

Evidence for CD4-enhanced Signaling through the Chemokine Receptor CCR5*

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Robert Staudinger^{‡§¶}, Sanjay K. Phogat^{||}, Xiaodong Xiao^{||}, Xiahong Wang[‡], Dimiter S. Dimitrov^{||},
and Susan Zolla-Pazner^{‡**}

From the [‡]Veterans Affairs New York Harbor Healthcare System and the Departments of [§]Neurology and ^{**}Pathology, New York University School of Medicine, New York, New York 10016 and the ^{||}Laboratory of Experimental and Computational Biology, Frederick Cancer Research and Development Center, NCI, National Institutes of Health, Frederick, Maryland 21702

The chemokine receptor CCR5 is constitutively associated with the T cell co-receptor CD4 in plasma cell membranes, but the physiological role of this interaction has not been elucidated. Here we show that detergent-solubilized, purified CCR5 can directly associate with purified soluble fragments of the extracellular portion of CD4. We further demonstrate that the physical association of CCR5 and CD4 in membrane vesicles results in the formation of a receptor complex that exhibits macrophage inflammatory protein 1 β (MIP-1 β) binding properties that are distinct from CCR5. The affinity of the CD4-CCR5 complex for MIP-1 β was 3.5-fold lower than for CCR5, but the interaction of CD4 and CCR5 resulted in a receptor complex that exhibited enhanced G-protein signaling as compared with CCR5 alone. MIP-1 β -induced G-protein activation was further increased by simultaneous stimulation of CD4 with its natural agonist, interleukin-16. Thus, the physical association of CD4 and CCR5 results in receptor cross-talk with allosteric CD4-dependent regulation of the binding and signaling properties of CCR5. Although the precise physiological role of the CD4 effects on CCR5-mediated signaling remains unknown, one can speculate that the cross-talk is a component of mechanisms involved in the fine tuning of immune system cell responses.

CD4 is a component of the molecular complex that facilitates the interaction of the T cell receptor with major histocompatibility complex (MHC) class II molecules; it also serves as the primary receptor for attachment of the human immunodeficiency virus-1 (HIV-1)¹ (1, 2). The chemokine receptor CCR5 serves as the entry cofactor for macrophage-tropic strains of HIV-1 (3–7). CD4 and CCR5 act in concert for HIV-1 entry by

a sequential, ordered, multistep mechanism. Both receptors are expressed on lymphoid cells but belong to unrelated receptor families. CCR5 is a member of the heptahelical G-protein-coupled receptors (GPCR), whereas CD4 belongs to the immunoglobulin superfamily of membrane receptors with a single transmembrane segment that contributes to signal transduction through its cytoplasmic association with the lymphocyte kinase Lck (8). GPCRs have been known to associate with each other, but only recently has hetero-oligomerization between unrelated receptors with direct coupling been demonstrated in co-localized neurotransmitter systems (9–14). Here, we present data that demonstrates that CD4 and CCR5, both of which are involved in leukocyte activation and HIV-1 infectivity, form a unique receptor complex that is distinct from CCR5 alone with respect to affinity for its ligand, macrophage inflammatory protein-1 β (MIP-1 β), and for G-protein signaling.

We have shown previously that CD4 and CCR5 are physically associated even in the absence of the gp120 glycoprotein (15). It has been demonstrated that this interaction is unique to CD4 and CCR5 and is mediated through the second extracellular loop of CCR5 and the first two domains of CD4. To investigate the interaction between CCR5 and CD4, we have now used human osteosarcoma HOS-CD4⁺-CCR5⁺ and HOS-CD4⁻-CCR5⁺ cells to study the pharmacological and biochemical properties of these two HIV-1 receptor molecules. Here we present data suggesting cross-talk between these molecules.

EXPERIMENTAL PROCEDURES

Materials—^[125I]MIP-1 β , ^[35S]guanosine-5'-(γ -thio)triphosphate (^[35S]GTP γ S) and soluble CD4 (sCD4) were from PerkinElmer Life Sciences. MIP-1 β was purchased from Peprotech (Rocky Hill, NJ). The CD4 antiserum T4-4 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The polyclonal anti-CCR5 antibody CKR-5 was purchased from Santa Cruz Biotechnology, and cyclohexyl-pentyl- β -D-maltoside (Cymal-5[®]) was from Anatrace (Maumee, OH). Recombinant two-fragment (first and second domains) soluble CD4 (D1D2-sCD4) was a gift by Dr. Ed Berger.

Binding Assays—HOS-CD4⁺-CCR5⁺ and HOS-CD4⁻-CCR5⁺ (5) were kindly provided by Dr. Dan Littman. Cell membranes were prepared as described (16). Briefly, cells were rinsed with phosphate-buffered saline, resuspended in lysis buffer (50 mM Hepes, pH 7.4, 1 mM EGTA containing protease inhibitor mixture (Sigma)), and then homogenized by 40 strokes with a tight pestle in a Dounce homogenizer. Nuclei and unbroken cells were then pelleted by low speed centrifugation (800 \times g for 10 min at 4 °C). The supernatant was centrifuged at 45,000 \times g for 30 min at 4 °C. The crude membrane pellet was washed once and then resuspended in above buffer with the aid of a Dounce homogenizer.

All binding studies, which are described in detail elsewhere (16), were performed at 20 °C in 20 mM Hepes, pH 7.4, 1 mM CaCl₂, 5 mM MgCl₂, and 1% bovine serum albumin in a final assay volume of 0.1–0.25 ml. ^[125I]MIP-1 β (72–272 pM) was incubated with 0.048–0.17 mg/ml membrane protein for 60 min. Receptor-bound radioligand was

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[¶] To whom correspondence should be addressed: Dept. of Neurology, New York University School of Medicine, 550 First Avenue, NBV 7W11, New York, NY 10016. Tel.: 212-686-7500, ext. 4418; Fax: 212-263-8228; E-mail: robert.staudinger@med.nyu.edu.

¹ The abbreviations used are: HIV, human immunodeficiency virus; GPCR, G-protein coupled receptor; MIP-1 β , macrophage inflammatory protein-1 β ; HOS, human osteosarcoma; sCD4, soluble CD4; ELISA, enzyme-linked immunosorbent assay; IL-16, interleukin-16; CHO, Chinese hamster ovary.

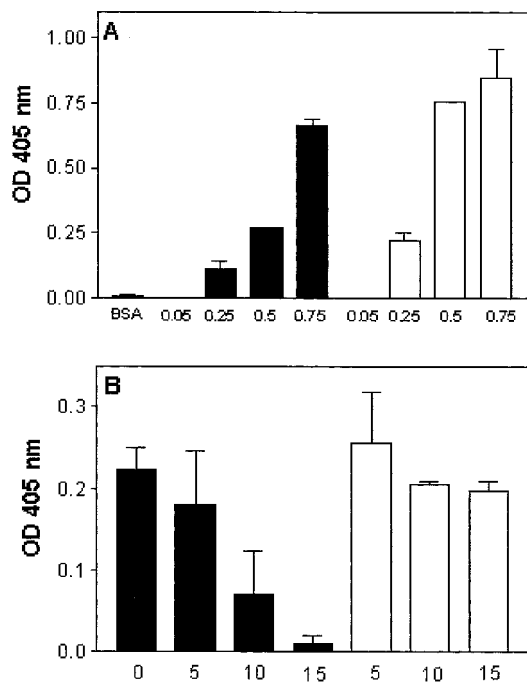


FIG. 1. Saturation binding of purified CCR5 to D1D2-sCD4 and four-domain sCD4. *A*, increasing concentrations (0.05–0.75 µg/ml) of D1D2-sCD4 (filled column) or sCD4 (open column) were coated on an ELISA plate and incubated with 1 µg/ml of purified CCR5 in a Cymal-5[®]-containing buffer as described under “Experimental Procedures.” *B*, 0.5 µg/ml of D1D2sCD4 was coated on the ELISA plate, and 1 µg/ml of purified CCR5 was added in the presence or absence of increasing concentrations (0–15 µg/ml) of either T4-4 (filled column) or OKT4 (open column) antibodies. Bound CCR5 was detected with the goat-anti-CCR5 antibody CKR5.

separated from unbound ligand by filtration through Whatman GF/C filters. Filters were washed twice with 4 ml of ice-cold incubation buffer containing 500 mM NaCl.

Solubilization and Purification of CCR5—CF2Th/synCCR5 cells were a kind gift from Dr. Joseph Sodroski. This canine thymocyte cell line stably expresses CCR5, which is expanded by the C9 tag TETSQVAPA (17). This tag is recognized by the 1D4 antibody, which was obtained from the National Cell Culture Center, National Institutes of Health. Cells were grown to confluency in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 4 mM glutamate, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.5 mg/ml G418, 0.5 mg/ml zeocin, and 3 µg/ml puromycin. Prior to harvesting, cells were incubated with 4 mM sodium butyrate for 40 h, washed with phosphate-buffered saline, and detached by treatment with 5 mM EDTA. Cells were solubilized for 30 min with 3 ml of solubilization buffer containing Cymal-5[®] as described (17) and then centrifuged for 30 min at 14,000 × *g*. The lysate was incubated with 1D4-Sepharose beads at 4 °C for 10–12 h. The Sepharose beads were then washed five times with 20 mM Tris-HCl (pH 7.5), 100 mM (NH₄)₂SO₄, 10% glycerol, and 1% Cymal-5[®]; the last wash included 500 mM MgCl₂. CCR5 was eluted from the Sepharose beads with washing buffer containing 200 µM C9 peptide (TETSQVAPA) and 500 mM MgCl₂. The concentration of harvested CCR5 was estimated by Coomassie Blue staining of an SDS polyacrylamide gel.

ELISA Assay for CCR5 Binding to CD4—0.05–0.75 µg/ml of D1D2-sCD4 or sCD4 was coated onto the ELISA plate in 50 mM carbonate, pH 9.6. Free binding sites were blocked with 4% milk in Tris-buffered saline. 1 µg/ml purified CCR5 was added in buffer containing 1% Cymal-5[®] and incubated for 12 h at 4 °C. Bound CCR5 was detected with the goat anti-CCR5 antibody CKR5 and an alkaline phosphatase-linked anti-goat antibody (Santa Cruz Biotechnology).

G-protein Activation—[³⁵S]GTPγS binding was carried out in 50 mM triethanolamine (pH 7.4), 5 mM MgCl₂, 1 mM EGTA, and 1 mM dithiothreitol containing 10 µM GDP and 15 µg of membrane protein at 20 °C as described in detail previously (18). The reaction was terminated by the addition of 4 ml of ice cold 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and filtration through Whatman GF/C filters.

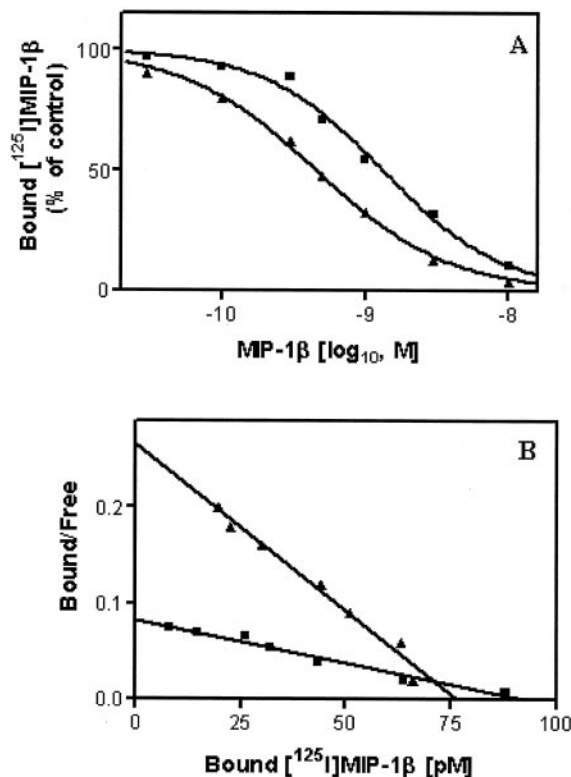


FIG. 2. Binding properties of [¹²⁵I]MIP-1β to CCR5 and CD4-CCR5. *A*, HOS-membranes expressing CCR5 (triangles) or coexpressing CD4 and CCR5 (squares) were incubated with 0.1 nM [¹²⁵I]MIP-1β in the presence of the indicated concentrations of MIP-1β. Half-maximal inhibition (IC₅₀) occurred at 0.45 nM and 1.45 nM, respectively. *B*, Scatchard transformation of displacement curves. *K_D* and *B_{max}* values were calculated by linear regression analysis. Coexpression of CD4 decreased the affinity of CCR5 for its ligand MIP-1β from 289 pM to 1.11 nM. The CCR5 density (*B_{max}*) was 1.5 pmol/mg and 1.9 pmol/mg in HOS-CD4⁻-CCR5⁺ and HOS-CD4⁺-CCR5⁺ cell membranes, respectively.

RESULTS

Detergent-solubilized Purified CCR5 Binds Specifically to Purified Recombinant sCD4—We have previously found that CD4 can be coimmunoprecipitated by anti-CCR5 antibodies from cells coexpressing the two molecules (15). To further study this interaction, we developed a binding assay using purified receptor proteins. Increasing concentrations of D1D2-sCD4 as well as full-length sCD4 were coated onto ELISA plates. Incubation with purified CCR5 showed a concentration-dependent binding of CCR5 to both D1D2-sCD4 and sCD4 (Fig. 1A). To further demonstrate the specificity of the CD4-CCR5 interaction, we used the polyclonal anti-CD4 antiserum T4-4 and the monoclonal anti-CD4 antibody OKT4 in these binding experiments. As illustrated in Fig. 1B, CCR5 bound specifically to D1D2-sCD4 as demonstrated by inhibition of CCR5 binding to CD4 by the anti-CD4 antiserum T4-4. The monoclonal anti-CD4 antibody OKT4, which was previously used to coimmunoprecipitate CD4 and CCR5 (15, 19), had no effect on the interaction of the two purified receptor molecules.

Binding Properties of CCR5 Are Distinct from the CCR5-CD4 Complex—We compared the ligand binding properties of CCR5 with those of CCR5-CD4 complexes. Competition binding experiments with [¹²⁵I]-labeled MIP-1β to membranes from HOS cells expressing either CCR5 or both CCR5 and CD4 are shown in Fig. 2A. Scatchard transformation revealed that the affinity of CCR5 for its ligand MIP-1β decreased by 3.5-fold when CD4 was coexpressed (Fig. 2B). We calculated by Scatchard analysis dissociation constants (*K_D*) of 278 ± 20 pM and 997 ± 115 pM for

MIP-1 β for HOS-CD4⁻-CCR5⁺ and HOS-CD4⁺-CCR5⁺ cell membranes, respectively. The CD4-CCR5 complex has therefore distinct pharmacological properties from CCR5, with the coexpression of CD4 resulting in a partial inhibitory effect on MIP-1 β binding to CCR5 due to a decrease in the affinity of CCR5 for its chemokine ligand. The CCR5 receptor density was similar in the two membrane preparations. The [¹²⁵I]MIP-1 β binding sites were 1.47 \pm 0.1 pmol/mg and 1.88 \pm 0.12 pmol/mg in HOS-CD4⁻-CCR5⁺ and HOS-CD4⁺-CCR5⁺ cell membranes, respectively. The CD4 receptor density in our HOS-CD4⁺-CCR5⁺ cell membranes was in excess of that of the CCR5 receptors with 9.43 \pm 0.4 pmol/mg as determined by [¹²⁵I]-gp120_{YU2} binding (data not shown). Interestingly, pre-incubation of HOS-CD4⁺-CCR5⁺ membranes with the T4-4 antiserum, which inhibits the association of CD4 and CCR5 (15), stimulated [¹²⁵I]MIP-1 β binding to 140%, whereas OKT4 had no effect (Fig. 3).

R5-tropic gp120, but Not X4-tropic gp120 or Interleukin-16 (IL-16), Inhibits MIP-1 β Binding to HOS-CD4⁻-CCR5⁺ Membranes—Gp120 and IL-16 are the known ligands to CD4. A natural ligand for CD4 IL-16 has been identified and charac-

terized as having a variety of biological effects similar to gp120 (20). R5-tropic gp120 has been shown to bind to CCR5 after interaction with CD4 and interfere with MIP-1 β binding to CCR5 (21, 22). We tested whether activation of CD4 by IL-16 would affect the binding properties of the CD4-CCR5 complex for MIP-1 β . As seen in Fig. 3, IL-16 did not affect the interaction of the CD4-CCR5 complex with MIP-1 β , confirming data previously reported (23). However, R5 tropic gp120 (ADA and YU2) interfered with MIP-1 β binding to HOS-CD4⁻-CCR5⁺ membranes, whereas X4-tropic gp120 had no effect.

CD4 Enhances CCR5-mediated G-protein Signaling in Co-transfected Cells—We next investigated whether there is a functional role for the physical interaction of these two receptor molecules and whether the physiologic response of the CD4-CCR5 complex is distinct from that of CCR5. For this purpose, we measured G-protein activation in HOS-CD4⁻-CCR5⁺ and HOS-CD4⁺-CCR5⁺ membranes by assessing [³⁵S]GTP γ S binding. GTP γ S interacts with the G-proteins with high affinity but is not hydrolyzed (24). G-protein activation is classically studied in membrane preparations, because guanine nucleotides cannot penetrate intact cells. As can be seen in Fig. 4, MIP-1 β (100 nM) accelerated the basal rate of [³⁵S]GTP γ S binding in HOS-CD4⁻-CCR5⁺ membranes, as expected for an agonist to CCR5. In HOS-CD4⁺-CCR5⁺ membranes, the addition of IL-16 further stimulated G-protein activation through CCR5. The rate constants were calculated assuming a pseudo-first order association as described (25) and are presented in Table I. IL-16 alone had no effect on CCR5⁺ or CD4⁺CCR5⁺ cell membranes but doubled the rate constant in the presence of MIP-1 β on CD4⁺CCR5⁺ membranes. Therefore, simultaneous occupation by the two agonists results in the most active signaling form.

DISCUSSION

Our results demonstrate that CD4 and CCR5 functionally associate on the plasma membrane and that co-receptors involved with immune stimulation and HIV-1 entry, which are members of unrelated receptor families, can interact. We provide biochemical and functional evidence for direct CD4-CCR5 cross-talk; CD4-CCR5 interaction leads to synergy such that CCR5 signaling is increased as a result of activation of CD4 by IL-16. The coexpression of CD4 itself had no effect on MIP-1 β -induced signaling of CCR5. Our data confirm a previous report wherein no effect of CD4 coexpression was found in chemokine-induced CCR5 internalization experiments (26) but also show that stimulation of CD4 enhances CCR5 function. The CD4-CCR5 complex is also pharmacologically distinct from CCR5 in that it is characterized by a lower affinity for binding to MIP-1 β . The association of CD4 and CCR5 at the cell membrane is reversible, because the T4-4 antiserum, which inhibits this interaction, stimulated MIP-1 β binding. Therefore, we suggest a model in which CD4, by associating with CCR5, allosterically modulates the binding properties of CCR5 for MIP-1 β , exhibiting decreased affinity for its ligand. Stimulation of CD4 with IL-16 does not further affect the binding of MIP-1 β to CCR5, but leads to enhanced signaling of CCR5. Mueller *et al.* (26) reported earlier a difference in MIP-1 β -induced internalization of CCR5 in CHO-CCR5⁺ and CHO-CCR5⁺-CD4⁺ cells. The

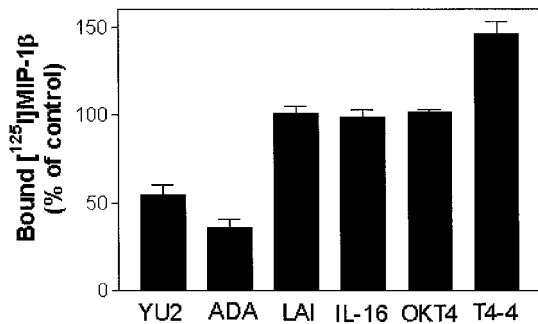


FIG. 3. Binding of [¹²⁵I]MIP-1 β to HOS-CD4⁺-CCR5⁺ membranes. R5-tropic gp120 (YU2 and ADA), X4-tropic gp120 (LAI) (100 nM each), IL-16 (5 μ g/ml), the anti-CD4 antibodies T4-4 (1:100) or OKT4 (10 μ g/ml) were pre-incubated with membranes for 1–6 h before the addition of [¹²⁵I]MIP-1 β . The effect on [¹²⁵I]MIP-1 β binding was assessed.

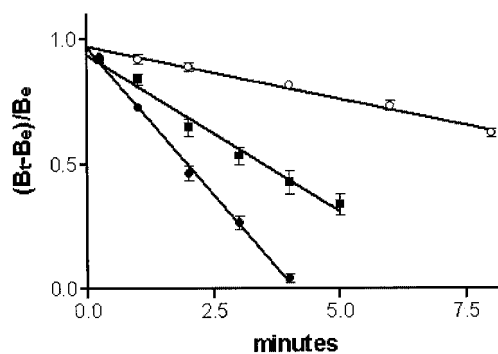


FIG. 4. Stimulation of [³⁵S]GTP γ S binding to HOS-CD4⁺-CCR5⁺ membranes. Before the addition of [³⁵S]GTP γ S, the membranes were incubated in the absence (open circles) or presence (filled squares) of 100 nM MIP-1 β or 100 nM MIP-1 β plus 5 μ g/ml IL-16 (filled circle). The reaction was terminated at the indicated time.

TABLE I
G-protein activation in membranes from HOS cells expressing CCR5 or both CCR5 and CD4

[³⁵S]GTP γ S binding to cell membranes was measured in the absence (control) or presence of 100 nM MIP-1 β , 5 μ g/ml IL-16, or 100 nM MIP-1 β plus 5 μ g/ml IL-16. The rate constants were calculated assuming a pseudo-first order association and are presented as mean from three independent experiments.

Cell membrane	Control	MIP-1 β (100nM)	IL-16 (5 μ g/ml)	MIP-1 β (100 nM) + IL-16
HOS-CD4 ⁻ -CCR5 ⁺	0.042 \pm 0.002	0.10 \pm 0.01	0.040 \pm 0.003	0.10 \pm 0.02
HOS-CD4 ⁺ -CCR5 ⁺	0.041 \pm 0.001	0.12 \pm 0.02	0.043 \pm 0.003	0.238 \pm 0.004

authors could demonstrate that this difference was attributable to the different levels of CCR5 expression in the two cell lines (8-fold difference in CCR5 levels). In the cell line selected for our experiments, CD4 coexpression did not significantly affect the level of CCR5. We determined a similar MIP-1 β binding site density in HOS-CD4⁻-CCR5⁺ and HOS-CD4⁺-CCR5⁺ membranes (1.47 versus 1.88 pmol/mg). Furthermore, we have shown earlier that, after uncoupling from the G-protein, CCR5 can no longer bind MIP-1 β (16). Therefore, the difference in affinity of CCR5 in HOS-CD4⁻-CCR5⁺ and HOS-CD4⁺-CCR5⁺ membranes cannot be attributed to a difference in the level of CCR5 expression in the two cell lines, because uncoupled CCR5 receptors do not exhibit detectable affinity for CCR5 (16). We chose a cell line for our experiments that expressed CD4 in excess of CCR5 so that, presumably, the CCR5 receptors are "saturated" with CD4. It will be interesting to determine whether there is a reciprocal effect of CCR5 on the binding and signaling properties of CD4.

It was previously reported that IL-16-induced chemotaxis is partially inhibited by pertussis toxin, and it was suggested that if a direct interaction of IL-16 with CCR5 exists, it could contribute to a CD4-induced migratory signal (23). Our data could provide an explanation for how CCR5 contributes to a CD4-induced signal.

Our findings may also have important implications for HIV-1 evolution and immunopathogenesis, because it has been suggested by many that a precursor of HIV-1 used CCR5 as the primary receptor (27–29) and that the close physical association of CD4 and CCR5 may have permitted the adaptation to CD4. HIV-1 has to react sequentially with its receptors to gain entry into a susceptible cell, and the formation of complexes between the HIV-1 receptors may make the entry process more efficient.

Finally, our data suggest a previously unknown signal transduction mechanism for chemokine receptors and immunoreceptors. If receptor interactions are a widespread phenomenon in cells of the immune system, the array of receptor complexes could be immense. Association of distinct receptor molecules would combine specificity with flexibility. Specificity would guarantee binding of the ligand to its receptor, but heterooligomerization would define a new level of functional diversity, depending on which receptor(s) are expressed by a particular cell and which ones form specific receptor complexes.

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