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Mutational analysis of a critical signaling domain of the human interleukin 4 receptor

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ABSTRACT **The human interleukin 4 receptor (hIL-4R) is a member of a superfamily of cytokine receptors defined by conserved features of their extracellular domains. The intracellular domains of the hIL-4R and of other members of this family lack any recognizable enzymatic motifs, though ligand-dependent tyrosine phosphorylation of these receptors has been observed. Recent studies have suggested that serine-rich and acidic domains within the cytoplasmic portions of cytokine receptors might be required for signal transduction. Using deletion and truncation mutants of the hIL-4R, we have explored an essential 39-amino acid signaling domain that is rich in acidic amino acid residues and in serine residues that form consensus phosphorylation sites for known serine/threonine kinases. To assess the contribution of these motifs to signaling, we engineered site-directed mutants of these residues. Surprisingly, cells expressing mutant hIL-4R lacking either the serine or the acidic amino acids retain the ability of cells expressing the wild-type receptor to proliferate in hIL-4. Furthermore, receptors in which all six cytoplasmic tyrosines are absent can function, suggesting that tyrosine phosphorylation of the receptor may be an epiphenomenon rather than a requisite event in signaling.**

Interleukin 4 (IL-4) is a T lymphocyte-derived cytokine that has pleiotropic effects on a variety of cells, particularly upon cells of hematopoietic origin (reviewed in ref. 1). Its receptor (2, 3) is a member of the "cytokine receptor superfamily" of type I transmembrane molecules that have two sets of paired cysteines with conserved spacing, fibronectin type III domains, and juxtamembrane Trp-Ser-Xaa-Trp-Ser motifs in their extracellular ligand-binding domains (reviewed in refs. 4–6). Some of the receptors in this superfamily exist as heterodimers or trimers, but the human IL-4 (hIL-4) receptor (hIL-4R) cDNA encodes a mature 800-amino acid protein that alone forms high-affinity binding sites when expressed in COS cells (3).

In spite of the conserved features of the extracellular portions of these receptors, it has been difficult to identify common motifs in their intracellular regions. While the cytoplasmic domains do not appear to be tyrosine kinases and in fact lack any known signal transduction motifs, ligand binding by these receptors has been reported to activate a wide variety of putative second-messenger events. It is unclear which of these processes are responsible for the link between ligand binding at the surface of the cell and the profound proliferative and differentiative effects that cytokines have upon cell function. One approach to answering this has been to use mutational analysis to identify receptor domains that are critical for signaling. Such domains have been identified for the hIL-2 receptor (hIL-2R) β chain (7), mouse erythropoietin receptor (8–10), mouse (11) and human granulocyte colony-stimulating factor (G-CSF) receptors (12), hIL-7 receptor (hIL-7R) (13), gp130 (14), and the common human accessory chain β_c (15). We initiated similar studies for the hIL-4R and are able to confirm recent observations (16, 17) that only a limited domain of the receptor is required for signaling; moreover, we used site-directed mutagenesis to test directly the contribution of evolutionarily conserved elements within that domain.

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MATERIALS AND METHODS

Cell Lines and Proliferation Assays. Ba/F3, an IL-3-dependent mouse pro-B-cell line (18), was maintained in RPMI 1640 medium supplemented with 10% (vol/vol) bovine calf serum and 10% (vol/vol) WEHI-3B-conditioned medium (IL3 medium). Cell proliferation was assessed by measurement of the reduction of sodium 3-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonic acid (XTT) (19) after 3 days of culture in IL3 medium, in COS cell supernatants containing hIL-4 (20), or in a medium containing a hIL-4 standard (National Institute for Biological Standards and Control, Hertfordshire, England).

hIL-4R Expression Constructs. First-strand cDNA was made from RNA prepared from human peripheral blood leukocytes (21) and used as the template for a polymerase chain reaction (PCR). The primers were oligonucleotides identical to the 5′ end of the coding portion of the published hIL-4R sequence (3) (nucleotides 176–193) in the sense orientation and to the 3′ end of the coding region and stop codon (nucleotides 2633–2653) in the antisense orientation. The 2.5-kb product was gel-purified by using GeneClean (Bio101) and was cloned into pGEM-7zf+ (Promega) to make the construct pGEM-hIL-4R. The nucleotide sequence of this product (United States Biochemical) agreed with that published for the hIL-4R except for two silent nucleotide changes (T → C at position 628 and C → T at position 1420), which may represent *Taq* polymerase errors or polymorphisms. cDNAs encoding two mutant hIL-4Rs of 96 and 198 authentic cytoplasmic amino acids were generated by restriction enzyme digestion of pGEM-hIL-4R as indicated in Fig. 1. The fidelity of these constructs was confirmed by sequencing. Subsequent truncation mutants were generated by using PCR to introduce stop codons at precisely nucleotides 1558, 1498, 1450, 1405, and 1348 to encode receptors of 205, 185, 169, 154, and 135 cytoplasmic amino acids, respectively (designated cy205, cy185, cy169, cy154, and cy135). Functional constructs were verified by mapping only and not by sequencing.

Site-directed mutants were generated by using recombinant PCR (22) to introduce changes in specific residues and to introduce new restriction sites to facilitate screening. The thermostable polymerase pfu (Stratagene), which has a proofreading exonuclease activity, was used for these reac-

Abbreviations: IL, interleukin; hIL, human IL; hIL-2R, hIL-7R, and hIL-4R, receptors for hIL-2, hIL-4, and hIL-7; XTT, sodium 3-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonic acid; AP, alkaline phosphatase; FACS, fluorescence-activated cell sorting.

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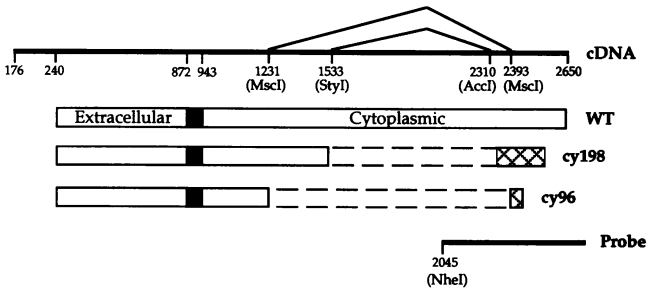

FIG. 1. Nucleotide and protein structure of the wild-type hIL-4R and two deletion mutants. At the top, the black line depicts to scale the coding region of the hIL-4R cDNA. The nucleotide numbers under the line refer to the published sequence (3): 176 is the first nucleotide encoding the initiator methionine, 240 is the first nucleotide encoding the N-terminal amino acid of the extracellular domain, nucleotides 872–943 encode the transmembrane domain, and the coding sequence ends at nucleotide 2650. The location of restriction sites used to engineer cDNAs for the deletion mutants is shown. The top boxed line shows the mature wild-type hIL-4R protein (WT) with the extracellular domain and 569-amino acid cytoplasmic domain labeled and the transmembrane domain indicated in black. The middle boxed line shows the deletion mutant cy198, a receptor with 198 authentic cytoplasmic amino acids and 78 novel amino acids (cross-hatched) created by a frameshift that was produced by restriction with *Sty* I and *Acc* I and religation. The bottom boxed line shows cy96, a receptor with 96 authentic cytoplasmic amino acids and 19 novel ones that was generated by restriction with *Msc* I and religation. At the bottom, the riboprobe used for the ribonuclease protection analyses is shown in relation to the wild-type and mutant molecules.

tions. The final products were screened for acquisition of the novel restriction sites and the presence of the site-directed mutations was confirmed by sequencing. The sense sequence of the oligonucleotide to mutate four serines was ATATATGGCGCCTTCTGTGCAGCGCCTGAGCCGCCAGGGATGACTTCCAGGAGGGA and to mutate eight glutamic acids was TTTGAGGCCCCGGTGGAGTGTCAGCAGGCCGGCCAGGTACAGCAACAAAAAGGGAGCTTCTGTGCATCG.

The wild-type and mutant receptor cDNAs were subcloned into the expression vector pGD, in which a Moloney murine leukemia virus long terminal repeat serves as the promoter for the gene of interest and a polycistronic message is produced with the polyomavirus thymidine kinase promoter driving a *neo* gene (23). For COS cell transfections, the expression vector pXM, which contains the adenovirus major late promoter and a simian virus 40 origin of replication, was used (24).

Transfection and Cell Cloning. Twenty-five micrograms of cesium chloride-purified receptor plasmid DNA was electroporated into 5×10^6 cells in 0.5 ml of cold PBS at 960 μF and 200 V in a Gene Pulser (Bio-Rad). The cells were plated in IL3 medium for 24 hr and then transferred at limiting dilution into IL3 medium containing 0.6 mg of G418 per ml. Clones were expanded for analysis.

hIL-4R mRNA Expression. RNA was purified from wild-type and transfected Ba/F3 and FDCP1 clones. A ^{32}P-labeled riboprobe was generated by using [^{32}P]UTP (Dupont/NEN) and T7 polymerase (Boehringer Mannheim) from a template made by restriction enzyme digestion of pGEM-hIL-4R at the unique *Nhe* I site (Fig. 1). The riboprobe was incubated with 10 μg of total cellular RNA overnight at 50°C, unhybridized RNA was digested with a mixture of RNases A and T1, and the residual probe was resolved by electrophoresis in 6% polyacrylamide gels.

Analysis of Cell Surface hIL-4R Expression. Transfected or wild-type Ba/F3 cells (1×10^6) were washed in Hanks' balanced salt solution (HBSS) and incubated for 1 hr at 4°C in HBSS containing 5% (vol/vol) COS cell supernatant containing hIL-4-alkaline phosphatase (AP) fusion protein (20). The cells were then washed and incubated with mouse anti-AP monoclonal antibody (Medix, Foster City, CA) at a 1:250 dilution followed by fluoresceinated goat anti-mouse IgG (SBA, Birmingham). Flow cytometry was subsequently performed. Alternatively, after binding with hIL-4-AP fusion protein and extensive washing, cells were lysed in 10 mM Tris·HCl, pH 8.0/1% Triton X-100, and the cell-associated AP activity was determined as described (25).

Analysis of Wild-Type and Mutant hIL-4R Proteins. Wild-type and mutant receptors in the pXM vector were transfected into COS cells by electroporation as described above. On day 3 after transfection, the cells were washed with methionine-free medium and then incubated with 400 μCi (1 μCi = 37 kBq) of [^{35}S]methionine for 4 hr. The cells were then washed and lysed in radioimmunoprecipitation assay (RIPA) buffer. Receptors were precipitated by incubation with biotinylated hIL-4 (R&D Systems, Minneapolis) for 2 hr at 4°C followed by incubation with avidin-conjugated agarose (Vector Laboratories, Burlingame, CA). The bound material was eluted in Laemmli buffer and applied directly to 7.5% polyacrylamide gels. The gels were dried and autoradiography was performed.

RESULTS

Analysis of the Expression and Function of the Wild-Type hIL-4R and Two Deletion Mutants. We engineered plasmids encoding the deletion mutants cy96 and cy198 (shown schematically in Fig. 1), transfected them into Ba/F3 cells, and analyzed six G418-resistant clones from each transfection. By ribonuclease protection using the probe depicted in Fig. 1, bands of the expected 611-nucleotide size were obtained from four of the six G418-resistant clones transfected with the wild-type receptor but not in RNA made from untransfected cells (Fig. 2). Three of six G418 resistant clones generated a protected fragment of ≈263 nucleotides as expected for cy96 and four of six G418-resistant clones generated a protected fragment of 346 nucleotides as expected for cy198 (Fig. 2).

To detect receptors upon the cell surface of clones that were making hIL-4R transcripts, cells were stained with an hIL-4-AP fusion protein. The cells were analyzed by flow cytometry. Examples of clones expressing wild-type and mutant hIL-4R are shown in Fig. 3. All of the clones that expressed mRNA of the appropriate size also expressed receptors on the surface of the cells, though the level of expression was variable. We did not perform formal Scatchard analyses of binding as it had previously been shown that a drastic truncation of the mouse IL-4R intracellular domain does not affect the affinity of the extracellular domain for ligand (2). Cell-associated enzymatic activity of the hIL-4-AP reagent reflected the FACS data and, as this assay was more rapid and sensitive than flow cytometry, it was used routinely for subsequent studies.

The FACS data suggested that Ba/F3 cells express modest amounts of transfected hIL-4R on the surface, as is commonly seen for cytokine receptors. To confirm that the hIL-4R constructs were encoding proteins of the corrected sizes, we studied them in metabolically labeled COS cell lysates (Fig. 4). A major specific band migrating with a mobility corresponding to the expected molecular mass of ≈125 kDa was seen in the lysate of cells transfected with wild-type hIL-4R, while major bands of 55 and 70 kDa appeared in cells transfected with cy96 and cy198, respectively. Less prominent bands of lower molecular mass were variably seen and may represent proteolytic degradation products. A nonspecific band of about 43 kDa precipitating in both sample and control lanes may be actin.

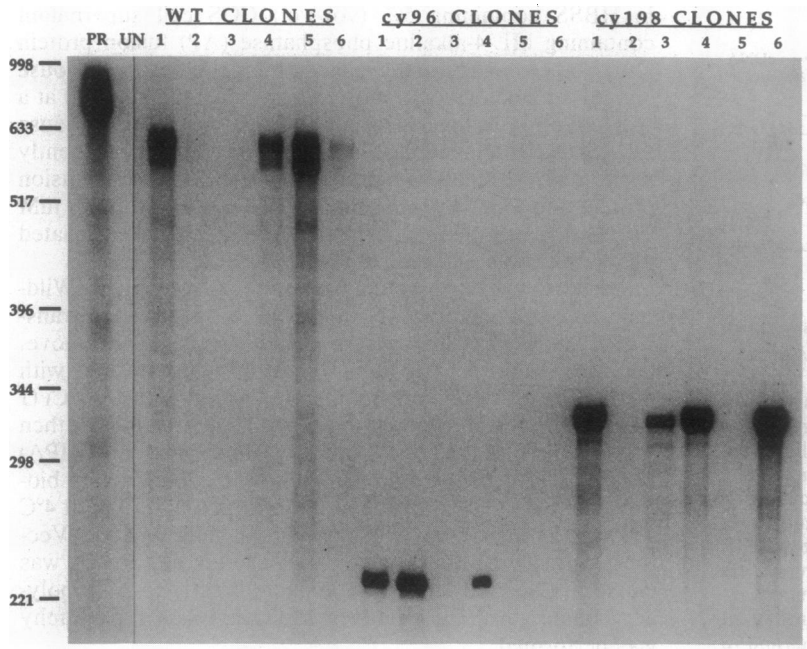

FIG. 2. Ribonuclease protection analysis of mRNA expression by Ba/F3 clones transfected with the wild-type hIL-4R or with deletion mutants. RNA was prepared from a pool of untransfected Ba/F3 cells and from stable clones of cells transfected with plasmid DNA encoding the wild-type hIL-4R, cy96, or cy198. Six clones of each were analyzed by ribonuclease protection with the riboprobe shown in Fig. 1, expected to protect transcripts of 611 nucleotides, 346 nucleotides, and 263 nucleotides for the wild-type receptor, cy198, and cy96, respectively. The undigested riboprobe is in lane PR. In lane UN, no visible bands are protected by RNA from untransfected Ba/F3 cells. Numbers on the left refer to nucleotides.

Each G418-resistant Ba/F3 clone was examined for its ability to proliferate in response to hIL-4. In preliminary experiments, proliferation as measured by the rate of XTT reduction was compared with the rate of DNA synthesis as measured by [^{3}H]thymidine incorporation, and a good correlation was found (data not shown). For subsequent experiments, the measurement of XTT reduction was routinely used. The untransfected parental cell line and G418-resistant clones that did not express hIL-4R mRNA and protein were able to grow only in medium containing mouse IL-3; in medium alone, or in medium supplemented with hIL-4, the cells rapidly died by apoptosis (data not shown). Clones expressing either the wild-type receptor or cy198 were able to proliferate in hIL-4 and in mouse IL-3 (Fig. 5). Although there was a range of hIL-4 responsiveness, no expressing clone was completely refractory to hIL-4. In contrast, no clone expressing cy96 responded to hIL-4. Thus, the region between cytoplasmic amino acids 96 and 198 contains elements required to transmit a proliferative signal.

PCR-generated Truncation Mutants Define a 39-Amino Acid Critical Domain. To narrow down the domain containing critical elements for signal transduction, constructs encoding progressively shorter mutant receptors—cy205, cy185, cy169, cy154, and cy135—were transfected into Ba/F3 cells

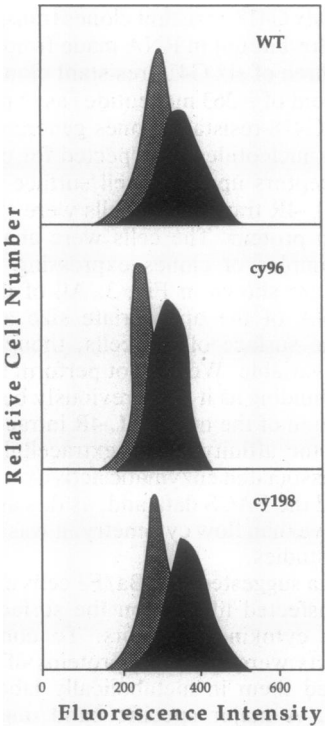

FIG. 3. Fluorescence-activated cell sorting (FACS) analysis of hIL-4 binding sites on transfected Ba/F3 cell clones. Clones of Ba/F3 cells transfected with cDNAs encoding the wild-type hIL-4R (*Top*), cy96 (*Middle*), and cy198 (*Bottom*) were incubated with the hIL-4-AP fusion protein, and bound ligand was detected by using a mouse anti-AP monoclonal antibody followed by a fluorescein-labeled goat anti-mouse antibody. Fluorescent cell cytometry was performed (black). For the controls, the hIL4-AP reagent was omitted (gray).

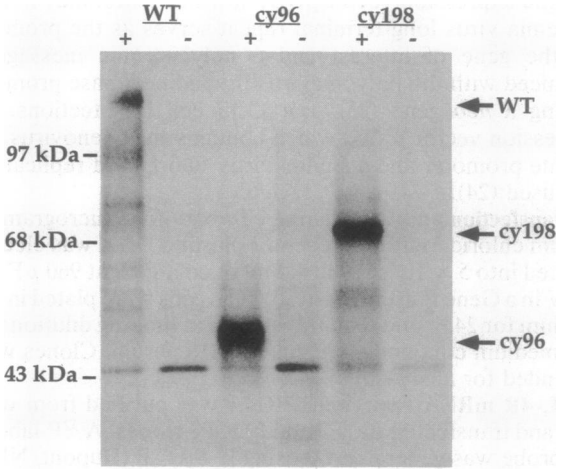

FIG. 4. Analysis of wild-type and mutant hIL-4R proteins. COS cells transiently transfected with the cDNAs for the wild-type hIL-4R (WT), cy96, or cy198 were metabolically labeled and lysed in Nonidet P-40, and the hIL-4R proteins were precipitated as described. The results of PAGE and autoradiography are shown. Each pair of lanes compares the specific precipitate (+) with the control precipitate in the absence of biotinylated hIL-4 (−). The mobility of molecular mass standards is indicated on the left, and the major protein bands corresponding to the expected receptor bands are indicated with arrows on the right.

and tested for surface expression and proliferation. All of these truncated receptors proved to be expressed and active in multiple G418-resistant clones (Fig. 6). Thus, the 39 amino acids included in cy135 but omitted in cy96 (shown at the bottom of Fig. 6) contain elements required for signaling. Since all of these truncation mutants delete the six cytoplasmic tyrosine residues of the hIL-4R, clearly those residues are not required for signaling. Truncation mutants shorter than 135 cytoplasmic amino acids—containing 111, 115, 120, 126, and 127 amino acids—were not expressed at detectable levels on the surface of G418-resistant Ba/F3 cell clones (data not shown). These mutants may be unstable or perhaps cannot be transported to the cell surface and were not studied further.

Testing Candidate Motifs by Site-Directed Mutagenesis. The 39-amino acid critical domain was examined for elements potentially involved in receptor signaling. Two features were identified: a very acidic stretch of five glutamic acids, a valine, and three more glutamic acids; and a serine-rich sequence that includes consensus phosphorylation sites for casein kinase II (paired serines in an acidic region) and glycogen synthase kinase 3 (Ser-Xaa-Xaa-Xaa-Ser). By recombinant PCR, we converted the acidic Glu_5-Val-Glu_3 sequence to Gln_2-Ala-Gly-Gln-Val-Gln_3, replacing most of the glutamic acids with glutamines (designated the "E- mutant," see Fig. 6). We also changed the serine-rich sequence Ser-Phe-Cys-Ala-Ser-Pro-Glu-Ser_2 to Ala-Phe-Cys-Ala-Ala-Pro-Glu-Ala_2, substituting alanines for the serine residues (designated the "S-mutant"). Six G418-resistant Ba/F3 clones transfected with each construct were selected and analyzed. Four clones of each were found to express the mutant hIL-4R. Although the number of cell surface hIL-4 binding sites was variable, each of these expressing clones was able to proliferate in response to hIL-4 (Fig. 7). Clearly, mutations of either of these candidate signal transduction elements did not abrogate signaling.

DISCUSSION

Analysis of deletion and truncation mutants of the hIL-4R indicates that the 39 amino acids between cytoplasmic residues 96 and 135 are critical for mitogenic signaling in Ba/F3 cells. This critical domain has two notable features: an extremely acidic glutamic acid-rich sequence, homologous to polyglutamate tracts that have been postulated to be protein-association domains (26); and a serine-rich sequence, encoding consensus motifs for phosphorylation by casein kinase II and glycogen synthase kinase 3. In a recent study, an internal deletion of the hIL-4R beginning at the same residue as our critical domain and continuing two residues beyond it was found to be essential for mitogenesis (16). These investigators postulated that the charged amino acids of this domain might be important but did not test those residues directly. One of their mutants and all five of our PCR-generated truncation mutants lack cytoplasmic tyrosines. Thus, although tyrosine phosphorylation of the hIL-4R has been observed (27, 28), it is not required for signal transduction in this system. A second group found that a mutant hIL-4R with an internal deletion of the first 133 amino acids of the cytoplasmic portion of the receptor is nonfunctional. These investigators also hypothesized that the polyglutamate motif might be important (17). In addition, they showed that an expressed receptor with an internal deletion of amino acids 233 through 348 does not signal. As we were able to generate functional truncated mutants that lack this domain, it presumably is not required for signaling *per se*, but its deletion may affect the conformation or accessibility of the more N-terminal critical domain.

To investigate the importance of the salient motifs within the critical domain directly, we used site-directed mutagenesis to make conservative mutations that would avoid imposing novel constraints upon the secondary structure of the receptor. Mutating eight glutamic acid residues to glutamine, glycine, and alanine within the most acidic portion of the critical domain did not abrogate signal transduction, nor did substitution of alanines for the four serines in the casein

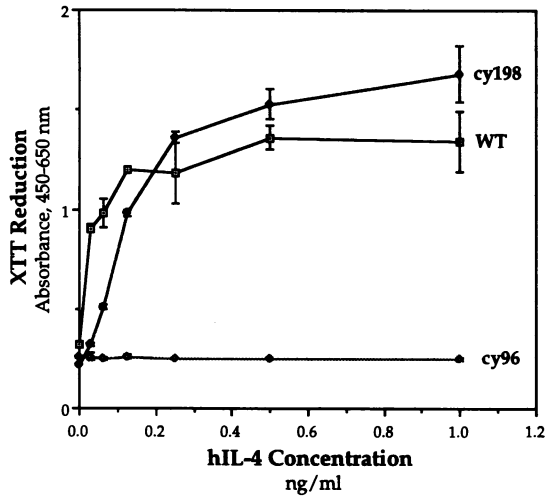

FIG. 5. hIL-4-dependent proliferation of transfected Ba/F3 clones. Clones expressing the wild-type hIL-4R (□), cy96 (◇), or cy198 (◆) were assayed for their ability to proliferate in response to hIL-4 after stimulation for 3 days followed by measurement of XTT reduction, as determined by the absorbance at 450 nm with background subtraction at 650 nm. For each hIL-4 concentration, the mean and standard deviation of triplicate wells are indicated.

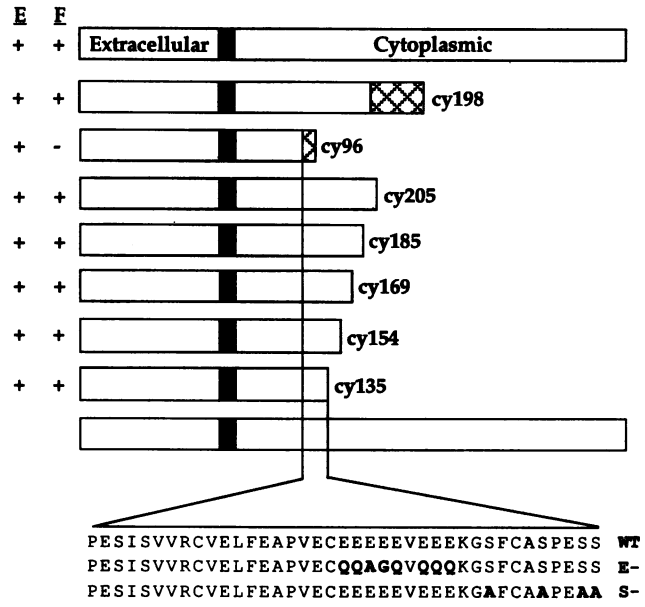

FIG. 6. Summary of expression and functional data for deletion and truncation mutants. The black boxes show the position of the transmembrane domain, and the cross-hatched boxes represent the novel amino acids generated in engineering the cDNAs for cy96 and cy198. "E" refers to expression as assessed by hIL-4-AP binding, and "F" refers to function as assessed by hIL-4-dependent proliferation. Six clones were analyzed for each construct, and the "+" signs signify that multiple positive clones were found. The location and sequence of the 39-amino acid critical domain, identified between cy96 and cy135, are indicated within the full-length receptor, as are the site-directed mutations of the E- and S-mutants (in boldface type).

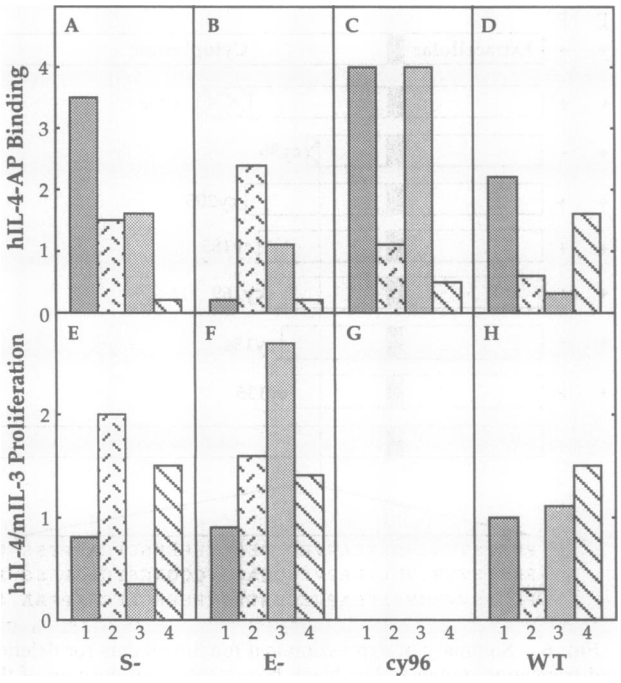

FIG. 7. Expression and function of S- and E-mutants. Four clones transfected with the cDNAs for the S- and E-mutants (*A* and *B*, respectively) shown in Fig. 6 were assayed for expression of hIL-4R. For comparison, the cell-surface expression of four clones expressing the cy96 mutation (*C*) or the wild-type hIL-4R (*D*) is shown. hIL-4-dependent proliferation of the same 16 clones is represented (*E–H*). To correct for the variable growth rate of each clone, hIL-4-dependent proliferation is expressed relative to proliferation in mouse IL-3.

kinase II and glycogen synthase kinase 3 consensus phosphorylation sites.

Critical signaling domains have been identified for other members of the cytokine receptor superfamily, and many also contain serine-rich or acidic elements. A study of deletion mutants of the hIL-2R β chain (7) and of a homologous region in the mouse erythropoietin receptor (10) demonstrated that serine-rich domains were required for proliferation. In a follow-up study, a single mutation of the kinase consensus site Ser-Pro-Gly-Ser-Ser to Arg-Pro-Gly-Ser-Ser in the cytoplasmic domain of the mouse erythropoietin receptor did not affect signaling (29), consistent with our findings for the hIL-4R. Interestingly, the serine-rich domains of both gp130 (14) and the hIL-7R (13) have recently been shown to be dispensable for signal transduction in transfected Ba/F3 cells.

The hIL-2R β chain has a domain rich in acidic amino acids that is not required for IL2-dependent proliferation but is essential for its association with $p56^{lck}$ in T cells (7, 30). While the physiological significance of this is not known, it is hypothesized that the acidic residues themselves play a role in this association. Acidic motifs exist in the critical domain of other cytokine receptors including the erythropoietin receptor, granulocyte colony-stimulating factor receptor, β_c, gp130, and hIL-7R, but their role in signaling has also not been tested directly. The β chains gp130 and β_c transduce signals from multiple ligand-binding α chains and have juxtamembrane cytoplasmic domains required for signaling that are characterized by hydrophobic residues and a PXP motif (14, 15). We did not test the homologous region in the hIL-4R, as it is proximal to the construct cy96 that had lost all signaling activity.

Thus 434 of the 569 cytoplasmic amino acids (76%) of the hIL-4R can be deleted without abrogating mitogenic signal transduction. These apparently superfluous residues may have an unknown regulatory role or they may play a role in signaling pathways in other cell types. We have confirmed that a region required for proliferation can be mapped to a small critical domain (in this case 39 amino acids). We have tested the importance of the casein kinase II and glycogen synthase kinase 3 consensus sites and the acidic polyglutamate motif within this critical domain directly. Receptors with nondisruptive site-directed mutations of those residues are still functional, conclusively demonstrating that they are not required to deliver a proliferative signal. Although tyrosine phosphorylation of the hIL-4R may occur subsequent to ligand binding, it is not essential since functional receptor mutants were constructed that lacked all cytoplasmic tyrosines.

Our thanks go to Alan D'Andrea, Leonard Zon, and Gregory Longmore for assistance with the Ba/F3 cell system; to Briggs Morrison for providing the hIL-4-CDM8 and hIL-4-AP-CDM8 constructs; to Juanita Campos-Torres in the Cell Sorter Lab; and to John Rush in the Biopolymers Facility. This work was supported in part by National Institutes of Health Grant 5 K08 HL02686-02.

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