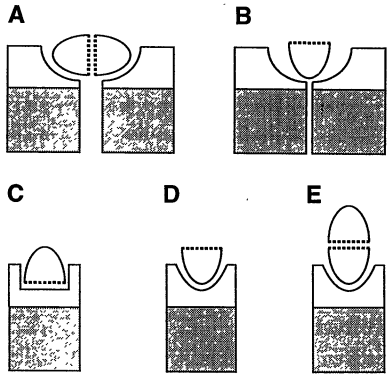

Fig. 6. Possible models for the interaction of IL-8 monomers and dimers with the receptor. In models A and B, the dimeric and monomeric forms, respectively, of IL-8 bind to a two-chain receptor. In model C, a monomer binds to a single-chain receptor through the region corresponding to the dimer interface. In model D, a monomeric IL-8 binds to a single-chain receptor, and in model E, a single-chain receptor binds the dimeric IL-8, but only one of the subunits participates in binding.

The results with the L25NMe analog demonstrate that a monomeric form of IL-8 is sufficient for receptor binding and full in vitro biological activity, and allow us to discuss models for the productive interaction of IL-8 with its receptor (Fig. 6). Model A, where dimeric IL-8 is required to cross-link two receptor molecules, is ruled out by the monomeric analog. In model C, IL-8 binds as a monomer; however, the interactions that normally occur at the dimer interface now facilitate receptor binding. This is unlikely, as the L25NMe modification does not permit the necessary H bonding. Model E, in which dimeric IL-8 binds to a single chain receptor, is not ruled out by the data; but in terms of energy used, dimerization at biologically relevant concentrations may not be favored. Model B, where the monomeric ligand binds two receptor chains at two distinct sites, is similar to the situation observed for growth hormone (9). However, receptor clustering is not generally considered a requirement for signal transduction by the seven transmembrane segment receptors. Therefore, model D, where monomeric IL-8 binds to a single chain receptor, seems the most likely mechanism of interaction.

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14. Molecular weights were determined by low-speed sedimentation equilibrium analytical centrifugation with Raleigh interference optics (Beckman Instruments, Palo Alto, CA). The molecular weights were calculated with the equation

$$\frac{2RT}{(1-\bar{\nu}\rho)}\,\frac{d(\ln Y)}{d(r^2\omega^2)}$$

where R is the gas constant, T is the temperature in kelvin, $\bar{\nu}$ is the partial specific volume, ρ is the density, Y is the concentration in fringe displacement units, and $d(\ln Y)/d(r^2\omega^2)$ is the slope from the plots in Fig. 3.
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23. Abbreviations for the amino acid residues are: C, Cys; E, Glu; F, Phe; I, Ile; K, Lys; L, Leu; R, Arg; and V, Val.

24. Supported by grants from PENCE, the Medical Research Council (MRC) of Canada, and the Swiss National Science Foundation. I.C.-L. is supported by an MRC Scholarship. The authors acknowledge P. Owen, P. Borowski, and L. Vo for assistance in protein synthesis, purification, and characterization; L. Hicks for sedimentation equilibrium; K. Oikawa for the fluorescence and CD studies; and G. McQuaid for the maintenance of the NMR spectrometers. M. Loetscher performed the binding assays. C. Davidson-Hall assisted with manuscript preparation.

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Requirement of Human Renal Water Channel Aquaporin-2 for Vasopressin-Dependent Concentration of Urine

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Concentration of urine in mammals is regulated by the antidiuretic hormone vasopressin. Binding of vasopressin to its V2 receptor leads to the insertion of water channels in apical membranes of principal cells in collecting ducts. In nephrogenic diabetes insipidus (NDI), the kidney fails to concentrate urine in response to vasopressin. A male patient with an autosomal recessive form of NDI was found to be a compound heterozygote for two mutations in the gene encoding aquaporin-2, a water channel. Functional expression studies in *Xenopus* oocytes revealed that each mutation resulted in nonfunctional water channel proteins. Thus, aquaporin-2 is essential for vasopressin-dependent concentration of urine.

The cloning of water-selective channels has begun to reveal the molecular basis of renal water transport. The water-selective channel, aquaporin-1 or CHIP28, is responsible for the constitutively high water permeability in the proximal tubule and the descending thin limb of the loop of Henle (*1*). In mammals, other mechanisms are needed to

Table 1. Osmotic water permeabilities of *Xenopus* oocytes injected with cRNAs encoding wild-type and mutant aquaporin-2. Removal of oocytes from female *Xenopus*, preparation, and monitoring of osmotic swelling were performed as described (*19*). Mutant cRNA- and water-injected oocytes were transferred from 200 mosM (osM_{in}) to 10 mosM (osM_{out}) modified Barth's medium diluted with water. To prevent premature rupture of the oocytes, Barth's medium was diluted only 1:1 for the wild-type cRNA-injected oocytes. Swelling was monitored with a Nikon Diaphot microscope (4× objective) equipped for video imaging (MagiCal, Joyce Loeble, Newcastle, United Kingdom). Oocyte images were captured at 1.7-s intervals for 1 min. All swelling experiments were performed at 22°C. Calculations of osmotic water permeability, P_f, of oocytes were done as described (*19*).

Injection	P_f(μm/s)*
5 ng of *aquaporin-2*	196 ± 26 (22)
Water control	20 ± 13 (16)
10 ng of R187C mutant	17 ± 11 (10)
10 ng of S216P mutant	18 ± 7 (11)
5 ng of *aquaporin-2* + 5 ng of R187C mutant	187 ± 38 (19)
5 ng of *aquaporin-2* + 5 ng of S216P mutant	192 ± 41 (18)

*Average ± SEM, with the number of assays in parentheses.

M	W	E	L	R	S	I	A	F	S	R	A	V	F L	A	E	F	L	A	T	20
L	L	F	V	F	F	G	L	G	S	A	L	N Q	W	P A	Q S	A S	L P	P	S	40
V	L	Q	I	A	M V	A	F	G	L	G	I	G	T I	L	V	Q	A	L	G	60
H	I V	S	G	A	H	I	N	P	A	V	T	V	A	C	L	V	G	C	H	80
V	S	V F	L	R	A	A	F	Y	V	A	A	Q	L	L	G	A	V	A	G	100
A	A	L I	L	H	E	I	T	P	A V	D E	I	R	G	D	L	A	V	N	A	120
L	S H	N	S N	T A	T	A	G	Q	A	V	T	V	E	L	F	L	T	L M	Q	140
L	V	L	C	I	F	A	S	T	D	E	R	R	G	E D	N	P L	G	T S	P	160
A	L	S	I	G	F	S	V	A T	L	G	H	L	L	G	I	H Y	Y F	T	G	180
C	S	M	N	P	A	R	S	L	A	P	A	V	V	T	G	K	F	D	D	200
H	W	V	F	W	I	G	P	L	V	G	A	I	L I	G	S	L	L	Y	N	220
Y	V L	L	F	P	P S	A	K	S	L	S Q	E	R	L	A	V	L	K	G	L	240
E	P	D	T	D	W	E	E	R	E	V	R	R	R	Q	S	V	E	L	H	260
S	P	Q	S	L	P	R	G	T S	K	A										271

Fig. 1. Deduced amino acid sequence (*14*) of the human aquaporin-2 protein. The amino acids that are different in the rat aquaporin-2 (*3*) are shown below the human sequence.

regulate the delicate water balance in response to the needs of the organism. This is accomplished in the kidney's collecting ducts where water resorption is regulated by the antidiuretic hormone arginine vasopressin (AVP). Binding of this hormone to its V2 receptor initiates a chain of signaling events that ultimately leads to the transient insertion of water channels in the apical membrane of principal cells and in the inner medullary collecting duct cells (*2*). The complementary DNA (cDNA) encoding a second water channel, aquaporin-2 or WCH-CD, was cloned which, on the basis of its exclusive expression in the collecting duct, could well be the vasopressin-regulated water channel (*3*).

Nephrogenic diabetes insipidus is a severe inherited disorder in which the kidney fails to concentrate urine in response to vasopressin (*4*). Mutations in the gene encoding the vasopressin V2 receptor have been identified in X-linked NDI (*5*). X-linked NDI is also characterized by a failure of the endothelium to release coagulation and fibrinolysis factors in response to the specific V2 agonist desamino-8-D-arginine vasopressin (dDAVP) (*4*, *6*). We have described a male patient with a variant of this disorder in which the abnormal dDAVP response is restricted to the kidney (*7*). Because the coding region for the vasopressin V2 receptor did not harbor any potentially harmful mutation in this patient (*8*), a mutation in another protein was considered. Such a defect could involve any protein that functions in intracellular signal transduction, in the expression or apical routing of the water channel in kidney cells, or in the water channel itself. We therefore tested by direct sequence analysis the hypothesis that in this variant NDI patient with an intact vasopressin V2 receptor, the disease is caused by mutation of the *aquaporin-2* gene.

We isolated human structural and genomic DNAs for *aquaporin-2* by screening kidney cDNA and cosmid libraries with a rat *aquaporin-2* cDNA probe (*9*). By fluorescent in situ hybridization on human lymphocyte chromosome spreads and Southern (DNA) blot analysis of human genomic DNA, the *aquaporin-2* gene was assigned to chromosome 12 (*10*). The cDNA was sequenced (*9*) and the deduced amino acid sequence showed an 89.7% identity with the rat aquaporin-2 protein (Fig. 1).

We used oligonucleotide primers based on the cDNA sequence to sequence the cosmid clone. Intron-exon boundaries of the *aquaporin-2* gene were identified at identical locations in related genes encoding the human erythrocyte water channel aquaporin-1 (*11*) and the major intrinsic protein of human lens fibers, MIP (*12*). On the basis of the deduced flanking intron sequences, primers were designed to amplify the four *aquaporin-2* exons from the patient's genomic DNA (*13*). We compared the sequence of those exons with the sequence derived from the cDNA, the cloned genomic sequence, and the genomic DNAs of two independent control individuals. We found one heterozygous point mutation in exon 3 and another heterozygous point mutation in exon 4 in the patient's DNA. The mutations were found on both strands sequenced, and both mutations caused nonconservative amino acid substitutions.

The C at position 559 was changed for T in exon 3, which resulted in a substitution of Cys for Arg^{187} (R187C) (*14*). This change is in one of the two most strongly conserved regions of the MIP family (*15*). A series of three invariant amino acids in this region, Asn-Pro-Ala is located just proximal to the substituted arginine (Fig. 2.). The arginine residue itself is conserved in 15 out of 17 members of the MIP family. Substitution of Pro for Ser^{216} (S216P) was caused by a conversion of T at position 646 to C in exon 4. This change is located in

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the sixth transmembrane domain (Fig. 2). In the other members of the MIP family, either serine or short-chain aliphatic amino acids are found at this position. The introduction of a proline residue might distort the α-helical structure in this part of the transmembrane region.

Direct sequencing of fragments of the *aquaporin-2* gene, amplified by the polymerase chain reaction from other family members, revealed that the R187C mutation was inherited from the father and the S216P mutation from the mother (Fig. 3). We were able to confirm this latter segregation event by restriction mapping because the T to C transition at position 646 created an Apa I restriction enzyme site (Fig. 3).

To investigate whether the mutations found in *aquaporin-2* resulted in nonfunctional water channels, we cloned wild-type *aquaporin-2* cDNA as well as both mutated *aquaporin-2* cDNAs into an expression vector, and all three constructs were transcribed in vitro (*16*). In vitro translation (*17*) showed that all three cRNAs produced a protein of the predicted size of 29 kD with equal efficiency (*18*). Injection of *Xenopus* oocytes with the cRNAs revealed that the mutant cRNAs failed to increase the osmotic water permeability, P_f, above that of the water-injected controls, whereas the wild-type cRNA increased the P_f value 10-fold (Table 1). Co-injection of either of the mutant cRNAs with the wild-type cRNA had no effect on the activity of the wild-type aquaporin-2 (Table 1). Thus, simultaneous expression of this nonfunctional aquaporin-2 had no functional consequence for the wild-type aquaporin-2, which is in keeping with the autosomal recessive inheritance observed in the family (Fig. 3). Our patient is, therefore, a compound heterozygote for two recessive mutations in the *aquaporin-2* gene.

Our findings demonstrate that aquaporin-2 is the vasopressin-regulated water channel in man. In NDI patients, mutations have now been identified at the first and last stages of the pathway for vasopressin-induced antidiuresis. Identification of patients with vasopressin-resistant diabetes who do not have mutations in either the gene encoding the vasopressin V2 receptor or the *aquaporin-2* gene may be of help in the identification of further elements essential for vasopressin-regulated water transport in the kidney.

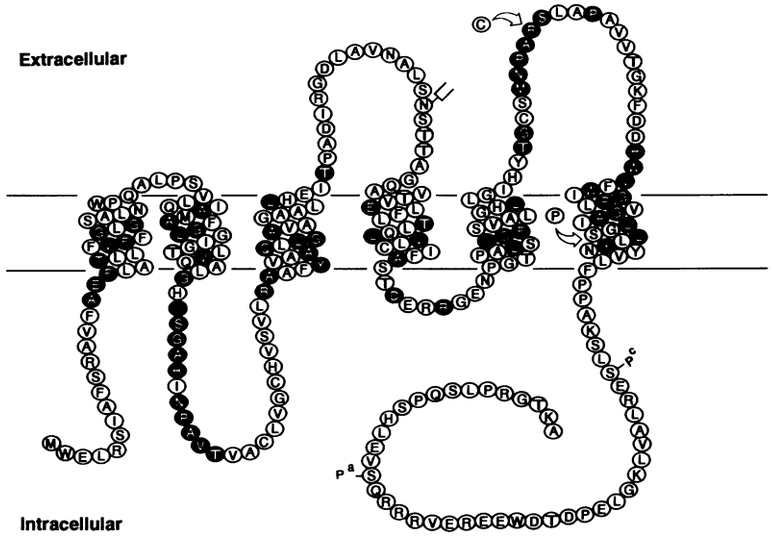

Fig. 2. Transmembrane orientation of the aquaporin-2 protein, based on the proposed topology of the rat homolog (*3*) and of the human aquaporin-1 protein (*1*). The two point mutations are indicated by arrows. The amino acid residues that are represented in the consensus sequence of the MIP family, which is conserved in at least 9 out of 17 analyzed members from bacteria, yeast, plants, and animals (*15*), are indicated by filled symbols. Consensus sequences for potential N-linked glycosylation sites (two-pronged symbol) and phosphorylation sites for protein kinase A (P^a) and protein kinase C (P^c) are indicated.

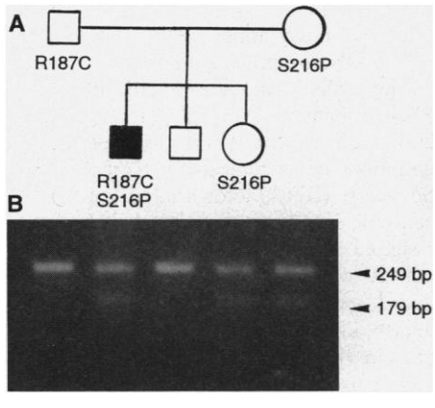

Fig. 3. (**A**) Inheritance of the R187C and S216P mutations in the family of the patient with a variant of NDI. Squares represent males; circles represent females. The shaded square represents the patient. Exon 3 and exon 4 of the *aquaporin-2* gene were sequenced directly from genomic DNA of all the family members. The heterozygous mutations found are indicated for each individual. (**B**) The PCR fragments for exon 4 of 249 bp were digested with Apa I and separated on a 2% agarose gel. The mother, patient, and daughter are all heterozygous for this mutation, as approximately half of the PCR product was reduced in size to 179 bp.

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9. A human cDNA clone was isolated from a lambda gt11 human kidney cDNA library (Clontech, Palo Alto, CA) by hybridization screening with an *aquaporin-2* cDNA probe from rat labeled with [α-^{32}P]deoxycytosinetriphosphate (*10*). The complete sequence as read by primer walking from both strands was obtained from a hybridization-positive clone with an insert of 0.9 kbp. An open reading frame of 813 bp was identified. Primers based on the cDNA sequence were used in direct sequencing of a cosmid containing the *aquaporin-2* gene to identify the intron-exon boundaries. The genomic sequence is deposited in GenBank, accession number Z29491. The polymerase chain reaction (PCR) and cycle sequencing were done as described (*5*).
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13. Genomic DNAs were isolated with the salt-extraction technique. The *aquaporin-2* exons were amplified with primers flanking the coding region of exon 1 (forward primer 5′-GCGAGAGCGAGTGCCCG-3′ and reverse primer 5′-CCCAGGACCTGCCCCTT-GT-3′); flanking exon 2 (forward primer 5′-CAGGA-AGATGGAGCCAGAGAG-3′ and reverse primer 5′-GTCCCTCTTGGGGTCTCTGTG-3′); flanking exon

3 (forward primer 5′-CTTCCTGCCCTGTCCT-3′ and reverse primer 5′-CCCCATCCCATGCTATTCCAG-3′); and flanking the coding region of exon 4 except for the terminal three codons (forward primer 5′-GTTGCCAAACCGCCCTCTCCGT-3′ and reverse primer 5′-GCGGCCCTCAGGCCT-TGGTA-3′). The same PCR primers were used in the cycle-sequencing reaction with the fluorescence-based Applied Biosystems model 373A DNA-sequencing system.

14. Abbreviations for the amino acid residues are: 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.
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20. We thank P. Krieg for the expression vector pT_7T_S. This study was supported by the Dutch Kidney Foundation (grants C91.1080, C92.1262, and C93.1299).

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Stat3: A STAT Family Member Activated by Tyrosine Phosphorylation in Response to Epidermal Growth Factor and Interleukin-6

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The STAT family of proteins carries out a dual function: signal transduction and activation of transcription. A new family member, Stat3, becomes activated through phosphorylation on tyrosine as a DNA binding protein in response to epidermal growth factor (EGF) and interleukin-6 (IL-6) but not interferon γ (IFN-γ). It is likely that this phosphoprotein forms homodimers as well as heterodimers with the first described member of the STAT family, Stat91 (renamed Stat1α), which is activated by the IFNs and EGF. Differential activation of different STAT proteins in response to different ligands should help to explain specificity in nuclear signaling from the cell surface.

Interferon signaling to the cell nucleus operates through phosphorylation on tyrosine of proteins that we have termed STATs (for signal transducers and activators of transcription) (*1–5*). The first proteins in the family to be described were a 91-kD protein (an 84-kD form of this lacks 38 COOH-terminal amino acids) and a 113-kD protein (*1, 2*). With the discovery of the family members described here, we suggest that the proteins be named in order of their description. Thus, Stat1α and Stat1β are the 91-kD and 84-kD proteins and Stat2 is the 113-kD protein. After IFN-α treatment of cells, both Stat1 (α and β) and Stat2 become phosphorylated (*3*), whereas after IFN-γ treatment Stat1 (α and β) but not Stat2 is phosphorylated (*4*). Each ligand thus elicits a specific STAT response. Upon IFN-γ–induced activation, Stat1 (α or β) forms a homodimer that binds DNA. The dimer is formed by intermolecular interaction of Src homology 2 (SH2) regions with phosphotyrosine residues (*6*). It also seems likely that IFN-α promotes interactions of Stat1 with Stat2 through tyrosine phosphorylation (*3, 6*).

The discovery that other polypeptide ligands [including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF-1), interleukin-10 (IL-10), ciliary neurotrophic factor (CNTF), and others] promote phosphorylation on tyrosine of Stat1 (*7–12*) raised the question of how transcriptional specificity might be maintained after cells encounter different ligands. Because the IFN-α and IFN-γ transcriptional response uses different STATs that share about 40% amino acid identity with each other (*2*), it seemed likely that additional family members might direct the formation of distinct DNA binding complexes in response to other signaling molecules. Indeed, CSF-1, IL-3, IL-4, IL-6, EGF, and CNTF all induce tyrosine phosphorylation of proteins or DNA binding activities that are distinct from the known Stat family members (*7–13*). Here, we describe a 92-kD mouse protein from the STAT family that is activated as a DNA binding protein by EGF and IL-6 but not by IFN-γ. We term this protein Stat3. A second family member, Stat4, has also been cloned by us and separately by others (*14, 15*).

We selected a 300-nucleotide probe (*16*) from the SH2 domain of the mouse Stat91 sequence by polymerase chain reaction and used this sequence to screen a mouse thymus complementary DNA (cDNA) library for clones representing possible family members. More than 10 such clones were partially sequenced, and all proved to be Stat1α or Stat1β. A number of placques that hybridized to the probe with less stringency were selected, subcloned, and sequenced (*14*). Of this group, about nine clones had identical sequences in the regions of overlap. This sequence was similar to but distinct from the Stat1 sequence. The longest clone contains both a stop codon and an ATG in a similar context compared to the translation start site of Stat1. With this ATG as a start codon, the new cDNA would encode a protein of 780 amino acids, similar in size to Stat1. The sequence of this cDNA, designated Stat3, had about 40 to 50% amino acid identity to Stat1 or Stat2, each of which is related to a similar degree (*2, 14*) (Fig. 1). In the same library screen, we also identified one other clone that is related but distinct from any of the above three genes and therefore named it Stat4. The complete sequences of Stat3, Stat4, and mouse Stat1 will appear elsewhere (*14, 17*).

Glutathione-S-transferase (GST) fusion products containing either 40 amino acids (amino acids 688 to 727) from the COOH-terminus or 158 amino acids (amino acids 2 to 159) from the NH_2-terminus of Stat3 were produced in bacteria, partially purified, and injected into rabbits to raise antisera (anti-Stat3c or anti-Stat3n, respectively) (*14*). From the A-431 or Hep-G2 cell extracts, the Stat3c antiserum precipitated predominantly a 92-kD protein and a less

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