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Sequential Mutations in the Interleukin-3 (IL3)/Granulocyte-Macrophage Colony-Stimulating Factor/IL5 Receptor β-Subunit Genes Are Necessary for the Complete Conversion to Growth Autonomy Mediated by a Truncated β_C Subunit

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An amino-terminally truncated β_C receptor (β_C-R) subunit of the interleukin-3 (IL3)/granulocyte-macrophage colony-stimulating factor/IL5 receptor complex mediates factor-independent and tumorigenic growth in two spontaneous mutants of a promyelocytic cell line. The constitutive activation of the JAK2 protein kinase in these mutants confirms that signaling occurs through the truncated receptor protein. Noteworthily, in addition to a 10-kb deletion in the β_C-R subunit gene encoding the truncated receptor, several secondary and independent mutations that result in the deletion or functional inactivation of the allelic β_C-R subunit and the closely related β_{IL3}-R subunit genes were observed in both mutants, suggesting that such mutations are necessary for the full oncogenic penetrance of the truncated β_C-R subunit. Reversion of these mutations by the expression of the wild-type β_C-R in the two mutants resulted in a fivefold decrease in cloning efficiency of the mutants in the absence of IL3, confirming a functional interaction between the wild-type and truncated proteins. Furthermore, expression of the truncated β_C-R subunit in factor-dependent myeloid cells did not immediately render the cells autonomous but increased the spontaneous frequency to factor-independent growth by 4 orders of magnitude. Implications for both leukemogenic progression and receptor-subunit interaction and signaling are discussed.

Adult hematopoiesis, in which the constant replenishment of short-lived mature blood cells from a pool of multipotential cells is tightly regulated yet able to respond rapidly to environmental changes, is controlled by at least two interacting systems. The maintenance of the stem cell pool and the production of lineage-committed precursors are thought to be controlled by the interaction of specialized cells of the bone marrow with hematopoietic precursors. This interaction is presumably regulated by the secretion of short-range regulatory factors or direct cell-cell interactions. In contrast, the proliferation of the committed precursors and their more differentiated progeny, as well as the induction of cell maturation, is primarily regulated by the interaction of a relatively large group of hematopoietic growth factors or cytokines that are released by auxiliary cells in hematopoietic tissues (e.g., T lymphocytes, endothelial cells, fibroblasts, and mononuclear phagocytes) and act at relatively large distances (2, 31).

These cytokines interact with the hematopoietic target cell through receptor molecules present on the cell surface in varying numbers. The receptor molecules transduce the binding of their cognate cytokines into cytoplasmic signals that eventually trigger a cascade of intracellular responses (11, 24). Although the receptor molecules of a few of these cytokines belong to the classical family of receptor tyrosine kinases (53), the majority make up a relatively new superfamily of receptors (3). This family is characterized by conserved amino acid sequence motifs in their extracellular domains: four conserved cysteine residues with relatively fixed spacing and a unique WSXWS sequence motif. Although no known catalytic motifs for signaling molecules (e.g., kinases, phosphatases, or nucleotide-binding proteins) have been identified in the cytoplasmic domains, functional assays have defined sequences that are conserved in several family members and are important for signal transduction (20, 34, 37, 42). The observation that several cytokines rapidly induce tyrosine phosphorylation has led to the hypothesis that the activated receptor recruits and/or activates protein tyrosine kinases for intracellular signaling. Indeed, the phosphorylation and/or physical association of different specific members of the Src and JAK families of tyrosine kinases by activated cytokine receptors has recently been reported (reviewed in references 22 and 24).

Many of the cytokine receptors are composed of at least two distinct subunits (23, 32, 52), one that is specific for a particular ligand and one that is common to several different receptor complexes. This sharing of receptor components may account for the large overlap of cytokine function observed. In this respect, the recently identified interleukin-3 (IL3)/granulocyte-macrophage colony-stimulating factor (GM-CSF)/IL5 receptor complex is of special interest. In both mice and humans, the receptors for IL3, GM-CSF, and IL5 are composed of a shared common β subunit (β_C-R subunit) and a distinct α subunit, which is specific for each cytokine and bind their ligand with low affinity (reviewed in reference 32). Although the β_C-R subunit has no binding capacity by itself, it forms high-affinity

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receptors for IL3, GM-CSF, and IL5 with their respective α subunits and is important for signal transduction. Interestingly, the mouse genome has two distinct β subunits (15, 19): the β_{IL3}-R subunit (previously known as AIC2A) is specific for the mouse IL3 receptor (IL3R), whereas β_C-R subunit (previously known as AIC2B) is equivalent to the human β_C-R subunit and is shared by the three receptor complexes (i.e., IL3R, GM-CSFR, and IL5R). Unlike the β_C-R subunit, the β_{IL3}-R subunit itself exhibits low binding affinity to IL3. The two mouse β-subunit genes have an unusually high degree of sequence identity (95% in the coding region) and appear to have been generated by a gene duplication of a common ancestral gene (14, 15).

Although still poorly understood, the complex controls of normal hematopoiesis regulate a precise and important balance between proliferation, differentiation, and self-renewal. However, cells do escape this control as a result of the accumulation of genetic lesions and contribute to various types of hematopoietic disorders, including chronic and acute forms of leukemia. Recent advances in the molecular analysis of chromosomal translocations characteristic for several types of leukemias have unveiled a large number of previously unknown genes implicated in normal and/or abnormal hematopoiesis (39, 44). Similarly, analyses of genes activated by the insertion of murine leukemia viruses in mouse model systems have also led to the identification of several hematopoietic regulatory proteins (26).

We have chosen an in vitro approach to identify genes and/or different mechanisms by which hematopoietic cells escape normal regulatory controls. We have previously described the isolation and characterization of autonomous mutants arising either spontaneously or after retroviral insertional mutagenesis of both mouse and human factor-dependent myeloid cell lines (48, 50). In the work described here, we have characterized in detail 2 mutants from a total of 11 factor-independent mutants of the murine D35 promyelocytic cell line (abbreviated Dind mutants) which were isolated at a frequency of greater than 2.4×10^{-7}. In contrast to the other nine mutants, which obtained factor-independent growth by an autocrine stimulation mechanism (e.g., secretion of IL3 or GM-CSF [21, 50]), these mutants have undergone alterations in the genes encoding the β subunits of the IL3/GM-CSF/IL5 receptor complexes. We demonstrate that the expression of a truncated and activated β_C-R subunit receptor in these cells mediates their factor-independent growth. Furthermore, our results establish that expression of at least the normal β_C-R subunit and perhaps also the β_{IL3}-R subunit interferes with signaling of the activated β_C-R subunit. Implications for leukemogenic progression and receptor signaling are discussed.

MATERIALS AND METHODS

Cell culture and transfection. All lines were maintained in Eagle's minimum essential medium (MEM; GIBCO) supplemented with 10% fetal calf serum. The D35 and FDC-P1 myeloid cell lines (8, 17) were grown with a final concentration of 5 to 15% WEHI-3B supernatant as a source of IL3. The retroviral helper cell lines GPE-86 and GP+env AM12 (29, 30) were used for the production of recombinant retroviruses.

Plasmid DNA was transfected by electroporation using a Bio-Rad Gene Pulser. For transfection, 4×10^6 cells were resuspended in 400 μl of medium with 20 μg of plasmid DNA and were pulsed with voltage and capacitance settings of 310 V and 960 μF. For the establishment of retroviral helper cell lines, GP+env AM12 cells were infected with viral particles released in the supernatant of electroporated GPE-86 cells. Twenty-four hours after infection, GP+env AM12 cells were selected for neomycin resistance (Neo^r) with 600 μg of G418 per ml. For Neo^r selection of infected Dind3 cells, 1.2 mg of G418 per ml was added to cultures.

Factor-dependent and -independent cell lines were cloned in 0.33% agar in MEM with or without growth factor at various cell densities. To determine the mutation frequency to factor-independent growth, a total of 10^7 to 10^8 cells were plated in 24-well plates as previously described (48).

Nucleic acid analysis. Preparation of DNA, Southern and Northern (RNA) analyses, and molecular cloning were performed as previously described (50). Total RNA was prepared by the guanidinium isothiocyanate procedure. Probes used for Southern and Northern analyses include the 5′ end (*Eco*RI-*Hin*dIII) or the 3′ end (*Hin*dIII-*Eco*RI) of the β_{IL3} receptor cDNA isolated from the original plasmid pAIC2-226 (23). To distinguish between β_{IL3}-R and β_C-R subunit transcripts, end-labeled oligonucleotides that spanned nucleotides (nt) 1935 to 1915 (β_{IL3}-R specific) and nt 1938 to 1918 (β_C-R specific) of the respective cDNA sequences (14) were used for hybridization (45°C, 18 h). Genomic DNAs of both wild-type and altered β_C alleles from D35 and Dind3 were molecularly cloned into Charon 18 and then subcloned into pBS-SK^- (Stratagene) and sequenced as described below.

For cDNA cloning, specific RNA transcripts were amplified by reverse transcriptase-dependent PCR. RNA (1.0 μg) was reverse transcribed into cDNA by using avian myeloblastosis virus reverse transcriptase and primed by a random primer (Boehringer Mannheim). Specific cDNA fragments were subsequently amplified with *Taq* polymerase, using the conditions originally described by Saiki et al. (41). Oligonucleotides for the amplification of the 5′ end of the altered β_C-R subunit cDNA spanned nt 1 to 20 and nt 1938 to 1918; for amplification of the 3′ end, oligonucleotides spanned nt 1579 to 1599 and nt 2955 to 2935 of the wild-type β_C-R subunit cDNA. Amplified products were cloned into the pCRII vector (Invitrogen, San Diego, Calif.) and sequenced by the dideoxy-chain termination method using an automatic sequencer (Applied Biosystems). To confirm the identified mutations, three independent amplified DNA fragments were sequenced from both ends.

Radioligand binding assays. Recombinant IL3 and GM-CSF were iodinated by using Iodogen (Pierce, Rockford, Ill.). Scatchard plot analyses using ^{125}I-labeled murine IL3 were carried out as described previously (45). For the GM-CSF binding assay, 10^6 cells were incubated at room temperature for 1 h in 100 μl of binding buffer (1 mg of bovine serum albumin per ml in Dulbecco's modified MEM containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.2]) including ^{125}I-labeled murine GM-CSF in the presence or absence of 500-fold unlabeled ligand. Cell-associated radioactivity was measured as previously described (35). The specific radioactivities of ^{125}I-labeled murine IL3 and ^{125}I-labeled GM-CSF were 9.5×10^5 and 9.3×10^5 dpm/pmol, respectively.

Immunoprecipitation and immunoblot analysis. Cells (2×10^7) were stimulated with 1 nM IL3 in 2 ml of MEM containing 10% fetal calf serum at 37°C for 5 min. Before stimulation, factor-dependent D35 and FDC-P1 cells were cultured in the absence of IL3 for 4 and 20 h, respectively. Cells were washed with cold phosphate-buffered saline and lysed in 1 ml of immunoprecipitation buffer (50 mM HEPES, 100 mM NaF, 10 mM $Na_4P_2O_7$, 4 mM Na_2MoO_4, 4 mM EDTA, 2 mM Na_3VO_4) containing 0.5% Nonidet P-40, aprotinin (2 μg/ml), leupeptin (10 μg/ml), and 1 mM Pefabloc (Boehringer) on ice for 30 min. Clear cell lysate was incubated with anti-JAK2 serum (Upstate Biotechnology, Inc., Lake Placid, N.Y.) at 4°C for 2 h, and the immunocomplex was precipitated with 10 μl of protein A-Sepharose. The proteins were electrophoresed on a sodium dodecyl sulfate–10% polyacrylamide gel and blotted onto an Immobilon-P membrane (Millipore). The membrane was then incubated with 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20 containing 3% bovine serum albumin at room temperature overnight and incubated with biotinylated antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.) at room temperature for 1 h. After incubation with streptavidin coupled with horseradish peroxidase (Sigma) at room temperature for 1 h, the membrane was subjected to the ECL detection system (Amersham).

Expression plasmids. In all cases, the pM5neo vector (27) was used as a eukaryotic expression vector. The expression vectors for the wild-type β_C-R subunit and β_{IL3}-R subunit were prepared by excision of the cDNA with *Eco*RI from the original plasmid pAIC2-522 (15) and plasmid pAIC2-226 (23), respectively, in which *Eco*RI linkers were inserted into the unique *Not*I site. To generate the expression vector for the truncated β_C-R subunit cDNA, the PCR-amplified 5′ and 3′ cDNAs subcloned into pCRII vectors (Invitrogen) were ligated together, using a common *Nde*I site. The full-length cDNA was then excised with *Eco*RI (site in vector) and subcloned into M5neo.

RESULTS

Two factor-independent mutants express an altered transcript encoding the β_C-R subunit of the IL3/GM-CSF/IL5 receptor complex. In contrast to nine other factor-independent D35 mutants, neither Dind2 nor Dind3 cell mutants released detectable levels of factor, thus excluding the possibility that the growth of these cells could be attributed to a mechanism of autocrine stimulation. It could, however, be predicted that mutations in proteins that convey the proliferation signal of either or both of these cytokines in the cell (e.g., receptor subunits, tyrosine kinases, or transcription factors) may be

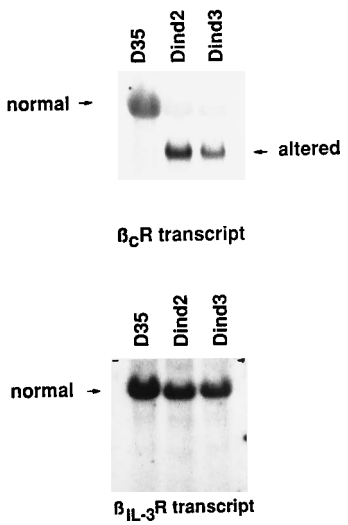


FIG. 1. Altered β_C-subunit transcript was detected in the Dind2 and Dind3 mutants by Northern blot analysis. RNA (15 μg per lane) from the parental and mutant cell lines was glycosylated, separated by agarose gel electrophoresis, and transferred to a nylon membrane. After deglycosylation, RNA was hybridized to oligonucleotides located in the cytoplasmic domain and specific for each subunit. The weak bands in the lanes containing Dind2 and Dind3 RNAs corresponding to full-length β_C-R subunit transcripts are due to unspecific hybridization to the 28S rRNA.

responsible for the acquired factor-independent growth. To determine if alterations had occurred in either of the β subunits of the IL3/GM-CSF/IL5 receptor complex, RNA from the parental and mutants cell lines was hybridized with the cDNA of β_{IL3}-R subunit, which recognizes transcripts of both subunits. Unlike the parental D35 cells, in which only the expected transcripts of approximately 4.5 kb were detected, the β_{IL3}-R subunit probe also hybridized with a 3.0-kb transcript in Dind2 and Dind3 cells. To determine the nature of the shorter transcript, oligonucleotides specific for each subunit were used in Northern blot analysis (Fig. 1). Oligonucleotides specific for the β_{IL3}-R subunit hybridized uniquely to the 4.5-kb transcript in all cell lines, whereas oligonucleotides specific for β_C-R subunit hybridized to the 4.5-kb transcript in the parental D35 cell line but only to the 3.0-kb transcript in the mutant cells. Furthermore, probes containing sequences encoding only the extracellular domain did not recognize the shorter transcript. Thus, both mutants express only truncated β_C-R subunit transcripts, in addition to the normal-length β_{IL3}-R subunit transcripts.

The altered β_C-R subunit transcript encodes a receptor subunit with intact transmembrane and cytoplasmic domains but a truncated and altered extracellular domain. In analogy with the oncogenic conversion of several known receptor molecules (4, 9, 47), we predicted that the altered β_C-R subunit transcript may encode a truncated receptor molecule that could stimulate cell proliferation in the absence of ligand binding. To determine the sequence of the truncated transcript, a cDNA copy was obtained from the Dind3 mutant by reverse transcriptase-mediated PCR. The 5′ portion of the transcript was amplified by using one oligonucleotide from the noncoding exon 1 and a second oligonucleotide from the cytoplasmic coding region. A second fragment encoding the remaining portion of the cytoplasmic domain was also amplified with appropriate oligonucleotide pairs (see Materials and Methods).

Sequence analysis of the resulting cDNA, presented in Fig. 2A, revealed several interesting characteristics of the altered transcript. As predicted from Northern analysis, the transcript did not contain any sequences encoding the wild-type extracellular domain. Indeed, identity with the normal transcript was found only in sequences originating from the noncoding exon 1 and then again in sequences originating from exon 11 (encoding the transmembrane domain) to exon 14 (containing the stop codon). No discrepancies with the published sequence were found in this region. Inserted between the sequences originating from exons 1 and 11 are located 168 nt of unknown origin. Thus, a deletion of 1,473 nt spanning exons 2 to 10, encoding the entire extracellular domain and including the predicted AUG start codon of the wild-type receptor, was replaced by an insertion of 168 nt.

Significantly, the newly acquired 168 nt introduce two AUGs, at positions 110 and 209, in the same reading frame as the normal receptor protein. As the surrounding sequences of both AUGs conform well to the consensus sequence for initiation of translation (24), one would predict that the first AUG would be used, generating a protein with a unique extracellular domain of 34 amino acids. Although the wild-type signal peptide has also been deleted, it can be predicted, on the basis of the negative and uncharged residues flanking the amino-terminal side of the hydrophobic transmembrane domain, that the de novo-synthesized protein would be transported through the endoplasmic reticulum and anchored to the cell membrane (54). In analogy with other type 1 signal-anchor proteins, in which the signal sequences function in targeting as well as in anchoring in the membrane, no proteolytic cleavage of the signal sequences would be expected to occur (18). The predicted protein would thus be anchored to the membrane with a unique extracellular domain and with transmembrane and cytoplasmic domains identical to those of the β_C-R subunit. A schematic representation of the predicted protein is shown in Fig. 2B.

The altered β_C-R subunit is transcribed from a gene with a 10-kb deletion between introns 1 and 10. To determine the genetic derivation of the altered β_C-R subunit transcript, genomic DNA analysis was undertaken. Southern blot analysis revealed gross rearrangements (Fig. 3). A common 9-kb *Hin*dIII fragment of both mutants was determined to contain the altered β_C-R subunit sequences. Molecular clones of the altered loci were compared by restriction and sequence analysis with wild-type clones. This analysis revealed that in both Dind3 and Dind2, an approximately 10-kb deletion had occurred between introns 1 and 10 in one β_C-R subunit allele (Fig. 4A). Sequence analysis of the recombination junction revealed sequence identity with the 168-nt unique sequences found in the cDNA of the altered β_C-R subunit (exon n in Fig. 4A). Thus, juxtaposition of sequences in introns 1 and 10 created altered splicing signals. The new exon is composed of sequences from the wild-type introns 1 and 10 and generates a translation start in frame with the transmembrane and cytoplasmic domains in the altered β_C-R subunit transcript. Significantly, sequence analysis of this region proved that the deletion junctions in Dind2 and Dind3 mutants were identical.

Large and independent deletions including both of the closely linked genes for the β_C-R subunit and β_{IL3}-R subunit have occurred in both mutants. As Northern blot analysis using a β_C-R subunit-specific oligonucleotide did not detect full-length transcripts in either Dind2 and Dind3 cells (Fig. 1), it would be predicted that deletions or alterations in both alleles had occurred. The complete loss of sequences specific for the β_C-R subunit in a 3-kb *Hin*dIII fragment in Southern blot analysis confirmed this prediction (Fig. 3). To characterize the genetic lesions that account for the loss of the second β_C-R

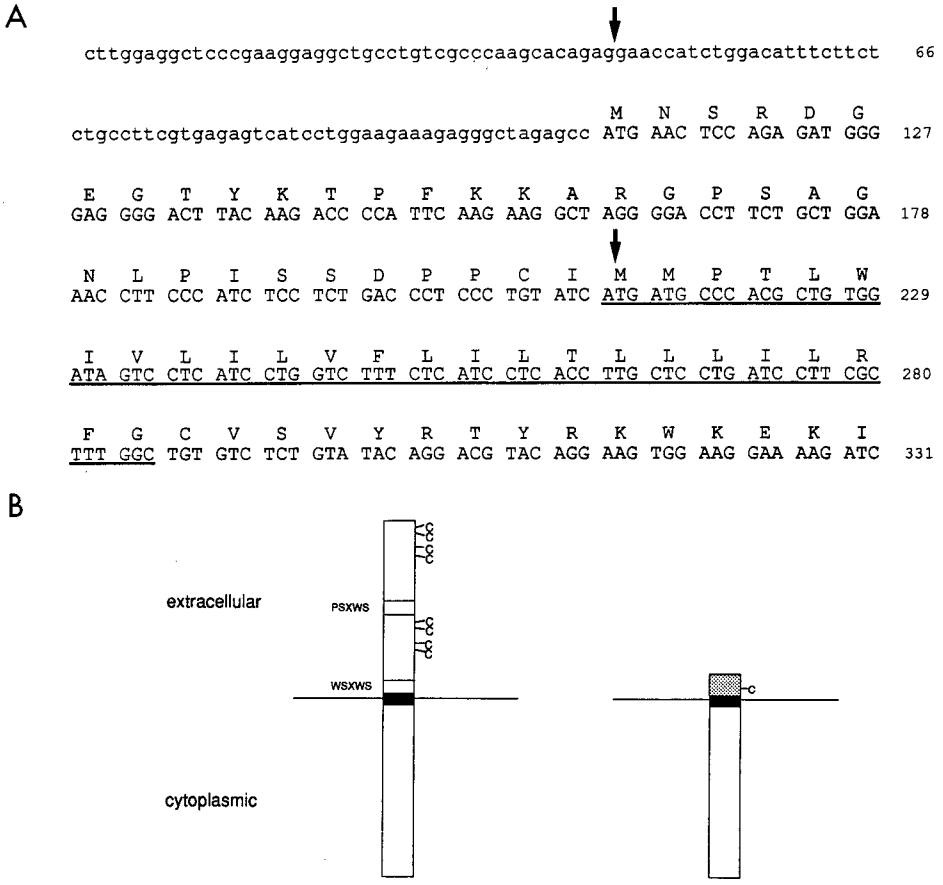


FIG. 2. Sequence analysis of a cDNA for the altered β_C-R subunit transcript predicts an amino-terminally truncated receptor protein. (A) Nucleotide sequence and predicted amino acid sequence of the 5′ end of the altered β_C-R subunit transcript. Sequences unique to the altered β_C-R subunit transcript and inserted between the sequences originating from the exons 1 and 11 of the wild-type β_C-R subunit gene are located between the two arrows. The transmembrane domain is underlined. The entire coding region was sequenced, but no differences between the sequences of the wild-type and the truncated β_C-R subunits in the cytoplasmic domain were found (references 14 and 15 and data not shown). (B) Schematic representation of the predicted truncated protein compared with the normal receptor protein. The transmembrane domain is denoted with a black box, and the predicted extracellular sequences unique to the altered β_C-R subunit are stippled. Conserved cysteine residues in the extracellular domain, as well as a newly introduced cysteine residue of the altered β_C-R subunit, are marked.

subunit allele, molecular clones containing the altered alleles were analyzed and compared with wild-type clones. On the basis of these results and those from Southern blot analysis, it was determined that a second alteration had occurred in Dind3, disrupting both the second allele of the β_C-R subunit gene and its neighboring gene encoding the β_{IL3}-R subunit. The recombination event juxtaposed sequences of intron 10 of the β_C-R subunit with the 3′ untranslated sequences of intron 14 of the β_{IL3}-R subunit (Fig. 5). Noteworthily, the two juxtaposed gene fragments were in a head-to-head orientation (5′ to 5′). Although it has been previously reported that these genes are located within at least 250 kb of each other, their orientation has not been assessed (14). On the basis of pulsed-field analysis of genomic DNA using probes specific for introns 1 of both genes, we predict that the normal genes are separated by approximately 50 kb and are oriented head to head (Fig. 5 and data not shown). Given this information, we conclude that an approximately 70-kb deletion has occurred in Dind3, resulting in the deletion of all or most of all of the transcribed and transcriptional control sequences of both the β_{IL3}-R subunit

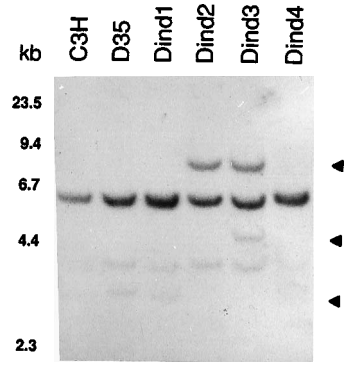
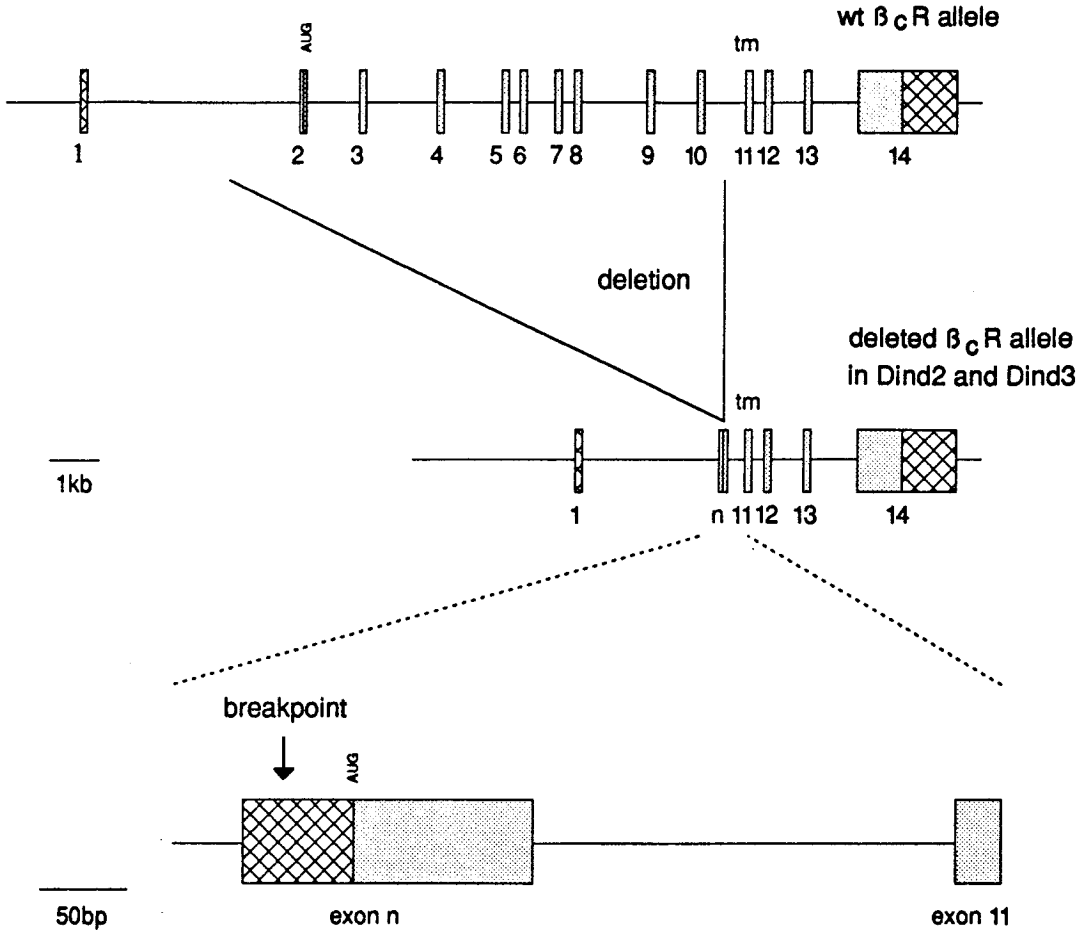


FIG. 3. Southern blot analysis reveals several shared and unique rearrangements in both the β_C-R subunit and β_{IL3}-R subunit genes in the Dind2 and Dind3 mutants. Genomic DNA (10 μg per lane) was digested with *Hin*dIII, size separated by gel electrophoresis, and transferred to a nylon membrane. DNA was hybridized with a full-length β_{IL3}-R subunit cDNA. Bands absent from or unique to either one or both of the Dind mutants are indicated with arrowheads.

A



B

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                                                intron | exon n
CCTGTCTTTCCTGAAAAACATGATAGAATAACTTCTTTTCAGGAACCATCTGGACATTTC   60
                                                3's.s.

TTCTCTGCCTTCGTGAGAGTCATCCTGGAAGAAAGAGGGCTAGAGCCATGAACTCCAGAG  120
  intron 1 | intron 10

ATGGGGAGGGGACTTACAAGACCCCATTCAAGAAGGCTAGGGGACCTTCTGCTGGAAACC  180

                                exon n | intron
TTCCCATCTCCTCTGACCCTCCCTGTATCAGTAAGCGATAGCCCATGCTCCTCATTTCAA  240
                                5's.s.

GAAGTCCCCCAGTTGACAAGCTCAGTCACCCCCCACCCAGGGTACTTGCTGCTGCTTAGG  300

GCAGAACTTCCCCATATTGAAGACAGGGAGCGGACCCAGGGTTTCTTCCCAGGTTCCTGG  360

CCTTTCTCACAGAGGGCATGGGAGACACTAGCTTCCTCCCTTAGCCTGCAGCTTGTGCCT  420

                                intron | exon 11
CCCATCTAATGCTGTTTCTCCTTGTTGACCAAGTGATGCCCACGCTGTGGATAGTCCTCA  480
                                3's.s.
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FIG. 4. Characterization of a 10-kb deletion in the β_C-R subunit gene of both Dind mutants by molecular analysis. (A) Schematic representation of the deletion characterized in the β_C-R subunit allele in both Dind2 and Dind3 mutants. The intron-exon structure was determined in part during this analysis or derived from the analysis of Gorman et al. (14). Noncoding regions are cross-hatched. The region encompassing the breakpoint junction in the altered β_C-R subunit is enlarged, and its sequence is shown in panel B. The newly created exon is indicated as exon n. wt, wild type; tm, transmembrane region. (B) Sequence analysis of the breakpoint junction between introns 1 and 10, which was determined to be identical in both mutants. The breakpoint junction is indicated with a thick vertical arrow. Intron-exon structure of the altered β_C-R subunit is indicated in boldface; the intron-exon structure of the normal β_C-R subunit gene is indicated underneath the sequence. Exon n of the altered β_C-R subunit genes contains the 168 nt encoding the extracellular domain of the truncated receptor, and exon 11 encodes the transmembrane domain. The AUG predicted to be used for translation start is underlined. Sequences conforming to the consensus sequence for 5′ and 3′ splice signals (s.s.) are indicated.

and β_C-R subunit genes. These results explain the absence of all wild-type β_C-R subunit transcripts in Dind3.

Similarly, on the basis of the complete loss or decreased intensities of distinctive bands in Southern analysis (Fig. 3 and data not shown), a large deletion which encompassed the second allele of the β_C-R subunit gene and its neighboring β_{IL3}-R subunit gene was also identified in Dind2. The breakpoints of this second deletion were outside the first exons (i.e., both genes were deleted in their entirety) and were not further characterized. Significantly, although the two large deletions in Dind2 and Dind3 encompass the same genes, they are not identical and thus are of independent origin.

Dind3 has lost high-affinity binding to both GM-CSF and IL3. Cells expressing only a truncated β_C-R subunit transcript lacking sequences encoding the normal extracellular domain should not exhibit high-affinity binding to GM-CSF, as the interaction of both the α and β receptor subunits is required for high-affinity binding (36). However, high-affinity IL3 binding would still be expected to occur, as the murine β_{IL3}-R subunit, for which normal-size transcripts were detected in both Dind2 and Dind3, is able to form high-affinity IL3R together with the α subunit or low-affinity IL3R with itself (19). To confirm the loss of high-affinity binding to GM-CSF but the retention of IL3 binding in these mutants, binding assays were performed. As expected, although the parental cell line exhibited high-affinity binding of GM-CSF (specific binding of 793 cpm at a concentration of 200 pM ^{125}I-labeled GM-CSF), no high-affinity GM-CSF receptors were detected in either Dind2 or Dind3. This is consistent with the deletion in both alleles of the β_C-R subunit gene and expression of only altered β_C-R subunit transcripts.

As would be expected from the lack of wild-type β_C-R subunit expression and the deletion of only one allele of the β_{IL3}-R subunit gene, Scatchard analysis demonstrated that the number of both high- and low-affinity IL3 receptors was lower in Dind2 cells than in the parental D35 cells: the D35 cells exhibited 3×10^3 high-affinity IL3R molecules (K_D = 416 pM) and 3×10^4 low-affinity IL3R molecules (K_D = 12.5 nM), whereas Dind2 showed only a few (<600) high-affinity IL3R and low-affinity IL3R molecules (Fig. 6A). However, unexpectedly, neither high- nor low-affinity IL3 binding sites could be detected in mutant Dind3 (Fig. 6A). These results suggest that in addition to the alterations in both alleles encoding the β_C-R subunit, alterations in the β_{IL3}-R subunit alleles in Dind3 had also occurred.

Loss of high-affinity binding can be attributed to mutations in the second β_{IL3}-R subunit gene allele in Dind3. Although Dind3 cells lost both detectable high- and low-affinity binding

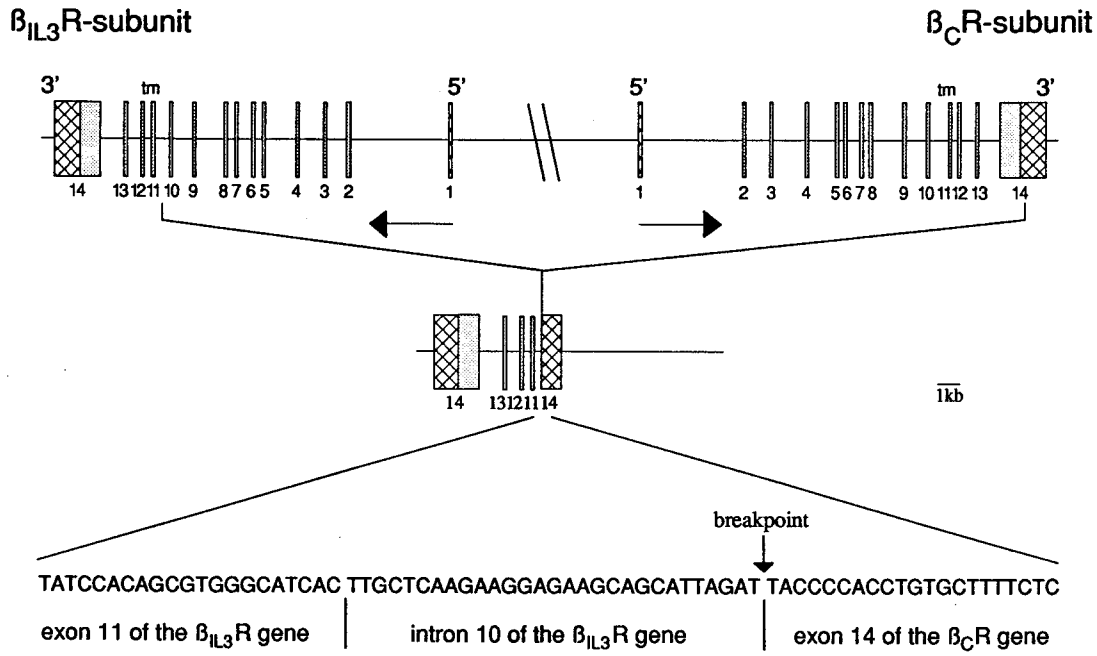

FIG. 5. Molecular analysis of the Dind3 mutant revealed a second deletion of about 70 kb, extending into both β_C-R subunit and β_{IL3}-R subunit genes. The proposed orientations and relative locations of the genes for the β_C-R and β_{IL3}-R subunits on murine chromosome 15 are depicted, as is the resulting juxtaposition of the two genes after a large deletion in Dind3. Sequence analysis of the breakpoint junction of the large deletion in Dind3 is shown. The breakpoint in the β_{IL3}-R subunit occurred in intron 10, 29 bp upstream of exon 11 (relative to normal gene transcription), whereas the breakpoint in β_C-R subunit occurred in the 3′ untranslated sequences of exon 14, 122 bp downstream of the stop codon and approximately 200 bp upstream of transcribed sequences with a high degree of identity to B2 elements (data not shown).

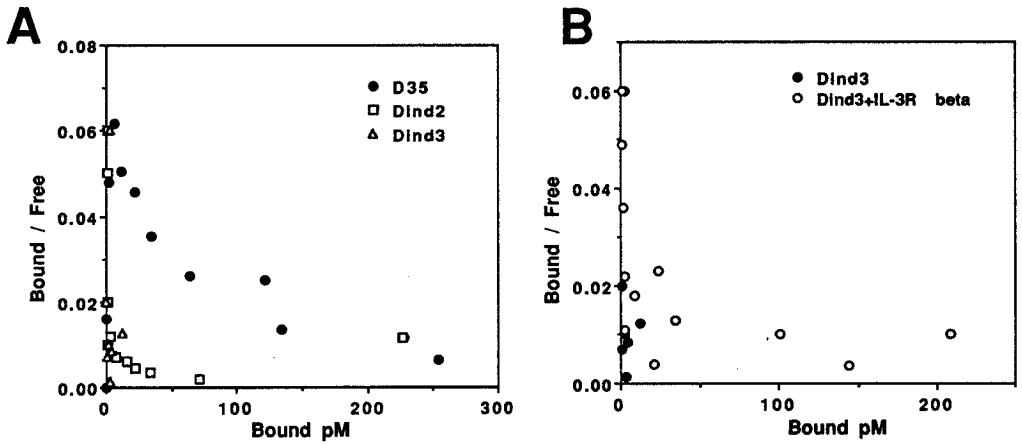

FIG. 6. Expression of the wild-type β_{IL3}-R subunit in Dind3 restores high-affinity binding. Scatchard analyses of binding analysis using ^{125}I-labeled murine IL3 of D35, Dind2, and Dind3 cells (A) and Dind3 and its transfectant expressing the β_{IL3}-R subunit (B).

sites to IL3, molecular and biochemical analysis did readily detect the underlying mutations. Genomic analysis of Dind3 described above revealed the nearly complete loss of one allele of the β_{IL3}-R subunit gene, due to a large 70-kb deletion, but no gross rearrangements were observed in the second allele of the β_{IL3}-R subunit gene in either mutant. Furthermore, neither Northern blot analysis nor flow cytometric analysis with an antibody specific for the β_{IL3}-R subunit (9D5 [35]) detected significant differences in transcript size or protein levels, respectively, in Dind3 cells compared with D35 cells (Fig. 1 and data not shown). Loss of high-affinity binding of IL3 could also be attributed to alterations in the IL3 α-subunit genes; however, no such alterations were detected by Southern analysis. To prove that the loss of binding was due to subtle mutations in the remaining β_{IL3}-R subunit gene, the wild-type cDNA was introduced into Dind3 cells by a retroviral vector (Fig. 7). As would be expected if the mutations in the β_{IL3}-R subunit gene were responsible for the loss of high- and low-affinity IL3 binding sites, Scatchard analysis demonstrated that both high-affinity sites due to β_{IL3}/α receptor interaction, in addition to the low-affinity binding sites of the β_{IL3}-R subunit alone, were restored by expression of the wild-type β_{IL3}-R subunit in the mutant cells (Fig. 6B). These experiments confirmed additional mutations in the Dind3 cell line affecting the β-subunit genes.

Expression of the wild-type β_C-R subunit in Dind3 cells results in a significant decrease in cloning efficiency in the absence of IL3. The clonal emergence of independent alterations in both Dind2 and Dind3, resulting in the loss of expression or functional inactivation of all or most of the related β-subunit genes, presumably following a common event (i.e., 10-kbp deletion resulting in a truncated β_C-R subunit), would indicate that these sequential events cumulatively confer a selective advantage to the growth of these mutants. To test whether the secondary loss of the wild-type β_C-R subunit was indeed an event that increased the phenotypic penetrance to factor-independent growth of the altered β_C-R subunit gene, retroviral vectors expressing the wild-type β_C-R subunit (Fig. 7) were introduced into Dind3 cells, and cell clonability with and without IL3 was monitored. Infected cells were selected with G418 and in the presence of IL3 and then cloned. The cloning efficiency in the presence of IL3 (either with or without G418) was determined to be approximately 22%, in agreement with results for uninfected Dind3 cells or control M5neo-infected Dind3 cells (Table 1). In contrast, the cloning efficiency of Dind3 expressing the wild-type β_C-R subunit receptor in the absence of IL3 was reduced by a factor of 4 to approximately 5% (Table 1). Significantly, no reduction in clonability in the absence of IL3 was observed in control infected or uninfected Dind3 cells. In conclusion, the ability of Dind3 cells to proliferate in the absence of IL3 is significantly reduced if wild-type β_C-R subunits are expressed. This finding provides direct evidence that sequential and secondary loss of the wild-type β_C-R subunit gene, and presumably also the β_{IL3}-R subunit genes, is

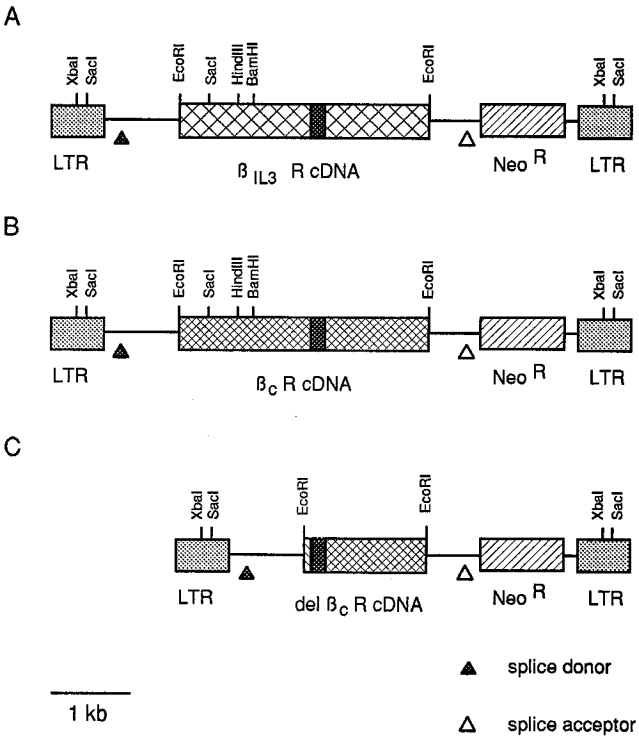

FIG. 7. Retroviral vectors were used to express normal and altered β-R subunits in hematopoietic cells. cDNAs of the wild-type β_{IL3}-R and β_C-R subunits, as well as the altered β_C-R subunit, were inserted into the unique cloning sites of M5neo, a retroviral vector based on myeloproliferative sarcoma virus, for efficient transfer and expression in hematopoietic cells (27, 49). M5neo without an inserted cDNA served as a negative control. LTR, long terminal repeat.

TABLE 1. Expression of the wild-type β_C-R subunit in Dind3 cells results in a significant reduction in cloning efficiency in the absence of IL3

Cell line	No. of cells plated	Expression under the following conditions:					
		−IL3 +G418[a]		+IL3 +G418		+IL3	
		No. of clones[b]	Cloning efficiency (%)	No. of clones	Cloning efficiency (%)	No. of clones	Cloning efficiency (%)
Dind3-β_CR-neo							
	100	3.4 ± 3.2	3	16.5 ± 3.5	17	21.5 ± 2.5	22
	330	27.5 ± 16.5	8	71.0 ± 24.0	22	96.0 ± 14.0	29
Dind3-neo							
	100	29.0 ± 3.0	29	22.5 ± 2.5	23	30.0 ± 2.0	30
	330	65.0 ± 3.5	21	55.0 ± 5.0	17	85.0 ± 4.0	26
Dind3							
	100	20.7 ± 3.6	21	ND[c]	ND	24.0 ± 1.0	24
	330	58.2 ± 12.8	18	ND	ND	70.8 ± 6.3	21

[a] Uninfected Dind3 cells were cloned in the absence of G418.
[b] Values represent averages of at least three independent experiments. Standard deviations are shown.
[c] ND, not determined.

necessary for the complete penetrance to factor-independent growth.

Expression of the altered β_C-R subunit in hematopoietic cells increases the mutation frequency to complete factor-independent growth. To demonstrate that expression of the truncated β_C-R subunit was the primary mutation leading to factor-independent growth of the Dind2 and Dind3 cells, factor-dependent cells were transduced with the altered β_C-R subunit cDNA within a retroviral vector containing the Neo^r gene and evaluated for factor-independent growth. For these experiments, the FDC-P1 myeloid progenitor cell line was chosen. Similarly to the D35 cell line, FDC-P1 cells are strictly dependent on either IL3 or GM-CSF for growth. Transfected FDC-P1 cells were selected by G418 resistance (Neo^r) in the presence of IL3 and then assayed for factor-independent growth. Removal of IL3 resulted in an immediate loss in cell proliferation within 48 h, but after 6 to 7 days, slowly proliferating factor-independent variants could be observed. The mutation frequency to factor-independent growth was determined to be approximately 3×10^{-4}, compared with 5×10^{-8} for FDC-P1 containing the Neo^r vector without the β_C-R subunit cDNA. Analysis of five independent clones transduced with the altered β_C-R subunit revealed significant variations in the mutation frequency to factor-independent growth, ranging from 10^{-3} to 10^{-7}. Clones with the lowest conversion frequencies expressed significantly lower transcript levels of the introduced $\Delta\beta_C$-R subunit than clones with a high frequency (data not shown). Three independent FDC-P1 $\Delta\beta_C$-R subunit-transfected clones exhibiting factor-independent growth were examined for alterations in the β_C-R subunit genes. Southern analysis using several different enzymes revealed no gross alterations. Similarly, Northern analysis using probes specific for both the extracellular and cytoplasmic domains did not reveal any differences in transcript size or number (data not shown).

In summary, although introduction and expression of the altered β_C-R subunit in FDC-P1 cells did not result in factor-independent growth of these cells in one step, the frequency at which factor-independent variants arose was increased on the average by 4 orders of magnitude. These results fully support our hypothesis that the oncogenic potential of the truncated β_C-R subunit is completely manifested only when secondary mutations have occurred. The lack of obvious alterations in expression or size of the wild-type β_C-R subunits in these transfectants suggest that different secondary mutations than observed for the Dind mutants are necessary. This difference may reflect the relatively high levels of expression of the introduced truncated β_C-R subunit or inherent differences in the cell lines.

Expression of the truncated β_C-R subunit results in constitutive phosphorylation of the JAK2 tyrosine kinase. Stimulation of cells with GM-CSF or IL3 leads to the tyrosine phosphorylation and activation of the tyrosine kinase JAK2 (46). Importantly, recent studies have demonstrated that JAK2 directly associates with the cytoplasmic domain of the β_C-R subunit necessary for the induction of mitogenesis, supporting the hypothesis that JAK2 is critical in coupling receptor activation to tyrosine phosphorylation and ultimately mitogenesis (38). To determine if the truncated β_C-R subunit also signaled through JAK2, both the spontaneous Dind2 and Dind3 mutants, as well as two factor-independent FDC-P1 clones transfected with M5-$\Delta\beta_C$-R, were assayed for tyrosine phosphorylation of JAK2 (Fig. 8). As expected, IL3 stimulation of both the parental D35 and the control FDC-P1neo cells resulted in phosphorylation of the JAK2 kinase, whereas no or very low levels of phosphorylated JAK2 were detected when IL3 was removed from cells. In contrast, in all Dind and FDC-P1 mutants expressing the $\Delta\beta_C$-R subunit, high levels of phosphorylated JAK2 were observed in the absence of IL3 stimulation. In concordance with expression of the β_{IL3}-R subunit in Dind2

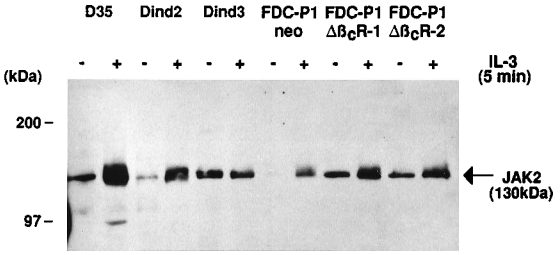

FIG. 8. Constitutive phosphorylation of JAK2 tyrosine kinase in factor-independent mutants expressing the truncated β_C-R subunit. JAK2 was isolated by immunoprecipitation from cells cultured without IL3 or stimulated with IL3 for 5 min and analyzed by Western blotting (immunoblotting) with an antiphosphotyrosine monoclonal antibody. All mutants were maintained without IL3, whereas the control D35 and FDC-P1neo cells were subjected to IL3 deprivation for 4 and 20 h, respectively. The residual level of JAK2 detected in D35 without IL3 is probably due to the relatively short withdrawal of IL3 before initiation of the experiment.

cells and wild-type β_C-R and β_{IL3}-R subunits in the $\Delta\beta_C$-R FDC-P1 transfectants, phosphorylation of JAK2 was increased in these cells when IL3 was added. No such activation was observed in Dind3 cells that do not express wild-type β subunits.

DISCUSSION

An amino-terminally truncated β_C-R subunit that mediates ligand-independent proliferation was identified in the autonomous mutants Dind2 and Dind3. The novel protein is transcribed from a mRNA arising from a β_C-R subunit gene in which a 10-kb deletion between introns 1 and 10 has occurred. Although both the predicted transmembrane and intracellular domains are identical to those in the normal β_C-R subunit, the entire extracellular domain has been replaced by 34 amino acids encoded by sequences originating from the normal intron 10. The ability of the truncated receptor protein to stimulate cell proliferation in the absence of ligand was confirmed by transfection into the factor-dependent myeloid cells FDC-P1. In all transfected FDC-P1 $\Delta\beta_C$-R clones, factor-independent variants arose at a frequency of up to 4 orders of magnitude higher than in control Neo^r transfectants. Furthermore, constitutive phosphorylation of the protein kinase JAK2 was observed in both the Dind2 and Dind3 mutants and in factor-independent FDC-P1 $\Delta\beta_C$-R transfectants. Verification that phosphorylation of JAK2 leads to its activation in these cells was demonstrated by confirming the transcriptional activation of STAT5, a direct target of the JAK2 kinase (unpublished results; 16, 55). Activation of JAK2 has recently been shown to correlate with the proliferation-induction associated with the membrane-proximal region of the β_C-R subunit after ligand stimulation (38, 43). Thus the truncated β_C-R subunit is able to mimic the activated receptor response in the absence of ligand.

How might receptor alterations result in ligand-independent receptor activation? In analogy with the receptor tyrosine kinases, it is predicted that the activation of the signal cascade by the cytokine receptor family after ligand binding is induced by conformational changes that occur after oligomerization of at least two receptor chains (24). The consequence of oligomerization may be the increased concentration of associated tyrosine kinase molecules (e.g., JAK), resulting in their cross-phosphorylation and associated activation of kinase activity. The critical mutations in the activated receptor molecule must therefore either mimic the conformational changes that normally occur during oligomerization or stabilize the oligomerization of the receptor subunits. In the case of the receptor complex mediating either IL3-, GM-CSF-, or IL5-induced signaling, neither the stoichiometry nor the composition of the active complex has been resolved. Recent evidence support a model of homodimerization of the β-R subunits for signal transduction, as has been shown for the shared signaling subunit gp130 of the various receptor complexes stimulated by ligands of the IL6 family (1, 6). However, it has not been clearly established if the role of the α subunit is to present the appropriate ligand to the β subunit and thereby induce and/or stabilize dimerization, or if it also directly interacts with signal transducers (38, 42, 51).

Supporting a model for β_C-R subunit homodimerization in receptor activation, we predict that the substitution of the wild-type extracellular domain with the 34 amino acids encoded in the altered β_C-R subunit transcript facilitates stable homodimerization of the truncated β_C-R subunit and the activation of associated tyrosine kinases, in particular JAK2. It has not yet been formally resolved if the α subunit participates in this receptor complex; however, as the entire extracellular domain, which is presumably the normal site of α- and β-subunit interaction, is deleted in the altered β_C-R subunit, it seems unlikely that oligomerization between the α and truncated β_C-R subunits would occur.

The importance of either the deletion of wild-type extracellular sequence or the composition of the de novo extracellular sequences of the altered β_C-R subunit in the predicted homodimerization is unknown. Of potential significance, the truncated β_C-R subunit contains a novel Cys residue in the extracellular domain, located two amino acids upstream of the transmembrane domain. In the activated erythropoietin receptor mutant R129C, the replacement of a Tyr residue with a Cys residue at position 129 has been shown to be the critical mutation, stabilizing by cysteine binding the homodimers that are formed (28, 56). Substitution analysis of the Cys residue in the altered β_C-R subunit is necessary to confirm its role in receptor activation. It is also of interest that both of the conserved WSYWS motifs are deleted in the altered β_C-R subunit. The importance of these conserved residues in signal transduction has been shown for a number of different receptors (12, 33, 37). Indeed, the duplication of these sequences in a recently described activated human β_C-R subunit was predicted to be a critical component of the activating mutation (7). Similarly, the significance of the preservation of the WSXWS motif directly upstream of the transmembrane domain in the truncation event resulting in the activation of the thrombopoietin receptor *MPL* has also been discussed (47). Thus, the loss of these conserved motifs in the truncated β_C-R subunit indicates that its function may be linked to dimerization rather than signal transduction and can be replaced with other extracellular sequences that facilitate dimerization.

Of special interest in the work described here is our observation that secondary mutations are necessary for the complete conversion to autonomous growth. The presence of secondary and independent mutations leading to the elimination of the allelic β_C-R subunit gene and its neighboring β_{IL3}-R subunit gene in both the Dind2 and Dind3 mutants, but not in the parental cell line or any of the other eleven Dind mutants, suggested a strong correlation with the altered phenotype of these mutants. Confirmation came from studies in which reversal of the deletions by expression of the wild-type β_C-R subunit in Dind3 cells resulted in a significant decrease in the cloning efficiency of these cells in the absence of IL3. Furthermore, expression of the $\Delta\beta_C$-R subunit in FDC-P1 cells did not immediately induce factor-independent growth, but required a lag time before autonomous variants exhibited factor-independent proliferation. Obviously, secondary mutations are necessary for the truncated β_C-R subunit to effectively stimulate proliferation. In FDC-P1 transfectants producing relatively high levels of the truncated β_C-R subunit (more than twofold higher than in the wild type), factor-independent variants arose at a frequency of between 10^{-3} and 10^{-4}. Such a high frequency suggests that physiological changes in the cellular environment may be sufficient for induction of cell proliferation by the $\Delta\beta_C$-R subunit (see below). In this light, it is perhaps significant that all activating receptor mutants of the cytokine family described to date have been identified within the context of a transducing retrovirus, in which expression levels are relatively high.

How might inactivation of wild-type receptor subunits facilitate the mitogenic response of the altered β_C-R subunit and thus the complete conversion to autonomous growth? The most probable explanation is that the wild-type β_C-R subunit interferes with the action of the truncated β_C-R subunit. One could envisage that this interference occurs at the level of either receptor-effector interaction or receptor oligomeriza-

tion. The latter possibility would assume that the wild-type β-R subunits are associated with the truncated β_C-R subunit, presumably through sequences in the transmembrane or cytoplasmic domains. This association decreases the pool of truncated β_C-R subunits that are able to form active homodimers. In support of this model, the formation of β_C-R subunit complexes in the absence of ligand has been reported (57). Alternatively, the wild-type β_C-R and β_{IL3}-R subunits sequester necessary effector molecules from the activated $\Delta\beta_C$-R subunit complex. In support of this model, recent studies have shown that JAK2 associates with nonactivated β_C-R subunits (38). It is also of interest that the cytoplasmic domain of the α subunit has been reported to be necessary for JAK2 activation (38). It could be envisaged that in the absence of α subunits in the $\Delta\beta_C$-R oligomerization complex, higher levels of JAK2 or other effectors are needed to provide stimulation. Significantly, no alterations in the expression of the wild-type β_C-R or β_{IL3}-R subunits was observed in factor-independent $\Delta\beta_C$-R FDC-P1 transfectants. The characterization of secondary mutations in these cells may reveal other mechanisms which promote the proliferative stimulation by the truncated β_C-R subunit.

Although the alteration of one allele and the loss of the second is a common occurrence in so-called tumor suppressor genes (i.e., genes encoding negative regulators of growth) during oncogenic progression, our results show that the loss of the wild-type allele is also an important event in the tumorigenicity initiated by oncogenes that stimulate proliferation and are thought to act in a dominant manner. Significantly, the loss of the normal allele associated with a mutated receptor allele in several human dysplasias has previously been reported; however, the significance of this event in tumorigenicity has not been established (10, 13, 40). In light of our results, similar interfering interactions could also be postulated.

We demonstrate here that a truncated version of the β_C-R subunit can stimulate proliferation in the absence of ligand, resulting in autonomous growth. In concordance with previous studies, the acquisition of autonomous growth in both Dind mutants and $\Delta\beta_C$-R transfectants was correlated with tumorigenic growth in vivo (18a, 27, 50). In light of the numerous examples of oncogenes generated by mutations in genes encoding regulators of proliferation and differentiation (5), this observation is perhaps not unexpected. More significantly, however, the results presented here underline the importance of secondary mutations necessary for the complete phenotypic penetrance of the mutated gene. The characterization of these secondary mutations not only sheds light on the sequential events involved in the multiple steps of carcinogenesis but also provides insight into the normal interactions between ligand-activated receptor subunits and effectors during signal transduction.

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