Targeting Combinatorial Transcriptional Complex Assembly at Specific Modules within the Interleukin-2 Promoter by the Immunosuppressant SB203580

The proximal promoter sequence of the interleukin-2 (IL-2) gene contains a series of composite sites or modules that controls much of its responsiveness to environmental stimuli. The integrated targeting of these modules is therefore a major mode of regulation. This report describes how multiple functional hierarchies, required for the recruitment of the p300 co-activator to the CD28RE/AP1 (TRE) module of the IL-2 promoter, are selectively disrupted in human T-cells by the immunosuppressive and anti-inflammatory actions of the p38 mitogen-activated protein kinase inhibitor (MAPK), SB203580. The molecular hierarchies targeted by SB203580 include the combinatorial interaction of NF-xB and CREB at the CD28RE/AP1 element coupled with the subsequent dynamic co-assembly and activation of p38. Several aspects of this targeting are linked to the ability of SB203580 to inhibit p38 MAPK-controlled pathways. Together, these results provide the molecular basis through which the combinatorial structure and context of the composite elements of the IL-2 promoter dictates mitogen responsiveness and drug susceptibility that are quantitatively and qualitatively distinct from the isolated action of single consensus sequences and/or transcriptional motifs.

It is now widely recognized that the control of gene expression occurs through a complex cascade of molecular events that ultimately mediate the assembly of active transcriptional complexes at specific genes (1, 2). This assembly process is dynamic and the information necessary for directing the targeting of these events is embedded within the primary sequence of promoters and 5` regulatory regions of every gene. New insights gained from sequencing of the human genome have made it clear that this embedded information constitutes a related, though separate, language or “code” that dictates how and when genes will be activated in response to environmental stimuli (2). The smallest units or packets of information in this code are the single binding motifs or recognition sequences that form the substrates for sequence-specific interactions of DNA binding transcription factors.

Although the practice of emphasizing individual protein-specific recognition sequences has formed a basis for thinking about the general structural organization of gene regulatory regions, it is now clear that this conceptualization is an oversimplification. The promoter sequence of many mammalian genes are highly structured and complex (3). The overwhelming plasticity in the response of the human genome to changes in its external environment is a direct reflection of this complexity. Thus, the concept of “one stimulus, one pathway, one transcription factor, and one gene” has limited utility when trying to understand gene regulation in response to diverse molecular signaling events (2).

The regulation of transcription can now best be described as a dynamic and hierarchical process that mediates the assembly and function of multiple factors at the regulatory elements of target genes. The rationale for the assignment of the factors within this hierarchy are based on properties that include, DNA binding affinity, protein-protein interactions, enzymatic activity, and relative abundance (1, 4). The first level in this hierarchy contains the sequence-specific DNA-binding proteins (e.g. AP-1,1 NF-xB, and CREB). Regulation of this hierarchy by molecular signaling events targets their nuclear accessibility, DNA binding affinity, and protein-protein interactions. The next level is represented by the transcriptional co-activator proteins or co-regulatory proteins (e.g. p300, BRG-1, and CARM). These factors have two very important roles in the hierarchy of transcriptional assembly. First, they influence chromatin structure through intrinsic enzymatic activities that can either covalently modify the chromatin or directly effect chromatin structure through ATP-dependent interactions with nucleosome structure. Second, they serve as important signal-regulated scaffolds that bridge and integrate interactions between DNA-bound factors and other transcriptional complexes. The final hierarchy contains the more abundant basal factors and type II RNA polymerase (polymerase II). Each of these hierarchies are selectively influenced by a complex series of intersecting and overlapping molecular signal cascades that achieve their final integration at the level of gene-specific promoters and 5` regulatory regions.

1 The abbreviations used are: AP-1, activator protein 1; TCR, T-cell receptor; CD28RE, CD28 response element; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, 12-O-tetradecanoylphorbol-13-acetate responsive element; PMA, phorbol myristate acetate; IL-2, interleukin-2; SB, cell permeable pyridyl imidazole; P/I, phorbol ester + ionomycin; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; aa, amino acids; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T-cells; HIV, human immunodeficiency virus; GALV, Gibbon ape leukemia virus; CAT, chloramphenicol acetyltransferase; UAS, upstream activation sequence; PKA, protein kinase A; CREB, cAMP-response element-binding protein.

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Several seminal observations that began to shed light on how the complex organization of promoter sequences could direct and integrate these intricate hierarchies of protein assembly were first provided by studies on the sequence structure and responsiveness of the interleukin-2 (IL-2) promoter in activated T-cells (5, 6). These initial studies clearly showed the IL-2 promoter to have a prototypical modular or "composite" organization comprised of what literally could be referred to as "modules within modules." The most important concept to arise from these studies was that few of these modules function independently. In fact, many studies suggested that multiple cooperative interactions (both positive and negative) are coordinated within the structure of the IL-2 proximal promoter sequences (7). The combinatorial "all or none" kinetics of the assembly of both T-cell specific and ubiquitous factors at the IL-2 promoter provides clear evidence of a thermodynamic linkage between these modules and events (8–10).

The modules that make up the structure of the proximal IL-2 promoter include: the NFAT/AP-1 site (nuclear factor of activated T-cells/activator protein 1) co-bound by NFAT and AP-1 dimers; the CD28RE/AP1 site (also known as CD28RRE/TRE site) co-bound by members of the NF-xB family and hetero- and homodimers of the AP-1 and CREB family; and the NFIL-2A site (also known as AP-1/Oct site) co-bound by octamer-associated factors and members of the AP-1 family (7, 11–16). Each one of these modules contains submodules that bind to individual transcription factors weakly in isolation, but with great synergy when bound in conjunction with factors at other neighboring modules or submodules (7, 12, 14, 17–19). A great deal of the cooperativity between factors is mediated by protein-protein interactions, not only between the DNA-bound components, but also by higher order interactions with other non-DNA binding molecules. Most of these non-DNA binding molecules are members of the second functional hierarchy of transcription factor assembly including p300 and CBP. Both p300 and CBP have been shown to have profound effects on the transcriptional activity of the IL-2 gene and other genes important in T-cell development (14, 20–22). Thus their intrinsic activity and coordinated recruitment to the IL-2 promoter are likely to be major targets for regulation by signal transduction events.

The p38 mitogen-activated kinase (MAPK) inhibitor, SB203580, was originally described as an inhibitor of inflammatory cytokine synthesis (reviewed in Ref. 23). Subsequent analysis of this class of drugs demonstrated that it could selectively inhibit the p38 MAPK family by competitive binding to the ATP pocket (24). Continued use of SB203580 in many systems has revealed it to have pleiotropic effects on a variety of signal pathways through mechanisms that varied depending on cell and tissue type in addition to the final drug concentration (25). Several years ago it was suggested that the immunosuppressive properties of SB203580 were in part because of its ability to suppress the production of IL-2 in activated T-cells, yet the mechanism underlying this suppression was not known (26).

In this report a transcriptional approach is used to map pharmacological actions of SB203580 at the IL-2 promoter in activated T-cells. Through this approach, we illustrate the concept that controlled gene expression is integrated by a multistep process that can be more easily conceived of in terms of how molecular signaling events impinge on the intrinsic hierarchical nature of transcriptional complex assembly at targeted genes and how the combinatorial nature of promoter structure forms the primary blueprint for this assembly. We first pinpoint modules within the IL-2 promoter that contribute most to its mitogen activation profile by mutational analysis and principal component analysis of the transcriptional activity of the IL-2 promoter and its isolated modules and submodules. After identifying the CD28RE/AP1 (TRE) module as one that shows high correlation with the activity and mitogen profile of the IL-2 promoter, we demonstrate that its transcriptional sensitivity to SB203580 is similar to the IL-2 proximal promoter in both transformed and primary human T-cells. We then dissect the contribution of the submodules of the CD28RE/AP1 (TRE) element to SB203580 sensitivity to demonstrate both NF-xB and CREB activity at the CD28RE/AP1 are targeted by SB203580. A major mechanism that integrates this level of targeting is shown to involve the activation-dependent phosphorylation of CREB on serine 133. Through in vivo activated kinase assays and enforced expression studies in both primary and transformed human T-cells, we show that the activity of p38 kinase plays a major role in the SB203580-mediated repression. Finally, kinetic analysis of CREB phosphorylation and CREB/p300 recruitment to the IL-2 promoter by chromatin immunoprecipitation, shows that an additional target of SB203580 is a dynamic CREB/p300 assembly that is kinetically correlated with a burst of CREB phosphorylation during T-cell activation. Results from this study demonstrate that the dynamic hierarchical assembly of higher order transcriptional complexes, coordinated by promoter modules, is a fundamental target for molecular signaling events. These findings emphasize how understanding the collective targeting of these assemblies will provide greater conceptual insights into mechanisms of signal integration than linkages between single pathways, solitary transcription factors, and isolated gene targets.

MATERIALS AND METHODS

Plasmids—The CD28RE-TRE-CAT, IL-2 CAT, NFAT-CAT, HIV-B CAT, and API-CAT reporter plasmids have been described (20). The NFIL-2A-CAT reporter was constructed by inserting 2 copies of the duplex sequence, 5'-AGCTTCAAGATTTTCTATTTACATACATATTTCAAAAG-3' and 5'-AGCTTTCTTTGAAAATGTGATATTGGAAAATCACTGTAAG-3', into the HindIII site upstream of the minimal fos promoter of EG6-CAT (27). The CD28RE/AP1-luc and CD28RE/AP1 mut-luc reporters have been previously described, and were a generous gift from Dr. Arthur Weiss (University of California, School of Medicine, San Francisco) (12). The CRE-CAT was a generous gift from Dr. Maria Laura Avantaggiati (Georgetown University Medical Center, Washington, D.C.). The UAS-CAT reporter plasmid contains 5 tandem copies of the UAS Gal4 recognition site linked to an E1B minimal promoter and its isolated modules and submodules. Mutations to the IL-2 promoter were introduced into the IL2-CAT reporter construct by site-directed mutagenesis using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). These base substitutions have been previously shown to abrogate transcriptional activity from CD28RE/A TR1 (TRE)-derived sequences (12, 30). The primer pairs used for the substrate is as follows: IL-2 M1 mutant, 5'-GGTTTGGGAGGTTTAAAGGAACGG-3' and 5'-AGAGCATAGTACCTTGTCATACAGAAGG-3'; and 5'-AGCTTTCCCTTCTCAAGATTTTCAAAAG-3'. IL-2 M2 mutant, 5'-GGTTTGGGAGGTTTAAAGGAACGG-3' and 5'-AGAGCATAGTACCTTGTCATACAGAAGG-3'; IL-2 M3 mutant, 5'-GGTTTGGGAGGTTTAAAGGAACGG-3' and 5'-AGAGCATAGTACCTTGTCATACAGAAGG-3'; IL-2 M5 mutant, 5'-GGTTTGGGAGGTTTAAAGGAACGG-3' and 5'-AGAGCATAGTACCTTGTCATACAGAAGG-3'; and IL-2 M6 mutant, 5'-GGTTTGGGAGGTTTAAAGGAACGG-3' and 5'-AGAGCATAGTACCTTGTCATACAGAAGG-3'.
formed as described (32). Inhibition by SB203580 (Calbiochem, San Diego, CA) was performed by pretreatment of transfected cells for 2 h prior to stimulation at a final concentration of 10 μM. Primary human T-cells were cultured from donor blood products by ficoll density gradient separation using lymphocyte separation medium (ICN, Aurora, OH). Red blood cells were removed by treatment with ACK lysis buffer (Quality Biological, Inc., Gaithersburg, MD). Lymphocytes were resuspended in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin G (sodium salt), 100 μg/ml streptomycin sulfate, 25 mM HEPES buffer (Gemini BioProducts, Woodland, CA) and incubated with 5% CO₂ at 37 °C overnight. Primary T-cells (2 × 10⁶) were washed in 37 °C × 1× Dulbecco’s phosphate-buffered saline without calcium and magnesium (Mediatech, Inc., Herndon VA) containing 0.5% bovine serum albumin and resuspended in 100 μl of supplemented human T cell Nucleofector™ Solution (Amxax Biosystems, Köln, Germany) per transfection. 5 μg of reporter was used for each transfection. Cells underwent nucleasefication, using the Amxax Nucleofector device at program U-14 and were placed in 37 °C media. Cells were harvested as described for Jurkat T-cells and assessed for luciferase activity as previously described (31). T-cell mitogens ionomycin (Calbiochem, San Diego, CA) and PMA (Sigma) were used at concentrations of 1 μM and 50 ng/ml, respectively, or as otherwise indicated. 

Antibodies—Antibodies against c-Rel, NFAT, ATF-1/CREB, and NF-xB p65 were from Santa Cruz (Santa Cruz, CA). Antibodies against phospho-p38, phospho-CREB, phospho-Jun, c-Jun, and p38 were from New England Biolabs (Beverly, MA). Immunoprecipitating antibodies for Rel family members were raised and affinity purified against a glutathione S-transferase-c-Rel (aa 1–538) fusion construct using a previously described procedure (33), and recognize Rel family members, p50, p65, and c-Rel. Antibodies against acetylhistone 3 were from Upstate Biotechnologies (Lake Placid, NY). Anti-p38 antibodies have been previously described (29). Relative phosphorylation was determined from densitometric measurement of autoradiographic exposures of Western blotted membranes processed by ECL detection (Amersham Biosciences) as previously described (32). The anti-CD28 monoclonal antibody 9.3 ascites were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA), and was used at a final dilution of 1:1000.

Determination of Interleukin-2 Concentration—Interleukin-2 concentrations were determined by enzyme-linked immunosorbent analysis using the Quantikine Immunoassay kit (R&D Systems) according to the manufacturer’s instructions.

Preparation of Nuclear and Whole Cell Extracts—Nuclear extracts were prepared from Jurkat T-cells as previously described (33). To prepare whole cell lysates, 2 × 10⁶ treated cells were washed 3 times in ice-cold PBS, and resuspended in 500 μl of buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 mM NaF at 4 °C. Lysates were sheared through a 22–25-gauge needle and clarified at 4 °C by microcentrifugation.

Immune Complex CREB Kinase Assay—Quantitation of activated MAPKAP kinase 2 from immunoprecipitates of Jurkat whole cell lysates was performed using the Upstate Biotechnology Inc. MAPKAP kinase 2 assay kit according to the manufacturer’s instructions. The CREB (S207/213) antibody (Calbiochem) was used at concentrations of 1 μg/ml for each transfection. Cells underwent nucleasefication, using the Amxax Nucleofector device at program U-14 and were placed in 37 °C media. Cells were harvested as described for Jurkat T-cells and assessed for luciferase activity as previously described (31). T-cell mitogens ionomycin (Calbiochem, San Diego, CA) and PMA (Sigma) were used at concentrations of 1 μM and 50 ng/ml, respectively, or as otherwise indicated. 

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transformed to a new vector space by minimization of covariance of input variables. The principal components of transformed data set are ordered by decreasing variance so that a three-dimensional projection along the first three principal components represents most of the information content of input data set.

For an input data set \( x \) of \( m \) observations of \( n \) dimensions, the procedure of principal component analysis involves setting up a covariance matrix, \( C \), defined by,

\[
C_{ij} = \frac{1}{n} \sum_{k=1}^{n} (x_{ik} - \bar{x}_i)(x_{jk} - \bar{x}_j)
\]

where the averages \( \bar{x}_i \) and \( \bar{x}_j \) are given by,

\[
\bar{x}_i = \frac{1}{n} \sum_{k=1}^{n} x_{ik} \quad \text{and} \quad \bar{x}_j = \frac{1}{n} \sum_{k=1}^{n} x_{jk}
\]

and the indices \( i \) and \( j \) take the values 1 . . . \( m \). The eigen vectors and eigen values of the covariance matrix \( C \) are computed by standard eigen value procedures such as singular value decomposition. The \( n \)th principal component of the input matrix \( x \) is normalized eigen vector of \( n \)th eigen value. The correlation matrix is equivalent to covariance matrix where the data set \( x \) is standardized to zero mean and unit variance. The first three major principal components of the transcriptional reporter data set were computed by minimizing Pearson correlation using the principal component analysis module of the Partek Pro Build 2001.10.22 software package (Partek Inc., St. Charles, MO) that accounted for 99.75% of the variance.

RESULTS

Prior studies have shown that IL-2 secretion can be inhibited in antigen-stimulated T-cells by treatment with the SB203580 p38 MAPK inhibitor (26, 35). The mechanism for this mode of repression has never been defined, although recent work has established that p38 MAPK is selectively activated in surface receptor-stimulated cells (35, 36). The Jurkat T-cell line is a human cell line derived from a patient with relapsed acute lymphoblastic leukemia (37). Although a transformed cell line, Jurkat cells maintain many of the basic molecular signaling pathways triggered during T-cell activation. It thus has served as a useful experimental model system for investigation of the transcriptional targeting of agents that influence T-cell function (38). Jurkat cells, like primary human T-cells respond to molecular signals that are initiated by cross-linking of the T-cell receptor and the CD28 class of co-receptors on its cell surface. This is normally achieved by physiological interaction with antigen-bound class II major histocompatibility complex molecules and the CD80/CD86 class of CD28 counter receptors. These cell surface-initiated signaling events can be mimicked \emph{in vitro} in Jurkat and primary T-cells by the addition of phorbol ester and ionomycin (P/I) in the presence of antibodies that cross-link the CD28 surface receptor (39, 40). Stimulation of Jurkat cells with P/I leads to a significant increase in IL-2 gene activation and secretion as determined by transcriptional reporter assays and IL-2 enzyme-linked immunosorbent analysis, respectively (Fig. 1). This induction is increased further by co-stimulation with antibodies that cross-link the CD28 receptors. As shown in Fig. 1, the transcriptional activation and production of IL-2, in both stimulated and co-stimulated cells, is blunted in the presence of SB203580.

SB203580 has been shown to repress a variety of different transcriptional pathways through both direct and indirect influences (23, 25). Most of these influences have been attributed...
to the action of p38 MAPK (23). Two ubiquitous transcriptional activation pathways that are particularly important in T-cell activation include the NF-xB pathway and the CREB/AP-1 pathways. The IL-2 promoter has been shown to have multiple binding sites for NF-xB family members and members of the AP-1 and CREB family of transcription factors (14–16, 41). The CD28RE/AP1 (TRE) (Fig. 2A) represents a single module in the IL-2 promoter that contains both xB and AP-1/CREB elements that have been shown to act cooperatively when measured by conventional transcriptional assays (12, 14). Interestingly, although each element within the CD28RE/AP1 module has been shown to be transcriptionally competent for independent function in the absence of the other (16, 30), the “in situ” mutation of either or both elements within the IL-2 promoter leads to near complete loss of activation of the IL-2 promoter (Fig. 2, B and C). This requirement is tightly restricted to the linked integrity of each element because mutation within the intervening sequence that joins the xB element to the AP1/CREB element of the IL-2 CD28RE/AP1 module has very little effect on the inducibility of the IL-2 promoter. The stringency of this combinatorial specificity is further demonstrated by the lack of variation between the species within the sequences of the CD28RE/AP1 module. As shown in Fig. 2B, this intervening sequence is the only region within the CD28RE/AP1 module that is not conserved between mouse and human.

Fig. 2 establishes that there is a rigorous sequence dependence within the CD28RE/AP1 module that is critical for directing the combinatorial targeting of xB and AP1/CREB factors to the IL-2 promoter during T-cell activation. Given prior studies on the effects of SB203580 in other systems, this dependence alone suggests that it should be able to disrupt the activity of the IL-2 promoter (36, 42). However, it is not clear how much the targeting of CREB and xB at the CD28RE/AP1 module contributes to the overall signaling response or “induction profile” of the IL-2 promoter. This issue is of particular relevance because several reports have shown that multiple regions within the IL-2 promoter can respond to CREB, AP-1, and xB transcriptional targeting during T-cell activation (12, 14, 30, 43–46).

To address this question, reporter assays were conducted in which the IL-2 promoter, its major composite modules, and consensus versions of its constituent submodules (see Table I) were challenged with 13 different combinations of T-cell mitogens (see Table II) and the profiles of their transcriptional response were compared. Each one of these combinations has been previously found to recapitulate multiple aspects of the molecular signaling cascades triggered during T-cell activation (39, 40). The reporters compared in this experiment were (see Table I): 1) the 600-bp proximal promoter of the IL-2 gene; 2) the IL-2 CD28RE/AP1 (TRE) site; 3) the IL-2 NF-AT site (ARRE-2, see Fig. 2A); 4) the IL-2 NF-IL-2A site (AP1/Oct, ARRE-1); 5) a consensus AP-1 site (GALV-AP1 (33)); 6) a consensus NF-xB site (HIV-long terminal repeat-derived (32)); 7) a consensus CRE site (cyclic AMP response element); 8) a hybrid fusion of the Gal4 DNA-binding domain with p300 (Gal4/p300 full-length) co-transfected with a Gal4 binding site reporter, Gal4 US, US with Gal4/p300 full-length; p300 FL.

### Table I

| Promoter elements with their synonym names |
|-------------------------------------------|
| 1  | IL-2, 600-bp proximal promoter of the IL-2 gene, −575 to +47 bp; +1 = transcription start |
| 2  | CD28RE-TRE, CD28RE/AP1 element; CD28RE/AP1 (TRE) |
| 3  | NFAT, NF-AT element; ARRE-2 |
| 4  | NF-IL2A, AP1/Oct element; ARRE-1; NF-IL-2A |
| 5  | AP1, consensus AP-1 site from GALV-LTR |
| 6  | HIV-xB, consensus NF-xB site from HIV-LTR |
| 7  | CRE, consensus cyclic AMP-response element, from somatostatin gene |
| 8  | p300 FL-UAS, co-transfected Gal4 binding site reporter, Gal4 US, US with Gal4/p300 full-length; p300 FL |
| 9  | p300 N-term UAS, co-transfected Gal4 binding site reporter, Gal4 US, US with Gal4/p300 N terminus; p300 N-term |

### Table II

| Mitogen combinations | Concentration |
|----------------------|--------------|
| 1                     | Unstimulated |
| 2                     | α-CD3        |
| 3                     | α-CD28       |
| 4                     | PMA          |
| 5                     | PMA, 50 ng/ml |
| 6                     | Ionomycin, 1 μM |
| 7                     | α-CD3 + α-CD28 |
| 8                     | PHA + PMA    |
| 9                     | PMA + α-CD3  |
| 10                    | PMA + α-CD28 |
| 11                    | PMA + Ionomycin |
| 12                    | PHA + PMA + α-CD28 |
| 13                    | PMA + Ionomycin + α-CD28 |

This approach generates a 9 × 13 matrix of multivariate transcriptional data that represents how the 9 different promoter elements are influenced by 13 different combinations of T-cell mitogens. In the absence of any other methods, the relative similarities and differences in these profiles would be hard to visualize (see Supplemental Materials Fig. 1). However, there are several powerful computational methods that employ dimension reduction strategies to help visualize trends in such multivariate (multidimensional) data (47, 48). By these methods the mitogen response profile of each gene regulatory element can be objectively compared (see Fig. 3).
The GALV consensus AP-1 site (4/H9262 (2/SB203580. Multiple p38 MAPK-dependent kinases such as that one or more of the regulatory pathways known to influence families of transcription factors. Thus, it is logical to presume that p38 signaling is a key mediator of IL-2 transcriptional activation and analyzed by principal component analysis using the Partek Pro analytical software package (see Materials and Methods). Shown is a three-dimensional plot where the data points (promoter elements) are dispersed according to the similarities in their mitogen response profiles. Views shown are horizontally rotated 45 degrees relative to one another.

Comparison of the mitogen activation profiles of the IL-2 promoter and its constituent modules by principal component analysis. Jurkat T-cells were transfected with the indicated CAT reporter plasmids (see Table I and “Materials and Methods”) driven by: the IL-2 promoter (base -575 to +47; +1 = transcription start) (4/μg); the CD28RE/TRE element (4/μg); the NF-AT site (4/μg); the AP1/Oct element (4/μg); the GALV consensus AP-1 site (4/μg); the HIV-κB site (4/μg); and a consensus CRE site (4/μg) in addition to combinations of the UAS-CAT reporter (2/μg) with either a full-length p300 (Gal4/p300 FL) or C-terminal-truncated p300 N-terminal p300 Gal4 fusion expression vector (Gal4/p300 Nterm) (3/μg). The transfected cells were exposed to 13 different treatment conditions (see Table II) including: 1) unstimulated; 2) α-CD3; 3) α-CD28; 4) PHA; 5) PMA; 6) ionomycin; 7) α-CD3 + α-CD28; 8) PHA + PMA; 9) PMA + α-CD3; 10) PMA + α-CD28; 11) PMA + ionomycin; 12) PHA + PMA + α-CD28; 13) PMA + ionomycin + α-CD28, using mitogens at the following concentrations: 1:1000 dilution of α-CD3; 1:1000 dilution of α-CD28 antibodies; 2 μg/ml PHA; 50 ng/ml PMA and 1 μg/ml ionomycin (see Table II). Data from the reporter assays were then normalized to fold activation and analyzed by principal component analysis using the Partek Pro analytical software package (see “Materials and Methods”). Shown is a three-dimensional plot where the data points (promoter elements) are dispersed according to the similarities in their transcriptional activity in response to the 13 different T-cell mitogen combinations (see Table I). Elements that are mapped close in the three-dimensional plots have greater similarity in their mitogen response profiles. Views shown are horizontally rotated 45 degrees relative to one another.

As demonstrated in Fig. 3, when compared with the five other modules and submodules of the IL-2 promoter, the CD28RE/AP1 (TRE) element (green) shows the greatest similarity to the IL-2 proximal promoter (pink) in its mitogen response.

Having established that the CD28RE/AP1 sequences make a major contribution to both the level and the character of the inducibility of the IL-2 promoter, the ability of SB203580 to inhibit the isolated CD28RE/AP1 element was tested and compared with known targets of SB203580. As shown in Fig. 4A, transcriptional activation of the CD28RE/AP1 (TRE) is inhibited by treatment with SB203580 in both phorbol ester/isonomycin (P/I)-stimulated and P/I plus anti-CD28 receptor antibody costimulated cells. Moreover, a dose-response curve (Fig. 4B) with increasing drug concentrations shows that both the CD28RE/AP1 element and the IL-2 promoter are significantly (greater than 2-fold) more sensitive to SB203580 than its classical target, ATF-2 (shown here in a Gal4/ATF-2 fusion reporter assay) (23). As expected, similar inhibition of the CD28RE/AP1 (TRE) is seen in primary human T-cells (Fig. 4C).

As mentioned above, several previous studies have shown that the CD28RE/AP1 (TRE) element is bound by and regulated by multiple members of the CREB, AP-1, and NF-κB families of transcription factors. Thus, it is logical to presume that one or more of the regulatory pathways known to influence the activity of these family members could be targeted by SB203580. Multiple p38 MAPK-dependent kinases such as MAPKAP kinase 2/3 have been shown to increase CREB phosphorylation in various cell types, including T-lymphocytes (36, 49–51). As demonstrated in Fig. 5A, both CREB-dependent transcriptional activity (using a Gal4 DNA binding domain CREB fusion) and the phosphorylation of CREB, at serine 133, is inhibited in P/I-stimulated and CD28 co-stimulated Jurkat cells by the addition of SB203580. Conversely, the transcriptional activity and the phosphorylation of c-Jun are unaffected by the SB203580 (Fig. 5B). A central role for the κB family member, c-Rel, in activation of the IL-2 promoter has been clearly demonstrated by several groups (19, 52, 53). Although SB203580 does cause inhibition of the NF-κB transcriptional activity in co-stimulated T-cells, there was no change in either the nuclear translocation of c-Rel or the degradation of IκBα (Fig. 5C). Recent data suggests that phosphorylation of the p65 κB family members plays a major role in a tumor necrosis factor-α-dependent activation of the IL-2 promoter through p38 MAPK (54). However, no change in p65 mobility was observed in lysates from cells activated in the presence or absence of SB203580 (data not shown).

As indicated above, multiple kinases capable of phosphorylating CREB and κB family members have been described (36, 55, 56). Those thought to directly increase CREB phosphorylation (on serine 133) include, MAPKAP kinase 2/3, MSK1, MSK2, RSK2 kinase, and protein kinase A. Of these kinases, MAPKAP kinase 2/3 and MSK-1 are known to be regulated specifically by p38 MAPK. They are thus likely targets for inhibition by SB203580. Accordingly, overexpression of p38...
MAPK in primary human T-cells leads to significant transactivation of the CD28RE/AP1 (TRE) element (Fig. 6A). Moreover, inhibition of the CD28RE/AP1 (TRE) element by SB203580 in activated Jurkat cells can be overridden by enforced expression of p38 MAPK (Fig. 6B). As expected, in vivo activation of a representative p38 MAPK-dependent CREB kinase, namely MAPKAP kinase 2/3, is significantly inhibited by SB203580 in both stimulated and co-stimulated T-cells (Fig. 6C).

Very recent studies have found that CREB phosphorylation proceeds through a stimulation-dependent “burst phase” followed by an “attenuation phase” where there is an initial increase in CREB phosphorylation followed by a gradual or abrupt decrease over time (57). As shown in Fig. 7, CREB phosphorylation during T-cell activation undergoes a rapid burst that is maximal within 30 min while CREB expression levels remain unchanged (see Supplemental Materials Fig. 3). Following this burst phase, the levels of phosphorylation attenuate rapidly, returning to near basal levels at 60 min. Phosphorylation time courses in both Jurkat and primary human T-cells show a substantial influence of SB203580 on the amplitude of CREB phosphorylation kinetics (Fig. 7, A and B).

A major consequence of CREB phosphorylation at serine 133 is an increase in its ability to recruit the transcriptional activators p300/CBP (58). p300/CBP is well known to have separate, but potent intrinsic transactivation domains in its N- and C-terminal portions. Furthermore, both p300 and CBP have been shown to be direct targets of signal transduction events in growth factor-stimulated cell lines and activated T-cells (20, 59). By using a hybrid fusion of the DNA binding domain of Gal4 with different fragments of p300 we were able to assess the sensitivity of the intrinsic transcriptional activity of multiple p300 domains to suppression by SB203580 during T-cell activation (Fig. 8). Interestingly the SB203580 sensitivity of the intrinsic transcriptional activity of p300 was domain and possibly conformation specific. Whereas the N-terminal domain of p300 (aa 1–743) was much more sensitive to SB203580 than the C-terminal domain (aa 1737–2414), the intrinsic tran-
scriptional activity of the full-length GAL4-p300 (aa 1–2414) was the most sensitive to repression. This higher sensitivity suggests the possibility that internal allosteric intramolecular interactions within p300 may play a role in the transcriptional inhibition by SB203580. Notably, although there is a significant increase in the amount and post-translational modification of p300 in whole cell lysates within 15 min after T-cell activation (see top inset, Fig. 8) there does not appear to be any alteration in the rate or amount of the modified forms with the addition of SB203580.

Consistent with the energetics of CREB phosphorylation during T-cell activation, the dynamic recruitment and assembly of CREB and p300 at the IL-2 promoter is also an in vivo target of SB203580 (Fig. 9). Jurkat T-cells were stimulated with phorbol ester and ionomycin in the presence and absence of a 1-h pretreatment with SB203580. Samples were withdrawn at 15-min intervals, formalin cross-linked, and analyzed by chromatin immunoprecipitation for in vivo protein-DNA interactions with IL-2 promoter sequences encompassing the CD28RE/AP1 module (Fig. 9). Fig. 9 shows the kinetic profiles for the association of CREB, p300, NF-κB subunits, and acetyl-histone 3 with the endogenous IL-2 promoter. There is a rapid, but transient association of CREB with the IL-2 promoter that peaks in 30 min (Fig. 9A), and like CREB phosphorylation in Jurkat cells (Fig. 7A), is nearly undetectable after 60 min. This rapid peak or “burst” in the association of CREB with the IL-2 promoter is significantly blunted by pretreatment with SB203580 prior to stimulation. A similar “burst and attenuation” is seen for the recruitment of p300 to the IL-2 promoter following stimulation (Fig. 9B). This association of p300 with IL-2 promoter sequences is similarly blocked by pretreatment with SB203580. Interestingly, members of the κB family appear to pre-exist at the IL-2 promoter in the absence of stimulation (Fig. 9C). Although this association increases after stimulation, it is not influenced by SB203580 treatment. This pre-bound status of κB factors at the IL-2 pro-
moter was unexpected. Nonetheless, a pre-stimulus association of NF-κB factors with endogenous genes has been reported previously for other promoters (60). Moreover, this NF-κB occupancy at the IL-2 promoter in unstimulated Jurkat is cell-specific because it does not occur in HeLa cells.2 Interestingly, although alteration in histone acetylation is a major hallmark of transcriptional activation, the status of histone 3 acetylation at the IL-2 promoter does not change during T-cell activation, nor is it affected by pretreatment with SB203580 (Fig. 9D).

DISCUSSION

The T-lymphocyte is continuously exposed to a wide variety of extracellular stimuli throughout the course of an antigen-mediated immune response. Thus, the primary signaling cascades that emanate from the dual TCR/CD28 receptor ligation are invariably gated by multiple overlapping signaling events initiated by other surface-receptor-mediated processes. Many of these processes either involve or are controlled by p38 MAPK (61). This co-mingling of signal transduction pathways creates a web of redundant, synergistic and antagonistic molecular events that achieve their final integration at the level of the targeted promoter. The combinatorial nature of promoter sequences and the protein complexes that assemble at them provides an adaptive surface that can accommodate this high level of complexity. Therefore it is not surprising that a drug, even with the high molecular specificity of SB203580, could exert such a multifaceted effect on the hierarchy of events that control transcription of the IL-2 promoter.

In this study we show that numerous combinatorial features of transcriptional events that occur at the IL-2 promoter are targeted by the action of SB203580 with very precise specificity and kinetics. Central to the specificity and sensitivity of this targeting is the modular nature of the promoter elements and the coordinated manner in which CREB, NF-κB, and p300-containing complexes are assembled and recruited at the CD28RE/AP1 (TRE) during T-cell activation. The site-directed mutagenesis experiments (Fig. 2) and the principal component analysis (Fig. 3) of the mitogen-activated transcriptional profile

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of the CD28RE/AP1 (TRE) elements show how essential this module is to the overall shape and amplitude of the transcriptional response of the IL-2 gene. The increased susceptibility of the CD28RE/AP1 (TRE) element to repression by SB203580, in comparison to a known direct target (i.e. ATF-2, Fig. 4B), is a clear demonstration of how the combinatorial nature of composite promoter modules adds increased sensitivity and selectivity to the transcriptional response. An indispensable feature in the assembly of complexes at the CD28RE/AP1 (TRE) is the phosphorylation of CREB. Accordingly, there is a high correlation between the kinetics of this phosphorylation (Fig. 7) and the assembly and recruitment of CREB and p300 at the IL-2 promoter (Fig. 9, A and B). Combined with the overlapping sensitivity of these events to SB203580 treatment, these collective observations provide convincing evidence for a pivotal role of these linked factors and their coordinated actions in the mechanism of transcriptional repression at the IL-2 promoter by SB203580.

Although prior studies have shown that SB203580 and other p38 MAPK inhibitors can repress activation of the NF-κB pathway, these effects are very small in activated Jurkat T-cells (Fig. 5C). The magnitude of repression is likely to be cell specific, as has been demonstrated in other cell types. The observations that NF-κB factors are re-associated with the IL-2 promoter in resting cells and that binding is only slightly increased during T-cell activation is very provocative and have been described for genes distinct from IL-2 in Jurkat and other cell types. The resistance of NF-κB occupancy to SB203580 is consistent with the minor effects that SB203580 exerts on the activity of the NF-κB reporter in activated Jurkat. Nonetheless, this exception does not rule out the possibility that profound NF-κB may be modulated by SB203580-dependent post-translational modifications that could influence the recruitment of transcriptional activators and/or repressors to the IL-2 promoter (60, 62, 63). The works of Shannon and colleagues (52) and other laboratories (64) have clearly shown that c-Rel plays a major role in the activation of the IL-2 promoter via the CD28RE/AP1 in surface receptor-stimulated T-cells. Moreover, this requirement for c-Rel can be satisfied by other NF-κB family members when phorbol ester and ionomycin are used as stimulants (64, 65). Therefore, the targeting of NF-κB by SB203580 at the IL-2 promoter may have differential influence on the mechanism of repression depending on the type of stimulus. These questions will be more easily addressed when antibodies with higher specificity and sensitivity to phosphorylated and acetylated forms of NF-κB family members become available. The differences in the dependence of NF-κB signaling on p38, and therefore SB203580 susceptibility, may well be determined by the type of inflammatory environment in which the activated T-cell lymphocyte is function.
and MAPKAP kinase 2/3 are likely to be redundant and will require dissection through extended analysis of available transgenic models combined with high resolution drug strategies (69, 70). A recent study suggests a primary role for MSK1 in p65 phosphorylation, however, this effect appears to be highly cell specific (54).

The concentration of kinase inhibitors used in this and other studies are important considerations. There is wide variation in the effective concentration necessary to see maximum SB203580 inhibition in many cell-based assays. In the case of p38, the published IC$_{50}$ have been shown to be as low as 100–500 nM (86). Although this efficiency is likely to vary from cell to cell based on permeability differences and the presence of non-specific drug binding capacity, the concentrations of SB203580 used in this study has been shown in other systems to produce some partial inhibition of the LCK tyrosine kinase and protein kinase B (86). Future studies correlating direct measurements of SB203580 IC$_{50}$ with the activity of p38 targets within the identical cell system will help to sort out this inherent ambiguity.

This report is the first to demonstrate a dynamic association of p300 and CREB with the IL-2 promoter. Although rapidly changing kinetics of association of co-activators like p300/CBP has been shown for other genes (71, 72) this observation is at odds with prior in vivo footprinting data that indicates that the IL-2 promoter is occupied by DNA-bound factors for several hours (10). In addition, work by Shannon and colleagues (68, 73) provides evidence that a nucleosome, positioned around the IL-2 promoter in resting cells, just that a dynamic change in its association (or remodeling of bound factors) is readily detected by the chromatin immunoprecipitation assay. Indeed, the time frame of this change is highly consistent with the observation that nascent IL-2 pre-mRNA is detectable within the first 45 min to 1 h after stimulation (75). Moreover, the dynamic association of p300 and CREB with the IL-2 promoter is abolished in the presence of cyclosporine A in a manner similar to the abrogation of the in vivo footprint of the proximal IL-2 promoter after cyclosporine A treatment (10). Thus the “all or none” observation is applicable in both kinetic frameworks (10). Another possibility is that the remodeling of nucleosomes at the proximal promoter may undergo some initial remodeling oscillations prior to going through a more sustained change, after the first hour, that is coupled to the committed initiation and elongation of the polymerase. These issues will be best addressed by kinetic studies using more extended time frames (0–6 h) and higher resolution (15-min intervals).

The targeting of the IL-2 promoter by SB203580 is not limited to the CD28RE/AP1 (TRE) module because recent data indicates that the transcriptional response of NFAT may also

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### Table: UAS-Luciferase Activity, Relative

| Repression (%) | SB203580 1-2414 | Unstim | Stim (P/I) | Sim + SB203580 |
|---------------|-----------------|--------|------------|----------------|
| 0%            | 140             | 155    | 215        | 215            |
| 50%           | 160             | 155    | 195        | 215            |
| 80%           | 180             | 160    | 215        | 215            |

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**FIG. 8.** The domain specific intrinsic transcriptional activity of p300 is inhibited by SB203580 in activated T-cells. Lower panel, Jurkat T-cells were co-transfected with 2 μg of a Gal4 UAS-luc reporter and 3 μg of the indicated Gal4p300 fusion constructs (aa 1–743, 969–1922, and 1737–2414; and full-length aa 1–2414). The transfected cells were then stimulated with P/I in the presence and absence of 10 μM SB203580. Cells were harvested and assessed for promoter activity. The level of SB203580 inhibition, by comparison to untreated samples, is shown, below the graph, as percent repression. Upper panel, whole cell lysates (4 μg) were prepared from Jurkat T-cell stimulated with P/I for 0, 15, 30, 45, and 60 min in the presence and absence of 10 μM SB203580 as indicated. Shown is an immunoblot of p300. Arrows indicate induced forms of p300 with differing mobility.
be affected (76), although it is not clear whether the effects of p38 are positive or negative (77). NFAT controlled transcription has been previously shown to be p300-dependent at the IL-2 promoter and in various cells types (20, 22, 78). Thus it is quite possible that a large portion of the effects of SB203580 on NFAT sites could be occurring at the level of the intrinsic activity of p300 (see Fig. 8). This is also consistent with the observation that the IL-2 promoter is more sensitive to SB203580 than either the direct ATF2 target or the CD28RE/AP1 (TRE) (Fig. 4B). There have been scattered reports that the intrinsic transcriptional activity of p300 may be a target of molecular signaling events (59, 79–82). Two particular studies provide some very intriguing evidence that p300/CBP is targeted for modulation by the activity of p38 MAPK. In one study the p38 MAPK-dependent MSK1 protein was shown to directly influence the activity of p300/CBP. In another study, p300 phosphorylation could be induced by C/EBP-β, a well known p38 MAPK target (80, 82). Preliminary experiments in which the in vivo phosphorylation of p300 is examined by phosphopeptide map analysis, demonstrates that numerous sites on p300 are phosphorylated in Jurkat T-cells after the addition of phorbol ester and ionomycin.3 Future studies will be directed at determining the functional influence of SB203580-sensitive p300 phosphorylation sites.

The rapid burst and quick attenuation of CREB phosphorylation during T-cell activation indicates that the dynamics of phosphorylation at serine 133 is tightly regulated. Recent description of this phenomenon by Montminy and colleagues (57) proposes the intriguing idea that these kinetics may be modulated by the interplay and association of histone deacetylases with specific protein phosphatases (57). Although the dynamics of CREB phosphorylation occurs with much slower kinetics in the choriocarcinoma cells lines described in that study, the notion that the dephosphorylation of CREB in activated T-cells could be mediated by histone deacetylases-protein phosphatase complexes suggests another mode of IL-2 regulation and may explain the ability of a protein phosphatase inhibitor, such as okadaic acid, to repress IL-2 expression (83). In the study by Canetttieri et al. (57) the addition of histone deacetylases inhibitors prolonged CREB phosphorylation. It may be that the dephosphorylation of CREB at the IL-2 promoter may be a necessary event in the remodeling of the complexes that assemble there. The finding that histone deacetylases inhibitors repress rather than stimulate the IL-2 promoter supports this idea (84, 85). Moreover, the role of possible CD28-activated phosphatases in this attenuation phase should be considered in light of the observation that SB203580 inhibition of CREB phosphorylation is greater with CD28 co-stimulation (Fig. 5A).

Finally the use of principal component analysis to profile the mitogen responsiveness of various promoters and promoter modules is a powerful approach to be used in conjunction with chromatin immunoprecipitation studies to unravel how molecular signaling events are integrated at promoter regions. Although applied to a rather small sample set in this study, the use of principal component analysis in larger high throughput studies, where mitogen profiles are compared in the presence of various inhibitory agents including SB203580, promises to yield a wealth of information on how promoter structure relates to its transcriptional targeting by molecular signaling cascades and pharmacological agents.

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Fig. 9. SB203580 treatment disrupts recruitment of the CREB and p300 to the IL-2 promoter. Jurkat T-cells were stimulated with P/I in the presence and absence of a 1-h pretreatment with 10 μM SB203580. At the indicated times points after stimulation (0, 15, 30, and 45 min) cells were formalin-fixed and analyzed by chromatin immunoprecipitation for the association of specific proteins with the CD28RE/AP1 (TRE) region of the IL-2 promoter with antibodies to: A, CREB; B, p300; C, NF-κB; and D, acetylhistone 3. For each section of this figure the top panel shows cells stimulated with P/I, the middle panel shows cells stimulated with P/I in the presence of 10 μM SB203580, and the bottom panel shows input control (10%) for each time point sample.

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