Molecular evolution of the human interleukin-8 receptor gene cluster

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Interleukin-8 (IL-8) is the prototype for a family of at least eight neutrophil chemoattractants whose genes map to human chromosome 4q13–q21. Two human IL-8 receptors, IL8RA and IL8RB, are known from cDNA cloning; IL8RA is a promiscuous receptor for at least two other related ligands, GROα and NAP-2. We now report cloning of the genes for IL8RA, IL8RB and a recently inactivated pseudogene of receptor A (*IL8RAP*). These form a cluster of only three genes in the superfamily of G protein-coupled receptors (GPCRs) and map to 2q34–q35. The coevolutionary diversity displayed by the IL-8 ligand–receptor complex — ligand promiscuity for IL-8, receptor promiscuity for IL8RA, gene duplication for both ligands and receptors and gene extinction in the case of *IL8RAP* — is unprecedented for the GPCR superfamily.

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Eukaryotic organisms have evolved the capacity to respond specifically to both exogenous signals such as photons of light or odorants, and endogenous signals such as growth factors or neurotransmitters. In the case of endogenous polypeptide signals, this has occurred by the concerted evolution of specific ligand and receptor gene pairs. Although the number of distinct receptors for a polypeptide ligand is usually one, pharmacologic analysis and molecular cloning have revealed a growing number of examples of ligand promiscuity, where a single ligand may bind to multiple distinct receptors. In fact, ligand promiscuity is a general property of certain other classes of ligands such as neurotransmitting amines[1]. Fewer examples of receptor promiscuity, where a single receptor binds to multiple distinct endogenous ligands, are known[2–7]. Ligand promiscuity evolves by replication of an ancestral receptor gene into a cluster of diverging receptor genes that may or may not be physically linked. We have found that the IL-8 receptor genes are a particularly rich example of the concerted evolution of the ligand-receptor relationship and the generation of signalling diversity.

IL-8 and the related ligands GROα, GROβ, GROγ, ENA-78, NAP-2, PF4 and γIP10 are 70–90 amino acid polypeptides with 26–51% sequence identity that are encoded by distinct genes clustered on human chromosome 4q13–q21 (refs 8–15; Table 1). All possess four conserved cysteine residues, the first two of which are separated by a single amino acid of variable identity. These "C-X-C" ligands are more distantly related to a second family of leukocyte chemoattractants where the first two cysteine residues are adjacent, the "C-C" ligands[8,9]. Despite 45–50% divergence of structure IL-8, GROα, and NAP-2 compete for similar binding sites on neutrophils and elicit similar functional responses[16–19]. Moreover, expression of IL-8 receptor A (IL8RA) in *Xenopus* oocytes reconstituted IL-8, GROα and NAP-2 signal transduction[20]. Expression of IL-8 receptor B (IL8RB) in human 293 kidney cells reconstituted IL-8 signal transduction[21]. The PF4 binding site appears to be distinct[19]. Binding data for the other C-X-C ligands have not yet been reported.

This suggested the possibility that the number of C-X-C ligands may exceed the number of distinct receptors capable of binding them. Since gene structure is highly conserved among GPCRs that bind the same prototype ligand[1,22,23] we examined this hypothesis by screening a human genomic DNA library at reduced stringency with an IL8RA cDNA probe.

The human IL-8 receptor gene family

Twenty-nine independent clones were identified. The melting temperature with respect to the probe divided the clones into two groups, those that melted at 55 °C in 5× SSPE and those that did not. The first group contained ten clones that cross-hybridized to a partial *Alu* repeat in the 3'-untranslated sequence of the probe. The second group contained nineteen clones that were divided into three subgroups according to restriction endonuclease digestion patterns. Subgroups I and II contained ten and three clones respectively, which encoded IL8RA[20] and IL8RB[21], respectively. Subgroup III contained six identical clones; the nucleotide sequence in the coding block of this gene is 88% identical to that of receptor A and 83% identical to that of receptor B. However, it is a pseudogene: three in-

Table 1 The human C-X-C family of neutrophil chemoattractant peptides

Ligand	Amino acid sequence	% identity to IL-8	Chromosomal localization	Binding competition
IL-8	S.A..KELRCLCIKTISKPFHPKFIKELRVIESGPHCANTEIIVKLSD.GRELCLDPKENWVQRVVEKFLKRAE.NS	100	4q13→21	YES
NAP2	-...----M----T-.GI---M-QS-E--GK-T--NQV-V-AT-K-.--KI----DAPRIKKI-Q-K-AGD-..-AD	51	NR	YES
GROα	A-V-..T------LQ-LQ.GI---M-QSVM-KSP-----Q--V-AT-KM.--KA--M-ASPI-KKII--M-NSDKS-	47	4q13→21	YES
GROβ	APL-..T------LQ-LQ.GI-L-M-QSVK-KSP-----Q--V-AT-KM.-QKA--M-ASPM-KKII--M--NGKS-	44	4q13→21	PRESUMED*
GROγ	A-VV..T------LQ-LQ.GI-L-M-QSVM-RSP-----Q--V-AT-KM.-KKA--M-ASPM--KII--I-NKGST-	44	4q13→21	PRESUMED*
ENA-78	AGPAA-VLR----V-LQ-TQ.GV---M-SM-Q-FAI--Q-SKV-VVAS-KM.-K-I----EAPFLKK-IQ-I-DGG..-KEN	39	NR	NR
PF-4	EAEEDGD-Q-L-V--T-.QVR-RH-TS-E--KA----PTAQL-AT-KM.--KI---LQAPLIKKIIK-L-...-..-	39	4q13→21	NO
γIP-10	VPL-RTV....--T--SINQ.-VM-RSLEK-EI-PASQF-PRV---ATMKKK-EKR--M-ESKAIKNLLKAVS-MSKR.-P	26	4q13→21	NR

* A murine homologue, MIP-2[36,37], competes with IL-8 for a common binding site on human neutrophils[19]
Sequences were aligned according to their four canonical cysteine residues (open boxes). Dashes indicate amino acids that are identical to the corresponding IL-8 residue; dots indicate gaps that were inserted to optimize the alignment. The single letter amino acid code is: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Binding competition refers to the ability of the listed ligands to compete with an IL-8 radioligand for binding to human neutrophils. NR, not yet reported.

frame stop codons and three frame shifts are found in the coding block. We designate this gene *IL8RAP* (IL-8 receptor A pseudogene). The coding sequence of *IL8RAP* was also obtained by polymerase chain reaction (PCR) amplification of genomic DNA from an unrelated individual. The same three frame shifts and in-frame stop codons were present as were initially found in the clones from the genomic library. Thus the sequence of the pseudogene is representative of the human gene pool. The 5' and 3' flanking sequence position identities between *IL8RAP* and *IL8RA* are 82% over 595 base pairs (bp) and 93% over 344 bp, respectively. The flanking sequences of *IL8RAP* are completely divergent from those of *IL8RB*. Although pseudogenes are frequently found in multigene families, two D_5 dopamine receptor pseudogenes are the only others that have been reported in the large superfamily of GPCRs[24-26].

All three genes lack introns in the coding sequence. When probes that included the complete coding sequence were hybridized at high stringency to human genomic DNA digested with *Pst*I, the same three fragments of 3.3, 1.8 and 5.0 kilobases (kb) were revealed in a complementary cross-hybridizing pattern (Fig. 1*a*). These bands were identical in size to hybridizing *Pst*I fragments of the genomic clones in subgroups I, II and III, respectively. Under low stringency conditions, no additional bands were seen (Fig. 1*b*). Hybridization analysis of human genomic DNA digested with *Eco*RI, *Eco*RV, *Hin*dIII or *Xba*I revealed fewer bands in each case[20].

It is unlikely that additional human genes exist for receptors that bind IL-8, GROα or NAP-2 for three reasons. First, in the GPCR gene superfamily gene structure is highly conserved when genes encode receptors that bind a common prototype ligand, even when the binding constants differ by several orders of magnitude. For example, the substance P receptor cDNA and the serotonin

Table 2 Similarity of the molecular evolution of *IL8RAP* with that of functional IL-8 receptor genes

Genes	Percent nucleotide divergence (%) and expected (E) vs observed (O) nucleotide changes									p-value
	Region 1			Region 2			Region 3			
	%	E	O	%	E	O	%	E	O	
IL8RAP vs *IL8RA*	29	10	24	16	60	76	6	59	30	<<.0001
IL8RA vs *IL8RB*	NA	NA	NA	15	NA	NA	16	NA	NA	NA
rIL8RB vs *IL8RB*	38	11	22	16	85	79	15	83	74	<.001

	Number of substitutions per synonymous (d_s) and non-synonymous (d_n) site								
	Region 1			Regions 2 and 3			Regions 1, 2 and 3		
	d_s	d_n	d_s/d_n	d_s	d_n	d_s/d_n	d_s	d_n	d_s/d_n
IL8RAP vs *IL8RA*	0.53	0.32	1.7	0.18	0.10	1.8	0.21	0.11	1.9
IL8RA vs *IL8RB*	NA	NA	NA	0.30	0.13	2.3	NA	NA	NA
rIL8RB vs *IL8RB*	1.9	0.35	5.4	0.49	0.08	6.1	0.53	0.10	5.3

The coding blocks of the indicated genes were aligned as described. The following regions were defined with reference to the 1070 nucleotides in the coding block of the IL8RAP sequence: Region 1, nucleotides 1–84; region 2, nucleotides 85–576; and Region 3, nucleotides 577–1070. In the upper half of the table, position differences were enumerated for each sequence comparison and the percent divergence was tabulated by region. Assuming that the nucleotide differences occurred by random substitution, contingency tables of expected and observed numbers of nucleotide differences were then constructed by region for each sequence comparison and chi-square was calculated. In the lower half of the table, the number of nucleotide substitutions per synonymous site and nonsynonymous site was estimated as described by Nei and Gojobori[33]. NA, not applicable: a meaningful analysis of nucleotide substitutions cannot be conducted because of numerous deletions in Region 1 of *IL8RB* with respect to *IL8RA*.

1A receptor gene were isolated by cross-hybridization to substance K receptor cDNA and β2 adrenergic receptor cDNA probes respectively, by virtue of ~65% nucleotide sequence identity in the respective coding regions[27-29]. Second, it is unlikely that the *Pst*I fragment size of a more extensively divergent putative fourth gene would coincide with those of *IL8RA*, *IL8RB* or *IL8RAP*, and therefore not be distinguishable by blot hybridization. Conversely, it is unlikely that a less divergent putative fourth gene with a *Pst*I fragment size identical to those of *IL8RA*, *IL8RB*, or *IL8RAP* would have been missed among the five million genomic clones that were screened since ten, three and six positive clones were isolated for *IL8RA*, *IL8RB* and *IL8RAP*, respectively. Third, the isolation of ten clones by virtue of cross-hybridization to a partial *Alu* repeat present in the 3'-untranslated sequence of the probe defines the sensitivity of the screening conditions: a putative fourth gene that contained ~87% nucleotide identity to the probe over as little as a 52 bp interval should have been identifiable.

Clustering of the human IL-8 receptor genes

The three *Pst*I fragments were mapped to human chromosome 2q33–qter by Southern blot analysis of DNA

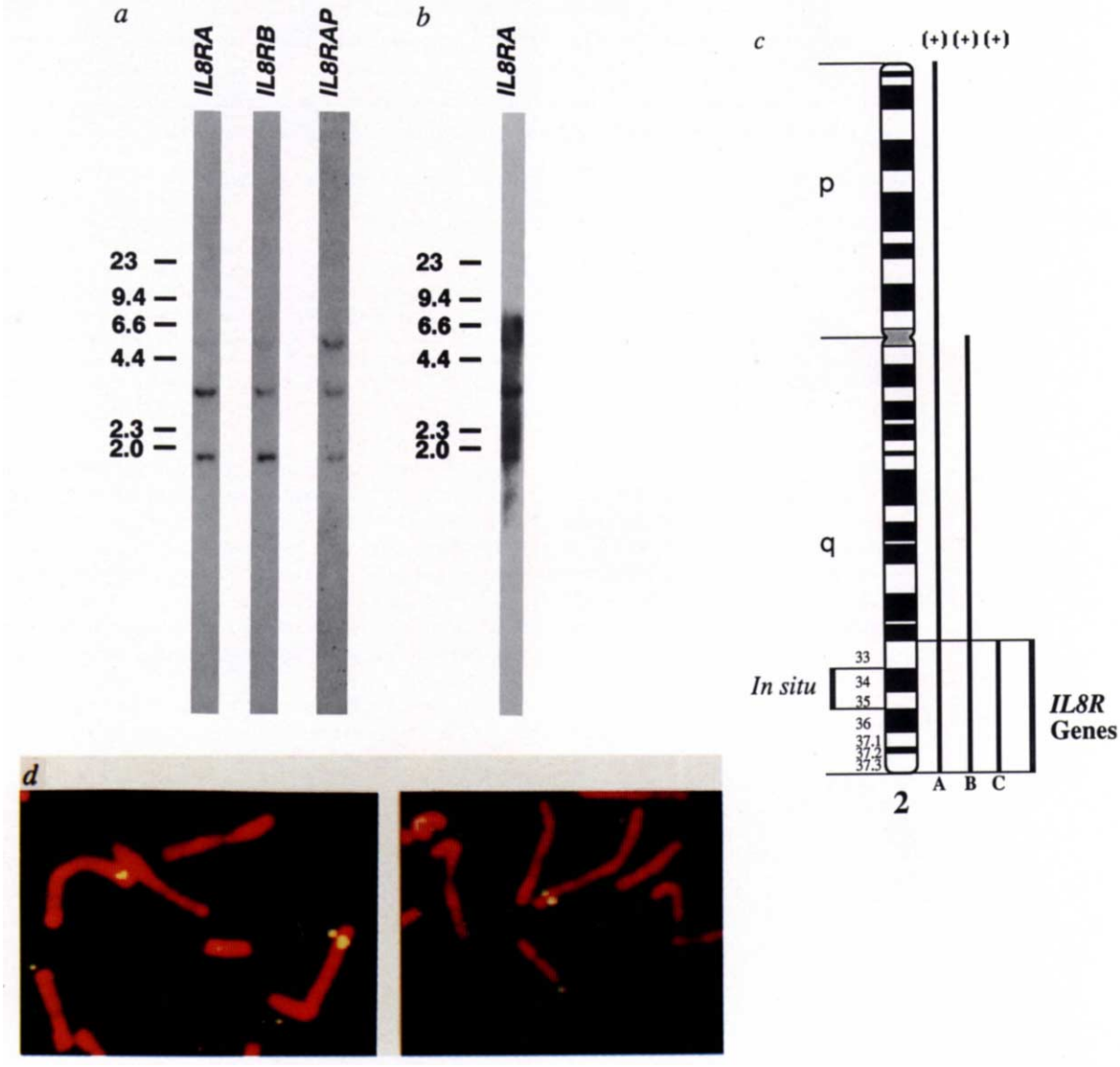

Fig. 1 Genomic analysis of the human IL-8 receptor gene cluster. *a,b,* Replicate blots of human genomic DNA digested with *Pst*I were hybridized with probes containing the coding region of the genes indicated at the top of each lane. The blots were washed at **(*a*)** high stringency (final wash at 68 °C in 0.1× SSPE for 1 h) or **(*b*)** low stringency (final wash at 45 °C in 5× SSPE for 1 h), then exposed to Kodak XAR-2 film in a Quanta III cassette at -80 °C for 12 hours to 3 days. In *a*, the exposures were chosen to emphasize the complementary cross-hybridizing pattern. No additional bands appeared after longer exposures. The position of DNA size standards is indicated at the left of each panel. *c,* Ideogram of G-banding patterns of human chromosome 2 and regional mapping of the IL-8 receptor gene cluster. The vertical bars on the right indicate an intact chromosome 2 (A), and the different portions of chromosome 2 present in these hybrids: B, XVIII-7B-3a Aza (2cen–qter); C, XIX-25A Agthg (2q33–qter). The right-hand brackets indicate the location of the gene by somatic hybrid cell analysis. The left-hand bracket shows the *in situ* hybridization results. *d,* Fluorescence *in situ* hybridization. The representative partial metaphases shown here are from an experiment using the IL8RB gene as the probe. In this experiment, 10 of 20 analysed metaphases had specific signals on at least one chromosome 2 at 2q34–q35. No other specific signals were seen.

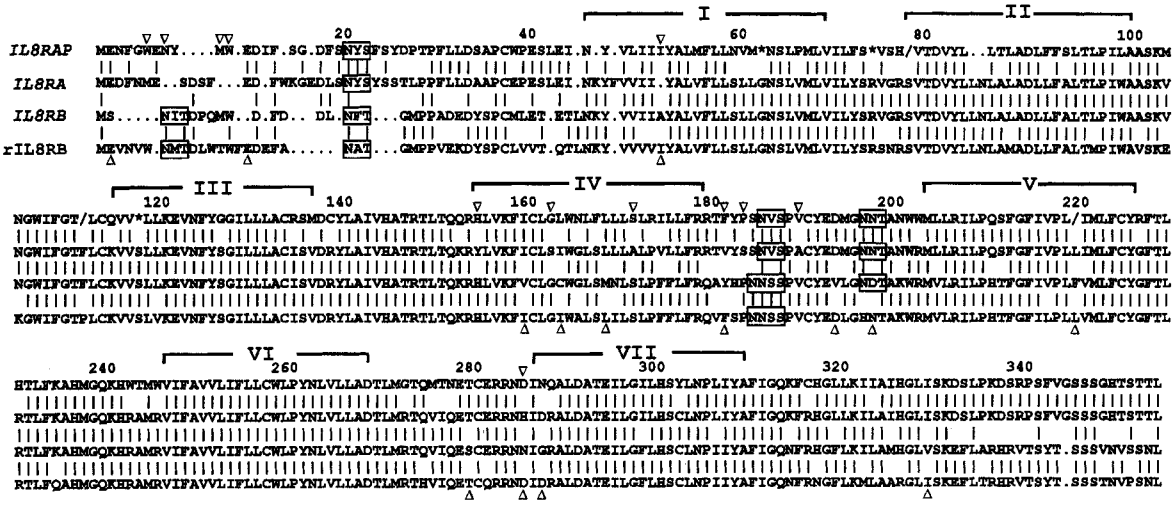

Fig. 2 Concerted evolution of the IL-8 receptors. Amino acid sequences were deduced from the coding sequences of *IL8RAP*, *IL8RA*, *IL8RB* and the rabbit homologue of IL-8 receptor B, rIL8RB[32]. The coding sequence of *IL8RAP* has been rectified at three frame shifts (/) to reveal its predicted ancestral structure; in-frame stop codons are designated by asterisks. Sequences were aligned as described and by inspection. Vertical bars indicate identical residues for each adjacent sequence position. Dots indicate gaps that were inserted to optimize the alignment. Abbreviations for the amino acid residues are as in the legend for Table 1. Arabic numbers above the sequence blocks refer to the *IL8RAP* sequence and are left justified. The location of the predicted membrane spanning segments I through VII are bracketed[38]. Open boxes designate predicted sites for N-linked glycosylation. Open triangles (△) indicate residues of rIL8RB that are distinct from those of its human homologue, *IL8RB*, but that are identical to the corresponding residue in one or both of the other more divergent sequences. Inverted open triangles (▽) indicate residues in the sequence deduced from *IL8RAP* that differ from its closest homologue, *IL8RA*, but that are identical to the corresponding residue of the sequence deduced from *IL8RB* and/or rIL8RB.

from rodent × human hybrid cell lines carrying different parts of chromosome 2 (Fig. 1*c*). All other chromosomes were excluded by at least two discordant hybrids. Fluorescent *in situ* hybridization (FISH) was performed with genomic probes containing the complete coding sequences for *IL8RA*, *IL8RB* and *IL8RAP* in Bluescript plasmid. Specific hybridization signals obtained with each of the three probes were localized on chromosome 2 at bands q34–q35 (Fig. 1*d*).

Interestingly, genes for two other neutrophil chemoattractant peptides, C5a and f-met-leu-phe, are also physically clustered, on human chromosome 19q13.3 (C. Gerard, personal communication). *FPRH1* and *FPRH2* are two additional human genes at 19q13.3 that encode putative receptors that are structurally related to the f-met-leu-phe receptor (C. Gerard, personal communication)[30]. In the large superfamily of GPCR genes, only two other examples of physical clustering are known: β2- and α1 adrenergic receptor genes on human chromosome 5q32-q34; and β1 and α2 adrenergic receptor genes on human chromosome 10q24-q26 (ref. 31).

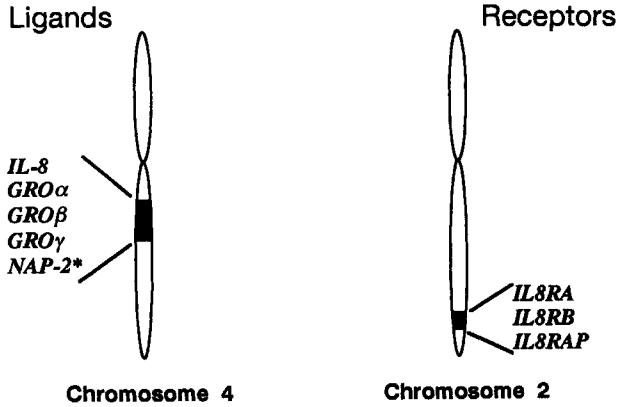

Fig. 3 Evolution of the human C-X-C ligand/receptor gene complex. C-X-C ligands that are known and predicted to interact with the human IL-8 receptors are shown. The asterisk, the chromosomal localization of NAP-2 has not yet been reported but is predicted to be on chromosome 4.

Evolution of the human IL-8 receptor gene family

DNA sequence analysis strongly suggests that the absence of a third functional receptor is due to the recent extinction of *IL8RAP*. If lethal mutation occurred soon after gene duplication, the distribution of nucleotide substitutions in *IL8RAP* compared to *IL8RA* should be random. Yet, the great majority of substitutions occur in the 5'-most half of the coding block (Table 2). Clustering of nucleotide substitutions is even more dramatic in a region defined by the first 84 nucleotides of the coding block of *IL8RAP*, designated as Region 1. This same pattern of nucleotide substitution is observed when the coding blocks of the two functional IL-8 receptor genes are compared with each other as well as with a functional rabbit homologue of *IL8RB*, (rIL8RB)[32]. In fact, the sequence of human *IL8RB* in Region 1 has been even more extensively altered from that of *IL8RA* by several small deletions. When the nucleotide substitutions that differentiate *IL8RAP* from *IL8RA* are analysed separately at synonymous and non-synonymous sites[33], a marked discrepancy is observed again between Region 1 and the rest of the region. The most striking difference occurs in the non-synonymous sites, 0.32 versus 0.10 substitutions per site. Again, the distribution of substitutions in each type of site is similar to that observed between *IL8RA* and *IL8RB*. The ratio of substitutions per synonymous site to substitu-

tions per non-synonymous site (d_s/d_n) is similar when one compares *IL8RA* to *IL8RB* and to *IL8RAP*, 2.3 vs 1.8. These values are in the range of those calculated for the most rapidly evolving mammalian genes[34].

Consistency in the pattern of molecular evolution of the pseudogene with the two human and one rabbit functional IL-8 receptor genes is further supported by comparison of the four predicted amino acid sequences (Fig. 2). Region 1 (as defined in Table 2) corresponds to the N-terminal segment in this alignment. It is predicted to be an extracellular domain of all GPCRs[1,22,23]. This segment has diverged substantially in the deduced sequence of all three functional receptors as well as that of the pseudogene. Yet a high content of acidic residues (~20%) has been retained in all. This is uncommon among other GPCRs[1,22,23]. Retention of this feature by the IL-8 receptors in an otherwise rapidly evolving domain could relate to the common need to bind a C-X-C ligand as these are all cationic proteins[8–11]. It also implies that *IL8RAP* was subjected to selective pressure after the gene duplication. Therefore *IL8RAP* probably encoded a distinct functional receptor for a C-X-C ligand long after the time of duplication (T_d) and until the time of non-functionalization (T_n). The sequence of a rabbit homologue of *IL8RA* is required to estimate T_d or T_n.

Conclusion

Thus, the human genome has expanded its repertoire of distinct genes encoding neutrophil chemoattractants while it has contracted the repertoire of genes encoding their receptors (Fig. 3). This example of receptor promiscuity stands in marked contrast to the numerous examples of ligand promiscuity found in the GPCR superfamily, including that of IL-8 for IL8RA and IL8RB. Receptor promiscuity is generally uncommon; notable examples outside of the GPCR superfamily are the α/β interferon receptor[2], the fibroblast growth factor (FGF) receptor[3], the IL-1 receptors[4,5] and the TNF receptors[6,7]. Interestingly, the genes for the α interferons and β interferon and those for two pairs of the FGFs (*FGF2* and *FGF5*, and *FGF3* and *FGF4*) are also physically clustered: the α and β interferon genes are on human chromosome 9 (ref. 2), and the genes for the two pairs of FGFs are on human chromosomes 4 and 11, respectively[35]. In the case of IL-1, genes for both ligands (IL-1α and IL-1β) and both receptors (IL-1 receptor Types I and II) are linked to human chromosome 2q12-q22[5].

Although experimental data are not yet available for GROβ and GROγ we predict that they too will bind to IL8RA because their structures are 88 and 85% identical to GROα, respectively (Table 1) and because a murine homologue, MIP-2 (refs 36,37), has been shown to compete for IL-8 binding sites[19]. The need for IL-8, NAP-2, GROα, and perhaps other related ligands to compete for only two potential binding sites imposes a fundamental limit at the receptor level on the potential diversity of neutrophil signal transduction networks that are activated by these ligands.

Note added in proof: The nucleotide sequence of the interleukin-8 receptor A pseudogene (*IL8RAP*) has been deposited at GenBank. The accession number is M98335.

Methodology

Genomic screening and hybridization conditions. A human placental genomic DNA library in the vector Lambda FIX (Stratagene, La Jolla, CA) was screened with a ^{32}P-labelled p2 cDNA encoding IL-8 receptor A[20] synthesized from random primers. Bacteriophage DNA was transferred to nitrocellulose filters in duplicate and hybridized with the probe in 6× SSPE (1× SSPE contains 150mM NaCl, 10mM NaH_2PO_4, and 1mM Na_2EDTA, pH 7.4), 50% formamide, 0.5% SDS, 10% dextran and 50 μg ml^{-1} of sonicated herring sperm DNA, at 35 °C for 12–16 h. Filters were washed in 5× SSPE, 0.1% SDS at 45 °C for 1 h (low stringency) and subsequently at higher stringencies (final wash: 0.1× SSPE, 0.1% SDS at 68 °C). The filters were exposed to XAR-2 film (Kodak) at -80 °C in a Quanta III cassette. Groups of positive clones bearing identical genes were identified by restriction mapping[39]. Appropriate fragments that hybridized to the probe were isolated by agarose gel electrophoresis, purified with Gene Clean (BIO 101), subcloned into pBluescript (Stratagene) and sequenced.

DNA sequencing and PCR amplification. DNA sequences were determined on both strands with sequence-based oligonucleotides (17 bases) by the dideoxynucleotide chain termination method[40]. The nucleotide sequence in the 3' untranslated region of the probe that has homology to a consensus *Alu* sequence[41] is: 5'-**AAAA**TCAGGCTGGCCAAC**GGGA**TGAAACCC**T**GTC-TCTACTAAAAATACAAAA -3'; nucleotides in bold refer to differences from the consensus sequence. The PCR amplification was carried out using specific primers (5' primer: 5'-AAAATGGAAAATTTTGGCTGGGAA -3'; 3' primer: 5'-TTAGAGAGTAGTGGAAGTGTGC CC -3') that flank the coding block of the IL-8 receptor pseudogene. The PCR amplification utilized 3 min at 97 °C followed by 30 cycles consisting of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s with a final extension at 72 °C for 10 min. The ends of the PCR product were filled-in using Klenow and ligated into the *Eco*RV site of pBluescript. The amplified DNA was sequenced as described. The DNA and protein sequences were compiled and analysed using the software package from the University of Wisconsin Genetics Computer Group[42] on a Cray supercomputer maintained by the National Cancer Institute Advanced Scientific Computing Laboratory, Frederick Cancer Research Facility, Frederick, Maryland.

Analysis of genomic DNA. Human genomic DNA (3 μg per lane) was digested with 6 U of *Pst*I restriction endonuclease (Boehringer-Mannheim) and fractionated by electrophoresis on an agarose gel (0.8%). After denaturation in alkaline solution the DNA was transferred to a Nytran filter by capillary action. The following probes were made: 1) p12, a 1.4 kb cDNA that extends from the start of the open reading frame through the poly(A) tail of IL-8 receptor A; 2) a 1615 base pair *Afl*III genomic fragment of IL-8 receptor B that extends from the start of the coding block through part of the 3'-untranslated region; and 3) the entire coding block of the IL-8 receptor pseudogene generated by PCR as described. Hybridization conditions were as described.

Chromosomal localization. The chromosomal assignments were made by using panels of somatic cell hybrids that contain different subsets of human chromosomes from fusion series XII, XV, XVII, XVIII, XIX, XXI, and 31 (ref. 43). Ten μg of genomic DNA samples extracted from parental control cells, and hybrid cell lines were digested with restriction enzymes, electrophoresed, transferred to Hybond nylon membranes (Amersham), and hybridized to ^{32}P-labelled p2 cDNA[20] probe as described[44]. Filters were rinsed twice in 2× SSC (1× SSC contains 150mM NaCl and 15mM sodium citrate·2H_2O, pH 7.0) and washed in 1× SSC, 1% SDS at 55–65 °C for 10–15 min. Autoradiography was carried out for 16–72 h at -40 °C with Kodak X-Omat AR film and two intensifying screens.

Fluorescence *in situ* hybridization. The following genomic DNAs containing complete coding blocks in pBluescript were biotin-11-dUTP labelled by nick translation using a kit (Boehringer Mannheim): a 2.7 kb *Xho*I fragment of IL8RA; a 4.0 kb *Hin*dIII fragment of IL8RB; and a 5.0 kb *Pst*I fragment of IL8RAP. Hybridization solutions consisted of 6.7 ng μl^{-1} labelled probe DNA, 200 ng μl^{-1} each of human and salmon sperm competitor DNA, 10% dextran sulphate and 2× SSC. Slides were hybridized overnight at 37 °C and pre- and post-hybridization washes were done as previously described[45] with

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the modification of room temperature 2× SSC washes post-hybridization. Chromosomes were identified by an R-banding pattern created by synchronization of the cells as described[46] with the exception of a BUdR release of the methotrexate block. A biotin/avidin/FITC detection system was used with chromosomes counterstained with propidium iodide (200 ng μl^{-1} final concentration). Only signals that were seen lying side-by-side on both chromatids of a chromosome were counted as specific. All single signals were assumed to represent random hybridization. A Zeiss Axiophot microscope equipped with the epifluorescence and a cooled charge-coupled device (CCD) camera (Photometrics PM512)/Macintosh computer system was used for imaging with software supplied by Tim Rand (Yale University). Photographs were taken on Kodacolor Gold ASA100 print film.

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