Evolution modulates the quantitative characteristics of protein interactions, and often uses combinations of weak interactions to achieve a particular specificity. We addressed how quantitative optimization might be used in the design of multidomain proteins, using a chimera containing epidermal growth factor (EGF) as a cell targeting element and interferon-alpha-2a (IFNα-2a) to initiate signal transduction. We first connected EGF and IFNα-2a via a linker that allows both ligands to bind to their receptors on a cell surface, then incorporated a series of mutations into the IFNα-2a portion that progressively decrease both the on-rate and dissociation constant of the IFNα-2a/IFNα receptor 2 (IFNAR2) interaction. Using this strategy, we designed chimeric proteins in which the activation of the IFNα receptor (IFNAR) in HeLa, A431 and engineered Daudi cells depends on the presence of EGF receptor (EGFR) on the same cell. The mutant chimeric proteins also inhibited proliferation of IFNα-sensitive cells in an EGFR-dependent manner. These results provide insights into the quantitative requirements for specific binding to multisubunit receptors, and illustrate the value of a quantitative approach in the design of synthetic-biological constructs.

Biological recognition events are often mediated by modular protein and nucleic acid segments that can be arbitrarily linked to give functional combinations. In the course of evolution, multidomain proteins have been repeatedly generated and constitute a large fraction of the proteins encoded by metazoan genomes. In the evolutionary improvement of such chimeras after a genetic rearrangement, an important but under-studied process is the quantitative optimization of the individual modules.

For many years, researchers have also constructed chimeric proteins with properties that derive from the parental modules. For example, one therapeutic approach has sought to use cell surface proteins as addresses to direct the delivery of specific molecules, such as toxins to tumor cells. Pastan’s group described a chimeric protein consisting of Pseudomonas exotoxin and IL-2, in which the IL-2 moiety directed the toxin to cells bearing IL-2 receptor (1); they later described a Pseudomonas exotoxin-TGFα chimeric protein that binds to EGFR (2-4), a hallmark of many tumors (5). Similar strategies have been adapted by many groups (6, 7). A universal problem with this kind of approach is that when any targeted agent is administered to a patient, unwanted effects will occur as the drug travels through the body before reaching its target.

We therefore sought a different strategy based on quantitative modulation of the signaling part of a targeted molecule. Our strategy builds on the ideas of Adam and Delbrück (8), who proposed that in biological systems, reaction rates are often enhanced by reduction of the dimension of a space in which diffusion occurs. Because a cell surface is effectively two-dimensional, we reasoned that an initial rapid binding reaction to one cell surface protein could drive a second, weak interaction on the same cell surface. We further reasoned that if we could tune the binding affinities of both the targeting agent and the activating ligand appropriately, we could develop a mutant chimeric protein that would show negligible activation on cells expressing just one of the relevant receptors. We set out to test this strategy using EGF as the targeting agent and IFNα-2a as the toxin.

By combining quantitative information about protein binding kinetics and affinities with structural information about protein ligands and
their cell-surface receptors, we developed a new class of artificial proteins that simultaneously bind to distinct cell surface receptors to create ligands with new cell-type specificities. These proteins, termed chimeric activators have the following general structure: an activity element containing at least one mutation, a linker and a targeting element (Fig. 1A). This design is analogous to natural designs that use multivalent interactions between ligands and cell-surfaces to ensure precise delivery of biological activities.

**Experimental Procedures**

**Cell lines and culture conditions.** Daudi, 293-T and HeLa cells were obtained from American type culture collection (Manassas, VA). A431 cells were a gift from Thomas M. Roberts. A431, 293-T and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Daudi cells were maintained in RPMI-1640 modified medium supplemented with 10% FBS, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Daudi cells were maintained in RPMI-1640 modified medium supplemented with 10% FBS, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Stable Daudi-pLPCX-EGFR (Daudi-EGFR) and Daudi-pLPCX cell lines were generated by retroviral infection of Daudi cells following the protocol of Pear et al. (http://www.stanford.edu/group/nolan/protocols/pro_helper_free.html). Infected Daudi cells were first selected with puromycin (1.5 µg/mL) and then cells were isolated with Dynabeads Pan mouse IgG from Invitrogen (Maryland, USA) pre-coated with anti-EGFR mAb following the manufacturer instructions. The constructs pLPCX and pLPCX-EGFR were kindly provided by Joan S. Brugge, Harvard Medical School (9).

**Antibodies:** anti-phospho-STAT1 (Tyr701) rabbit pAb and anti-EGFR rabbit pAb were purchased from Cell Signaling Technology (Beverly, MA); PhoshoDetect™ anti-phospho-EGFR (Tyr1068) rabbit pAb, anti-EGFR mouse mAb (EGFR.1), anti-EGFR mouse mAb (528) and anti-EGFR rabbit pAb were purchased from Calbiochem (San Diego, CA); anti-actin mouse mAb was purchased from Chemicon International (Temecula, CA); anti-human IFNα/β R1-phycoerythrin mouse mAb was purchased from R&D systems (Minneapolis, MN); anti-EGFR R-Phycoerythrin-conjugated mouse mAb was purchased from BD Biosciences (San Jose, CA).

**Gene synthesis, protein expression and purification.** The coding sequence for the ‘wild-type’ chimeric activator, INFα-2a-(Gly4-Ser)₇-EGF, consisting of the 165 amino acids of mature INFα-2a (GI:2781226), the 35 amino acid linker and the 53 amino acids of mature EGF (GI:24987355), was synthesized by Top Gene Technologies (Quebec, Canada). This sequence was codon optimized for expression in *P. pastoris*. The sequence was sub-cloned (with XhoI and XbaI restriction sites) into the pPICZα A vector (Invitrogen), which includes the alcohol oxidase promoter and the α-factor leader sequence, a c-myc epitope tag and a His₆ tag for purification. The final sequence of the constructs was confirmed by DNA sequencing. Approximately 20 µg of the DNA construct were linearized with *Pme I* prior to transformation of *P. pastoris* X33 (Mut⁺) and KM71H (Mut⁵) cells. The electroporation method of the EasySelect™ *Pichia* expression kit (version H; Invitrogen) was used for transformation, and the transformants were plated on MD and MM agar plates to screen for methanol utilization (Mut) phenotype. Several Mut⁺ and Mut⁵ clones were put on plates with high zeocin concentrations (0.5 to 1 mg/ml) to select for clones with multiple integration events. A Mut⁵ clone was selected for the protein expression. Transformants were grown and induced with methanol according to the instructions from Invitrogen. The INFα-2a-(Gly4-Ser)₇-EGF chimeric activator was secreted into the medium and was purified with the ProBond™ purification system (for purification of polyhistidine-containing recombinant proteins, version K, Invitrogen). Purity was checked by Coomassie blue stain and by immunoblotting against EGF. The final yield was approximately 1 mg per ml of cell culture.

We constructed variants of INFα-2a-(Gly4-Ser)₇-EGF containing the IFNα–2a mutations K133A, R144A, and R149A by standard recombinant DNA techniques using the Quick-Change™ site-directed mutagenesis kit from Stratagene (CA, USA). To produce control proteins, a set of *Pichia* expression vectors encoding IFNα–2a wild-type and mutant proteins lacking the linker and EGF but containing the C-myc and His₆ tags were also constructed by analogous techniques. The correctness of the constructs was confirmed by DNA sequencing.
The transformation, expression and purification of the different constructs were performed as described above.

**Cell Stimulation, protein extraction, immunoblotting and immunoprecipitation.** A431 and HeLa cells were seeded in 60 mm plates, Daudi and Daudi-EGFR were seeded in 50 mL bottles. Once they reached confluence the growth medium was replaced with fresh DMEM and cells were incubated with anti-EGFR mouse mAb 528. After 2 h treatment cells were tested for STAT1 stimulation as described above.

**EGFR stimulation:** cells were stimulated with human recombinant EGF from E. coli (Cell Signaling Technology), the chimeric activators, and Interferon-α A (Calbiochem) or vehicle (as negative controls) for 5 min at 37 °C and 5% CO₂. Stimulations were terminated by washing the cells once with ice-cold phosphate-buffered saline, and cells were lysed in NP40 lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, 25 mM NaF, 10 mM β-glycerophosphate, 250 μM Na₃VO₄ supplemented with protease inhibitor cocktail tablets (Roche)). Cells were solubilized for 15 min at 4 °C in lysis buffer follow by centrifugation at 15,000g for 15 min at 4 °C, the detergent extracts (supernatant) were then subjected to immunoprecipitation. Lysates were incubated with anti-EGFR mouse mAb 528. EGFR was immunoprecipitated with a mixture of Protein A and G-Sepharose beads (Amersham Bioscience) for 1 h. at 4 °C. Beads were washed with NP-40 lysis buffer and boiled in SDS sample buffer. Proteins were resolved on 8% SDS–polyacrylamide, transferred to nitrocellulose, and detected by immunoblotting with anti-phosphoSTAT1(pTyr701) antiserum.

**Neutralization of EGF receptor and STAT1 stimulation.** HeLa cells were seeded as previously described. Once they reach confluence the medium was changed for fresh DMEM and cells were incubated with anti-EGFR mouse mAb 528. After 2 h treatment cells were tested for STAT1 stimulation as described above.

**Anti-proliferative assay.** The anti-proliferative activity of IFNα–2a–linker–EGF chimeras, corresponding IFNα–2a proteins, and commercial IFNα A on Daudi and Daudi-EGFR cell lines was assayed as follows. Proteins that had been previously filtered thorough a 0.2 μm PDVF filter unit (Millipore, Ireland) and then quantitated were serially diluted. Twenty serial dilutions were prepared in flat-bottomed 96-well plates for each tested protein. Daudi and Daudi-EGFR cells grown in RPMI-1680 medium were added (3 x 10⁴ cells in 100 μL) to each well and were grown for an additional 60 h. in the presence of the different proteins. The number of living cells was then determined using a cell staining kit (Cell proliferation reagent WST-1, Roche) based on the colorimetric detection of the cleavage of the tetrazolium salt WST-1 into formazan. The WST-1 reaction solution was added according to the manufacturer’s recommendation for a period of 4 h, after which the absorbance at 450 nm and 650 nm (reference wavelength) was recorded in an ELISA reader (Victor³V, PerkinElmer).

**RESULTS**

**Quantitative rationale for design of chimeric activators.** The design of chimeric activators with a desired cell specificity requires an understanding of the on-rates, off-rates, and equilibrium constants of each of the receptor-binding elements. An important aspect of ligand-receptor interactions is that kₘₐₓ is primarily limited by diffusion. The off-rate for ligand-receptor interactions is often slower than the process of receptor-mediated endocytosis, and in such cases kₐₜ is not relevant to signaling. As a result, the binding of a chimeric protein composed of a targeting element and an activity element might not be significantly influenced by the supposed targeting element. For example, EGF and IFNα–2a both have similar on-rates (Table 1) (10, 11), so that fusion of EGF to IFNα–2a may
have very little effect on the binding of the latter to its receptor. We imagined that for a chimeric activator to have a cell specificity driven by the targeting element, one or both of the following conditions should be met: the on-rate of the activity element should be lower than that of the targeting element, or if the off rates are faster than the internalization rate, then the equilibrium constant \(K_{on}/K_{off}\) of the targeting element should be higher than that of the activity element. Once such a chimeric activator binds to a cell surface via the receptor for the targeting element, binding of the activity element to its receptor should in principle be driven by its high local concentration relative to its receptor (Fig. 1C and D).

A rationally designed set of chimeric activators were constructed, consisting of wild-type and mutant forms of IFN\(\alpha-2a\) as the activating element, and EGF as the targeting element (Fig. 1A). Our choice of these elements was based on the following considerations: 1) the three-dimensional structures of the EGF/EGFR and the IFN\(\alpha-2a\)/IFNAR2 complexes have been solved or modeled (12-14), allowing us to choose appropriate positions for linking and to design a linker of the right length; 2) a precise characterization of the on-rates, off-rates, and \(K_2\)s of a series of IFN\(\alpha\) alanine-scanning mutants was previously reported (11), so that we could choose a series of IFN\(\alpha-2a\) mutants with step-wise reductions in on-rates and affinity (Table 1); and 3) the on-rates and binding constants of wild-type EGF and IFN\(\alpha\) are similar, so that the effect of mutations should be significant. The on-rates of both proteins are thought to be faster than diffusion-limited, because they are driven by charge-complementarity between these ligands and their receptors (11, 16). Another consideration was that the molecules described here could serve as a proof-of-concept for a protein drug, and that targeting might improve the therapeutic index of a toxic molecule only by a limited amount. Thus, the fact that IFN\(\alpha\) already has a preferential activity against abnormal cells such as cancer cells and virus-infected cells made it an attractive candidate (17).

For the chimeric activator concept to work, the targeting element and activation element must be able to bind simultaneously to their respective receptors. The published structure of EGFR with a ligand and a model of the IFN\(\alpha-2a\) structure indicate that in these receptor-ligand complexes, the EGF is about 90 Å from the cell surface, and the IFN\(\alpha-2a\) is about 50 Å from the cell surface, so that a linker of at least 40 Å is needed to bridge the two elements. We therefore selected a standard glycine-serine linker of 35 amino acids ([Gly,Ser]a), with a length of roughly 120 Å; Fig. 1B) (18). Four chimeric activators were constructed (Table 1). These consist of wild-type or mutant IFN\(\alpha-2a\) at the N-terminus, followed by the 35-amino acid linker, EGF, a myc epitope tag, and a (His)6 purification tag at the C-terminus. The IFN\(\alpha-2a\) mutations used were K133A, R144A, and R149A, which allow the protein to fold correctly but cause stepwise reductions in the on-rate and equilibrium binding of IFN\(\alpha-2a\) for its receptor (Table 1) (11). The chimeric activators carrying wild-type INF\(\alpha-2a\) and these mutant forms were termed CA-wt, CA-K133A, CA-R144A and CA-R149A, respectively.

We expressed these chimeric activators and the corresponding individual IFN\(\alpha-2a\) and IFN\(\alpha-2a\) mutants in the yeast Pichia pastoris according to published methods, and purified them from culture supernatant (19, 20). All proteins were epitope-tagged and His\(\alpha\)-tagged for further detection and purification. The proteins were expressed at high levels and recovered at high purity (see Experimental Procedures).

**Functionality of parts within chimeric activators.** The biological activity of the targeting element, EGF, was verified in the chimeric activators by testing its ability to stimulate phosphorylation of tyrosine-1068 in EGFR. Activation of EGFR was evaluated in HeLa cells by treating them with the four chimeric proteins, a commercial recombinant EGF as a positive control, and IFN\(\alpha-2a\) tagged with c-myc and His\(\alpha\) as a negative control. Fig. 2 illustrates the induction of phosphorylation of the EGFR by the different chimeric proteins as well for the rEGF control (lanes 2 to 7). Cells treated only with PBS (vehicle) or with tagged IFN\(\alpha\) do not show detectable activity (lanes 1 and 8). To test the activity of the IFN\(\alpha\) portion of the chimeric activators, we examined by Western blot the phosphorylation of STAT1 tyrosine 701 (a consequence of IFN-\(\alpha\) receptor activation; Fig. 3 and Supplementary Fig. 1 online). These
experiments demonstrated that the EGF and IFNα-2a components of the chimeric proteins were both active and that the mutations K133A, R144A, and R149A reduced the activity of the tagged IFNα-2a to a degree similar to the reductions previously reported (11).

**Activity of IFNα-2a-EGF chimeric activators depends on EGFR.** We next tested whether the IFNα-2a-EGF chimeric proteins could signal through IFNAR in an EGFR-dependent manner by comparing their activity with the activity of the corresponding IFNα-2a (mutant) protein in cells expressing both EGFR and IFNAR. We used two different epithelial cell lines with varying levels of EGFR expression: A431 cells, which show high expression of EGFR (~2x10^4 EGFR/cell), and HeLa cells, which show much lower expression of EGFR (2x10^3 EGFR/cell) (21). Quantification by FACs analysis confirmed the expression levels of EGFR and IFNAR (Supplementary Table 1 online). The results indicated that an IFNα-2a-EGF chimeric activator (without a mutation) and IFNα-2a alone induced interferon signaling with about equal efficiency (Fig. 3A and B, lanes 4-9; see also supplementary Fig. 1 online), and that the IFNα-2a mutant proteins K133A, R144A, and R149A, which respectively are reduced by 10x, 40x, and 200x for IFNAR binding (Table 1), show correspondingly low levels of IFNα signaling (Fig. 3A and B, lanes 13-15, 19-22 and 26-29).

The chimeric activators with EGF fused to the mutant IFNα-2a showed higher levels of IFNα signaling than the corresponding IFNα-2a mutants alone, however. For example, in HeLa and A431 cells pSTAT1 was activated when cells were treated with CA−R144A and CA−R149A (Fig. 3A and B, lanes 16-18 and 23-25) but not when the cells were treated with the corresponding IFNα-2a mutants, even at higher concentrations (Fig. 3A and B, lanes 22 and 29). No synergistic effect was seen when cells were treated with a combination EGF and any of the various IFNα-2a mutants (data not shown).

As a second way to test whether the enhancement of IFNα signaling depends on binding to EGFR, we introduced EGFR into the Daudi cell line, which does not normally express this receptor (Supplementary Fig. 2 online) and compared IFNα signaling in this engineered line to that seen in the parent cell line. The Daudi cell line is derived from a human Burkitt’s lymphoma cell, and its proliferation and survival are inhibited by IFNα (22). The function of the chimeric activators on Daudi and Daudi-EGFR cells clearly depended on the presence of the receptor for the targeting element (Fig. 3C and D). The CA−R149A protein stimulated STAT1 phosphorylation only in Daudi-EGFR cells (Fig. 3D, lanes 23-25) and not in the Daudi parental cell line (Fig. 3C, lanes 26-29). The CA−wt and IFNα−2a proteins were active in both cell lines, as expected, whereas the IFNα−2a(R149A) mutant showed only slight activity at high concentrations. Chimeric activators containing the intermediate-strength mutations CA−K133A and CA−R144A gave intermediate levels of signaling in Daudi cells, consistent with their predicted properties, and showed quantitative enhancements in signaling relative to IFNα−2a(K133A) and IFNα−2a(R144A) in Daudi-EGFR cells (Fig. 3D, lanes 10-22).

The activities of the chimeric activators differed on the various cell lines (Fig. 3). We hypothesize that these cell-type differences were due to differences at the level of receptor expression, although the HeLa, Daudi, and A431 cell lines have different tissue origins and oncogenic mutations, so that any comparison of results between these lines can only be suggestive. Daudi cells have 10-20 times more IFNAR molecules at the cell surface than do HeLa and A431 cells ( Supplementary Table 2 online), which may explain the greater level of IFNAR activation in Daudi cells, for example in response to CA−R144A and IFNα−2a(R144A).

As an additional control, we tested whether signaling by IFNα−2a(mutant)-EGF chimeric activators could be inhibited by an antibody against the receptor for the targeting element (Fig. 4). Pre-treatment of HeLa cells for 2 h. with the mouse monoclonal anti-EGFR antibody 528 inhibits the activation of the STAT1 signaling pathway by the K144A and R149A mutant chimeric activators (Fig. 4, lanes 13-14 and 17-18), but not the activation caused by the non-chimeric IFNα−2a and mutants. Neither the wild-type IFNα−2a nor the CA−wt showed inhibition (Fig. 4, lanes 5-6, 9-10), as expected. The 528 antibody does not itself activate the EGF or the IFNα signal cascades. Treatment with mAb 528 appeared to
have no effect on signaling induced by the CA-K133A protein. Since the IFNα element retains significant binding to the IFNAR, it is possible that this chimera was able to displace the antibody from the EGFR during the 30-minute course of the experiment.

EGFR-dependent inhibition of cell proliferation by chimeric activators. The ability of the chimeric proteins to suppress the proliferation of Daudi and Daudi-EGFR cells is shown in Fig. 5. In both cell lines, a dose-dependent growth inhibition was observed. When Daudi cells were treated with the chimeric activators and their IFNα−2a counterparts, essentially no difference in their IC₅₀ was found (Fig. 5A-C). Furthermore, the ability to inhibit proliferation diminished according to the ability to bind to IFNAR and activate STAT1.

The chimeric activators, relative to their IFNα counterparts, showed enhanced inhibition of cell proliferation and survival of Daudi-EGFR cells (Fig. 5E and F). The chimeric activators containing the mutations K133A and R144A were about 10-fold and 100-fold more potent, respectively, than the corresponding IFNα−2a mutants alone. CA−R149A weakly inhibited proliferation in a pattern that could not be fit to a sigmoid curve (data not shown) and its activity relative to the corresponding IFNα−2a mutant therefore could not be quantified.

The behavior of the Daudi-EGFR cells was somewhat affected by the presence of EGFR, which may contribute pro-survival signals. The IC₅₀ of wild-type and mutant IFNαs were about 5- to 6-fold higher in Daudi-EGFR (Fig. 5). The maximal extent of proliferation inhibition was also less than for cells treated with IFNα mutants alone. These results are consistent with previous observations that EGF can antagonize the antiproliferative and pro-death effects of IFNα (23).

DISCUSSION

In the experiments described here, we addressed whether the cell-type specificity of an extracellular signaling protein could be altered by a combination of genetic fusion followed by quantitative modulation. The results have implications for understanding the natural design of multisubunit proteins and ligand-receptor interactions, as well as for the design of artificial proteins targeted to cancer cells and other types of disease-causing cells.

The goal of our particular design was to alter the cell-type specificity of IFNα so that it would only activate its receptor on cells bearing EGFR. We attached EGF to IFNα−2a with a flexible linker, so that both modules could simultaneously bind to their receptors. We also mutated IFNα−2a so that its ability to bind to its receptor would be significantly reduced, using a set of previously defined mutations that reduce the equilibrium binding of IFNα−2a for its receptor by 10-, 40- and 200-fold (11). We hypothesized that the resulting chimeric protein would be essentially unable to bind directly to IFNARs, but would bind to EGFR with high affinity. By virtue of the high local concentration of the chimera on the cell surface, the IFNα−2a module would then be able to bind to its receptor.

Several lines of evidence indicated that the resulting chimerized, mutated proteins induced IFNα signaling in an EGFR-dependent manner. First, a comparison of IFNα−2a(mutant)-EGF chimeras with their unchimerized IFNα−2a(mutant) counterparts in HeLa and A431 cells, which express both EGFR and IFNAR, showed that the chimeric proteins were more potent in inducing STAT1 phosphorylation, which results specifically from the activation of IFNAR (Fig. 3). As expected, the differential effect was particularly pronounced for chimeric activators carrying mutations that significantly reduce IFNα binding to its receptor. In contrast, IFNα−2a-EGF (CA-wt) was essentially indistinguishable from wild-type IFNα−2a; this result is expected because the binding of IFNα to its receptor is quantitatively similar to that for EGF. Second, the activities of the chimerized and unchimerized proteins were compared on Daudi cells and Daudi cells engineered to express EGFR. These results indicated that the enhanced stimulation of STAT1 phosphorylation by the EGF chimeras depended on the presence of EGFR on the cell surface. The improved selectivity of the mutant chimeric activators seen with HeLa and A431 cells was reproduced in Daudi-EGFR cells, but not in parental Daudi cells. Third, the stimulation of STAT1 phosphorylation could be inhibited by an anti-EGFR antibody.
The chimeric activator proteins described here induced a biological response in an EGFR-dependent manner. The proliferation and survival of Daudi cells is inhibited by IFNα-2a. We found that Daudi cells expressing EGFR were more sensitive than parental Daudi cells to IFNα-2a(mutant)-EGF chimeric activators by up to more than order of magnitude (Fig. 5). In a therapeutic context, the Daudi-EGFR cells may be considered to represent target cells, the parental Daudi cells may represent non-target cells where receptor activation results in side effects, and the differential effect corresponds to the therapeutic index of a protein drug. Thus, by reducing the binding of a given activator and attaching it to a targeting element, we can improve the therapeutic index by more than an order of magnitude.

A natural extension of our approach would be to use a tumor-specific antibody as a targeting element. The molecular designs reported here depended in part on structural models of both the IFNα/IFNAR2 and EGF/EGFR complexes. Use of antibody V regions in chimeric activators will become more feasible as more structures of antibody-receptor complexes are solved (24, 25).

In summary, we have constructed artificial signaling molecules based on quantitative principles that may reproduce the design of natural systems. We envision that further analysis of these principles may allow the improvement of protein therapeutics and a deeper understanding of the forces that shape natural biological system design.

REFERENCES


FOOTNOTE
1. The abbreviations used are: CA, chimeric activator; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IFNα-2a, interferon-alpha-2a; IFNAR, IFNα receptor; IL-2, interleukine-2; Mut+, methanol utilization wild-type; Mut–, methanol utilization slow; TGFα, tumor growth factor α; STAT-1, signal transducer and activator 1.
2. We would like to thank Dr Rebecca Ward and Jessica Hurt for their careful proofreading and helpful comments. P.A.S. is supported in part by grants from NIH and funds from Harvard University. P.C. is supported by a Fulbright-M.E.C (Spain) postdoctoral fellowship.

FIGURE LEGENDS

Fig. 1. A. General structure of chimeric activators, showing a ‘targeting element’ connected by a peptide linker to an ‘activity element’ with a mutation that reduces binding to the receptor for the Activity Element. B. Molecular model of the IFNα–2a-EGF chimeric activator (space-filling structure), showing how the IFNα–2a and EGF components can simultaneously interact with their receptors (ribbons). Models for EGF/EGFR complex (12, 13), and the IFNα–2a/IFNAR2 complex (14, 15), are shown with the C-termini of the receptor extracellular domains at the bottom; in each case, these C-termini are followed by the membrane-spanning segment of the receptor. C-D. Mechanism of specific binding of chimeric activators to target cells. D. The chimeric activator binds poorly to non-target cells because the intrinsic binding affinity of the mutant activity element to its receptor is low. D. In contrast, the targeting element binds to receptors on a target cell at a high rate. After the targeting element complexes with its receptor, the activity element is in a high local concentration relative to its receptor, so that the activity element can then bind and stimulate signal transduction.
Fig. 2. EGFR activation upon treatment with IFNα−2a-EGF chimeric activators. HeLa cells were stimulated for 5 minutes with vehicle (PBS; lane 1) EGF (lane 2), chimeric activators containing wild-type EGF linked to wild-type or mutant IFNα−2a (lanes 3-7), or wild-type IFNα−2a protein expressed from Pichia pastoris in the same manner as the chimeric activators (lane 8). EGF receptor immunoprecipitates from stimulated HeLa cell lysates were separated by SDS-PAGE, then immunoblotted for Tyr1068-phosphorylated EGF receptor (top) or total EGF receptor (bottom).

Fig. 3. STAT1 activation upon treatment with IFNα−2a-EGF chimeric activators. Starved HeLa (A), A431 (B), Daudi (C), or Daudi-EGFR (D) cells were incubated for 30 minutes with PBS (vehicle), commercial IFNα A, EGF, IFNα/EGF chimeric activator proteins containing wild-type or mutant IFNα-2a (CA−wt CA−K133A, CA−R144A, or CA−R149A), or the corresponding wild-type or mutant IFNα−2a proteins produced from Pichia (see Experimental Procedures). Lysates were prepared and immunoblots performed as described in Experimental Procedures probing with an anti-STAT1(pTyr701) antibody, and with an anti-actin antibody as a loading control. (See also Supplementary Figure 1, which shows similar immunoblots in which lanes were scanned with a densitometer and the phospho-STAT1 signal normalized to the actin signal.) HeLa, A431, and Daudi-EGFR cells express both EGFR and IFNAR, while Daudi cells express only IFNAR.

Fig. 4. Neutralization of EGFR. HeLa cells were pre-treated for 2 hours with 1 µg/mL of mAb 528, a mouse monoclonal antibody which prevents EGF from binding to EGFR (even-numbered lanes), followed by treatment for 30 minutes with vehicle (lanes 1-2), or 1.5 nM of commercial IFNα A (lanes 3-4), IFNα/EGF chimeric activator proteins containing wild-type or mutant IFNα-2a (CA−wt CA−K133A, CA−R144A, or CA−R149A), or the corresponding wild-type or mutant IFNα−2a proteins (lanes 7-8, 11-12, 15-16, and 19-20).

Fig. 5. EGFR-dependent anti-proliferative activity of IFNα−2a-EGF chimeric proteins. Daudi and Daudi-EGFR cells were grown for 60 hours in the presence of various concentrations of IFNα−2a-EGF chimeric proteins or the corresponding IFNα−2a proteins purified from Pichia. The relative number of viable cells was determined by the production of formazan, which absorbs at A460, from tetrazolium (see Experimental Procedures). Curves were fit to the data using a 4-parameter fit (Microcal Origin 5.0).

Supplementary Fig. 1. Normalized dose-response of STAT1 phosphorylation in HeLa cells treated with IFNα−2a-EGF chimeras and IFNα−2a proteins. These data represent an independent replication of the experiment shown in Figure 3, using a wider range of protein concentrations. HeLa cells were incubated with vehicle (PBS), recombinant human IFNα-A (1.5 nM), recombinant human EGF (17 nM), IFNα−2a-EGF chimera, and the corresponding wild-type and mutant IFNα−2a proteins expressed in Pichia, and STAT1 Tyr701 phosphorylation was assayed as described in Figure 3 and in the Experimental Procedures. The bar graph at the top of the figure represents the normalization of densitometry scans of the immunoblot representing STAT1 phosphorylation to the immunoblot representing total actin (bottom). Densitometry was performed using AlphaEasy FC software.

Supplementary Fig. 2. EGF-responsiveness of an engineered, stable Daudi-EGFR cell line. Daudi cells that stably express EGFR were generated by retroviral transduction as described in the Experimental Procedures. Parental Daudi and Daudi-EGFR cells were treated with EGF for 5 minutes at 37 °C, and then assayed for total EGFR using the mouse antibody EGFR.1 (left panel) or for phospho-EGFR using a rabbit antiserum specific for pTyr1068 (right panel). From left to right, lanes are: M, molecular weight markers in kiloDaltons as indicated; parental Daudi cells vehicle or EGF; and Daudi cells transduced with pLPCX-EGFR as described in Experimental Procedures.
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<td></td>
<td>$538 \times 10^{-9}$</td>
<td>IFNα–2a (R149A) – (Gly–Ser); – EGF (CA-R149A)</td>
</tr>
</tbody>
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Table 1. On-rates, off-rates, and dissociation constants of EGF, and wild-type and mutant IFNα2.
Figure 1.
Figure 4.

<table>
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<th>Vehicle</th>
<th>rhIFNα</th>
<th>CA-wt</th>
<th>IFNα-2a</th>
<th>CA-K133A</th>
<th>IFNα-2a (K133A)</th>
<th>CA-R144A</th>
<th>IFNα-2a (R144A)</th>
<th>CA-R149A</th>
<th>IFNα-2a (R149A)</th>
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<td>mAb 528</td>
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</tr>
</tbody>
</table>
Figure 5.

A) Daudi

B) CA-K133A

C) CA-R144A

D) Daudi-EGFR

E) CA-K133A

F) CA-R144A

IC₅₀ CA-wt = 0.5 nM
IC₅₀ IFN-α-2a = 0.2 nM
IC₅₀ CA-K133A = 1.5 nM
IC₅₀ IFN-α-2a (K133A) = 1.5 nM
IC₅₀ CA-R144A = 10 nM
IC₅₀ IFN-α-2a (R144A) = 10 nM

Absorbance at 450 nm

Concentration (M)
Enhancement of cell-type specificity by quantitative modulation of a chimeric ligand
Pablo Cironi, Ian A. Swinburne and Pamela A. Silver

J. Biol. Chem. published online January 29, 2008

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