Heparin Decreases Activator Protein-1 Binding to DNA in Part by Posttranslational Modification of Jun B

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Abstract Heparin is a potent inhibitor of the proliferation and migration of vascular smooth muscle cells. This agent selectively inhibits the transcription of tissue-type plasminogen activator and interstitial collagenase, probably by decreasing the binding of activator protein-1 (AP-1) to phorbol ester-responsive elements in the promoters of these genes. Decreased AP-1 binding is not due to a direct inhibition by heparin, since heparinase digestion of nuclear extracts prepared from heparin-treated smooth muscle cells does not restore AP-1 binding activity. Treatment of cells with heparin suppresses the expression of Jun B, one of the components of AP-1. The major effect of heparin is at the level of posttranslational modification of Jun B. Results from pulse-chase labeling experiments show that the newly synthesized Jun B is rapidly converted to a higher-molecular-weight form and that conversion is suppressed by heparin. Evidence is presented suggesting that the heparin-inhibited event is phosphorylation of Jun B. (Circ Res. 1994;75:15-22.)

Key Words • Jun B • posttranslational phosphorylation • tissue-type plasminogen activator • interstitial collagenase • activator protein-1

Proliferation and migration of vascular smooth muscle cells (SMCs) contribute to the restenosis of arteries subjected to angioplasty, and effective treatment of this disorder should regulate these responses to injury. Heparin may be an appropriate drug for this purpose because it inhibits SMC growth and migration both in vitro and in vivo. It is of interest to understand how heparin produces these inhibitory effects, since heparin-like glycosaminoglycans secreted by endothelial cells may be endogenous inhibitors of SMCs in vivo. The mechanism of heparin action is still not known, even though there is a vast literature describing its effects. Heparin has been shown to affect a number of cellular functions: it decreases the expression of oncoproteins, it decreases the binding of different growth factors, it enhances the activity of a growth inhibitor, and it alters the deposition of matrix proteins. It is not clear whether these effects represent different pathways or whether they are related to a common pathway that is modulated by heparin. It is also not known whether heparin enters the cells and acts directly or whether it acts indirectly via secondary mediators to produce these effects.

To understand the mechanism of heparin action, we have identified a pathway that is modulated by heparin and hope to determine the molecular basis of the action of heparin by unraveling this pathway. We have previously shown that heparin selectively inhibits the expression of two proteases, tissue-type plasminogen activator (TPA) and interstitial collagenase, in an in vitro mitogenesis model using baboon SMCs. Heparin also decreases the expression of TPA in rat SMCs induced to migrate by arterial injury. Further investigation has suggested that heparin inhibits the transcription of TPA and collagenase by decreasing the binding of a transacting factor, activator protein-1 (AP-1), to the phorbol ester-responsive element (TRE). A consensus TRE sequence is present in the promoter of collagenase gene, and a similar, but not identical, sequence is found in TPA. The binding of AP-1 to the TRE is sufficient to activate the transcription of collagenase. Hence, the inhibitory effect of heparin on TPA and collagenase may be related to its inhibitory effect on AP-1 binding.

It has been suggested that heparin decreases AP-1 binding by direct inhibition in HeLa cells. We have examined this possibility in baboon SMCs and have found that digestion of the nuclear extracts with heparinase does not restore the decreased AP-1 binding. This result suggests that the decrease in AP-1 binding is not by direct inhibition. We also examine the possibility that heparin decreases AP-1 binding by inhibiting the expression of one or more proteins of the AP-1 complexes.

Materials and Methods

Materials

Heparin (isolated from porcine intestine mucosa), phorbol 12-myristate 13-acetate (PMA), heparinase (isolated from Flavobacterium heparinum), and cell culture media were obtained from Sigma Chemical Co. Recombinant heparinase was a generous gift from Dr Robert Rosenberg, MIT. Artic shrimp alkaline phosphatase (SAP) was obtained from USB. Antibodies and blocking peptides to Jun B and c-Jun were purchased from Santa Cruz Biotechnology, Inc. and Oncogene Science. Reagents used for RNA isolation and Northern
blotting were of molecular biology grade. Radioisotopes were obtained from Amersham Corp.

Cell Culture
Aortic SMCs isolated from normal baboons were used between passages 10 and 18. After the cells had been growth-arrested for 3 days in serum-free medium (Dulbecco’s modified Eagle’s medium/F-12 Ham [1:1] containing 6 μg insulin per milliliter, 5 μg transferrin per milliliter, 1 mg ovalbumin per milliliter, 200 U penicillin per milliliter, and 200 μg streptomycin per milliliter), the experiments were started by the addition of fresh serum-free medium containing PMA (5 ng/mL)+heparin (100 μg/mL).

Gel Retardation Assays
Nuclear protein extracts prepared from SMCs were used for gel retardation as described previously.15

Supershift Gel Retardation Assays
Nuclear extracts were preincubated with 2 μg of the Jun B antibody (described in Western blotting section) for 10 minutes at room temperature before the addition of the reaction buffer containing the 32P-labeled probe. The probe for the gel retardation assay was a synthetic oligonucleotide containing the binding sequence for AP-1 from the human collagenase promoter region (5'-AAAGCATGATCAGACACTCTGCG-3').

Northern Analysis
Total RNA was isolated from SMCs20 and used for Northern blotting as described in our previous report.14 cDNA probes for jun B (1.2-kb mouse DNA),21 c-jun (1.8-kb mouse c-jun),21 and glyceraldehyde-3-phosphate dehydrogenase (1.2-kb human cDNA)22 were labeled by nick translation. Signals were detected by autoradiography and quantified by phosphorimaging.

Western Blotting
Nuclear proteins were used for the Western blotting.23 The antibodies to Jun B and c-Jun are polyclonal antibodies raised against peptides corresponding to residue 45-61 of murine Jun B and residue 73-87 of c-Jun. These antibodies are specific to Jun B and c-Jun and do not cross-react with each other or with Jun D. The secondary antibody was alkaline phosphatase-conjugated rabbit antibody. The signal was detected by incubating the membrane in nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Continuous and Pulse-Chase Labeling
For continuous-labeling experiments, growth-arrested SMCs were incubated in serum-free medium lacking methionine for 1 hour before addition of PMA+heparin. Cells were treated with PMA+heparin for 1 hour and then labeled with 100 μCi/mL of [35S]methionine for 3 hours. For pulse-chase labeling experiments, SMCs that have been incubated in methionine-free medium were treated with PMA+heparin for 2 hours and then pulsed with 200 μCi/mL of [35S]methionine for 30 minutes. Cells were chased for 0, 10, 20, 40, 80, 160, and 210 minutes in medium without [35S]methionine and containing 18 mg/L unlabeled methionine. For immunoprecipitation under non-denaturing conditions, cells were lysed in RIP buffer (9.1 mmol/L Na2PO4, 1.7 mmol/L NaHPO4, 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.02% sodium azide, pH 7.25) with 1 mg/mL bovine serum albumin, 0.2 U/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. For immunoprecipitation under denaturing conditions, the cells were lysed in 50 mmol/L Tris, pH 7.5, 0.5% SDS, and 70 mmol/L β-mercaptoethanol and then boiled for 10 minutes. The boiled mixture was diluted with 4 vol RIP without SDS.24 Cell debris were removed by centrifuging at 14,000g for 30 minutes at 4°C. Aliquots of the supernatant were used to determine the incorporation of [35S]methionine into total cellular proteins by trichloroacetic acid precipitation. An equal amount of trichloroacetic acid–precipitable radioactivity from each sample was used for the immunoprecipitation. The supernatants were precleared with protein A agarose. The precleared supernatant was incubated with the Jun B antibody (1 μg/mL) for 1 hour at 4°C, followed by the addition of protein A agarose and continued incubation overnight at 4°C. The immunoprecipitates were washed four times with RIP buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Labeled proteins were detected by fluorography, and the signals were quantified by phosphorimaging.

Dephosphorylation by Alkaline Phosphatase
After washing four times with RIP buffer, the 35S-Jun B immunoprecipitates were washed three times with 100 mmol/L Tris, pH 8.8, 10 mmol/L MgCl2, and 0.2 U aprotinin per milliliter before the addition of 5 U SAP and incubated at 37°C overnight.

Rephosphorylation of SAP-Treated 35S-Jun B Immunoprecipitates
Nuclear or cytoplasmic extracts prepared from PMA-stimulated SMCs were used as a source of kinases to phosphorylate SAP-treated samples. SAP was inactivated by incubation at 65°C for 15 minutes. After inactivation of SAP, samples were rephosphorylated by either nuclear or cytoplasmic extracts in 20 mmol/L HEPESS, pH 7.5, 15 mmol/L MgCl2, phosphatase inhibitors (0.15 mmol/L sodium molybdate, 0.15 mmol/L β-glycerophosphate, 15 mmol/L sodium fluoride, and 0.3 mmol/L sodium orthovanadate), and 1.5 mmol/L ATP at 30°C for 30 minutes. After phosphorylation, the reaction mixture was washed once with RIP buffer before analysis by SDS-PAGE.

In Vitro Phosphorylation Using [γ32P]ATP
Nuclear extract isolated from cells treated with PMA for 4 hours was phosphorylated in the presence of [γ32P]ATP at 30°C for 30 minutes. After phosphorylation, the nuclear extracts were lysed under denaturing conditions before immunoprecipitation with the Jun B antibody.

Statistical Analysis
A paired t test was used to analyze the differences between PMA and PMA+heparin treatments. Values are presented as mean±SEM.

Results
Effect of Heparinase on AP-1 Binding
We have previously shown that PMA increases AP-1 binding in SMCs and that heparin decreases the induced AP-1 activity. To determine whether the decreased AP-1 binding was due to a direct inhibition by heparin in the extracts, nuclear extracts from SMCs were pre-digested with heparinase before the binding assays. Digestion with heparinase of nuclear extracts prepared from heparin-treated cells did not restore the decreased AP-1 binding (Fig 1, lane 4 versus 5). Hence, the decreased AP-1 binding was not due to a direct inhibition by heparin. We have examined the possibility that heparinase inhibitory activity might be present in the nuclear extracts by deliberately adding exogenous heparin to the extracts. The addition of 17 μg/mL exogenous heparin abolished the AP-1 binding (lane 6 versus 4), and digestion with heparinase restored it (lane 7 versus 6). A similar experiment was also performed in nuclear extracts prepared from PMA-treated
cells to determine the activity of heparinase (lanes 8 through 10). Direct addition of exogenous heparin abolished AP-1 binding (lane 9 versus 8), and digestion with heparinase restored the binding (lane 10 versus 9). These results were obtained by using either recombinant (shown) or commerically available (not shown) heparinase.

**Effect of Heparin on jun and fos mRNAs**

In a time-course study, jun B and c-jun mRNAs increased transiently after PMA stimulation; the level peaked at 2 hours and was still slightly elevated at 4 hours (Fig 2). The responses of jun B and c-jun mRNAs to PMA were different in magnitude. PMA increased

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**Fig 1.** Effect of heparinase on activator protein-1 (AP-1) binding. Nuclear extracts prepared from smooth muscle cells treated with phorbol 12-myristate 13-acetate (PMA) and heparin for 4 hours were used for the gel retardation assays. Digestion with recombinant heparinase was performed by addition of the enzyme (2 U) to the nuclear extracts in the presence of 1 mmol/L phenylmethylsulfonyl fluoride and 5 mmol/L CaCl$_2$ and incubated at 37°C for 1 hour before the gel-retardation assays. Nuclear extracts were prepared from cells subjected to the following conditions: medium alone (lane 1), PMA without heparinase digestion (lane 2) and with digestion (lane 3), and PMA+heparin without heparinase digestion (lane 4) and with digestion (lane 5). Whether there was heparinase-inhibitory activity in the nuclear extracts prepared from PMA+heparin-treated cells was examined by direct addition of exogenous heparin (17 μg/mL) to the extracts in the absence (lane 6) or presence (lane 7) of heparinase. A similar experiment was also performed to determine the activity of heparinase (lanes 8 through 10). Nuclear extracts were prepared from PMA (lane 8) and by direct addition of exogenous heparin (17 μg/mL) to the extracts in the absence (lane 9) or presence (lane 10) of heparinase (n=4 experiments).

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**Fig 2.** Northern analysis of jun B and c-jun in the presence of phorbol 12-myristate 13-acetate (P) and heparin (H). Total RNA was isolated from smooth muscle cells treated with either P or P+H for 0, 1, 2, and 4 hours. The blot was reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPD) to normalize the amount of RNA loaded into each lane. jun B mRNA by 6±1.2-fold and c-jun mRNA by 2±0.3-fold (n=5). Heparin slightly decreased the PMA-induced jun B and c-jun mRNAs by 33±10% and 37±13%, respectively (n=5). jun D mRNA was constitutively expressed and was not modulated by PMA or heparin (data not shown). We previously have shown that PMA increases c-fos mRNA and that heparin has no significant effect on the induction. fos B mRNA was not detected in either unstimulated cells or PMA-stimulated cells (data not shown).

**Heparin Decreases Jun B Expression**

Heparin decreased expression of Jun B as determined by Western blot analysis. It was found mainly in the nuclear fraction; only a trace amount was found in the cytoplasmic fraction (data not shown). Antibody specific to Jun B detected two specific polypeptides, a major band at 43 kD and a minor band at 39 kD, plus a nonspecific band at 49 kD (Fig 3). The two specific bands could be blocked by a Jun B peptide (amino acid 45-61), against which the antibody was made (Fig 3). This region (amino acid 45-61) is specific to Jun B and has little homology with the c-Jun or Jun D sequence. In addition, these two bands were not observed with antibodies specific to c-Jun or Jun D (data not shown). The level of Jun B slightly increased at 2 hours, reached a maximum at 4 hours, and remained high at 6 hours after stimulation with PMA. Heparin decreased PMA-induced Jun B at all time points (Fig 3). The inhibitory effect on Jun B was specific to heparin, since other

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**Fig 3.** Western analysis of Jun B expression in the presence of phorbol 12-myristate 13-acetate (P) and heparin (H). Nuclear proteins isolated from smooth muscle cells treated with serum-free medium (SF) alone, P, and P+H for 2, 4, and 6 hours were used for Western blotting. The level of Jun B was also determined in cells treated with P+chondroitin-4-sulfate (CS). Two Jun B polypeptides were detected at 43 kD and 39 kD. The specificity of these two bands was determined by incubating a Western blot containing nuclear proteins prepared from cells treated with P for 4 hours with the Jun B peptide (amino acid 45-61).
glycosaminoglycans such as chondroitin-4-sulfate (Fig 3) or chondroitin-6-sulfate (data not shown) did not reduce Jun B expression.

The increase in Jun B induced by PMA was due to new protein synthesis, since the increase could be blocked by cycloheximide (data not shown). This was further confirmed by metabolically labeling SMCs with [35S]methionine for 3 hours, followed by immunoprecipitation with the Jun B antibody (Fig 4, left panel). The total amount of [35S]methionine incorporated into cellular proteins determined by precipitation with trichloroacetic acid was not different in cells treated with either PMA or PMA + heparin. Similar to the results obtained with Western blotting, immunoprecipitation of Jun B detected both 43-kD (major) and 39-kD (minor) bands (Fig 4, left panel). These two bands were specific to Jun B, since they could be blocked by the Jun B peptide (data not shown). Furthermore, both bands were detected by immunoprecipitation under nondenaturing or denaturing conditions, which suggested that these two bands were not a result of coimmunoprecipitation. PMA increased the labeling of the 43-kD and 39-kD Jun B bands by 3.5±0.6- and 3.0±0.4-fold, respectively, above the serum-free control (n=9). Heparin decreased the PMA-induced 43-kD Jun B by 61±9% (P<.005, n=9) and the 39-kD Jun B by 38±22% (P<.01, n=9) (Fig 4, left panel). Heparin had no effect on the efficiency of immunoprecipitation of Jun B, since addition of heparin (20 μg/mL) directly to the assay did not affect the amount of Jun B recovered (data not shown).

The effect of heparin on c-Jun expression was determined by immunoprecipitation (Fig 4, right panel). The antibody to c-Jun detected a 40-kD c-Jun band and a 43-kD nonspecific band. The 40-kD band is specific to c-Jun, since it could be completely eliminated by the blocking peptide (residue 73-87) (data not shown). The level of c-Jun was not significantly increased by PMA (22±17%, n=8). Heparin slightly decreased the level of c-Jun (Fig 4, right panel).

Jun B in AP-1 Complexes

Whether heparin decreases Jun B in the AP-1 complexes was determined by supershift gel-retardation assay. The presence of Jun B in the complexes was detected by the formation of a higher-molecular-weight complex with the antibody, as indicated by a supershift band (Fig 5, lane 5). This supershift band was specific to Jun B, as it could be eliminated by the Jun B blocking peptide (Fig 5, lane 9 versus 8). Also, the Jun B supershift was not a result of artifactual shift due to the addition of excess antibody, since this supershift band was not observed in the presence of an irrelevant antibody such as NF-kB (data not shown). PMA increased the level of Jun B in the AP-1 complexes, and
heparin suppressed the induction by ≈70% (Fig 5, lane 5 versus 6). These results indicate that the decreased Jun B expression by heparin (Fig 4, left panel) corresponded to a reduced level of Jun B in the AP-1 complexes.

**Effect of Heparin on Posttranslational Modification of Jun B**

The small reduction of jun B mRNA by heparin (33±10%) could not account for the decrease in the protein (61±9%), suggesting that the major effect of heparin on Jun B was at the posttranscriptional level. To explore this possibility, we next examined the effect of heparin on the synthesis, posttranslational modification, and degradation of Jun B with pulse-chase labeling experiments. Cells were treated with PMA and heparin for 2 hours, labeled for 30 minutes with [35S]methionine, and then chased with unlabeled methionine medium for various periods. [35S]-labeled Jun B was recovered by immunoprecipitation and analyzed by SDS-PAGE.

Results from pulse-chase experiments showed that the 39-kD Jun B was the precursor to the 43-kD Jun B. The 39-kD Jun B was the predominant species after a brief labeling (Fig 6a, 0 minutes), and it rapidly converted to the 43-kD form during the chase (Fig 6a). This conversion was illustrated by the 43-kD-to-39-kD ratio at various periods. In PMA-stimulated cells, the ratio increased from 0.8 to 3.1 after 80 minutes of chase (Fig 6b). The 39-kD Jun B was the predominant species after brief labeling (Fig 6a, 0 minutes), whereas the 43-kD Jun B was the predominant species after continuous labeling (Fig 3). This difference was due to the rapid conversion of the newly synthesized 39-kD Jun B to the 43-kD form.

Heparin slowed the conversion of the 39-kD Jun B to the 43-kD Jun B (Fig 6a). Results from three experiments showed that heparin consistently decreased the apparent conversion of the 39-kD Jun B to the 43-kD Jun B, as demonstrated by a lower ratio at a range of time points (Fig 6b). The decreased conversion in the presence of heparin also was reflected in the longer half-life of the 39-kD Jun B, which was estimated to be 71 versus 56 minutes in PMA-stimulated cells. In this particular experiment, heparin appeared to decrease the amount of Jun B synthesized (Fig 6a, 0 minutes). However, average results from three experiments showed that heparin had no effect on the newly synthesized 39-kD Jun B (1±17%, P=NS), and it only had a small effect on decreasing the 43-kD Jun B (25±14%, P=NS) at 0 minutes.

**Posttranslational Phosphorylation of Jun B**

Whether the posttranslational modification of the 39-kD Jun B to the 43-kD Jun B was due to phosphorylation was addressed by performing dephosphorylation and rephosphorylation studies. Dephosphorylation using alkaline phosphatase should lead to the disappearance of the 43-kD Jun B and the reappearance of the 39-kD Jun B, whereas rephosphorylation of the alkaline phosphatase–treated Jun B should result in the reappearance of the 43-kD Jun B. [35S]Jun B immunoprecipitates were used in both studies in order to follow the conversions between the 39-kD Jun B and the 43-kD Jun B.

[35S]Jun B was isolated by immunoprecipitation from PMA-stimulated SMCs, which were metabolically labeled with [35S]methionine. Dephosphorylation with SAP of the [35S]Jun B caused the disappearance of the 43-kD Jun B and the reappearance of the 39-kD Jun B (Fig 7a, lane 1 versus 2). This change was best illustrated by comparing the 43-kD-to-39-kD ratio before and after SAP treatments. The ratio was 2.2 before treatment with SAP, and it decreased to 0.6 after treatment. The decrease in the ratio was not observed with heat-inactivated SAP or in the presence of phosphatase inhibitors (p-nitrophenyl phosphate and β-glycerophosphate) (data not shown), indicating that the changes were not due to proteolysis. Similar results were also observed with calf intestine alkaline phosphatase (data not shown). Thus, the conversion of the 39-kD Jun B to the 43-kD Jun B was due to phosphorylation.

Nuclear or cytoplasmic extracts isolated from PMA-stimulated SMCs were used as a source of kinases to rephosphorylate SAP-treated [35S]Jun B immunoprecipitates. SAP-treated [35S]Jun B was incubated at 65°C for 15 minutes to inactivate SAP and then phosphorylated with either nuclear or cytoplasmic extracts in the presence of unlabeled ATP. The control was a SAP-treated sample that had not been incubated at 65°C to inactivate SAP before being phosphorylated with nuclear extracts. Rephosphorylation with either nuclear or cytoplasmic extracts caused an increase in the 43-kD Jun B and a decrease in the 39-kD Jun B, as shown by an increase in the 43-kD-to-39-kD ratio from 0.6 to 1.2 and 1.1 (Fig 7a, lane 2 versus 3 and 4). This change was not observed in the control sample with active SAP during rephosphorylation, as shown by the ratio of 0.7 (Fig 7a, lane 5).
The sum of the 43-kD and 39-kD Jun B signals was compared between samples treated with and without SAP. The amount recovered from the SAP-treated sample was 87±8% (P=NS, n=3) of the nontreated sample, indicating that SAP treatment did not cause proteolysis. However, incubation of the SAP-treated 32P-Jun B immunoprecipitates with either nuclear or cytoplasmic extracts resulted in a decrease of the sum of the 43-kD and 39-kD Jun B signals; the amount recovered was 60% of the untreated sample. Although some proteolytic activity was present in the extracts, the increase in the ratio observed in the SAP-treated samples phosphorylated with the extracts (Fig 7a, lane 3 and 4) was due to an actual increase in the 43-kD Jun B and a decrease in the 39-kD Jun B. This indicated that the increase in the ratio was not a result of selective proteolytic degradation of the 39-kD Jun B.

In vitro phosphorylation of nuclear extracts isolated from PMA-stimulated SMCs was performed using [32P]ATP. 32P-Jun B in the nuclear extract was recovered by immunoprecipitation using the denaturing condition and then analyzed by SDS-PAGE. Results showed that 32P-Jun B migrated only as the 43-kD band and that no band of 39 kD was detected (Fig 7b). These observations suggested that the 43-kD band is the phosphorylated form of Jun B at steady state.

The effect of heparin on phosphorylation of Jun B was determined by in vitro phosphorylation of nuclear extracts isolated from PMA+heparin-treated SMCs. Results showed that the amount of 32P-Jun B (43 kD) recovered from PMA+heparin–treated cells was less than that from PMA-treated cells (Fig 7c). These results supported the notion that heparin decreases the phosphorylation of Jun B.

Discussion

Heparin inhibits the expression of TPA and collagenase in part by lowering the cellular activity of transcription factor AP-1.15 In the present study, we examined the effect of heparin on components of AP-1 complexes. Jun B and c-Fos, but not c-Jun, Jun D, or Fos B, were induced by PMA. Heparin suppressed the expression of Jun B as demonstrated by Western blot analysis and immunoprecipitation. Heparin reduced the amount of Jun B in the AP-1 complexes, as demonstrated by supershift gel-retardation assay. We also have investigated the effect of heparin on c-Fos by Western blotting and immunoprecipitation. Because the c-Fos antibodies (obtained from two different sources) did not work in either assay, we could not quantify the effect of heparin on c-Fos. However, results obtained from the supershift gel retardation assay showed that the c-Fos supershift band was not reduced by heparin (unpublished data).

The effect of heparin on Jun B expression was both at the transcriptional and posttranscriptional levels. It had a small, but significant, influence in decreasing Jun B mRNA induced by PMA, which probably explains the decrease in synthesis of Jun B protein when it was measured with short labeling times. The major effect of heparin was at the level of posttranslational modification of Jun B. Western blot analysis and immunoprecipitates obtained from continuous-labeling experiments detected two species of Jun B, a major band at 43 kD and a minor band at 39 kD. Results from pulse-chase labeling experiments are consistent with the 39-kD Jun B being the precursor and the 43-kD Jun B the posttranslational modified product in PMA-stimulated cells. The conversion of 39-kD to 43-kD Jun B was suppressed by heparin and seemed to be due to phosphorylation, on the basis of results obtained from both dephosphorylation and rephosphorylation studies. Consistent with these conclusions, it has been shown in 3T3 cells that newly synthesized Jun B is rapidly phosphorylated, resulting in an increase in the apparent molecular weight.24 These observations suggest that an important influence of heparin is on the posttranslational phosphorylation of Jun B.

Unlike c-Jun, phosphorylation of Jun B has not been well characterized. However, sequence comparisons show interesting differences in potential phosphorylation sites in Jun B. At least five phosphorylation sites have been identified in c-Jun:26-30 two of them (Ser 63 and Ser 73) are located in the N-terminus, and the other three (Thr 231, Ser 243, and Ser 249) are adjacent to the DNA-binding domain in the C-terminal portion of the molecule. It is not clear which enzymes phosphorylate c-Jun in vivo, although in vitro studies have shown that c-Jun can be phosphorylated by various kinases. For instance, mitogen-activated protein kinase (MAPK).29,31
p34\textsuperscript{cdc2}, and a 67-kD protein\textsuperscript{32} have been demonstrated to phosphorylate Ser 63, Ser 73, and Ser 243, respectively, whereas casein kinase-II (CK-II)\textsuperscript{30} and glycogen synthase kinase-3 (GSK-3)\textsuperscript{26,34,35} phosphorylate Thr 231 and Ser 249, respectively. Phosphorylation of the N-terminus increases the transactivating activity of c-Jun, whereas phosphorylation of the C-terminus decreases the DNA binding activity of c-Jun.\textsuperscript{26-28,30,35} Whether phosphorylation modulates Jun B in the same manner as c-Jun is not clear. Others have shown that MAPK phosphorylates the N-terminus of c-Jun but not Jun B\textsuperscript{36} and that GSK-3 phosphorylates the C-terminus of both c-Jun and Jun B.\textsuperscript{35} By sequence inspection, Jun B appears to have multiple potential CK-II and MAPK phosphorylation sites. Results obtained from labeling serum-stimulated 3T3 cells with \textsuperscript{32}P show that Jun B is phosphorylated at a much higher level than is c-Jun.\textsuperscript{24} The possibility that Jun B is phosphorylated at multiple sites and that heparin affects phosphorylation of specific sites might explain the mutated effect of heparin on the overall phosphorylation of Jun B. It will be important to identify the heparin-modulated phosphorylation site(s) in Jun B. We speculate that the magnitude of the effect of heparin might be larger when we examine the phosphorylation of these sites.

An intriguing possibility is that heparin affects Jun B phosphorylation by inhibiting one of the kinases. A recent report has shown that heparin reduced MAPK activity by inhibiting the activation of the enzyme in rat SMCs.\textsuperscript{37} Heparin also might inhibit CK-II, since it is a potent competitive inhibitor of CK-II with \( K_i \) of 1.4 nmol/L.\textsuperscript{38} CK-II is a constitutively expressed enzyme and is found mainly in the nucleus.\textsuperscript{39} It is conceivable that heparin might enter the nucleus and inhibit CK-II. Busch et al\textsuperscript{19} concluded from heparitinase experiments using HeLa cells that heparin was present in the nuclear extract and was interfering with AP-1 binding. We performed similar experiments with baboon SMCs and found that heparitinase digestion of the nuclear extracts from heparin-treated cells did not restore the decreased AP-1 binding. On the other hand, trace amounts of heparin might be present in the nucleus at levels sufficient to inhibit CK-II but too low to inhibit AP-1 binding to DNA. On the basis of the \( K_i \) value, we calculate that the amount of heparin required to inhibit CK-II by 50\% is \( \approx 20 \) ng/mL, which is at least 150-fold less than the amount (3 \( \mu \)g/mL) needed to inhibit AP-1 binding.\textsuperscript{15} In fact, heparin has been shown to bind to SMCs and be internalized.\textsuperscript{40} Furthermore, small amounts of heparan sulfates have been recovered from nuclear preparations.\textsuperscript{41} Recent studies suggest that the mechanism for nuclear translocation of heparin may depend on the binding to other proteins. A likely candidate is fibroblast growth factor (FGF), which has a nuclear translocation sequence and a high affinity for heparin.\textsuperscript{42} The nuclear translocation of basic FGF has been shown to occur in bovine endothelial cells.\textsuperscript{43} It is possible that after internalization, heparin binds to cytoplasmic basic FGF and is transported into the nucleus.

CK-II phosphorylates a number of nuclear and cytoplasmic proteins, many of which are involved in gene expression and protein synthesis.\textsuperscript{44} For example, phosphorylation of serum-responsive factor by CK-II increases the on and off rates of binding to the serum-responsive element, which then leads to an increase in transcription of c-fos.\textsuperscript{45} According to the hypothesis proposed here, heparin, which has been shown to decrease c-fos gene expression in rat SMCs,\textsuperscript{8} may act by inhibiting CK-II phosphorylation of serum-responsive factor. Likewise, the small effect of heparin on decreasing \( jun \) B mRNA found in the present study may be due to an effect on CK-II-phosphorylated transcription factors. Thus, it would be of interest to find out if the influence of heparin is on selected CK-II phosphorylated proteins or if it affects all of them.

What is the role of Jun B in activating the expression of AP-1-responsive genes? The current biochemical evidence suggests that because of its decreased DNA-binding activity, Jun B is not as potent as c-Jun in activating gene transcription.\textsuperscript{46} In cell types such as HeLa cells, in which c-Jun is the major protein of the Jun family, Jun B most likely does not play a significant role in activating AP-1-responsive genes. However, the situation may be different in other cell types in which the content of Jun B is higher, since it will be able to form abundant heterodimers with Fos proteins. The DNA-binding affinity of Jun B:c-Fos or Jun B:Fos B is comparable to that of c-Jun complexes.\textsuperscript{47} The importance of Jun B in collagenase and TPA gene expression in baboon SMCs is currently under investigation.

In conclusion, we have demonstrated that heparin decreases AP-1 binding in part by decreasing Jun B expression. Heparin probably affects the posttranslational phosphorylation of Jun B. We hypothesize that heparin inhibits the kinases that phosphorylate Jun B. It will be important to identify the kinases that phosphorylate Jun B in vivo, to determine the actual phosphorylation sites in Jun B, and to examine the effect of heparin on phosphorylation of these sites.

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