, 1993)). Cell receptor binding affinity has generally been cited as the major determinant of local cell function dose-response relationships. The work in this thesis contributes to growing evidence that ligand properties governing receptor/ligand trafficking dynamics leading to ligand depletion and receptor downregulation are even more important (Ciardelli, 1996). This leads to the concept of a cell-level dynamic analysis, involving “matrix” criteria related to multiple ligand/receptor binding properties within diverse cellular compartments, to understand what properties would yield more effective cytokines (Lauffenburger, et al., 1998); this could be employed in high-throughput combinatorial manner or toward rational design.

The research described in this thesis focuses on understanding how the potency of therapeutic proteins can be altered by varying molecular properties that govern receptor/ligand trafficking dynamics. For IL-2, the fact that the three receptor chains play differing roles in binding, trafficking, and signaling provides an opportunity to explore enhancement of potency by increasing binding to one of the chains relative to the others. (Actually, the γ-chain significantly binds ligand only in complex with β-chain,
so we can simplify the system for many purposes by considering only binding to the βγ-chains.) For instance, evidence that the α-chain is important for binding but not for signaling and internalization permits us to successfully predict that an IL-2 variant possessing reduced affinity for the βγ-chains will demonstrate greater potency because of diminished IL-2 depletion and IL-2R downregulation, as described in the modeling results in Chapter 4. As in previous work with the EGF/EGFR system, this is a counterintuitive prediction, because this alteration decreases the ligand's affinity for the chains that generate signaling.

Furthermore, the fact that different cell types in the immune system bear different chains suggests the possibility of modulating cell type activation. NK cells, for example, do not generally express the α-chain so an IL-2 mutant with higher α-chain affinity but lower βγ-chain affinity could be predicted to selectively activate T-lymphocytes but not NK cells, and vice versa. Stimulation of natural killer (NK) cells by IL-2 creates nonspecific inflammatory responses that hamper the utility of IL-2 at high doses. These effects occur upon IL-2 binding to the intermediate-affinity IL-2R-βγ complex, which is endogenously expressed on NK cells. The 2D1 variant binds the βγ complex with a lower affinity \textit{versus} IL-2 at cell-surface pH. Therefore, the efficacy of 2D1 is enhanced at both low and high concentrations: through decreased depletion at low concentrations where T cells are stimulated, and through reduced NK cell stimulation at high concentrations (see Figure 5.1). The insight gained from our study of the basis of the enhanced bioactivity of 2D1, combined with the quantitative rigor provided by the mathematical model could lead to the rational design of IL-2 variants with further improvements in therapeutic potency.
It should be further beneficial to combine improvements at the local dose-response level with improvements at the systemic level. Thus, it can be additionally informative to study binding, trafficking, and potency properties of PEGylated IL-2 and/or other therapeutic cytokines, to see how chemical modification may have positive or negative effects at the local cell level. The goal of future studies can thus be aimed at learning whether the local dose-response potency of cytokines can be improved by altering their structure in a way to maximize signaling while diminishing endocytic ligand depletion and receptor downregulation. Experimental methodologies aimed at gaining quantitative information concerning the relationship of cytokine/receptor binding properties to trafficking dynamics have been outlined here, and can be applied to other systems. The mathematical model proposed in Chapter 4 further aids in predicting how particular alterations in binding properties might result in improved trafficking dynamics and cell response potency.
Figure 5.1 Window of therapeutic efficacy.

The efficacy of 2D1 is enhanced at both low and high concentration: through decreased depletion at low concentrations where T cells are stimulated, and through reduced NK cell stimulation at high concentrations.
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Interaction of IL-2Rβ and γc Chains with Jak1 and Jak3: Implications for XSCID and XCID. Science, 266: 1042-1045.


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Developed a mathematical model based on both data and correlations to estimate drying characteristics throughout a tablet-coating column. Investigated the sterile filtration efficiency for a protein complex used as part of a vaccine formulation.

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Investigated the effects of external environment on the stability and activity of an industrial enzyme. Contributed to the efforts of protein engineering to enhance lipase performance in detergent formulations.
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Initiated the process of isolation and purification of an acyl-CoA synthetase enzyme from *P. oleovorans*. Optimized bacterial growth conditions in a stirred-tank bioreactor, and developed an enzymatic activity assay in Professor Jay Keasling's biochemical engineering laboratory. (Fall 1994)

University of California, Berkeley
Applied the Pedersen Reaction, a methodology using stereospecific reductive coupling, to the stereoselective synthesis of diols from protected amino-aldehyde precursors. Synthesized, purified, and characterized new molecules in Professor Steven Pedersen's chemistry laboratory. (June 1992-May 1993)

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