Activation of NF-κB by Interleukin 2 in Human Blood Monocytes

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Abstract
We report here that interleukin 2 (IL-2) acts on human blood monocytes by enhancing binding activity of the transcription factor NF-xB to its consensus sequence in the 5′ regulatory enhancer region of the IL-2 receptor α chain (p55). Similarly, IL-2 activates NF-xB in the human monocytic cell line U 937, but not in resting human T-cells. This effect is detectable within 15 min and peaks 1 h after exposure to IL-2. Enhanced NF-xB binding activity is followed by functional activation in that inducibility of the IL-2 receptor α chain is mediated by enhanced NF-xB binding and that a heterologous promoter containing the NF-xB consensus sequence (−291 to −245) of the IL-2 receptor α chain gene is activated. In addition, IL-2 is capable of increasing transcript levels of the p50 gene coding for the p50 subunit of the NF-xB transcription factor, whereas mRNA levels of the p65 NF-xB gene remained unchanged.

Introduction
The high affinity IL-2R consists of α and β chains, but only the β chain participates in signaling of IL-2 (1). Molecular cloning and functional analysis of the IL-2R β chain have recently identified a small region of the cytoplasmic domain sufficient for signal transduction. This region has also been postulated to represent an active site required for association with a second messenger in the signal transduction cascade and to potentially act via a phosphorylation signal (1). In lymphocytes, such a second messenger molecule interacting with the α chain was recently identified (2). Human blood monocytes constitutively express the IL-2R β chain (p75) (3), whereas expression of the IL-2R α chain (p55) by human blood monocytes is inducible upon culture with IL-2 that can be further enhanced when IL-2 is combined with γ-interferon (4). The underlying molecular mechanism, including the signal transduction pathway mediating this induction, is, however, unknown. We have examined the nuclear basis of the action of IL-2 on IL-2R α chain expression.

Results
Treatment of human PBMO is associated with IL-2R α chain expression by these cells (1). Whereas resting monocytes did not display transcripts for the α chain of the IL-2R by Northern blot analysis, treatment with IL-2 was associated with accumulation of IL-2R α chain transcripts (data not shown). To study the effect of IL-2 on the transcriptional activation of the IL-2R α chain gene, nuclear run-on assays were performed. As expected from Northern blot data, resting monocytes constitutively transcribed only very low levels of the IL-2R α chain gene. However, treatment of PBMO with IL-2 results in strong transcriptional activation of the IL-2R α chain gene (Fig. 1). These data indicate that enhanced expression of the IL-2R α chain is at least in part due to transcriptional activation of this gene by IL-2 in PBMO.

The next set of experiments was designed to study the molecular mechanism mediating this transcriptional activation. Deleted forms of the IL-2R α chain promoter were constructed (Fig. 2A), linked to the hGH as a reporter, and transiently transfected into human monocytes. Monocytes transfected with the p(−317) IL-2R α promoter fragment responded to IL-2 with an approximately 8-fold increase in hGH activity (Fig. 2B), whereas monocytes transfected with the p(−225) IL-2R α promoter fragment failed to synthesize hGH upon IL-2 treatment. In addition, various other deleted promoter constructs (p(−179), p(−141), p(−50)) also did not respond to IL-2 with enhanced hGH release (data not shown). These data suggested that an IL-2-responsive element located between positions −317 and −225 is responsible for transcriptional activation of this gene in PBMO.

Recent studies have demonstrated that an enhancer element located at position −291 to −245 is crucial for activation of the IL-2R α chain promoter (5, 6). The enhancer element was subcloned in front of the heterologous herpes thymidine kinase promoter, linked to the human growth hormone gene as a reporter. This construct, termed p(IL-Rα)TKGH, was transiently transfected into human monocytes, the human monocytic cell line U 937, and resting human T-cells. This IL-2R α chain construct responded to IL-2 in monocytes and U 937 cells, but not in T-cells, with an approximately 20-fold enhancement of hGH activity (Fig. 3). Control experiments with a plasmid containing a mutant form of this enhancer p(IL-2R α mt)TKGH or a control plasmid without this enhancer region (pTKGH) failed to display enhanced hGH activity in the presence or absence of IL-2 in cultures of transfectants (Fig. 3). These data indicate that this region contains an IL-2-responsive element which is functional in monocyctic cells but not in T-cells.
EMSA assays were used to determine whether IL-2-induced nuclear factors could bind to a double stranded oligonucleotide corresponding to this region. As shown in Fig. 4A, nuclear proteins from resting monocytes displayed a faint retarded band when incubated with this fragment. Complex formation was enhanced when nuclear proteins from monocytes exposed to IL-2 for 15 min were analyzed. Binding activity of this factor peaked after 30 to 60 min and returned to baseline levels within 2 h upon exposure to IL-2. Comparable results were obtained when the monocytic cell line U 937 was analyzed (Fig. 4A), whereas resting T-cells displayed high constitutive binding of this factor as compared to resting monocytes, which were, however, only weakly, if at all, able to be enhanced by IL-2 (Fig. 4A). In addition, the IL-2-mediated induction of complex formation by monocytes was dose dependent and detectable in the range of 100 to 1000 units/ml of IL-2. No significant dose dependency was observed below 100 units/ml (Fig. 4B). Furthermore, treatment of monocytes with the protein synthesis inhibitor cycloheximide for 30 min prior to addition of IL-2 for 1 h did not interfere with IL-2-mediated induction of NF-xB binding activity (data not shown).

The enhancer region (position −291 to −245) contains a recognition site for the transcription factor NF-xB (6, 7). Competition assays with a double stranded oligonucleotide containing the consensus sequence of the NF-xB transcription factor of the xB immunoglobulin gene were able to completely inhibit binding of monocyte- and T-cell-derived nuclear proteins, whereas an unrelated oligonucleotide containing the AP-1 consensus sequence did not interfere with complex formation (Fig. 5A).

In addition, this competition assay also revealed that monocyte- and T-cell-derived nuclear proteins gave different patterns of retarded bands, indicating size heterogeneities of the proteins involved. Therefore, the molecular size of NF-xB-binding proteins was analyzed by UV cross-linking experiments. As shown in Fig. 5B, T-cell-derived proteins appeared to be 50, 75, and 85 kD in size, whereas monocyte-derived NF-xB protein was only 50 kD in size. In additional experiments, EMSA were performed with nuclear proteins from untreated and IL-2-exposed monocytes and NF-xB protein purified from human placenta. Purified p50 protein comigrated with the IL-2-inducible monocyte-derived nuclear factor.

In additional experiments, the effects of IL-2 on mRNA accumulation of the p50 gene coding for one binding subunit of NF-xB and the p65 gene coding for the xB receptor-binding subunit were investigated (8). As shown in Fig. 6, resting monocytes failed to display p50 mRNA on Northern blot analysis of total cytoplasmic RNA. When, however, poly(A) selected RNA was used, a faint signal for p50 and p65 became detectable in resting monocytes (data not shown).

Treatment of monocytes with IL-2 was associated with a significant increase of the 3.9-kilobase p50 mRNA levels at 12 h. Maximal levels were achieved by 24 h, whereas longer duration of IL-2 exposure (48 h) caused down-regulation of p50 transcript levels. In contrast, treatment with IL-2 did not alter transcript levels of the p65 gene during this time course (data not shown).

Discussion

NF-xB translocates into the nucleus following activation by a variety of stimuli such as active phorbol esters, the tax protein of the human T-cell leukemia virus I, double stranded RNA, interleukin 1, or tumor necrosis factor α (9). We report here that IL-2 also has to be considered as a potent activator of NF-xB binding activity. Moreover, our data indicate that expression of the β chain by monocytes is sufficient to allow IL-2 to dissociate the transcription factor NF-xB from its cytoplasmic inhibitor and generate binding activity of NF-xB to its consensus sequence within the IL-2α chain promoter. Enhanced binding activity of NF-xB to its recognition site translates into functional activation, in that a heterologous promoter containing the IL-2α chain enhancer element is transcriptionally activated upon IL-2 treatment in human monocytes, whereas resting T-cells fail to respond to IL-2 with enhanced NF-xB binding activity or functional activation. In addition, monocyte- and T-cell-derived nuclear proteins that bind to the IL-2α chain (−291 to −245) differ not only in their inducibility by IL-2, but also in their molecular size. This observation might indicate that cell type-specific expression of distinct members of the NF-xB family mediates signal-dependent induction of this molecule. In this context, recent work is of note demonstrating that inducible activities of different members of the NF-xB family are controlled by distinct mechanisms (10).

Monocytes respond to IL-2 by releasing inflammatory cytokines such as IL-1 and tumor necrosis factor α (11, 12) and increased cytotoxicity (13). IL-2 was further found to induce monocytes to express several protooncogenes, including c-fos, c-myc, c-pim, and c-hms (14, 15). The signal transduction pathway following binding of IL-2 to its surface binding sites, however, is presently unknown. Recent reports have indicated that common signal transduction pathways such as protein kinase C translocation, calcium mobilization, and phosphatidylserine turnover are not directly involved in IL-2 signal transduction (16-18). Our data, now for the first time, show that IL-2 is a potent activator of the signal transduction molecule NF-xB, even in the absence of functional high affinity IL-2R, and that exposure of monocytes to IL-2 also leads to accumulation of transcripts of the p50 gene coding for a DNA-binding subunit of NF-xB. Transient increase of p50 transcripts, however, occurred after evidence for enhanced binding activity of NF-xB protein. In addition, IL-
Fig. 2. A, schematic representation of various deleted promoter constructs of the IL-2R α chain promoter linked to the human growth hormone gene (hGH) as a reporter. Black box, the IL-2R α chain promoter enhancer element from position −293 to position −245; below, sequence with the NF-κB recognition site underlined. B, hGH synthesis of monocytes transfected with p[−317] IL-2R α (+317) or with p[−223] IL-2R α (−225) in the presence (open columns) or absence (closed columns) of IL-2.

Fig. 3. IL-2 induces activity of a heterologous promoter containing the NF-κB consensus sequence. Monocytes (Mo), U 937 cells, and T-cells were transiently transfected with the heterologous promoter construct containing the wild-type [p[−267]/−244]; p[IL-2R α]TKGH] or a mutant [p[−267]/−244], p[IL-2R α, mt]TKGH], or no fragment [pTKGH] inserted 5′ of the herpes thymidine kinase promoter followed by the hGH gene. Cells were maintained in medium in the presence (closed columns) or absence (open columns) of IL-2. hGH activity in culture supernatants was assessed by enzyme-linked immunosorbent assay. Shown is a representative experiment. Four additional transfections gave comparable results. For protocol, see “Materials and Methods.”
2-mediated activation of NF-κB was detectable in the presence of protein synthesis inhibition, suggesting that preexisting protein was activated by IL-2. Taken together, these results suggest that IL-2 is effective in regulating NF-κB by two independent mechanisms at two levels. Alternatively, activation of NF-κB binding might be associated with autoinduction of the p50 gene.

Further studies are needed to clarify whether activation of NF-κB is also sufficient to mediate other effects than the expression of IL-2R α chain or whether the formation of the high affinity IL-2R engaging NF-κB is necessary to allow the initiation of other effects of IL-2 by human blood monocytes. In this context, a recent study (19) is of note, indicating that the α chain primarily acts in order to facilitate the interaction of IL-2 with the β chain of the IL-2R, thereby increasing its affinity for IL-2.

Materials and Methods

Cell Separation. Leukocyte preparations were obtained from buffy coats of blood donated by consenting healthy volunteers through the courtesy of the Blood Bank, University of Freiburg, Freiburg, Germany. Monocytes were isolated as described previously (20). Briefly, buffy coats were diluted with minimum essential medium (Seromed Biochrom, Berlin, Germany), washed, and centrifuged over a Ficoll-Hypaque cushion (Seromed Biochrom). Interphase cells were then collected and subjected to two subsequent rosetting steps with aminoethylisothiouronium bromide-treated sheep red blood cells (Flow Laboratories, Meckenheim, Germany) for depletion of T-lymphocytes, as described (20). Monocytes were further purified by two adherent steps of the E-rosette-negative cell fraction. Monocytes obtained in this way were >97% pure, as assessed by α-naphthyl acetate esterase staining and immunofluorescence analysis using anti-CD14 monoclonal antibody (Becton Dickinson, Heidelberg, Germany).

Cell Culture. Human monocytic leukemia U 937 cells (American Type Culture Collection, Bethesda, MD), T-lymphocytes, and monocytes, prepared as described above, were cultured in RPMI 1640 medium (Seromed
monocytes were harvested after the appropriate culture time as indicated. Cells were resuspended in guanidine (Sigma) and extracted with an equal volume of acetate-EDTA-equilibrated phenol. This mixture was incubated at 60°C for 25 min with frequent vortexing. The aqueous phase was recovered after centrifugation and extracted once with an equal volume of phenol-chloroform and twice with chloroform. The resulting RNA was precipitated overnight at −20°C with 2.5 volumes of ethanol. The total RNA from each sample (40 μg) was then electrophoresed in a 1% agarose gel containing 20 mM sodium borate, pH 8.3, 0.5 mM EDTA, and 3% formaldehyde. The RNA was then transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Germany) in 10X SSC (1.5 M sodium chloride and 150 mM sodium citrate) using capillary blotting overnight. The blots were then baked and prehybridized at 65°C in 7% SDS, 10X Denhardt's (1X Denhardt's = 0.02% Ficoll, 0.2% bovine serum albumin, and 0.02% polyvinyl pyrolidone), 5X SSC, and 20 mM salmon sperm DNA (Sigma). The blots were probed with the EcoRI fragment of the pSP65 IL-2R α chain (kindly provided by T. Waldman, National Cancer Institute, Bethesda, MD); the NotI fragment of pBKS-p50 (kindly provided by S. Ghosh, Whitehead Institute, Cambridge, MA); or the HindIII/XbaI fragment of p8c CMV-p65 (kindly provided by P. Baeuerle, Genzentrum, University of Munich, Martinsried, Germany) radiolabeled by random priming with [α-32P]CTP (>6000 Ci/mmoll) (Amersham, Arlington Heights, IL). The blots were washed at 55°C in 1% SDS-1X SSC and autoradiographed with Kodak XAR film (Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen. For nuclear run-on assays, 106 cells were lysed in RSB [10 mM Tris-HCl, 5 mM KCl, 3 mM MgCl2, containing 0.5% Nonidet P-40 (Sigma)] and washed once in ice-cold phosphate-buffered saline. Nuclei were incubated at 26°C in 15% glycerol-70 mM KCl-2.5 mM MgCl2-10 mM EDTA, 4 mM concentrations each of ATP, CTP, and GTP, 2 mM

Biochrom) supplemented with 10% low endotoxin fetal calf serum (Hazeltom, Vienna, UT) in the presence of 2 mM L-glutamine and 1% penicillin-streptomycin in 5% CO2 in air at 37°C. Some of the cultures were treated with recombinant human IL-2 (Cetus Corp., Emeryville, CA) at 10 to 500 units/ml for the indicated time periods. Cells were also treated with 10 μg/ml cycloheximide (Sigma, Munich, Germany).

Isolation of Total Cellular RNA, Northern Blot Analysis and Run-on Assay. Medium or IL-2 (100 units/ml)-treated

![Fig. 6](image-url) Induction of p50 mRNA by IL-2 in monocytes. Monocytes were treated with 100 units/ml recombinant human IL-2 for 4, 12, 24, and 48 h and harvested thereafter. Total cytoplasmic RNA was isolated, and 40 μg RNA were hybridized to the p50 complementary DNA probe (NF-κB). Rehybridization with actin controls for comparable RNA loading in single lanes.
UTP, 0.5 mM dithiothreitol, 60 units/ml RNAsin (Boehringer Mannheim, Mannheim, Germany) in the presence of 100 μCi of [32P]UTP 3000 Ci/mmol (Amersham Buchler, Braunschweig, Germany) for 30 min. The mixture was digested with DNase I and proteinase K, extracted once with trichloroacetic acid and phenol-chloroform, and precipitated in 70% ethanol buffer before hybridization of 5 × 10^6 cpm/ml of hybridization buffer [50% formamide-2× SSC-1× DS-5× Denhardt’s solution [1× Denhardt’s solution = 0.2% Ficoll-0.02% bovine serum albumin (Fraction V; Sigma)-0.02% polyvinyl pyrolidone] -5 μg/ml tRNA]. Filters contained 10 μg each of linearized plasmid immobilized on nitrocellulose (Schleicher and Schuell). After hybridization at 42°C for 3 days, filters were rinsed in 2× SSC at 55°C, 2× SSC containing 10 μg/ml RNAse A at 37°C, and, finally, 0.5× SSC at 55°C for 30 min each time, and were exposed to Kodak X-Omat films for 10 days.

**Electrophoretic Mobility Shift Assay.** Nuclear proteins from untreated and IL-2-treated cells were prepared as described previously (21). A double stranded oligonucleotide, corresponding to position −291 to −245 of the IL-2 α chain (6), was synthesized and end labeled using the Klenow large fragment polymerase (Boehringer Mannheim). Nuclear proteins (10 μg) and labeled DNA (10,000 cpm) were incubated in a buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1.0 μg polydeoxyinosinoc-deoxycytidylic acid (Sigma), and 12% (v/v) glycerol at room temperature for 30 min. Samples were then analyzed on a 5% polyacrylamide gel (Sigma). The gel was dried and autoradiographed. For competition assays, 25-fold molar excess of unlabeled double stranded oligonucleotide containing the NF-κB consensus sequence of the human κB immunoglobulin gene (ACAAGGACCTTCCGCT) or the AP-1 consensus sequence of the human collagenase gene (GATCCCCGTAC TCGT) was added prior to incubation. For comigration experiments, NF-κB protein purified from human placenta (kindly provided by P. Bäuerle, Genzentrum, University of Munich, Martinsried, Germany) was incubated with the end-labeled NF-κB oligonucleotide.

**Preparation of Promoter Constructs and Transfection Assays.** The −317/+100 HindIII/KpnI fragment of the IL-2 α chain promoter (a kind gift of W. C. Greene, National Cancer Institute, Bethesda, MD) was blunt ended and subcloned into the HindIII site of pOGH (22). Deleted deletion were made by restriction enzyme digestion of the HindIII/KpnI IL-2 −291 α chain promoter fragment as follows: XmnI yielding the p(−225) fragment, MnlI, yielding p(−179), EcoRI, yielding p(−141), and EcoRl, yielding p(−50) IL-2 α chain promoter fragments. The fragments were blunt ended and subcloned into the HindIII site of pOGH.

A synthetic oligonucleotide corresponding to the −291 to −245 fragment of the IL-2 α chain promoter containing the wild-type NF-κB recognition sequence (GGGGAATCTCCC) or a mutant NF-κB recognition (GGCTCAATCTCCC; mutated bases are underlined) site were subcloned into the HindIII site of the pTKGH vector containing the heterologous herpes thymidine kinase promoter linked to the human growth hormone gene as a reporter gene (22). These constructs were termed p(−225)αTKGH or p(−141)αTKGH, respectively. Monocytes, U 937 cells, and T-cells were transiently transfected using the DEAE-dextran method (23) with p(−225)αTKGH, p(−141)αTKGH, or the control plasmid ptkGH not containing the enhancer element, maintained in SCM for 24 h, and then cultured for an additional 24 h in SCM in the presence or absence of IL-2 (500 units/ml). A β-galactosidase expression vector (24) was cotransfected to monitor transfection efficiency. Human growth hormone activity was assessed in cell-free supernatants using a commercially available enzyme-linked immunosorbent assay (Eurogenetics N.V., Tessen-derlo, Belgium).

**UV Cross-linking.** UV cross-linking was performed as described previously (25). Briefly, the IL-2 α chain oligonucleotide was body labeled with the Klenow large fragment polymerase (Boehringer Mannheim) in the presence of 5 mM dATP, 5 mM [32P]UTP (Boehringer-Mannheim), 5 mM 5’-bromodeoxyuridine triphosphate (Sigma), and [α-32P]dCTP (Amersham Buchler). Nuclear proteins from IL-2-treated monocytes and T-lymphocytes were incubated with 100 fmol body-labeled oligonucleotide as described above and then subjected to UV irradiation (254 nm) for 20 min at 4°C. Proteins were separated under reducing conditions on a 12% SDS gel. A rainbow marker (Amersham Buchler) was used as a size marker. The gel was dried and autoradiographed at −70°C using an intensifying screen.

**References**


