yielded Mg/Ca of 8.50 mmol/mol ±0.04 (1σ) for DCP and 8.10 mmol/mol ±0.2 (1σ) for AA. To normalize the two data sets, 0.40 mmol/mol was added to the ratios measured by AA.
31. The weak correlation between Mg/Ca and VPI suggests that dissolution may have a minor effect on the Mg/Ca ratio of *Krithe* shells. However, because of the nature of the water masses influencing this site, dissolution is likely to covary inversely with temperature. Thus the weak correlation observed may simply be a residual temperature signal. Furthermore, the full range of Mg/Ca values occurs at the intermediate VPI value of 4. The lack of a significant correlation between *Krithe* Sr/Ca ratios and the VPI of Site 607 ostracode samples, as well as the high and nearly constant down-hole values of *Krithe* Sr/Ca ratios, indicate that burial diagenesis (recrystallization) is not an important factor in these samples. Recrystallization of biogenic calcites in deep-sea sediments typically leads to a fourfold decrease in the original Sr/Ca ratios over a period of 10^7 years (*46*).
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Designer Cytokines: Targeting Actions to Cells of Choice

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Some growth factors are therapeutically useful partly because restricted expression of their receptors limits their action to particular cell types. However, no unique stimulatory factor is known for many clinically relevant cell types, such as $CD34^+$ hematopoietic stem cells. Here, soluble α receptor (Rα) components for interleukin-6 (IL-6) and ciliary neurotrophic factor (CNTF) were targeted in an active form to cells expressing surface markers such as CD34 or CD45, thereby rendering those cells responsive to IL-6 or CNTF. The targeting of Rα components may provide the means to create "designer" cytokines that activate a desired cell type expressing a specific cell surface marker.

Cytokines and growth factors regulate vital biological processes such as induction and regulation of immune responses, cellular proliferation, differentiation, and apoptosis and are of potential use in the treatment of human diseases (*1*). Proteins such as erythropoietin, granulocyte colony-stimulating factor, and thrombopoietin are therapeutically promising at least in part because restricted expression of their receptors limits their sites of action. However, many other cytokines, such as leukemia inhibitory factor (LIF), have not proven to be useful therapeutically because of their pleiotropic effects resulting from the widespread expression of their receptors (*2, 3*). LIF induces the differentiation of myeloid M1 cells, activates the proliferation of DA1.1 myeloid cells, increases the number of megakaryocyte progenitors in vivo, inhibits differentiation of embryonic stem (ES) cells, activates the cholinergic switch of sympathetic neurons, and promotes bone remodeling. Although some of the actions of LIF would be therapeutically desirable, others are deleterious; mice injected with cells secreting large amounts of LIF die after 2 to 10 weeks (*4*). However, if the action of LIF could be restricted to new bone formation (*4, 5*), inhibition of adipogenesis (*6*), or stimulation of $CD34^+$ hematopoietic stem cells (*7*), this more specific cytokine could have therapeutic value.

CNTF, a member of the cytokine family that encompasses LIF, IL-6, oncostatin M, IL-11, and cardiotrophin-1 (*8, 9*), is in a sense a cell-specific counterpart to LIF. The action of CNTF is more restricted because unlike LIF, the receptor for which consists solely of the signal-transducing components LIFR and gp130 (*10, 11*), CNTF additionally requires a specificity-determining Rα component termed CNTFRα to which it must bind before interacting with gp130 and LIFR (*12, 13*). Thus, although both CNTF and LIF induce heterodimerization of the signal transducers gp130 and LIFR and lead to identical downstream signal transduction events, the sites of CNTF action are restricted to cells that express CNTFRα in addition to gp130 and LIFR (*14*). CNTFRα, which is anchored to the cell membrane by a glycosyl-phosphatidylinositol linkage, does not appear to participate directly in transducing the signal (*15*). Similar to CNTF, both IL-6 (*16*) and IL-11 (*17*) require a specificity-determining Rα component (IL-6Rα and IL-11Rα, respectively); the complex of Rα and cytokine initiates signal transduction by inducing gp130 to homodimerize (*13, 18*). Thus, in general terms, these three Rα components function as required specificity components for their cognate cytokines (Fig. 1A).

We report here a way to exploit these Rα components to create new, specific cellular targets for CNTF or IL-6. We achieved this by targeting soluble Rα (sRα) components to the surface of any cell expressing the common signal-transducing receptor components gp130 and LIFR. Rα targeting relies on a unique feature of the Rα components: Although the soluble extracellular domains of most receptors function as antagonists (*19, 20*), soluble CNTFRα (sCNTFR) and IL-6Rα (sIL-6R) function agonistically by binding their cognate cytokine and then interacting with their signal-transducing components on the cell surface (*15, 21*). Thus, LIF-responsive cells that express gp130 and LIFR, but not CNTFRα, become CNTF-responsive in the presence of sCNTFR. Similar to LIF, the combination of CNTF and sCNTFR is not cell-selective; high concentrations (up to 2.5 μg/ml) of sRα must be used because it is not concentrated on the cell surface. In contrast, addition of a cell surface binding domain to an sRα, such as a monoclonal antibody, should

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not only concentrate the sRα at the surface of target cells, but should also allow selective activation of cells that express the appropriate surface marker (Fig. 1B).

To test whether Rα components could be targeted to the cell surface and retain activity, we created constructs encoding chimeric proteins containing the extracellular domain of either IL-6Rα or CNTFRα fused to the Fc region of human immunoglobulin G1 (IgG1) [termed IL-6R–Fc and CNTFR-Fc, respectively (*22*)] and tested their ability to potentiate tyrosine phosphorylation of LIFR and gp130 on cells engineered to express either Fc receptors (FcγRI) or an irrelevant protein. Although we observed little tyrosine phosphorylation of gp130 when COS cells were incubated with IL-6 alone, addition of saturating sIL-6R along with IL-6 induced phosphorylation of gp130 regardless of whether COS cells were transfected with plasmids encoding FcγRI or a control protein (Fig. 2A). In contrast, stimulation with IL-6R–Fc and IL-6 conferred only weak gp130 phosphorylation in COS cells expressing the control protein but resulted in robust tyrosine phosphorylation of gp130 in COS cells expressing FcγRI (Fig. 2A). Washing the FcγRI-expressing COS cells after the addition of IL-6R–Fc had minimal effect on subsequent IL-6–mediated phosphorylation of gp130, whereas no gp130 phosphorylation was observed after washing of control COS cells or of FcγRI-expressing COS cells treated with untargeted sIL-6R (Fig. 2A) (*23*). This result is consistent with the notion that binding of IL-6R–Fc to FcγRI results in stable cell surface targeting of the Rα in an active state.

Targeting of CNTFR-Fc was evaluated in CNTF-unresponsive HepG2 cells that were stably transfected with a control plasmid or one encoding FcγRI. Our results were analogous to those obtained with targeted IL-6R–Fc: CNTFR-Fc markedly potentiated CNTF-induced tyrosine phosphorylation of LIFR and gp130 only when the target cells expressed FcγRI and this enhanced response persisted despite washing of the cells after the addition of CNTFR-Fc (Fig. 2B).

In the absence of targeting, both IL-6R–Fc and CNTFR-Fc were less active than their untagged counterparts (Fig. 2, A and B). Although the reason for this is unclear, the reduced activity provides an advantage by increasing the therapeutic index because the inherent activity of the untargeted Rα-Fc chimeric proteins is lower.

One prediction of the targeting model (Fig. 1B) is that a targeted sRα would be

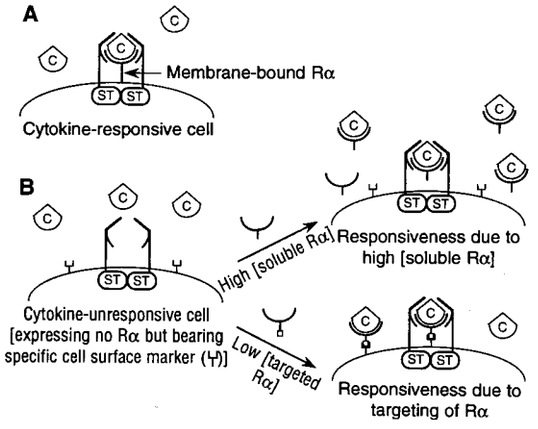

Fig. 1. Soluble Rα targeting. (**A**) A cell responsive to cytokines requiring an Rα component expresses that component on its cell surface. The complex competent for signal transduction for CNTF, IL-6, and IL-11 is composed of the appropriate Rα, the signal-transducing components (ST), and cytokine (C). (**B**) A cell that is nonresponsive to cytokines that require an Rα can be rendered maximally responsive by the addition of a high concentration of sRα. The same cell can also be rendered responsive with a low concentration of an engineered sRα that is targeted to bind to the cell surface in an active form.

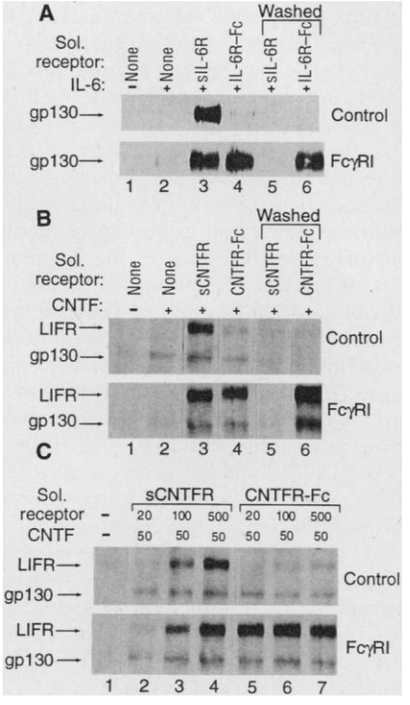

Fig. 2. Cell surface targeting of Rα-Fc fusion proteins to cells bearing the high-affinity Fc receptor. (**A**) COS7 cells were transiently transfected with pCDM8-derived expression vectors encoding either human nerve growth factor (control) or the human high-affinity IgG receptor [FcγRI (*26*)]. Two days after transfection, the cells were incubated in serum-free RPMI (Irvine Scientific) for 2 hours before treatment with human IL-6 (50 ng/ml, R & D Systems) ± sIL-6R (1 μg/ml, R & D Systems) or IL-6R–Fc [~2 μg/ml (*22*)] as indicated. The cells were preincubated with the soluble (Sol.) receptor (5 min) before stimulation with the cytokine for 5 min at 37°C; where indicated, the cells were washed three times with RPMI plus bovine serum albumin (1 mg/ml) before the addition of cytokine. Proteins were immunoprecipitated from cell lysates with an antibody to gp130 and immunoblotted with an antibody to phosphotyrosine (*27*). (**B**) Stable transfectants of HepG2 cells expressing either FcγRI or *neo* (control) were tested for their response to CNTF (50 ng/ml) with or without 0.5 μg of sCNTFR (*15, 28*) or CNTFR-Fc per milliliter (*22*). Receptor complexes were immunoprecipitated with an antibody to LIFR (*27*) and immunoblotted as in (A). (**C**) HepG2-expressing FcγRI or control HepG2 cells were treated with various amounts of either sCNTFR or CNTFR-Fc (nanograms per milliliter) as indicated and CNTF (50 ng/ml). Samples were processed and immunoblotted as in (B).

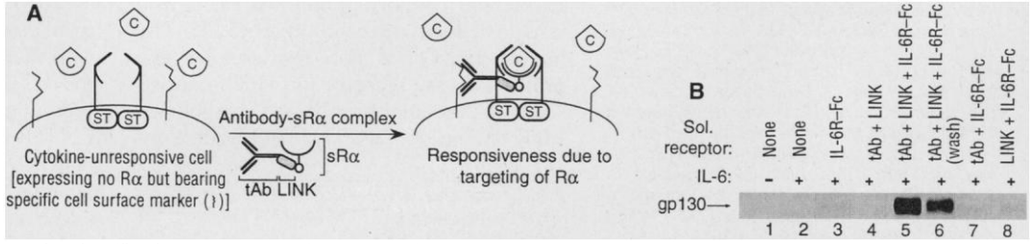

Fig. 3. Antibody-mediated targeting of sRα components. (**A**) Antibody-mediated targeting of Rα-Fc to a cytokine-unresponsive cell. The cell expresses the signal-transducing (ST) components and a cell surface marker to which a monoclonal antibody is available but does not express the appropriate Rα. Binding of the targeting complex to the cell surface marker through themonoclonal antibody concentrates the sRα at the cell surface in an active form, thus rendering the cell responsive to the cytokine. (**B**) SK-ES cells were starved in RPMI for 2 hours and then incubated with the indicated combinations of the three components of the targeting complex: tAb (anti-LNGFR, Boehringer Mannheim), LINK (protein A-G, Pierce), and IL-6R–Fc. The components (1 μg/ml) were added sequentially in the indicated order at 5-min intervals before stimulation with IL-6 (50 ng/ml). Cell lysates were processed and immunoblotted as in Fig. 2A.

more potent than an untargeted sRα in mediating cytokine responses. Whereas 500 ng/ml of sCNTFR was required for near-maximal phosphorylation of LIFR and gp130 on either the control HepG2 or FcγRI-expressing HepG2 cells (Fig. 2C), as little as 20 ng/ml of CNTFR-Fc gave maximal activity on FcγRI-expressing HepG2 cells (Fig. 2C). In contrast, the equivalent amount of CNTFR-Fc had little effect on CNTF activity in control HepG2 cells (Fig. 2C). Therefore, providing a binding site that allows the sRα component to be concentrated at the cell surface results in an increase in its apparent activity.

Monoclonal antibodies that recognize cell surface markers provide a potential means to selectively target various cell types. We therefore tested whether sRα-antibody complexes could serve as targeting reagents (Fig. 3A) and render a cell cytokine-responsive by concentrating the Rα at the cell surface in an active form. Targeting complexes were created by coupling of an appropriate targeting antibody (tAb) to an Rα-Fc through a protein A-G bridge (termed LINK) (Fig. 3A). IL-6R–Fc was targeted to the low-affinity nerve growth factor receptor (LNGFR) on SK-ES cells, which constitute a human neuroepithelioma cell line that does not normally respond to IL-6 (Fig. 3B). Whereas addition of IL-6R–Fc alone or in combination with either the LINK or the tAb (anti-LNGFR) resulted in an equal but small amount of IL-6–dependent phosphorylation of gp130, inclusion of the complete targeting complex (tAb·LINK·IL-6R–Fc) resulted in robust gp130 phosphorylation (Fig. 3B). The enhancement of gp130 phosphorylation was largely retained even when cells that had been incubated with the LNGFR-targeted complex were subsequently washed before the addition of IL-6, indicating that the LNGFR-targeted complex was stably associated with the cell surface (Fig. 3B).

We investigated the effectiveness of antibody-mediated sRα targeting to the hematopoietic cell markers CD34 and CD45 in an assay of TF-1 cell proliferation. TF-1 cells constitute an erythroleukemic line that expresses CD34 (*24*) and CD45 and proliferates in the presence of LIF, but lacks CNTFRα and therefore responds poorly to CNTF unless sCNTFR is also present (*15*). Complexes targeted to CD34 or CD45, and nontargeting complexes that either lacked antibody or contained an irrelevant antibody, were compared for their ability to stimulate TF-1 proliferation in the presence of CNTF. CNTFR-Fc targeted to either CD34 or CD45 enhanced CNTF-dependent stimulation of TF-1 cell proliferation compared with either of the nontargeted CNTFR-Fc complexes (Fig. 4A). Both a decrease in the median effective concentration (EC_{50}) and an increase in the maximum level of cell proliferation were observed for the targeted complexes relative to the nontargeted complexes. Control experiments showed that neither the antibodies nor the LINK had any intrinsic effect on the proliferation of TF-1 cells either in the presence or absence of CNTF (*25*). Furthermore, neither targeting nor nontargeting antibodies had any effect on TF-1 cell proliferation when added with an sCNTFR that lacked the Fc domain (Fig. 4B), demonstrating that the targeting effect is observed only when the sCNTFR is attached to the antibody and thereby concentrated at the cell surface.

Our results demonstrate the feasibility of creating selective sites of action for CNTF or IL-6 by targeting their specificity-determining components CNTFRα or IL-6Rα. Targeting an Rα to the cell surface in an active form required little optimization; every cell surface marker that we attempted to use as a docking molecule successfully mediated an enhanced cytokine response. If it proves desirable to eliminate the normal responses to CNTF or IL-6 when targeting, it may be possible to engineer compensating mutations in the cytokine and the Rα that render them specific only for their engineered counterparts and not for the naturally occurring molecules. Thus, Rα targeting may be a potential way to create "designer" cytokines that are tailored to stimulate cells of choice, especially those for which a unique cytokine may not exist.

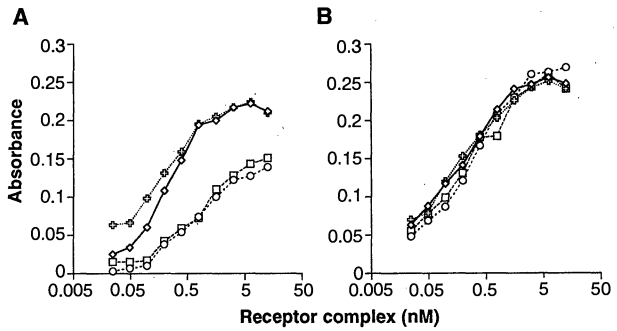

Fig. 4. Stimulation of TF-1 cell proliferation by CD45- or CD34-targeted sCNTFR. The absorbance plotted is the difference between the values at 570 and 650 nm. (**A**) CNTFR-Fc was coupled to monoclonal antibodies to CD45 (Biosource, Camarillo, California), CD34 (Oncogene Sciences, Cambridge, Masschusetts), or the flag peptide (M2; UBI, Lake Placid, New York) through a protein A-G bridge by co-incubation of each component (10 μg/ml) in serum-free RPMI. The resulting noncovalent complexes [CD45·LINK·CNTFR-Fc (◇); CD34·LINK·CNTFR-Fc (+); and M2·LINK·CNTFR-Fc (○)] were used in combination with CNTF (5 ng/ml) in a 3-day proliferation assay (*28, 29*). Also tested was the LINK·CNTF-Fc complex alone (□). (**B**) The activity of nontargeted sCNTFR (+ LINK; □) was not affected by antibodies to CD45 (CD45·LINK and sCNTFR; ◇), CD34 (CD34·LINK and sCNTFR; +), and M2 (M2·LINK and sCNTFR; ○).

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22. IL-6R–Fc and CNTFR-Fc chimeric proteins were engineered by fusion in-frame of the DNA sequences encoding either the first 358 amino acids of IL-6Rα followed by Ala-Gly, or the first 334 amino acids of the CNTFRα gene followed by Ser-Gly, to the hinge-C_{H2}-C_{H3} portion of human IgG1. The chimeric genes were subcloned into mammalian expression vectors or into baculovirus expression vectors and used for protein expression in the corresponding system.
23. In agreement with these results, protein immunoblots with antibody to IL-6R–Fc showed that COS cells expressing FcγRI that had been incubated with sIL-6R–Fc and washed bound and retained IL-6R–Fc, whereas identically treated COS cells expressing the control protein did not (*25*).
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29. The TF-1 proliferation assay was performed in RPMI plus immunoglobulin-depleted serum. The degree of proliferation is directly proportional to the absorbance difference between 570 and 650 nm measured in the assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The maximum absorbance achieved with the use of the targeted complexes containing CNTFR-Fc was equivalent to that observed for sCNTFR, both of which were essentially identical to the proliferation induced by LIF, indicating that most cells responded to the targeting complexes.

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