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Role of a STAT Binding Site in the Regulation of the Human Perforin Promoter

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The pore-forming protein perforin is preferentially expressed in NK and cytotoxic T cells. To investigate the molecular regulation of human perforin gene transcription, the activity of the human perforin promoter was analyzed in human NK and T cell lines using various promoter fragments linked to a luciferase reporter gene. A core promoter was identified within 55 bp upstream of the transcription start site. This promoter region contains a guanine/cytosine box and has basal activity in YT, Kit225-k6, and Jurkat cells. A strong enhancer activity was identified between positions −1136 and −1076, a region that includes a STAT-like element. This enhancer region was active in YT cells, which have constitutive perforin expression and activated STAT3 protein, but not in Kit225-k6 or Jurkat cells, which do not have constitutive perforin expression. Mutation of the STAT binding site resulted in a dramatic down-regulation of promoter activity. Electrophoretic mobility shift assays, using a probe containing the STAT element of the perforin promoter, indicated that this element can bind STAT3 from YT cells. Moreover, the STAT element was shown to bind STATS5a/b induced by IL-2 as well as STAT1a induced by IL-6 in human NK cells. Together, these results suggest that STAT proteins play a key role in perforin gene transcription and provide a model by which cytokines can regulate perforin gene expression.

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Cytotoxic lymphocytes have the ability to kill malignant or infected cells by releasing cytotoxic proteins contained in their cytoplasmic granules. One of these proteins, perforin, is exclusively expressed by NK and cytotoxic T cells (1). Perforin is responsible for forming pores in the target cell membrane (2–4) and plays a central role in the killing process as demonstrated by the significant impairment of NK and T cell cytolytic activity in perforin deficient mice (5). Although resting NK cells constitutively express perforin, it can only be detected in T cells after activation (6). Although it is believed that the control of perforin expression occurs at the transcriptional level, the molecular mechanisms responsible for that control have not been completely characterized.

Human and mouse perforin genes have been cloned, and the structure of the 5′-flanking regions was analyzed (7–9). It was found that the general organization of the genes is very similar with three exons and a large intron separating the 5′ untranslated region from the coding region. At the nucleotide level, mouse and human perforin genes are very homologous, especially in a region proximal to the cap site (10). In that region, several potential regulatory elements of the promoter are conserved, suggesting that human and mouse genes are regulated in a similar fashion. However, many of the conserved sequences are not related to known regulatory elements, and the human perforin 5′-flanking region contains certain sequence elements, which are absent from the mouse sequence, that could represent species-specific regulatory elements.

Studies of mouse perforin promoter activity in perforin-positive and -negative cells revealed a core promoter that was not cell type specific, flanked by several cis-acting elements that restricted reporter gene expression to perforin-positive cells (11). Upstream regulatory elements were found that could enhance or suppress perforin promoter activity in perforin-positive or -negative cells, respectively. An Ets-homologous binding site found in the proximal region of the mouse promoter was shown to bind Ets-related proteins in CTLL-R8, a perforin-positive cell line (12). More recently, a critical role for Sp1- and Ets-related transcription factors in maintaining CTL-specific expression of mouse perforin was reported (13). Interestingly, it was also proposed that Ets family members expressed exclusively by nonkiller cells can silence perforin expression (14), further strengthening the role of these transcription factors in regulation of perforin expression.

Because a functional analysis of the human perforin promoter has not been performed, it remains to be established if the human perforin promoter is controlled in a similar fashion by Ets-related proteins or other transcription factors. STAT proteins are transcription factors that transfer signals generated by cytokine receptors from the cell surface to the nucleus and direct gene regulation by binding to a cis-acting STAT binding site (15, 16). Several cytokines, including IL-2, IL-4, IL-6, IL-7, IL-12, and IFNs, have been shown to enhance perforin expression in different systems (17–20). In addition, the importance of STAT1 and STAT4 proteins in cell-mediated immunity has
been documented (21, 22). Therefore, STAT proteins represent transcription factors that could be involved in perforin gene regulation. In this study, a functional analysis of the human perforin promoter region was performed, and evidence for an important role of a STAT binding site in up-regulating perforin gene transcription in perforin-positive cells is presented.

**Materials and Methods**

**Cell lines and reagents**

YT cells, a human NK-like cell line, and Jurkat cells, a human T cell line, were cultured in complete medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin). The Kit225-kb IL-2-dependent human T cell line was cultured in complete medium containing 20 U/ml human rIL-2 (Chiron, Emeryville, CA). Antisera against STAT1, STAT3, and STAT5a/b were kindly provided by Dr. Andrew C. Lamer (Food and Drug Administration, Center for Biologics Evaluation and Research, Division of Cytokine Biology, Bethesda, MD).

**Purification and stimulation of NK cells**

PBMCs were separated on Ficoll-Hypaque (ICN, Costa Mesa, CA) as previously described (23). Leukocyte suspensions were washed in HBSS and resuspended in RPMI 1640 containing 1% FCS. Monocytes and B cells were removed by adherence to plastic dishes followed by passage through a nylon wool column. Highly enriched populations of NK cells (>90% CD3−) were obtained from PBMC by centrifugation of nylon wool nonadherent cells on discontinuous density gradients of Percoll (Pharmacia Biotech, Uppsala, Sweden). Contaminating CD3+ cells in NK cell fractions were removed with anti-CD3 mAbs coupled to magnetic beads. Cells were washed and resuspended in RPMI 1640 medium, incubated at 37°C for 30 min in the absence of FCS, and immediately stimulated with 1000 U/ml IL-2 or 100 U/ml IL-6 for 15 min.

**Construction of reporter plasmids**

 Fragments of the human perforin promoter region were amplified from genomic DNA by PCR using a common 5′ reverse primer (5′-GCTCCTGGAAATTTCTGGCCATC-3′) containing an EcoRI site and various 5′ forward primers containing a HindIII site. The forward PCR primers used were: P1, 5′-GGTGGAGGAAGCTTACGCTCAG-3′; P3, 5′-CCCGAGAAGCTTCCATACAAGC-3′; P5, 5′-TGACCTAGAAGCTTACGCTCAG-3′; P6, 5′-TGGAGGGAAGCTTACGCTCAG-3′; P7, 5′-GAGGAGGTGGGGAAGCTTACGCTCAG-3′; and P8, 5′-GCTGCTGAAGCTTACGCTCAG-3′. The PCR-derived fragments were digested with HindIII and EcoRI, cloned into pBluescript-KS (Stratagene, La Jolla, CA), and sequenced entirely using the Sequenase II kit (United States Biochemical, CA) at positions 1079 to 2113.

Each fragment was excised from recombinant pBluescript-KS clones with KpnI and BamHI and subcloned upstream of the luciferase gene in the promoter-less and enhancer-less pGL3-basic vector (Promega, Madison, WI) at the KpnI and BglII sites. The P2 construct was generated from the P1 clone by excision of the region upstream of the P2 site (position −1136). The P4 construct was generated from the P3 clone by excision of the region upstream of the NcoI site (position −835). The P9, P10, and P11 constructs were generated from the P8 clone by excision of the region upstream of the NcoI site (position −835). The P9, P10, and P11 constructs were generated from the P8 site by excision of the region upstream of the NcoI site (position −835). The P9, P10, and P11 constructs were generated from the P8 site by excision of the region upstream of the NcoI site (position −835). The P9, P10, and P11 constructs were generated from the P8 site by excision of the region upstream of the NcoI site (position −835). The P9, P10, and P11 constructs were generated from the P8 site by excision of the region upstream of the NcoI site (position −835).

The potential STAT binding site TTCGGAGAA at the 5′ end of the P2 construct was mutated to TGCCGACAA using the transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA), and the mutated clone was verified by sequencing.

The β-galactosidase (β-gal) reporter plasmid was constructed as follows: complementary oligonucleotides consisting of three copies of a potential STAT binding site-containing segment of the human perforin 5′-flanking region (5′-TGGGGCCAGATTCCGAGAAGACAGCAT-3′; position −1009 to −1073) were synthesized with HindIII and SalI restriction sites at the 5′ and 3′ ends, respectively, and annealed to form dsDNA. The fragment was subcloned upstream of a thymidine kinase (TK) promoter linked to the β-gal gene. The construct was verified by sequencing.

**Transient transfection**

Transient transfections were performed by the DEAE-dextran method as previously described (24). Briefly, for each point, 4 × 104 cells were incubated with 5 µg of indicated reporter gene constructs in RPMI 1640 with 50 mM Tris-HCl (pH 7.5) containing 350 µg/ml DEAE-dextran for 1 h at 37°C with periodic agitation. Cells were washed twice with RPMI 1640, 50 mM Tris-HCl, resuspended in complete medium, and incubated for 48 h.

**Luciferase and β-gal assays**

Cells were washed with PBS, pelleted, and lysed by vortex mixing for 15 s in 100–200 µl of reporter lysis buffer (Promega). A clear lysate was obtained by centrifugation at 12,000 × g. Luciferase activity was measured from 20 µl of supernatant using the luciferase assay system (Promega) according to the manufacturer’s directions. For β-gal assays, 80 µl of cell lysate were preincubated at 50°C for 1 h and β-gal activity was measured by a standard colorimetric assay using the chlorophenol red β-galacto-pyanoside substrate. Luciferase units were corrected for protein content and normalized for β-gal activity.

**Preparation of nuclear extracts**

Cells were washed once with ice-cold PBS and once with buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 1 mM Na3VO4, 25 mM NaF, 10 mM Na-pyrophosphate, and 25 mM p-nitrophenylphosphate) and lysed in buffer A containing 0.5% Nonidet P-40 (Sigma, St. Louis, MO). Lysate was placed on ice for 10 min and centrifuged at 4000 × g at 4°C for 4 min to remove cytoplasmic proteins. Nuclear proteins were extracted from the pellet in high salt buffer (410 mM KCl, 25% glycerol, and 0.2 mM EDTA in buffer A). Insoluble material was removed by centrifugation at 15,000 × g for 10 min. Protein concentration was measured with a Bio-Rad protein assay (Hercules, CA) and samples were stored at −70°C until use.

**Electrophoretic mobility shift assays (EMSA)**

Double-stranded oligonucleotides containing potential STAT and purine binding transcription factor-PUF-like motifs from the 5′-flanking region of human perforin gene (CTGCGATTTCAGAAGGTTGGGCACTGCGGAGAGATGGCCAGAAGCAGAC) were synthesized with 32P-labeled DNA probe. Cold competition experiments were performed by including unlabeled probes. Results were visualized by autoradiography after 1–4 days exposure at −70°C.

**Results**

**Mapping of human perforin promoter transcriptional activity and localization of NK-specific elements**

Previous reports have indicated that the human perforin promoter region contains many putative cis-acting regulatory elements (9, 10). To study the role of these elements in perforin gene regulation, we have generated luciferase reporter constructs containing 11 different perforin promoter fragments, cloned into the promoter-less and enhancer-less luciferase reporter plasmid pGL3. The size and location of the fragments used are shown in Fig. 1. These constructs were transfected into YT cells, an NK-like cell line that has constitutive transcription of the human perforin gene (26) and two human T cell lines, Jurkat and Kit225-k6, that do not express perforin. Fig. 1 shows the relative luciferase activity of the promoter constructs in transiently transfected cells. The P11 construct (−55 bp) had a 3-fold increase in activity when compared with the empty vector in YT cells and 7- to 8-fold in Jurkat and Kit225-k6.
cells, indicating the presence of a core promoter element in this fragment. Constructs with increasing lengths of 5’ sequence produced no significant peak of promoter activity in Jurkat or Kit225-k6 cells. However, in YT cells, constructs P1 and P3 (−1366 and −1076 bp) showed a 34- and 25-fold increase, respectively. The highest promoter activity was observed with construct P2, containing 1136 bp upstream of the transcription start site. The activity of this construct was about 65 times higher than the empty vector control (Fig. 1). These results suggest that cell-specific transcription factor(s) regulate perforin promoter activity and that an enhancer sequence is located in the region from −1136 to −1076 of the perforin promoter.

Identification of a STAT binding site in the perforin promoter

A computer-assisted search of the −1076 to −1136 region of the perforin promoter revealed the presence of two known transcription factor binding sites, STAT and PuF. To investigate whether YT cells contain specific trans-acting factors that are able to bind to these sites, EMSA were performed with a probe containing nucleotides −1141 to −1076 of the perforin promoter. Two DNA-protein complexes were detected in YT cells (identified as C1 and C2 in Fig. 2, lane 1). Specific oligonucleotides were used for competition assays to identify the elements involved. As expected, a full-length unlabeled probe completely eliminated the C1 and C2 bands, indicating that these complexes were specific (Fig. 2, lane 2). Interestingly, an unlabeled oligonucleotide (TGGGGCCAGATTCCGAGAGACACGAT) containing the putative STAT element present in the labeled probe was able to block the formation of complex C1 but not C2, indicating that the STAT-containing region is only involved in the formation of complex C1 (Fig. 2, lane 3). On the other hand, the addition of an unlabelled oligonucleotide (CTGCAGTTTTCTAGAAGAGGGTGCGGAG) containing the PuF-like element did not prevent the formation of either complex, indicating that PuF binding proteins are not involved in the formation of these complexes (Fig. 2, lane 4). EMSA analysis of Jurkat cells with the probes described above did not result in the detection of any complexes, consistent with the lack of promoter activity in these cells (data not shown).

Supershift analyses were performed to identify the DNA binding proteins involved in the formation of complex C1 in YT cells. As shown in Fig. 2 (lane 5), addition of anti-STAT3 antiserum supershifted complex C1, revealing the presence of STAT3 in that complex and further demonstrating the role of the STAT binding element in the formation of the C1 complex. NRS had no effect on the formation of either complex. Collectively, these data provide evidence that STAT proteins can bind to the perforin promoter, forming complex C1, whereas unknown proteins are involved in the formation of complex C2.

**FIGURE 1.** Activity of perforin promoter constructs in YT, Kit225-k6, and Jurkat cell lines. Luciferase constructs, shown in the left panel, were transiently cotransfected with a CMV-β-gal construct in the indicated cell lines by DEAE-dextran. Two days after transfection, cells were harvested, lysed, and lysates were tested for luciferase and β-gal activity. The luciferase units presented were adjusted for β-gal activity.

**FIGURE 2.** Binding of activated STAT3 to the STAT element of the perforin promoter. A total of 5 μg of nuclear extracts from YT cells were incubated with a radiolabeled perforin probe corresponding to nucleotides −1141 to −1076 before running on an 5% polyacrylamide gel. Competition was done with the same unlabeled probe (lane 2), the STAT binding site-containing probe (lane 3) or the PuF-like-containing probe (lane 4). Supershift analysis was done by adding anti-STAT3 antiserum (lane 5) or NRS (lane 6).
responding to nucleotides activated NK cells were incubated with a radiolabeled perforin probe corresponding to nucleotides \(-1141\) to \(-1076\) before running on a 5% polyacrylamide gel. Competition was done with the same unlabeled probe (lane 2), a STAT binding site containing probe (lane 3), or the PuF-like element-containing probe (lane 4). Supershift analysis was done by adding the indicated anti-STAT antisera (lanes 5–7). A total of 5 µg of nuclear extract from IL-6-activated NK cells were incubated with the probe described in A. Antiserum against STAT1α was used to supershift the observed complex.

Because YT is an NK-like cell line, it may differ from freshly isolated NK cells. Therefore, the ability of the perforin STAT element to bind STAT proteins from IL-2-activated human NK cells was studied. As shown in Fig. 3A (lanes 1–4), specific complexes with the perforin STAT, but not with the PuF-like element, were observed with NK cell nuclear extracts. Contrary to the results obtained with YT cells, inhibition of complex formation with anti-STAT Abs demonstrated that activated STAT5a/b and not STAT3 were binding to the STAT element (Fig. 3, lanes 5–7). To investigate whether STAT1α is capable of binding to the perforin STAT element, we stimulated human NK cells with IL-6, which has been shown to activate STAT1α. As shown in Fig. 3B, IL-6 stimulation results in the detection of a complex that can be blocked by the addition of anti-STAT1α antiserum. This indicates that multiple STAT proteins are capable of binding the perforin STAT element and that different STAT proteins may be used by different perforin-positive cells to regulate perforin gene transcription.

**Suppression of perforin promoter activity by mutation of the STAT binding site**

Having demonstrated that STAT proteins bind a specific sequence of the perforin promoter, the functional relevance of that observation was then evaluated. The STAT binding site contained in the P2 construct was altered by site-directed mutagenesis so that it could no longer be recognized by STAT proteins. We have previously shown that this mutation completely abrogates STAT binding activity (25). YT cells were transfected with wild-type or mutated P2 constructs and luciferase activity was measured. As shown in Fig. 4, destroying the STAT binding site resulted in a dramatic reduction of promoter activity to the level of the core promoter (P11). This result clearly indicates the requirement for STAT proteins in perforin promoter activity and suggests the presence of a suppressor element in the region between \(-1076\) and \(-1136\) bp.

**Enhancer activity of the perforin promoter STAT element**

To test the transcription-enhancing activity of the perforin STAT element, a TK-β-gal reporter plasmid containing three repeats of the STAT binding element was generated. YT cells were transfected with the STAT containing vector or control vector with the TK promoter and β-gal gene alone and stimulated with 1000 U/ml of IL-2 for 24 h before measuring β-gal activity (Fig. 5). The transcriptional activity of the STAT containing vector was notably higher than the control vector; this finding is consistent with our previous result that constitutively activated STAT3 from YT cells is able to bind the perforin STAT element (Fig. 2). The IL-2 stimulation only increased the activity of the STAT containing vector about 2-fold. Regardless, these data demonstrate that the perforin STAT element can function as an enhancer in a heterologous promoter.

**Discussion**

Previous studies with the mouse perforin gene have indicated an important role for Ets-related transcription factors in the control of perforin transcription (11–14). There are two Ets-related sites in the mouse perforin promoter, one in the core promoter region adjacent to the guanine/cytosine box at position \(-43\) and a second in

**FIGURE 3.** Binding of activated STAT proteins to the STAT element of the perforin promoter. A, A total of 5 µg of nuclear extracts from IL-2-activated NK cells were incubated with a radiolabeled perforin probe corresponding to nucleotides \(-1141\) to \(-1076\) before running on a 5% polyacrylamide gel. Competition was done with the same unlabeled probe (lane 2), a STAT binding site containing probe (lane 3), or the PuF-like element-containing probe (lane 4). Supershift analysis was done by adding the indicated anti-STAT antisera (lanes 5–7). B, A total of 5 µg of nuclear extract from IL-6-activated NK cells were incubated with the probe described in A. Antiserum against STAT1α was used to supershift the observed complex.

**FIGURE 4.** Suppression of perforin promoter activity by mutation of the STAT binding site. The potential STAT binding site TTCCGAGAA at the 5' end of the P2 construct was mutated to TGCGGACAA using site-directed mutagenesis. YT cells were transfected and tested for luciferase and β-gal activity as described in Materials and Methods.

**FIGURE 5.** Enhancer activity of the perforin promoter STAT binding site on a heterologous promoter. Three copies of the sequence TGGGCCTCAGATTTCCGAGACGACCAT, containing the STAT binding site, were cloned upstream of the TK promoter and β-gal gene. The control vector consisted of the TK promoter and β-gal gene alone. Constructs were transiently transfected in YT cells by DEAE-dextran and cultured for 2 days in the presence or absence of IL-2. Cells were lysed and β-gal activity was measured.
an upstream site at position −497. The upstream Ets-related site was shown to enhance expression of the mouse perforin promoter in CTLL cells that have constitutive perforin expression. There are two similarly spaced Ets-like sites in the human promoter (−34 and −610). No increase in human promoter activity was observed with constructs containing the upstream Ets-related site (constructs P4–P6 vs P7, Fig. 1), suggesting that the Ets-related sites may only be important for basal promoter activity or cell type-specific restriction of promoter activity in the human. However, a strong enhancer activity was detected in construct P2 that increased promoter activity about 65-fold in YT cells, but not in Jurkat and Kit225-K6 cells, suggesting that cell-specific transcription factors are binding to this region of the promoter. This region of the human promoter contains a potential STAT binding site (TTCCGAGAA) at −1081. DNA protein binding assays confirmed that the putative STAT element from the human promoter is capable of binding STAT proteins. In YT cells, the STAT3 protein was found to bind to this element (Fig. 2), whereas in IL-2-activated human peripheral blood NK cells, STAT5a/b proteins were detected (Fig. 3A) and STAT1α was detected after IL-6 stimulation (Fig. 3B). Interestingly, the mouse perforin promoter contains a similar site (TTCTGAGAA) at position −802. Mouse perforin promoter constructs containing this potential STAT binding site showed increased transcriptional activity only in perforin-positive cells (11, 13), suggesting that this element may also be active in the mouse perforin promoter.

The mouse and human STAT elements are flanked on the 5′ side by purine-rich sequences. Interestingly, the human gene contains a single PuF-like element in this region. PuF is encoded by the nucleoside diphosphate kinase-B gene that has been postulated to suppress tumor metastasis. In addition, PuF has been shown to act as a transcriptional activator of the myc promoter in which it binds to a tandem repeat of GGTTGGG (27). An oligonucleotide corresponding to this region did not produce any DNA-protein complexes in either YT or peripheral blood NK cells, suggesting that no activated PuF or PuF-like proteins are present in these cells or that the single PuF motif is not sufficient to confer transcription factor binding.

Mutation of the STAT binding element demonstrated the essential role of the element in driving perforin promoter activity in YT cells. It is of interest to note that removal of STAT binding revealed a strong suppressor activity present in the −1076 to −1136 region of construct P2, because the mutant had transcriptional activity that was greatly decreased when compared with construct P3. A lower m.w. complex was observed with a probe spanning this region (C2; Fig. 2), that could not be inhibited by competition with oligonucleotides containing either the STAT site or PuF-like element. Additional experiments will be necessary to determine whether this complex is associated with the inhibitory activity detected in this region.

The identification of a functional STAT binding element in the human perforin promoter and a potential STAT binding element in the mouse promoter provides a possible molecular explanation for the ability of cytokines to modulate perforin expression. Two cytokines that have been demonstrated to increase perforin expression, IL-2 and IL-7 (6, 19), have also been demonstrated to activate and induce heterodimerization of the STAT5a/b isoforms in human peripheral blood T cells (28, 29). Similarly, IL-2 stimulation of NK cells predominantly activates STAT5 isoforms (25). Our results with primary human NK cells have demonstrated the ability of STAT5a/b from IL-2-activated NK cells to bind to the perforin STAT element. Although the activation of human perforin gene transcription by STAT3 in YT cells aided in the identification of a STAT-responsive element in the perforin promoter, STAT5a/b probably represents the physiologically important activator in response to IL-2 stimulation. Indeed, although IL-2 can induce STAT3 activation in human peripheral blood NK cells (25), no STAT3 could be detected using the perforin promoter STAT binding site (Fig. 3A) and treatment of YT cells with IL-2 also leads to the activation of STAT5a/b (Ref. 30 and data not shown). IL-6 treatment is known to activate STAT1α and STAT3 (31), and it has also been shown to induce perforin transcription (18). In the present study, STAT1α activated by IL-6 treatment was shown to bind to the perforin STAT element. We have also observed the ability of the perforin STAT element to bind to STAT1α, STAT3, and STAT5 induced in fresh human T cells (data not shown). Therefore, it appears that the perforin STAT element is responsive to multiple STAT proteins. This correlates with the ability of various ILs to enhance perforin expression although they induce the activation of different STAT family members.

This study provides strong evidence that transcriptional activation of the perforin gene in human NK cells involves STAT protein activation. Because perforin has been shown to be crucial for NK lytic activity (4), these results provide a molecular explanation for the cytokine regulation of NK lytic activity.

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