Trans-Repressor Activity of Nuclear Glycosaminoglycans on Fos and Jun/AP-1 Oncoprotein-mediated Transcription

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Abstract. Heparin blocks the phorbol ester-induced progression of nontransformed cells through the G0/G1 phase (Wright, T.C., L. A. Pukac, J. J. Castellot, M. J. Karnovsky, R. A. Levine, H.-Y. Kim-Park, and J. Campisi. 1989. Proc. Natl. Acad. Sci. USA. 86: 3199–3203) or G1 to S phase (Reilly, C. F., M. S. Kindy, K. E. Brown, R. D. Rosenberg, and G. E. Sonenshein. 1989. J. Biol. Chem. 264:6990–6995) of the cell cycle. Cell cycle arrest was associated with decreased levels of stage-specific mRNAs suggesting transcriptional regulation of cell growth. In the present report, we show that heparin selectively repressed TPA inducible AP-1-mediated gene expression. Heparin-induced trans-repression was observed in primary vascular smooth muscle cells, as well as in the transformed HeLa cell line and in nondifferentiated F9 teratocarcinoma cells. Inhibition of AP-1-mediated trans-activation occurred with heparin and pentosan polysulfate but not with chondroitin sulfate A or C. Heparin-binding peptides or heparitinase I addition to nuclear lysates of heparin-treated cells allow enhanced recovery of endogenous AP-1-specific DNA binding activity. We propose a model in which nuclear glycosaminoglycans play a trans-regulatory role in altering the patterns of inducible gene expression.

Several laboratories have recently demonstrated that cell matrix glycosaminoglycans (GAGs)1 and heparin potently suppress entry of cells into S-phase in response to mitogens by arresting them early in the cell cycle (Reilly et al., 1986; Benitz et al., 1986; Wright et al., 1989; Reilly et al., 1989; Jackson et al., 1991). Inhibition of cell growth was not due to the sequestration of serum nutrients, mitogens, and growth factors (Reilly et al., 1986; Benitz et al., 1986) but rather involved the high affinity binding of heparin (Kd ~10−9) to specific cell surface receptors (Castellot et al., 1985; Resink et al., 1989) and internalization of the GAGs characterized by a t1/2 of 15–20 min in primary cell cultures. Inhibition of cell migration and proliferation in vivo after heparin perfusion has also been reported (Clowes and Karnovsky, 1977; Guyton et al., 1980). Subsequent studies by Fedarko and Conrad (1986) and Ishihara et al. (1986) demonstrated that the cellular uptake of specific GAG subfractions by hepatocytes resulted in their nuclear sequestration and in cell cycle arrest. Heparin has been reported to selectively inhibit the mitogenic stimulation of cells by TPA (12-O-tetradecanoyl phorbol β-acetate [phorbol 12-myristate β acetate]) and serum but not by EGF (Wright et al., 1989; Reilly et al., 1989; Pukac et al., 1990). The antiproliferative response was not associated with a decrease in the level of inducible protein kinase C (PKC) activity (Wright et al., 1989; Pukac et al., 1990) suggesting that the intracellular GAG blocked events distal to PKC. Wright et al. (1989) and Pukac et al. (1990) found that the effects of heparin on cell proliferation, induced by TPA or serum, correlates with attenuated levels of c-fos and c-myc mRNA. The expression of these immediate early, or competence class genes, including c-jun, is known to be stimulated by various mitogens and growth factors (Cochran et al., 1983; Kelly et al., 1983; Greenberg and Ziff, 1984; Kruijer et al., 1984; Müller et al., 1984; Greenberg et al., 1985; Verma, 1986; Gilman et al., 1986; Almendral et al., 1988; Chiu et al., 1988; Lamph et al., 1988; Curran and Franza, 1988; Verma and Sassone-Corsi, 1989; Vogt and Bos, 1989), and to precede the onset of protein synthesis required for entry into S phase or the development of a more differentiated cellular phenotype. However, under certain conditions their expression or overexpression results in neoplastic transformation (Miller et al., 1984; Schütte et al., 1989).

The proto-oncogene products, Fos and Jun/AP-1, associate to form a heterodimeric trans-activator complex which binds with high affinity and specificity to AP-1-like promoter elements (Angel et al., 1987; Bohmann et al., 1987; Lamph et al., 1988; Rauscher et al., 1988; Sassone-Corsi et al., 1988; Chiu et al., 1988). The AP-1 site, also described as the TPA-responsive element (TRE), is characterized by the patin-

1. Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; FB and JB, Fos and Jun/AP-1 DNA-binding domain peptides; GAG, glycosaminoglycans; HBP-1, heparin binding peptide; PPS, pentosan polysulfate; SMC, smooth muscle cells; TK, thymidine kinase; TRE, TPA responsive element.
dromic consensus base sequence TGAG/CTCA. Expression of genes containing the TRE is regulated by the interaction of the Fos-Jun/AP-1 trans-activating complex with this DNA element. TREs have been identified in the 5' regulatory domains of both the c-fos and c-jun genes and both have been implicated in their autoregulatory function (Gilman et al., 1986; Sassone-Corsi et al., 1988; Kouzarides and Ziff, 1988; Nakabeppu et al., 1988; Angel et al., 1988b; Lucibello et al., 1989; Schuermann et al., 1989). The promoter region of c-fos is responsive to a variety of mitogens and growth factors (Treisman, 1985; reviewed in Verma, 1986). Cell stimulation is followed by a rapid elevation in c-fos and c-jun gene expression which, at least in the case of c-jun, is mediated through interaction of the activated Fos-Jun/AP-1 complex with a TRE in the 5' region of the genome. When translated, these oncogenes bind to and activate other genes containing the TRE in a coupled amplification process required to induce cellular proliferative or developmental events (Müller et al., 1984; Treisman, 1985; Lau and Nathans, 1987).

Upon stimulation, a functional dimer of Fos with Jun/AP-1 occurs requiring the leucine-zipper domain of each protein (Landschultz et al., 1989; Schuermann et al., 1989; Turner and Tjian, 1989; Busch and Sassone-Corsi, 1990a). Binding of the Fos-Jun dimer to the TRE is directed by the adjacent basic DNA-binding domain (Gentz et al., 1989; Nakabeppu and Nathans, 1989; Busch and Sassone-Corsi, 1990b). We noted that these DNA-binding domains contained sequences similar to those previously reported to exhibit high affinity heparin binding (Cardin and Weintraub, 1989; Jackson et al., 1991). The fact that these transcription factors are heparin-binding proteins and that nuclear heparins have been previously identified, prompted us to examine the role and specificity of heparin in the attenuation of nuclear responses to mitogen. In these studies we have measured the level of inducible Fos-Jun/AP-1 nuclear activity and demonstrate a direct nuclear inhibitory role for glycosaminoglycans on TRE-regulated gene expression.

Materials and Methods

Cell Culture

HeLa cells and mouse F9 testicular teratocarcinoma cells were routinely cultured in DME supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and 10% FCS in an atmosphere of 5% CO2 for HeLa cells and 7% CO2 for F9 cells. Cells were grown in flasks (model T75; Falcon Labware, Oxnard, CA) and passaged 1:3 every 2-4 d with 0.1% trypsin treatment to release cells from the culture plates. Cells for transfection experiments were seeded similarly in 100-mm plates (Falcon Labware); cells from one T75 flask were divided into 20 100-mm plates. Cells were split in the above medium and allowed to attach overnight after which the medium was changed to that supplemented with low serum (0.1% FCS) before each experiment.

Vascular smooth muscle cells (SMC) were isolated enzymatically from two to three thoracic aortas of male Sprague-Dawley rats (150 g) according to a modified method of Ives et al. (1978). Briefly, aseptically obtained aortic strips were predigested at 37°C in serum-free DME containing collagenase (1 mg/ml) and elastase (0.5 mg/ml) for 30 min. The luminal side of the aorta was gently scraped to remove the endothelium and the medial layer was separated from the adventitia. The medial layer was rinsed and digested for an additional 2 h with a fresh enzyme mixture at 37°C. Cells were grown in DME supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 25 mM Hepes at 37°C with 5% CO2 in humidified air. Subculture was performed every 7 d after the culture had reached confluence. Cultures between the fifth and ninth passages were used in the present study.

Cell Transfections and Chloramphenicol Acyl Transferase (CAT)

Transient transfections were performed on HeLa, F9, and SMC in cultures seeded at round 30% confluence in 100-mm Petri dishes in MEM containing 2 mM glutamine, 10% FCS and 100 U/ml penicillin, and 100 µg/ml streptomycin as indicated and allowed to attach overnight. Cells were re-fed with the same medium containing 0.1% FCS for the indicated period of time with or without 200 µg/ml heparin (porcine gut mucosal, No. 820-5077 MG; Gibco Laboratories, Grand Island, NY) as described in the figure legends. Cells were washed and re-fed with the same medium in the absence of heparin and transfected with the indicated plasmid(s) for 12 h using the calcium phosphate coprecipitation method as described elsewhere (Gorman, 1985). Cultures were then washed and re-fed with the same medium before TPA treatment as indicated. After phorbol ester treatment, cells were harvested by mechanical scraping and CAT assays were performed with [3H]chloramphenicol as substrate at 37°C for 2 h as described (Gorman, 1985). 50 µg of cell protein per assay was used as determined with the protein assay kit (BioRad Laboratories, Cambridge, MA) with gamma globulins as protein standards. CAT assays were terminated by extracting each reaction mixture with 500 µl of ethyl acetate. Extracts were evaporated to dryness, resolubilized in 30 µl of ethyl acetate and resolved on silica gel thin layer chromatography plates with a developing solution of chloroform/methanol (95:5, vol/vol). Quantitation was achieved by cutting out the corresponding mono- and diacylated chloramphenicol spots and measuring the radioactivity. CAT-specific activities were determined based on a [14C]chloramphenicol specific activity of 60 mCi/mmol.

DNA Binding Assay

HeLa cell nuclear lysates were prepared exactly as described by Dignam et al. (1983) from a pool of four similarly treated cultures of HeLa cells (at ~50% confluence in 100-mm petri dishes) grown in 0.1% FCS supplemented medium for 48 h. To assay lysates for TRE binding activity, first they were dialyzed at 4°C against Dignam's buffer D (Dignam et al., 1983). The assay consisted of 10 µg of protein per lysate, 1 ng (0.2 pmol) of 32P-labeled synthetic metallothionein IIA TRE which contained the consensus AP-1 binding site (GTGAGTCAG; Lamph et al., 1988), and 2 µg of poly[dI-dC] unless stated otherwise, to reduce nonspecific binding activity. Assays were performed in 20 µl of TM buffer (50 mM Tris-HCl, pH 7.9, 12.6 mM MgCl2, 1 mM EDTA, 1 mM DTT 20% glycerol). Each tube was gently vortexed, incubated at room temperature for 20 min, and resolved on 4% polyacrylamide native gels prepared and electrophoresed in 0.25% TBE (1X TBE: 50 mM Tris-Borate, pH 8.3, 1 mM EDTA) at 150 V for 1 h as described (Busch and Sassone-Corsi, 1990b). Gels were dried and autoradiographed by exposing to film for 4-12 h.

Heparin Binding Assays

Solid-phase assays of [125I]-labeled heparin binding to the Fos and Jun/AP-1 DNA-binding domain peptides were performed in Immulon 4 Removewell microwell plates (Dynatech Laboratories, Inc., Alexandria, VA). [125I]-labeled heparin was prepared as described by Cardin et al. (1987). Typically, 1-10 µg of each peptide in 50 µl were absorbed to the wells for 3 h at room temperature. Residual peptide was removed by aspiration, and the wells were washed five times with 250 µl PBS. In saturation binding experiments, excess amounts of [125I]-labeled heparin in 50 µl PBS were added to each well and allowed to incubate 8 h at room temperature. In each assay, the radiiodinated ligand was removed by aspiration and the wells were extensively washed with successive 250-µl aliquots of PBS until residual counts in the wash were near background. The amount of bound ligand was determined by gamma counting. In the absence of peptide, no concentration-dependent [125I]-heparin was bound (<3,000 cpm) to the well surfaces (data not shown).

Results

Negative Trans-regulation of Jun/AP-1-mediated Transcription by Heparin

Transient transfection studies have been used to study inducible promoter function for a wide variety of transcription factors. As a starting point to investigate the role of nuclear GAGs on phorbol ester-inducible cellular functions, we ex-
Figure 1. (A) Transient transfection assays were performed using a TRE/TK-CAT reporter gene construct in which the metallothionein IIA TRE (TGAGTCA) precedes the herpes thymidine kinase heterologous promoter (bases -109+-57) and the bacterial CAT DNA sequence contained in the plasmid (pTEN) as described by Visvader et al. (1988). (B) Cotransfection experiments were performed with the TRE/TK-CAT reporter gene construct and the expression vector for Jun/AP-1, pSV-c-jun, which contains the SV-40 early gene promoter and polyadenylation sequence flanking the c-jun DNA sequence as previously described (Lamph et al., 1988). (C) Heparin pretreatment of cells reduced trans-activation through the TRE. HeLa cells were seeded at ~30% confluence and re-fed after attachment in medium containing 0.2% FCS with (lanes 1, 5, and 6) or without (lanes 2-4) heparin (200 μg/ml) for 24 h before transfection with the reporter plasmid TRE/TK-CAT alone (lanes 1-3, and 5) or cotransfected with pSV-c-jun (lanes 4 and 6). Control cells in the absence (lane 7) or presence (lane 8) of heparin were transfected with RSV-CAT (Angel et al., 1987b). Equimolar amounts of reporter plasmid and pSV-c-jun expression plasmid or the control plasmid, pTEN (lanes 1-3, 5, 7, and 8), were transfected at a total of 20 μg of DNA per 10-cm culture dish. After a 12-h transfection period, cells were washed twice with medium and re-fed under the same conditions with (lanes 1, 3, 5, and 6) or without (lanes 7) hepaticin at 200 μg/ml. TPA (75 ng/ml) was then added for 1 h, and 5 h later cells were harvested (lanes 2 and 5). CAT assays were performed for 2 h at 37°C using 50 μg of cell protein per assay as described (Gorman, 1985). (D) Heparin suppression of pSV-c-jun-induced TRE/TK-CAT transactivation is a time-dependent process. HeLa cells were pretreated with heparin for 18, 24, 30, and 36 h before cotransfection with the TRE/TK-CAT reporter plasmid and pSV-c-jun expression plasmid. Control lanes 1, 3, 5, and 7 were not exposed to heparin. After heparin treatment for 18 h (lane 2), 24 h (lane 4), 30 h (lane 6), or 36 h (lane 8) cells were washed twice with medium without heparin, re-fed in the absence of heparin and transfected with equal molar concentrations of TRE/TK-CAT and pSV-c-jun as described in C. Parallel control plates without earlier exposure to heparin were transfected at 18 h (lane 1), 24 h (lane 3), 30 h (lane 5), and 36 h (lane 7). After a 12-h transfection period, cells were washed twice with medium and re-fed in the absence of heparin. After a total of 24-h post-heparin incubation, cells were harvested and frozen in liquid nitrogen and kept stored at -70°C until the final time point was harvested. CAT assays were run simultaneously as described in Methods and Materials.
24 h either in the presence (Fig. 1 C, lanes 1, 5, 6, and 8) or absence (Fig. 1 C, lanes 2–4, and 7) of heparin as described in Materials and Methods. Cells were transfected with either TRE/TK-CAT (Fig. 1 C, lanes 1–3, and 5), RSV-CAT (Fig. 1 C, lanes 7 and 8) reporter genes alone, or with both TRE/TK-CAT and pSV-c-jun (Fig. 1 C, lanes 4 and 6). TPA was added as indicated (Fig. 1 C, lanes 2 and 5) for 1 and 5 h later cells were harvested and assayed for CAT expression. The results show that heparin did not activate CAT expression in cells transfected with TRE/TK-CAT alone (Fig. 1 C, lane 7) indicating that GAGs do not induce an immediate–early gene response in these cells. In the absence of heparin, activation of TRE/TK-CAT expression occurred in cells treated with TPA (Fig. 1 C, compare lane 2 with 3) and in those cotransfected with the expression vector pSV-c-jun which produces high levels of Jun (Fig. 1 C, compare lane 3 with 4). In contrast, cells pretreated for 24 h with heparin and then stimulated with TPA (Fig. 1 C, compare lane 2 with 5) or cotransfection with pSV-c-jun (Fig. 1 C, compare lane 4 with 6) showed no induction of TRE/TK-CAT expression. Similar attenuation was not observed in cells transfected with the RSV-CAT reporter gene (Fig. 1 C, lanes 7 and 8). These results demonstrate that heparin shows some selectivity towards Jun/AP-1-mediated trans-activation through the TRE. Since heparin did not inhibit CAT expression in cells transfected with the RSV-CAT reporter gene, this rules out the possibility that the transduction efficiencies of TRE/TK-CAT were altered as a result of heparin pre-treatment.

**Time-dependent Trans-Repression by Heparin**

In the next experiment, HeLa cells were pretreated with GAGs for increasing periods of time before transfection to determine the rate of heparin uptake and transcriptional repression of Jun/AP-1–induced TRE/TK-CAT expression. As is demonstrated in Fig. 1 D, up to 18 h of heparin treatment (Fig. 1 D, lane 2) did not significantly effect pSV-c-jun–induced CAT expression relative to untreated control cells (Fig. 1 D, lane 1). However, attenuated CAT expression was observed by 24 and 30 h of heparin exposure (Fig. 1 D, compare lane 3 with 4, and lane 5 with 6, respectively). At 36 h (lane 7 and 8), the expression in both control and heparin-treated cells had dropped significantly, presumably due to poor transfection efficiencies, as cells continued to divide and approached confluence. Thus, the inhibition of TRE/TK-CAT expression in HeLa cells by heparin required their continuous exposure to GAGs for ~18–24 h. Moreover this trans–regulatory response did not result in growth arrest in the HeLa transformed cell line. The time required to observe the onset of trans–repression is consistent with that observed previously for the heparin-induced changes in the collagen phenotype expressed in unsynchronized vascular SMCs (Majack and Bornstein, 1985) and the level of hepatic triglyceride lipase expression in HepG2 cells (Busch et al., 1989).

**Heparin Mediates Antiproliferative and Trans-Repressional Responses in Rat Vascular SMCs**

Heparin treatment of HeLa cells is not associated with an antiproliferative effect (Fig. 1 D and Busch, S. J., unpublished results) in contrast to that reported previously for primary SMC cultures (Pukac et al., 1990). As HeLa cells are a transformed cell type, we examined whether GAG–mediated trans-repression of AP-1 activity would accompany an antiproliferative response in primary rat vascular SMCs. In this experiment the TRE/TK-CAT reporter gene was transiently transfected into SMC with or without a 24-h heparin pretreatment. After transfection some cultures received their only heparin treatment (Fig. 2, lane 6) or a secondary treatment (Fig. 2, lane 5), 15 min before TPA induction. Whereas heparin pretreatment did not alter basal levels of TRE/TK-CAT expression (Fig. 2, lane 2), it completely abolished the TPA inducibility (Fig. 2, compare lane 3 with 4). In contrast to HeLa cells, which requires an 18–24-h exposure to heparin for trans-repression, a 15-min exposure before phorbol ester treatment was sufficient to abolish TRE/TK-CAT induction by TPA in SMC (Fig. 2, lanes 5 and 6). The antiproliferative effects of 200 μg/ml heparin was determined in parallel experiments by measuring [3H]thymidine incorporation. Consistent with previous reports, SMC required 50 min–2 h for half-maximal inhibition of this proliferative response with an associated EC50 of 50 μg/ml.

**Heparin Inhibits the Phorbol Ester Stimulation of AP-1 in Nondifferentiated F9 Cells**

We next examined the ability of heparin to effect TRE–inducible promoter function in murine F9 testicular teratocarcinoma stem cells, a nondifferentiated stem cell line. This cell line has low basal AP-1 activities (Chiu et al., 1988) that allow transfection to precede heparin treatment without generating excessive background levels of CAT expression. As shown in Fig. 3, there is little or no Fos–Jun/AP-1 intrinsically active present in uninduced F9 cells in either the absence or presence of heparin (Fig. 3, lanes 1 and 2, respectively). In contrast, cotransfection with pSV-c-jun plus TRE/TK-CAT resulted in strong induction of CAT expression in the presence of TPA (Fig. 3, lane 3) and was repressed by >90% in cells treated with heparin for 24 h before phorbol ester stimulation, (Fig. 3, lane 4). TPA treatment accom-
Fig. 3. c-jun/AP-1 transactivation of TRE/TK-CAT expression is blocked in nondifferentiated F9 cells by posttransfection heparin treatment. F9 embryonal tetracarcinoma stem cells were grown and transfected as described in Materials and Methods with TRE/TK-CAT and control plasmid pTEN (lanes 1 and 2), or cotransfected with an equimolar concentration of TRE/TK-CAT and pSV-c-jun (lanes 3 and 4). After a 12-h transfection period, cells were washed twice with medium and re-fed with the same plus (lanes 2 and 4) or minus (lanes 1 and 3) 200 μg/ml of heparin. After 24 h cells were exposed to TPA (75 ng/ml) for 1 h, and 5 h later cells were harvested and assayed for CAT activity.

panying pSV-c-jun cotransfection optimized CAT expression in this cell line (Busch, S. J., unpublished results). These data show that heparin strongly attenuates phorbol ester-inducible gene expression mediated through a TRE promoter in distinct cell types; in SMChaving a nontransformed and differentiated phenotype, in HeLa cells that exhibit a transformed and partially differentiated phenotype, and in F9 stem cells with a transformed and nondifferentiated phenotype.

Selective Trans-Repression by Heparin and Pentosan Polysulfate

We have demonstrated the promoter selectivity of GAG-mediated transcriptional repression in Fig. 1 by showing a lack of trans-repression of the RSV promoter CAT reporter gene construct in HeLa cells. To further examine the specificity of GAG-attenuated promoter function, we compared heparin with several other GAGs for their ability to produce an altered transcriptional response in HeLa cells. In Fig. 4, TRE/TK-CAT expression was determined in both uninduced and TPA-induced HeLa cells without earlier GAG treatment (NONE), or after a 24-h pretreatment with either heparin, chondroitin sulfate A, pentosan polysulfate, or chondroitin sulfate C. As shown, TPA induced a similar level of CAT expression in the control, chondroitin sulfate A-, and chondroitin sulfate C-treated cells. In contrast, heparin and pentosan polysulfate-treated cells had both a diminished background (open bars) and TPA-induced level of transcriptional activity (hatched bars). There was no significant difference in total cell numbers in cultures grown in the absence or presence of heparin or pentosan polysulfate (PPS), which again suggests that suppressed Fos-Jun/AP-1 transcriptional activity does not correlate with growth arrest in HeLa cells. These data further suggest that trans-repression of the Fos-Jun/AP-1 transcription factor complex is not a general property of sulfated polysaccharides but is selective for heparin and its homopolysaccharide analogue, PPS.

Heparin Competes for Promoter Binding by Interacting with the Basic DNA-binding Domain of Fos-Jun/AP-1

Glycosaminoglycans have been shown to enter the cell

Figure 4. Specificity of GAGs on TPA-inducible TRE/TK-CAT. HeLa cells seeded at 20% confluence were re-fed in normal medium containing 0.2% FCS and either no GAG (NONE), or 200 μg/ml of heparin (H), chondroitin sulfate-A (Cs-A; No. c8529, Sigma Chemical Co., St. Louis, MO), pentosan polysulfate (PPS; No. p8275, Sigma Chemical Co.), or chondroitin sulfate-C (Cs-C, No. c4384, Sigma Chemical Co.). After incubating 24 h in the presence of the indicated GAG, cells were washed twice and the medium was replaced with normal medium containing 0.1% FCS and no GAG. Cells were then transfected for 12 h with 10 μg of TRE/TK-CAT, washed, and re-fed with medium containing 0.1% FCS and stimulated with TPA (75 ng/ml, ●) or with the carrier solvent (7 μl ethanol, ○) for 6 h. Cells were then harvested and assayed for CAT activity as described in Materials and Methods. (A) A qualitative evaluation by radioautographs from a typical experiment. Uninduced levels of CAT expression are compared with TPA-induced levels after no pretreatment (lane 2), heparin pretreatment (lane 4), chondroitin sulfate A pretreatment (lane 6), pentosan polysulfate (lane 8), or chondroitin sulfate C (lane 10). (B) CAT-specific activity was quantitated and is shown as the mean of three experiments as described in Materials and Methods.

Jun/AP-1 transcription factor complex is not a general property of sulfated polysaccharides but is selective for heparin and its homopolysaccharide analogue, PPS.
Figure 5. (A) Heparin reversibly blocks Fos-Jun/AP-1 binding to the TRE in vitro. HeLa cell nuclear lysates were prepared from TPA-induced cells by the method of Dignam et al. (1983) and assayed for TRE binding activity of intrinsic Fos-Jun, in the presence and absence of heparin added in vitro. In lanes 6-10, a heparin-binding peptide (HBP1, amino acid sequence KSKTSKRKIR) was included to demonstrate reversibility of binding inhibition. Binding assays contained 10 ng (protein) aliquots of the induced lysate, 2 ng of poly[dI-dC], and 1 ng of 32P-TRE without heparin (lanes 1 and 6) or with the indicated amount of heparin without HBP1 (lanes 1-5) or with HBP1 (lanes 6-10). (B) Fos and Jun/AP-1 DNA-binding domain peptides bind heparin. In vitro heparin binding to synthetic polypeptides representing the Fos (residues 139-161) and Jun/AP-1 (residues 257-279) DNA-binding domains (synthesized as previously reported in Busch and Sassone-Corsi, 1990b), was measured with 125I-heparin prepared exactly as described by Cardin et al. (1987). Peptides were adsorbed to 96-well Immulon 4 Removawell™ plates at increasing concentrations (1 ng-10 µg) for 3 h at room temperature. Wells were then washed 5 times with 250 µl PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) to remove trace unbound peptide. An excess of 125I-heparin was then added to each coated well and incubated 8 h at room temperature as described in Materials and Methods. After removal of unbound 125I-heparin, and after five washes, bound 125I-heparin was quantitated by gamma counting. (C) Fos (FB) and Jun/AP-1 (JB) DNA-binding domain peptides compete for inhibitory heparin in vitro. DNA-binding assays were performed with TPA-induced HeLa cell nuclear lysates and a 32P-labeled TRE in the absence (lane 1) or presence (lanes 2-7) of 200 ng heparin without FB or JB peptide addition (lanes 2 and 5) or after adding FB (lanes 3 and 4) or JB (lanes 6 and 7). Nuclear lysates (10 µg) were added to 10 µl of 2× TM (see Materials and Methods) containing the indicated concentrations of heparin and peptide (poly[dI-dC] was excluded) and preincubated together for 10 min before addition of 32P-TRE. Mixtures were then incubated at room temperature for 20 min before gel resolution.
through high affinity receptor-mediated processes (Castellot et al., 1985b; Resink et al., 1989), and in the case of primary hepatocytes, specific GAG fractions have been shown to concentrate in the nucleus (Fedarko and Conrad, 1986; Ishihara et al., 1986, 1987). To determine whether the attenuated promoter function observed in the previous experiments was because of the presence of an inhibitory nuclear GAG, the relative level of TPA-inducible TRE-binding activity in HeLa cell nuclear lysates was examined by polyacrylamide gel DNA-binding assays. We first examined heparin's ability to compete for promoter binding activity upon direct addition of the GAG to the assays. In Fig. 5 A (lanes 2–5), addition of heparin to nuclear lysates from TPA-induced cells blocked the intrinsic Fos–Jun/AP-1 binding activity (arrow) to the TRE in a dose-dependent manner. The induced binding activity was shown to be because of specific interactions by Fos–Jun/AP-1 with the TRE based on the ability of unlabeled TRE to specifically compete for binding and anti-Fos and anti-Jun antibodies to block TRE complex formation (Busch and Sassone-Corsi, 1990b). The observed inhibition by heparin was completely reversed in the presence of HBP-1, a heparin-binding peptide (Fig. 5 A, lanes 6–10) (Martin et al., 1988). These findings show that the presence of heparin in the nuclear lysate completely inhibits the promoter binding activity of endogenous Fos/Jun and that a competing heparin-binding peptide protects against inhibition by GAG. In reviewing the activity with HBP-1, attention was then focused on the heparin-binding properties of the Fos/Jun DNA binding regions. Peptide regions of Fos (FB-1, residues 139–161) and the Jun (JB, residues 257–279) representing the DNA-binding domains were synthesized. These peptides were selected as the most likely domains to bind heparin based on a comparison of their amino acid sequences to those of known heparin-binding proteins (Cardin et al., 1987; Jackson et al., 1991). Furthermore, if heparin bound to these domains in the intact Fos and Jun proteins it would be expected to block DNA binding competitively. To test this hypothesis, first we examined the ability of 125I-labeled heparin to bind directly to FB-1 and JB (Fig. 5 B). As is shown, the Fos (FB-1) and Jun (JB) peptides bound heparin and exhibited similar heparin-binding capacities. We then tested these peptides in DNA-binding assays for their ability to reverse heparin competition for TRE binding (Fig. 5 C).

A TPA-induced nuclear lysate prepared from HeLa cells exhibited high levels of endogenous Fos–Jun/AP-1 binding activity (Fig. 5 C, lane 1, arrow) which was completely inhibited by the addition of heparin (10 ng/μl) (Fig. 5 C, lanes 2 and 5). FB-1 and JB DNA-binding domain peptides, like HBP-1, when titrated into each binding assay (Fig. 5 C, lanes 3 and 4, and 6 and 7, respectively) reversed the inhibition by heparin. These results demonstrate that inhibition of Fos–Jun/AP-1 binding to the TRE by heparin in vitro is reversible by the heparin-binding Fos and Jun/AP-1 DNA-binding domain peptides.

**Heparin Inhibits TPA Induction of Nuclear AP-1 Binding Activity**

To determine if heparin has an effect on the TPA-inducible levels of TRE binding activity in intact cells, HeLa cell nuclear lysates were prepared and the endogenous TRE binding levels in these lysates were determined from control (untreated and uninduced) cells (Fig. 6 A, lane 1), untreated and TPA-induced cells (Fig. 6 A, lane 2), and heparin-treated and TPA-induced cells (Fig. 6 A, lane 3). As demonstrated, the intrinsic levels of Fos–Jun/AP-1 binding activity was strongly activated in untreated TPA-induced cells (Fig. 6 A, lane 2). However, after heparin pretreatment, TPA was unable to induce an equivalent level of TRE binding activity (Fig. 6 A, lane 3). The addition of an equivalent concentration of heparin to all cell cultures just before harvest (see legend to Fig. 6 A) eliminates the possibility that the nuclear GAG arrive artifactually during lysate preparation. These data suggest that blocked transactivation of TRE/TK-CAT (see Figs. 2–5) correlates with reduced levels of TRE-promoter binding activity in response to TPA treatment in cells preconditioned with heparin.

In Fig. 6 A, heparin treatment inhibited Fos–Jun/AP-1 binding activation in pretreated cells by either a direct or indirect mechanism. To determine if the inhibitory activity was active in nuclear lysates of heparin-pretreated cells, the nuclear lysate from lane 3 (see Fig. 6 A) was added to that from TPA-induced cells (Fig. 6 A, lane 2) and binding activity was measured. As is shown in lane 4, addition of the inhibited nuclear lysate to the TPA-induced lysate abolished the strong TRE binding activity originally observed (Fig. 6 A, lane 2). These data demonstrate that the inhibitor of TRE binding is present in excess and is active in the nuclear lysates of heparin-treated cells. Addition of nuclear lysates from untreated control cells did not inhibit the TRE binding activity in nuclear lysates of TPA-induced cells (data not shown). To establish whether or not Fos–Jun/AP-1 binding activity was present but was inhibited by a nuclear heparin in lysates of GAG-pretreated cells, the GAG-inhibited nuclear lysate (Fig. 6 A, lane 3) was preincubated with the FB-1 and JB peptides before assay and TRE binding activity determined. As shown in Fig. 6 B, both FB-1 (lanes 2–6) and JB (lanes 8–11) allowed a partial recovery of TRE binding activity when compared to nuclear lysates of TPA-treated cells alone (compare to lane J). These findings support the conclusion that GAGs or some other factor(s) present in the nuclear lysates of heparin-conditioned cells recognizes and binds to the DNA-binding domain of Fos and Jun/AP-1 and is responsible for the attenuation of phorbol ester-inducible Fos–Jun/AP-1 binding and trans-activation activity through a TRE promoter element.

**Direct Evidence for Heparin in the Nucleus**

Both the nuclear lysate mixing experiments described above and the nuclear reconstitution experiments in which heparin and peptides were exogenously added are consistent with heparin's ability to effect nuclear transcription factor function. However, it is also possible that heparin-treatment stimulates the cells to synthesize or stimulate an inhibitory protein that could effect nuclear AP-1 function by interacting with the DNA binding domains of Fos and Jun/AP-1. To provide direct evidence for the presence of nuclear GAG, nuclear lysates prepared from HeLa cells were digested with highly purified heparinase and then endogenous TRE binding activity was determined. This enzyme digests glycosaminoglycans enriched in heparan sulfate to release uronic acid. In Fig. 6 C, nuclear lysates were prepared from HeLa cells and analyzed for TRE binding activity as described, without (Fig. 6 C, lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and...
8) a predigestion with heparitinase. Heparitinase digestion on untreated control nuclear lysates caused a twofold enrichment in binding activity. This enrichment was observed in control cells whether or not heparin was added to the medium before cell harvest suggesting that GAGs are intrinsic to the nucleus and compete with DNA for binding to transcription factors. Heparitinase digestion of heparin-treated control cells showed a similar increase in binding activity as observed in untreated controls (Fig. 6 C, lanes 7 and 8). Heparin pretreatment again prevented phorbol ester induction of TRE binding activity (Fig. 6 C, compare lane 3 with 5) with a much greater increase in binding activity after heparitinase digestion than seen in controls (Fig. 6 C, compare lane 2 with 6). Heparitinase enhancement was also observed in uninduced heparin-treated cells (Fig. 6 C, lanes 7 and 8). In lanes 9–11, heparitinase digestion of inhibitory GAG is demonstrated in which a synthetic polypeptide corresponding to the yeast AP-1 binding protein GCN4 (residues 221–281 that compose the core sequence sufficient for protein dimerization and DNA binding) forms a complex with the TRE (Fig. 6 C, lane 9) which was blocked upon addition of heparin (Fig. 6 C, lane 11). When heparitinase was added to GAG-inhibited GCN4, binding activity was restored (Fig. 6 C, lane 10). These data suggest that inhibition of transcriptional activation of cellular Fos and Jun is a direct result of nuclear heparin sulfate (which are targeted by heparitinase) content and that untreated control cells also exhibit nuclear GAG activity. Heparin treatment must increase the concentration of nuclear GAG to levels sufficient to trans-repress TPA-inducible AP-1–associated gene transcription.

Discussion

Expression of the proto-oncogenes c-fos and c-jun/AP-1 can be induced by several agents (Greenberg et al., 1985; Bohmann et al., 1987; Kouzarides and Ziff, 1988; Nakabeppu et al., 1988; Schuermann et al., 1989). The phorbol ester, TPA, while not sufficient to induce transformation in cells can activate the protein kinase C pathway (Nishizuka, 1986); increase binding of the Fos–Jun/AP-1 transcription factor complex to a TRE (Angel et al., 1988b; Turner and Tjian, 1989); induce the c-fos gene through a TRE found in its promoter or through the dyad symmetry element (Treisman, 1985; Verma, 1986; Visvader et al., 1988; Verma and Sassone-Corsi, 1989), which is known to bind the nuclear factor SRF (Treisman, 1985; Gilman et al., 1986); and induce c-jun expression (Angel et al., 1987, 1988b; Visvader et al., 1988). Transfection of cells with the Jun/AP-1 expression vector pSV-c-jun mimics the response to TPA, and results in elevated TRE binding activity and expression of genes that contain this element in their promoter. In the present studies, cotransfection with the pSV-c-jun expression vector and the TRE/TK-CAT reporter gene construct was used to monitor specifically Jun-mediated activity in cells after heparin treatment. The results of Fig. 1 show that heparin treatment of HeLa cells for 18–24 h blocks TPA-induced and pSV-c-jun–induced trans-activation through a TRE without a comparable effect on Rous sarcoma viralLTR promoter. These findings suggest that GAG-mediated transcription regulation may be promoter selective. Studies are currently being expanded to compare regulatory responses to a variety of constitutive and inducible transcription factor–specific promoter elements.

Our results in SMC (Fig. 2) are consistent with the suggestion by Wright et al. (1989) that heparin inhibits TPA-induced SMC proliferation by decreasing coupled proliferation-responsive gene expression. Pukac et al., (1990) demonstrated that heparin does not alter protein kinase C activity to effect a change in growth rate. These authors suggested that transcriptional rates of the c-fos and c-myc induced by TPA may have been affected. In contrast to HeLa cells, primary vascular SMC showed trans-repression of inducible AP-1–mediated gene expression and growth arrest suggesting that heparin–inhibitable Fos and Jun/AP-1 activity is coupled to re-entry of arrested cells into G1-phase of the cell cycle. Moreover, the rapid response to heparin by these cells when compared to HeLa cells may reflect the large number of high affinity receptors (105/cell, Kd 10-9 M, Castellot et al., 1985b) on the SMC surface. Heparin also blocked inducible AP-1–mediated gene expression in nondifferentiated F9 cells suggesting

Figure 6. (A) TPA-inducible Fos-Jun/AP-1 binding activity is inhibited in heparin-pretreated cell nuclear lysate. HeLa cells were grown in 10-cm plates to ~60% confluence in DME as described in Materials and Methods. Cells were seeded and allowed to attach overnight in normal medium followed by a 24-h initial treatment period in 0.2% FCS supplemented medium (+ or – heparin, 200 μg/ml) and a second treatment period for 1 h (+ or – TPA, 75 ng/ml). After TPA treatment, all plates were adjusted to 200 μg/ml with heparin, washed twice with PBS, and nuclear lysates were prepared exactly as described by Dignam et al. (1983). Binding assays were conducted as described in Materials and Methods. Cells were prepared under one of the following three conditions: 0.2% FCS supplemented medium (control, lane 1), medium with a 1-h TPA (lane 2) treatment before cell harvest, or medium containing 200 μg/ml heparin for 24 h, followed by a 1-h TPA treatment (lane 3) before cell harvest. In lane 4, the nuclear lysates from TPA-induced cells (lane 2) and the heparin-pretreated TPA-treated cells (lane 3) were combined before assay. (B) Fos (FB-1) and Jun/AP-1 (JB) DNA-binding domain peptides allow recovery of Fos-Jun/AP-1 binding activity in nuclear lysates prepared from heparin-treated, TPA-induced HeLa cells. DNA binding assays were performed on the same lysates as in A; TPA-induced HeLa cell nuclear lysate (lane 1) or on the inhibited lysates from heparin-pretreated TPA-treated HeLa cells in the absence (lanes 2 and 7) or presence of FB-1 (lanes 3–6) or JB (lanes 8–11). Nuclear lysates were mixed with the indicated concentration of peptide for 10 min before addition of 32P-TRE in 10 μl of 2× TM (see Materials and Methods). Mixtures were incubated for 20 min (in the absence of poly [dI.dC]) and resolved as described in Materials and Methods. (C) Heparitinase digestion increases recovery of TRE binding activity in nuclear extracts. Nuclear extracts were incubated at 37°C in 1× TM containing 1 mM PMSF with or without 10 U of type I heparitinase (Sigma Chemical Co.) for 1 h before addition of poly dI.dC and 32P-TRE. Mixtures that were incubated with (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and 8) heparitinase were then resolved as described in Materials and Methods. Lanes 1 and 2, control untreated cells; lanes 3 and 4, TPA-treated cells; lanes 5 and 6, heparin plus TPA-treated cells; lanes 7 and 8, heparin-treated cells. Lanes 9–11, the yeast GCN4 core peptide (residues 221–281) with TRE binding activity (lane 9) is inhibited by the direct addition of heparin to the binding reaction (lane 11) which is prevented with the simultaneous addition of heparitinase (lane 10).
that the heparin-responsive pathway is a ubiquitous cellular pathway. This conclusion is consistent with a number of studies in which nondifferentiated or dedifferentiated cells in culture respond to GAG treatments to develop or regain a differentiated phenotype (Jackson et al., 1991).

The fact that in HeLa cells inhibition of TPA-induced Fos–Jun/AP-1 activity by heparin was not associated with cell cycle arrest demonstrates that c-jun and c-fos activation (competence gene expression) is not required for continuous growth in these cells. Castellot et al. (1985a) reported earlier that heparin growth sensitivity is a cell-specific phenomena in which both proliferation rates and gene expression profiles are affected. SMC were found to be the most sensitive and transformed cell types the least. Our results comparing the rate at which heparin can attenuate the nuclear transcriptional response in SMC and HeLa cells in which 15 min of heparin treatment was sufficient to abolish a TPA response in SMC while 18–24 h was required in HeLa cells (compare Fig. 1 with 2) may be explained by a difference in the number of heparin receptors on the cell surface although to date, the number of heparin-binding sites on HeLa cells has not been determined. Castellot et al. (1985a) and others (reviewed in Jackson et al., 1991) have also reported alteration of cellular mRNA profiles in response to GAG or matrix-component treatments. The fact that transcriptional activation through the AP-1 promoter element is one of the earliest transcriptional events activated in response to growth factors and mitogens and since heparin can only block induction by certain stimulatory agents (Wright et al., 1989; Pukac et al., 1990) may suggest that nuclear GAGs function to allow for selective processes to dominate at certain times in development.

In the current study, we conclude that nuclear GAGs alter the level of TPA-inducible gene expression by interfering with the TRE binding activity of the Fos–Jun/AP-1 transcription factor complex. While the binding analysis of nuclear lysate preparations still suffers from the potential argument that heparin arrival in the nuclear lysate occurred at the time of cell lysis, we have controlled for that possibility by introducing heparin to all cells just before lysis. The conclusion that nuclear GAG inhibit by direct transcription factor interaction is based on the presence of inhibitory activity in heparin-pretreated cell nuclear lysates (Fig. 6). This activity was reversible with heparin-binding peptides, including the Fos and Jun basic DNA-binding domain peptides and by digestion with the GAG-specific enzyme, heparitinase. The Fos and Jun DNA-binding domain peptides as well as that of the homologous yeast transcription factor GCN4 (Sauder et al., 1991), are known to possess intrinsic a-helical structure (Patel et al., 1990; Busch and Sassone-Corsi, 1990a) and affinity for their promoter element (Busch and Sassone-Corsi, 1990b). The results of the binding experiments support the conclusions from the transient transfection studies and provide evidence for a possible mechanism of action in which nuclear GAG act as a direct competitive inhibitor of DNA thereby blocking the trans-activation response. Castellot et al. (1986) reported the inhibition of SMC proliferation by GAG required the presence of a 3-O-sulfated pentasaccharide. Similarly, Resink et al. (1989) found that pentosan polysulfate and heparin were competitive for the same cellular binding site and that both were inhibitory to vascular SMC proliferation. That pentosan polysulfate blocked the induction of TRE/TK-CAT expression while chondroitin sulfate A and C did not, shows a specificity for GAG structure which may be related to the degree and/or pattern of sulfation. As the 3-O-pattern of sulfation is characteristic of both heparin and PPS but not chondroitin sulfate A and C, we suggest that this structural feature is required for trans-repression. This type of structural information may be important for future studies designed to develop anticancer drugs for specific nuclear transcription factor targets.

Transcriptional repressors are known to play an important role in growth and development (Levine and Manley, 1989; Ohkuma et al., 1990). Controlled growth and development requires precisely regulated levels of gene expression, in particular for those encoding the oncproteins (Bishop, 1985). Nuclear GAG interactions with transcription factors provides the cell with a novel method to both regulate the activity of and stabilize nuclear proteins. By attenuating the transcriptional activation of some inducible transcription factors, coupled gene reactivation might be similarly attenuated. This may account for the coupled trans-repression observed for both c-fos and c-myc described previously (Müller et al., 1984; Wright et al., 1989). By stabilizing pools of transcription factors, GAG protect them from degradation thereby providing a reservoir of readily available activity. Alternatively, at times when the concentration of GAGs reach competitive levels in the nucleus, transcriptional activity may be attenuated and certain auto-inducible transcription factor activity spikes may be blocked. In primary cultures of nontransformed cells, such regulatory molecules may be required to prevent cell cycle re-entry, perhaps, to allow cells to develop or maintain a more specialized phenotype. The general outcome of such cell matrix- or GAG-mediated trans-regulatory events is that cells express an altered phenotype.

The sequence of events involved in such GAG-mediated regulation of nuclear transcription factors is unclear. The many steps involved in the regulation and accumulation of nuclear GAGs include: selective synthesis (Fritze et al., 1985; Bassols and Massquè, 1988; Carey and Evans, 1989) and secretion of both free (Piepknorn et al., 1989) and attached chains to cell surface and cell matrix proteoglycans (Ishihara et al., 1987; Jalkanen et al., 1987; Carey and Evans, 1989); cellular secretion of heparitinases, proteinases, and phospholipases (Castellot et al., 1982; Jalkanen et al., 1987; Ishihara et al., 1987; Nakajima et al., 1987, 1988) required for release and uptake of GAGs; specific binding and internalization of GAGs by cell surface receptors (Hoover et al., 1980; Castellot et al., 1985b; Resink et al., 1989); and transport and metabolism in the cytosol and translocation into the nucleus (Castellot et al., 1985b; Fedarko and Conrad, 1986; Ishihara et al., 1986; Herbert and Maffrand, 1989). Regulation at any or all of these steps may precede regulation of transcription factor activities by selective nuclear interactions with GAG.

As depicted in Fig. 7, nuclear uptake of GAGs is likely to be a highly selective and tightly regulated process, otherwise the nuclear content of GAG would have a heterogeneous composition depending on what was available in the cellular matrix. This is apparently not the case for hepatocytes (Ishihara et al., 1986; Fedarko et al., 1986, 1989). It has also been demonstrated that changes in composition of cell surface-associated GAGs to a more suppressive form occur as
Figure 7. A proposed model for nuclear glycosaminoglycan modulation of transcription factor activity. Internalized GAG are transported to the nuclei by some as yet undefined process where they bind to specific nuclear proteins. In this model the Fos-Jun/AP-1 transcription factor complex is bound up and as such is rendered stable and inactive for binding to DNA. The factors or conditions which reverse this process may include decrease nuclear GAG concentrations possibly because of endogenous heparitinase activity, protein-protein interactions, and phosphorylation events.

a function of cell density in tissue culture (Fritz et al., 1985; Ishihara et al., 1987; Fedarko et al., 1989) or in vivo (Thesleff et al., 1987).Suppressive effects on cell migration and proliferation have also been observed in vivo after heparin infusion (Clowes and Karnovsky, 1977; Guyton et al., 1980; Beniz et al., 1986; Thesleff et al., 1987). The observation that the "nearest neighbor" cell type influences cellular proliferation and differentiation by production of specific proteoglycans and glycosaminoglycans (Morrison-Graham and Weston, 1989) has important implications in the role of these matrix molecules in embryogenesis and growth perhaps as they relate to regulating the timing and pattern of gene expression required for the development of a differentiated phenotype. Our study provides evidence that heparin and similar sulfated polysaccharides play an antagonistic role to modulate inducible-transcription factor function. We propose that such attenuation helps to promote differentiation by stabilizing a specific cell phenotype.

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