Post-transcriptional Regulation of Syndecan-1 Expression by cAMP in Peritoneal Macrophages

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Abstract. Syndecan-1 is a cell surface heparan sulfate proteoglycan that is proposed to serve in cell–cell adhesion, cell-matrix anchorage, and growth factor signaling. Its expression is temporally and spatially regulated during epithelial-mesenchymal interactions in many developing tissues. In some cases, this regulation appears to be achieved at the level of transcription. However, induction of syndecan-1 expression in the embryonic kidney mesenchyme is suggested to occur at the level of mRNA translation (Vainio, S., M. Jalkanen, M. Bernfield, and L. Saxén. 1992. *Dev. Biol.* 152:221-232). To identify a system in which the regulatory mechanisms controlling syndecan-1 expression can be studied, cells of the monocyte-macrophage lineage, which regulate the expression of many cell surface receptors, were screened for syndecan-1 expression. The syndecan-1 gene is active in blood monocytes as well as resident and thioglycollate-elicited mouse peritoneal macrophages, but expression of the proteoglycan is regulated at two levels. First, elicited macrophages accumulate nine-fold more syndecan-1 mRNA than do resident macrophages or circulating blood monocytes. Another member of the syndecan family of proteoglycans, syndecan-4, shows a distinct pattern of expression, suggesting that this regulation is specific for syndecan-1. Second, utilization of the mRNA for syndecan-1 production encounters a post-transcriptional block in the elicited macrophages that can be overcome by triggering agents such as E-type prostaglandins or dibutyryl cAMP, which raise intracellular cAMP levels. Dibutyryl cAMP does not induce syndecan-1 expression in resident peritoneal macrophages, which lack a pool of stored mRNA. This suggests that this agent promotes the post-transcriptional utilization of stored syndecan-1 mRNA. The induced proteoglycan appears at the cell surface as a ~100-kD heparan sulfate-rich isoform of syndecan-1. This suggests that a cAMP-dependent post-transcriptional control mechanism may be present in a variety of tissues when syndecan-1 expression is regulated.

SYNDECAN-1 is the prototypical member of a family of integral membrane heparan sulfate proteoglycans that are expressed in different combinations by most cell types (reviewed by Bernfield et al., 1992). The defining features of these glycoproteins are: (a) highly conserved transmembrane and cytoplasmic domains, and (b) decoration with heparan sulfate glycosaminoglycans. Although the function of the cytoplasmic domain is unclear, the presence of four invariant tyrosines suggests roles in targeting and/or anchorage to cytoplasmic molecules, such as components of coated pits or the cytoskeleton. Indeed, syndecan-1 exhibits a polarized distribution along the basolateral surface of epithelial cells, and is proposed to be anchored to the cytoskeleton (Rapraeger et al., 1986).

Many of the suggested roles of the syndecans are inferred from the biological properties of heparan sulfate. This glycosaminoglycan binds proteins in the extracellular matrix (ECM)[1] such as interstitial collagens, fibronectin, tenascin, and thrombospondin, which suggests that the syndecans may serve to anchor cells to the ECM (Jackson et al., 1991; Bernfield et al., 1992). In addition, syndecan-1 has been identified as a low affinity binding site for basic FGF (Kiefer et al., 1990) and heparan sulfate at the cell surface is essential for receptor binding and biological activities of FGFs (Rapraeger et al., 1991; Yayon et al., 1991; Olwin and Rapraeger, 1992).

Although progress has been made toward elucidating the structures and activities of syndecans, the mechanisms controlling the expression of these molecules are not known.

1. *Abbreviations used in this paper:* dbcAMP, dibutyryl cAMP; dbcGMP, dibutyryl cGMP; ECM, extracellular matrix; LPS, lipopolysaccharide; MØ, macrophage.

Several examples of the temporal and spatial regulation of syndecan-1 expression have been reported. B lymphocyte progenitors express the proteoglycan when attached to the stromal matrix of the bone marrow and lose it coordinately with their maturation and release into the circulation (Sanderson et al., 1989). Secreting plasma cells regain the molecule when they become anchored within the interstitial matrices of the lymph node and duodenum, and use syndecan-1 to bind type I collagen in vitro (Sanderson et al., 1989; Sanderson et al., 1992b). During wound healing, coordinate induction of syndecan-1 mRNA and proteoglycan expression is observed in rapidly proliferating keratinocytes adjacent to wound margins (Elenius et al., 1991). Syndecan-1 expression is regulated both spatially and temporally during epithelial-mesenchymal interactions in the developing tooth (Vainio et al., 1991), kidney (Vainio et al., 1992), and limb (Solursh et al., 1990). In each of these tissues, syndecan-1 is transiently lost from the epithelium as it changes shape, but regained when this tissue becomes morphologically stable. Syndecan-1 is also induced in the proliferating mesenchyme. In the developing tooth, this appears to involve transcriptional activation of the syndecan-1 gene (Vainio et al., 1991). In the developing kidney, syndecan-1 is induced in the metanephric mesenchyme by interaction with the metanephric duct (Vainio et al., 1992). A notable characteristic of this induction is that syndecan-1 mRNA is expressed in the uninduced mesenchyme, but not utilized until the inductive period. This suggests that an important mechanism for regulating syndecan-1 expression in the kidney, and perhaps other tissues, is at the level of mRNA translation.

Macrophages provide a useful system to begin studying this important regulatory mechanism. These cells participate in a multitude of interactions with other cells, the ECM, chemical mediators, and pathogens. Not surprisingly, macrophages express roughly 50 types of cell surface receptors, many of which are regulated during the development and activation of these cells (Adams and Hamilton, 1984; Gordon, 1986). Furthermore, macrophages are engaged in several activities that likely require the regulated expression of heparan sulfate proteoglycans, including anchorage to and directed migration through ECM (Gordon, 1986; Springer, 1990) and participation in wound healing and angiogenesis through the production and release of heparan sulfate-binding growth factors (Baird et al., 1985; Rappolee et al., 1988; Knighton and Fiegel, 1989; Higashiyama et al., 1991; Stein and Keshav, 1992).

In the present study, circulating blood monocytes, resident and thioglycollate-elicited murine peritoneal macrophages were screened for syndecan-1 mRNA and proteoglycan expression. This analysis revealed that the syndecan-1 gene is active in each of these cell types, but the expression of this proteoglycan is regulated at two levels. First, the syndecan-1 mRNA is specifically accumulated by macrophages during inflammatory recruitment. In contrast, mRNA encoding syndecan-4 is expressed at the same level by each of these cell types, indicating that the induction of syndecan-1 expression is specific. Secondly, utilization of the syndecan-1 mRNA for proteoglycan expression encounters a post-transcriptional block that can be overcome by agents, such as prostaglandin E_2, that raise intracellular cAMP levels. The mechanism controlling syndecan-1 expression during macrophage activation may be indicative of a mechanism op-

erative in many cell and tissue types, including stratifying keratinocytes (Sanderson et al., 1992a) and condensing mesenchymal cells in the developing kidney (Vainio et al., 1992), that demand the temporal regulation of this molecule.

Materials and Methods

Reagents

cDNA probes for mouse syndecan-1 (4-19b) (Saunders et al., 1989) and mouse syndecan-4 (pMB176) were generously provided by Dr. Merton Bernfield (Joint Program in Neonatology, Harvard Medical School, Boston, MA).

Rat mAb 281.2, specific for the core protein of mouse syndecan-1, was isolated as described previously (Jalkanen et al., 1985). Rabbit anti-actin serum was obtained from Sigma Chemical Co. (St. Louis, MO). Nonspecific rat IgG, FITC-conjugated rabbit anti-rat IgG, and alkaline phosphatase-conjugated goat anti-rat and goat anti-rabbit IgGs were purchased from Jackson Immunoresearch (West Grove, PA).

Dibutyryladenosine 3':5'-cyclic monophosphate (dbcAMP), dibutyrylguanosine 3':5'-cyclic monophosphate (dbcGMP), PMA, forskolin, prostaglandins E_1 and E_2, and lipopolysaccharides (LPS) were obtained from Sigma Chemical Co. Cholera toxin was purchased from ICN Biomedicals (Irvine, CA). Interferon-γ (1,000 U/ml) was generously provided by Dr. Donna Paulnock (Department of Medical Microbiology, University of Wisconsin, Madison, WI).

Cell Culture

Mouse (NMuMG) mammary gland epithelial cells were maintained at low passage in DME (Gibco Laboratories, Gaithersburg, MD) containing 10% (vol/vol) FBS.

Peripheral blood mononuclear leukocytes were isolated from mouse blood according to Boyum (1968): blood was aspirated via heart puncture of anaesthetized male C57Bl/6 mice (Harlan Sprague Dawley, Indianapolis, IN) and diluted into RPMI-1640 (Gibco Laboratories) containing 20 U/ml heparin. Cells were carefully layered onto histopaque (1.077 g/ml, Sigma Chemical Co.) and centrifuged at 400 g for 30 min. The white cell interface was collected, washed with medium, and monocytes were isolated by adherence to tissue culture dishes coated with FBS (Kumagai et al., 1979).

Peritoneal exudate cells were elicited by intraperitoneal injection of 10–12 wk old male C57Bl/6 mice with 1 ml Brewer's thioglycollate broth (Difco, Detroit, MI) 4–5 d before harvesting cells. Resident and thioglycollate-elicited macrophages were isolated by peritoneal lavage with RPMI-1640 followed by adherence for 30–60 min to tissue culture dishes. Adherent monocytes, resident and elicited peritoneal macrophages were washed vigorously with medium after the attachment period. Routinely, >95% of the cells were positive for the macrophage-specific antigen MAC-1 by immunofluorescent staining (Ho and Springer, 1984). Primary cultures were used for experiments immediately after their isolation.

RNA Isolation and Northern Analysis

The isolation of total and poly(A)+ RNA from cell monolayers has been described previously (Badley et al., 1988; Sambrook et al., 1989). For RNA slot blot analysis, aliquots of 1 μg/lane poly(A)+ RNA were applied to a nitrocellulose membrane (Schleicher and Shull, Inc., Keene, NH) and baked. For Northern analysis, aliquots of 5 μg of total RNA/lane were separated on a formaldehyde-containing 1% agarose gel by electrophoresis at 40 V overnight and blotted onto a Nytran membrane (Schleicher and Shull, Inc.).

Blots were sequentially probed with the 1.4-kb EcoRI-HindIII fragment from the 4-19b syndecan-1 cDNA, the 368-bp BamHI-EcoRI fragment from the pMB176 syndecan-4 cDNA, and the 1.3 kb PstI fragment from the pGPDN5 GAPDH cDNA. Inserts were oligolabeled with [α-^{32}P]dCTP (>3,000 Ci/mmol, DuPont, Wilmington, DE) using the Prime-a-Gene system (Promega Corp., Madison, WI), and isolated on columns of Sephadex G-50 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) (Sambrook et al., 1989).

Blots were prehybridized at 42°C for 4 h in 50% formamide, 5× Denhardt's, 0.1% SDS, 5× SSPE, and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed at 42°C for 16 h in the same buffer containing 2.5× Denhardt's and 10^6–10^7 cpm/ml ^{32}P-oligolabeled cDNA. The membranes were washed in 6× SSPE, 0.5% SDS at room temperature (2

× 15 min), and in 1× SSPE, 0.5% SDS at 60°C (1 × 60 min). The 0.24–9.5-kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used to determine the molecular size of the hybridizing RNA species.

Isolation of Proteoglycans and Digestion of Glycosaminoglycans

Confluent cell monolayers were lysed and sonified in 8 M urea, 0.2% Triton X-100, 10 mM Tris-HCl, pH 8.0 containing protease inhibitors. DEAE-Sephacel (Pharmacia LKB Biotechnology) was combined with lysates (100 µl per 10^7 cells) and mixed overnight at 4°C. Beads were washed with TBS and eluted with 1 M NaCl in heparitinase digestion buffer (100 mM Hepes, pH 6.5, 10 mM CaCl₂, 0.5% CHAPS, 0.2 mg/ml BSA). Eluted proteoglycan samples were collected as described (Sanderson et al., 1992b).

Enzymatic cleavage of heparan sulfate and chondroitin sulfate glycosaminoglycan chains was achieved by diluting proteoglycan samples 2× with ddH₂O followed by incubation twice for 2 h at 37°C with 1 mU/ml of heparinase III (heparitinase, ICN Biochemicals, Cleveland, OH), or with 50 mU/ml of chondroitinase ABC (ICN Biomedicals), or with both enzymes.

Gel Electrophoresis and Immunoblotting

Electrophoresis of proteoglycan samples was performed in SDS-polyacrylamide gradient (3.5–15% T; 7.5% C) gels in TrisHCl/Borate buffer, pH 8.8 (Koda et al., 1985). Electrophoresis of proteoglycan core protein samples was performed as described by Laemmli (1970) in 7% SDS-polyacrylamide gels. Samples were then electroblotted onto cationic nylon (Zeta Probe-GT, BioRad, Rockville Center, NY) at 60 V for 4 h. Membranes were fixed with 0.05% glutaraldehyde in PBS for 30 min (Sanderson et al., 1989). Blocking was achieved by incubation for at least 2 h at 37°C in TBS containing 5% nonfat powdered milk, 25 µg/ml heparin, and 0.02% sodium azide.

Blots were probed for syndecan-1 by incubation with 1 µg/ml mAb 281.2 in TBS containing 5% non-fat powdered milk (Blotto) for 2 h at 37°C. Membranes were washed 5 × 10 min with TBS containing 0.1% NP-40, once for 10 min in blotto, then further incubated for 60 min in blotto containing alkaline phosphatase-conjugated goat anti-rat IgG (1:3,000). After washing as above, blots were incubated 10 min in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris HCl, pH 9.5). Finally, the membranes were incubated at 37°C for 5–10 min in LumiPhos chemiluminescent substrate (Boehringer Mannheim, Indianapolis, IN) and exposed to x-ray film (Kodak XAR-5).

Immunostaining

Immunolocalization of syndecan-1 was done with mAb 281.2. Resident or thioglycollate-elicited mouse peritoneal macrophages were cultured 9 h on glass coverslips in the absence or presence of 1 mM dibutyryl cAMP, washed, then fixed in 4% formaldehyde. For permeabilization, cells were extracted in 0.2% Triton X-100 for 5 min at room temperature. Cells were blocked in PBS containing 10% bovine serum and incubated 30 min with 25 µg/ml mAb 281.2 or nonspecific rat IgG in the same. After thorough washing, cells were incubated 30 min with FITC-rabbit anti-rat IgG (1:40 in PBS containing 10% bovine serum). After washing fivefold with PBS, coverslips were mounted on glass slides in Immumount (Shandon Southern Instruments, Inc., Sewickley, PA) and viewed on a Nikon Microphot-FX microscope. Cells incubated with nonspecific rat IgG showed no staining. Pictures were recorded on Kodak Tri-X Pan film exposed at ASA 800.

Results

Elicited Peritoneal Macrophages Accumulate Syndecan-1 mRNA

Mouse blood monocytes, resident peritoneal macrophages, and thioglycollate-elicited peritoneal macrophages were screened for syndecan-1 mRNA in an initial effort to determine whether expression of this heparan sulfate proteoglycan is regulated during macrophage activation. Slot blot analysis of poly A⁺ RNA reveals that the syndecan-1 gene is active in all three cell populations (Fig. 1). However, the level of expression is dramatically different. Circulating
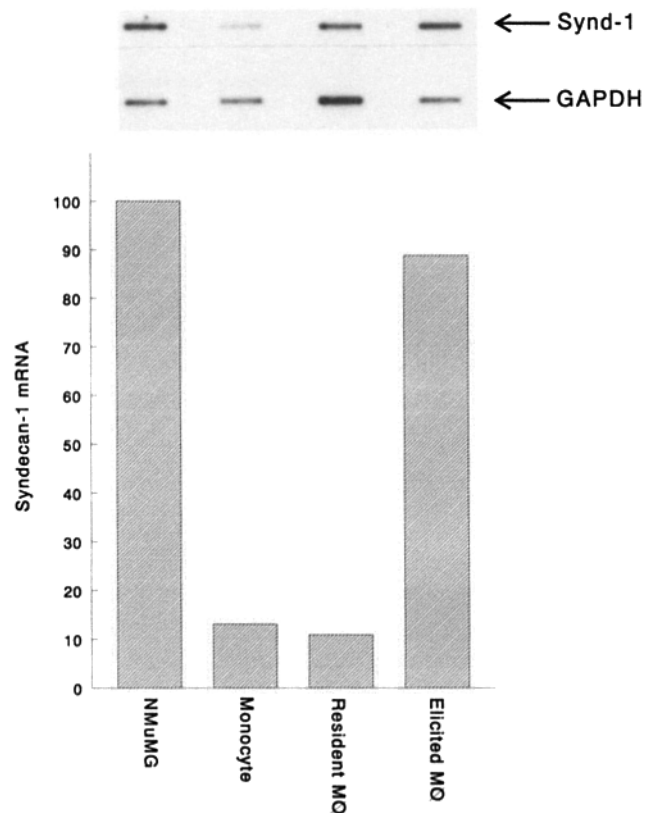

Figure 1. Syndecan-1 mRNA accumulates in thioglycollate-elicited peritoneal macrophages. Approximately 1 µg poly A⁺ RNA from cultured NMuMG cells, blood monocytes, resident and elicited mouse peritoneal macrophages was applied to a nitrocellulose membrane. The blot was probed with a 1.4-kb EcoRI-HindIII fragment of the 4-19b syndecan-1 cDNA and washed in 1 × SSPE plus 0.5% SDS at 60°C. After autoradiography, the blot was stripped, reprobed with a 1.3-kb PstI fragment of the pGPDN5 cDNA encoding glyceraldehyde phosphate dehydrogenase (GAPDH), and washed as above. Scanning densitometry was performed and the relative abundance of syndecan-1 mRNA calculated after correcting for loading differences between samples.

blood monocytes express only low levels of the mRNA. The proteoglycan is also expected to be scarce in these cells, as monocytes from a variety of sources reportedly lack heparan sulfate proteoglycans (Kolset and Gallagher, 1990). The resident peritoneal macrophages also express low levels of the transcript, similar in amount to the peripheral blood monocytes. In contrast, macrophages recruited to the peritoneal cavity during thioglycollate-induced inflammation express ∼ninefold higher levels of syndecan-1 mRNA. Indeed, the elicited peritoneal macrophages accumulate as much syndecan-1 mRNA as NMuMG mammary epithelial cells (Fig. 1), which express ∼10⁶ copies per cell of this heparan sulfate proteoglycan (Rapraeger et al., 1985; Jalkanen et al., 1985). Finally, elevation of syndecan-1 mRNA levels in elicited macrophages reflects the coordinate accumulation of two transcripts of 2.6 and 3.4 kb (Fig. 2). Thus, the macrophages express both mRNA species associated with syndecan-1 production in other cell types.

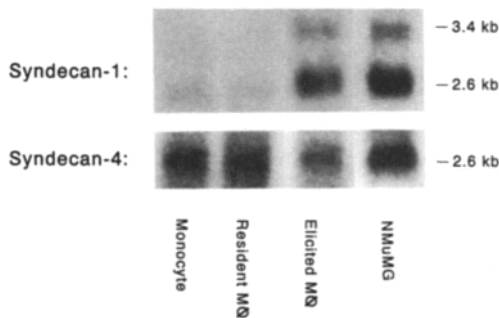The regulation of syndecan mRNA accumulation that accompanies peritoneal macrophage recruitment during in-

Figure 2. Syndecan-1 and Syndecan-4 show distinct patterns of expression during macrophage activation. Total RNAs (5 μg) from cultured NMuMG cells, blood monocytes, resident and thioglycollate-elicited peritoneal macrophages were size-separated, transferred to a nylon filter, and probed with a 1.4-kb EcoRI-HindIII fragment of the 4-19b syndecan-1 cDNA and washed in 1 × SSPE plus 0.5% SDS at 60°C. After autoradiography, the blot was stripped, reprobed with a 368-bp BamHI-EcoRI fragment of the pMB176 syndecan-4 cDNA and washed as above. Sizes of hybridizing RNA molecules were determined by comparison with a 0.24-9.5-kb RNA ladder. This identified two syndecan-1 transcripts, at 3.4 and 2.6 kb, and a single 2.6-kb syndecan-4 transcript.

flammation appears to be specific for syndecan-1. Another member of the syndecan family, syndecan-4, is also expressed by cells of the monocyte-macrophage lineage; several macrophage-like cell lines accumulate syndecan-4 mRNA, and this is the sole detectable heparan sulfate proteoglycan expressed at the surface of P388D1 macrophage-like cells (Yeaman and Rapraeger, 1993). To determine whether primary cultures of monocytes and macrophages express syndecan-4 mRNA and whether its expression mirrors that of syndecan-1, northern analysis for each of these core protein transcripts was performed. In addition to the characteristic syndecan-1 transcripts, blood monocytes and resident peritoneal macrophages accumulate a 2.6 kb syndecan-4 transcript (Fig. 2). This mRNA is also expressed by elicited peritoneal macrophages. However, in contrast to syndecan-1, the abundance of syndecan-4 mRNA remains relatively uniform after macrophage recruitment. After correcting for loading differences between samples, densitometric scanning reveals that the level of syndecan-4 mRNA in the elicited macrophages decreases to 80% of the level in the resident macrophages. However, this occurs as syndecan-1 mRNA undergoes a ∿ninefold increase in the elicited macrophages. Therefore, syndecan-1 is specifically accumulated by elicited peritoneal macrophages, suggesting that expression of individual members of this proteoglycan family are differentially regulated.

Elicited Peritoneal Macrophages Do Not Express Syndecan-1 Proteoglycan

Elicited peritoneal macrophages and NMuMG epithelial cells accumulate similar amounts of syndecan-1 mRNA (Fig. 1). In the NMuMG cells, mRNA expression is coupled to the production of high levels of this proteoglycan, which is located predominantly at the cell surface (Fig. 3; Rapraeger et al., 1985; Jalkanen et al., 1985). We anticipated, therefore, that the elicited macrophages would express high levels of syndecan-1 at the cell surface. Yet attempts to isolate the proteoglycan have shown little, if any, accumulation of this mol-
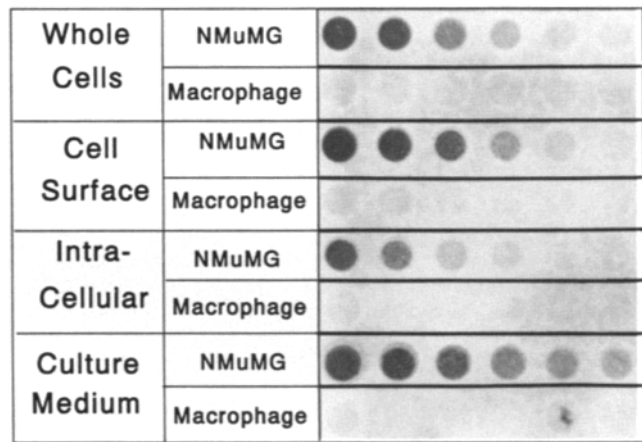

Figure 3. Dot immunoassay for syndecan-1 protein in cultures of NMuMG epithelial cells and thioglycollate-elicited peritoneal macrophages. NMuMG cells and thioglycollate-elicited peritoneal macrophages were cultured for 24 h then extracted in 8 M urea plus 1% Triton X-100 for analysis of syndecan-1 in whole cell lysates. Alternatively, cells were incubated with 25 μg/ml trypsin on ice for 10 min to remove cell surface material before extraction. Serial threefold dilutions of whole cell lysates, trypsin-released material (*cell surface*), material remaining with the cells (*intracellular*), and material accumulated in the culture medium were applied to a cationic nylon membrane. The blot was blocked and probed for syndecan-1 with mAb 281.2 as described in Materials and Methods. The first dot in each row of samples represents material obtained from 10⁶ cells.

ecule in these cells. Macrophage cell lysates are negative in dot immunoassays that detect 100-fold less syndecan-1 than that in the NMuMG cells (Fig. 3). Furthermore, immunostaining of cells permeabilized with Triton X-100 shows that neither syndecan-1 proteoglycan nor nascent core protein is present in the macrophages (Fig. 6 *b*). Finally, the proteoglycan does not accumulate in the culture medium of macrophages, suggesting that it is not rapidly shed from the cell surface (Fig. 3). Thus, although inflammatory recruitment of macrophages into the peritoneal cavity is accompanied by an accumulation of syndecan-1 transcript, the macrophages do not appear to translate this mRNA into protein.

Exogenous dbcAMP Triggers the Expression of Syndecan-1 by Elicited Peritoneal Macrophages

Because elicited peritoneal macrophages fail to express syndecan-1 despite accumulating abundant mRNA, it is possible that the cells require an additional triggering signal for proteoglycan production. A variety of macrophage activation agents and second messenger analogs were screened for the ability to induce syndecan-1 expression in the macrophages (data not shown). This identified dbcAMP as such a trigger. The membrane-permeable cAMP analog induces production of a proteoglycan that reacts with mAb 281.2, which is specific for syndecan-1 (Jalkanen et al., 1985). The macrophage isoform of syndecan-1 migrates in a heterodisperse distribution during SDS-PAGE, with a median mass of ∿100 kD compared to protein standards, and is thus smaller than the 160-kD form expressed by NMuMG epithelial cells (Fig. 4). Syndecan-1 produced by macrophages bears predominantly, if not exclusively, heparan sulfate glycosaminoglycan
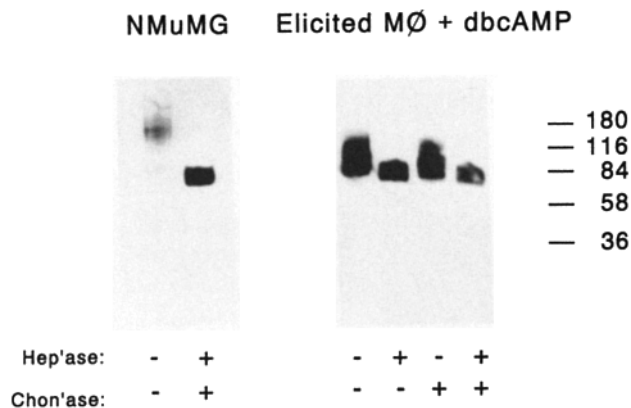
NMuMG   Elicited MØ + dbcAMP

— 180
— 116
— 84
— 58
— 36

Hep'ase: −   +   −   +   −   +
Chon'ase: −   +   −   −   +   +

Figure 4. dbcAMP induces expression of syndecan-1 proteoglycan by elicited peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages were cultured in the presence of 1 mM dbcAMP for 9 h. Proteoglycans were isolated from NMuMG cells and dbcAMP-treated macrophages as described in Materials and Methods. After digestion without or with heparinase III (*Hep'ase*) and/or chondroitinase ABC (*Chon'ase*), samples corresponding to ~4 × 10⁶ cells/lane were fractionated by electrophoresis in SDS-polyacrylamide gradient gels and transferred to a Zeta Probe-GT cationic nylon membrane. The membrane was fixed, blocked, and probed for syndecan-1 with mAb 281.2 as described in Materials and Methods. Nondigested syndecan-1 migrates in a heterodisperse distribution, with a median mass of ~160 kD in NMuMG cells and 100 kD in macrophages, compared with protein standards. Enzymatic digestion of glycosaminoglycan chains reveals the same ~80 kD core protein in each cell type.

chains, as treatment with heparitinase reduces its size to a core protein of ~80 kD, identical in size to the core protein expressed by the NMuMG cells. Treatment with chondroitinase ABC has little, if any, effect on this proteoglycan (Fig. 4). Therefore, treatment of elicited peritoneal macrophages with dbcAMP promotes the expression of syndecan-1 bearing primarily heparan sulfate glycosaminoglycan chains.

To define the optimal conditions to employ dbcAMP, it was desirable to remove the glycosaminoglycan chains from syndecan-1 before electrophoresis because the intact proteoglycan is heterodisperse and difficult to visualize and quantify on immunoblots. Furthermore, the core protein has a defined size which is diagnostic for syndecan-1, especially when detected by mAb 281.2, which is specific for this core protein. Therefore, intact proteoglycan was isolated on DEAE Sephacel beads and treated with heparitinase prior to SDS-PAGE and immunoblotting as described in Materials and Methods.

Dibutyryl cAMP appears to induce the utilization of a stored pool of syndecan-1 mRNA in elicited peritoneal macrophages. Macrophages cultured in the absence of the analog for up to 48 h fail to express detectable amounts of syndecan-1 proteoglycan (not shown). However, macrophages exposed to dbcAMP 2 h after their isolation (Fig. 5) or following 26 h in culture (not shown), are rapidly stimulated to express the proteoglycan. The induction of syndecan-1 expression by dbcAMP is both dose- and time-dependent. Treatment of macrophages with 100 μM dbcAMP induces production of syndecan-1, but the abundance of the proteoglycan increases substantially with tenfold higher concentrations of the analog (Fig. 5 *A*). Because 1 mM dbcAMP is at the upper end
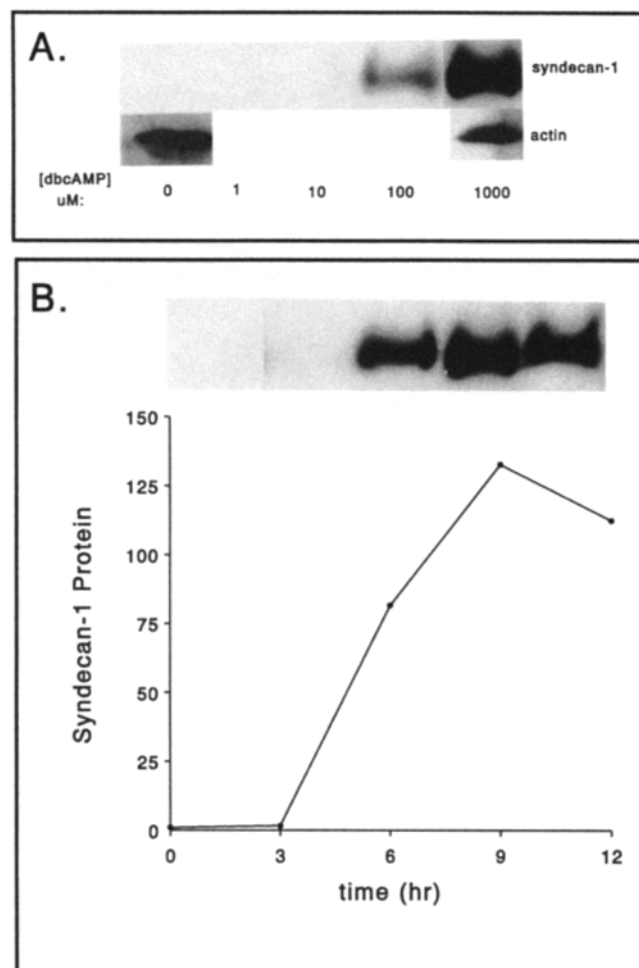

Figure 5. Induction of syndecan-1 expression by dbcAMP is dose- and time-dependent. In *A*, elicited peritoneal macrophages were cultured for 9 h in the presence of increasing concentrations of dbcAMP, as indicated. In *B*, the cells were cultured in the presence of 1 mM dbcAMP for 0, 3, 6, 9, or 12 h. Actin abundance in macrophages cultured 9 h in the absence or presence of 1 mM dbcAMP was assessed by immunoblot analysis with a polyclonal anti-actin antiserum (1:1,000) after electrophoresis of whole cell lysates in 7% acrylamide gels. For quantification of syndecan-1 expression, proteoglycans were isolated from equal numbers of cells (2.5 × 10⁶ cells/lane) and digested with heparinase III and chondroitinase ABC as described in Materials and Methods. Samples were fractionated by electrophoresis in 7% SDS-polyacrylamide gels and transferred to cationic nylon. Membranes were fixed, blocked, and probed for syndecan-1 with mAb 281.2 as described in Materials and Methods. In panel B, scanning densitometric analysis was performed and the relative abundance of syndecan-1 in each lane was calculated. Note that the abundance of actin is unchanged under conditions in which syndecan-1 induction is maximal, indicating that the dbcAMP treatment does not promote a global increase in protein synthesis in the macrophages.

of concentrations employed in cell regulation studies, higher concentrations were not tested. However, this concentration induces syndecan-1 expression on macrophages to levels similar to those observed on NMuMG cells (cf. Fig. 4).

Kinetic analysis of syndecan-1 induction reveals that the abundance of the proteoglycan increases in a linear fashion, after a 3-h lag, and reaches maximal levels 9 h after addition
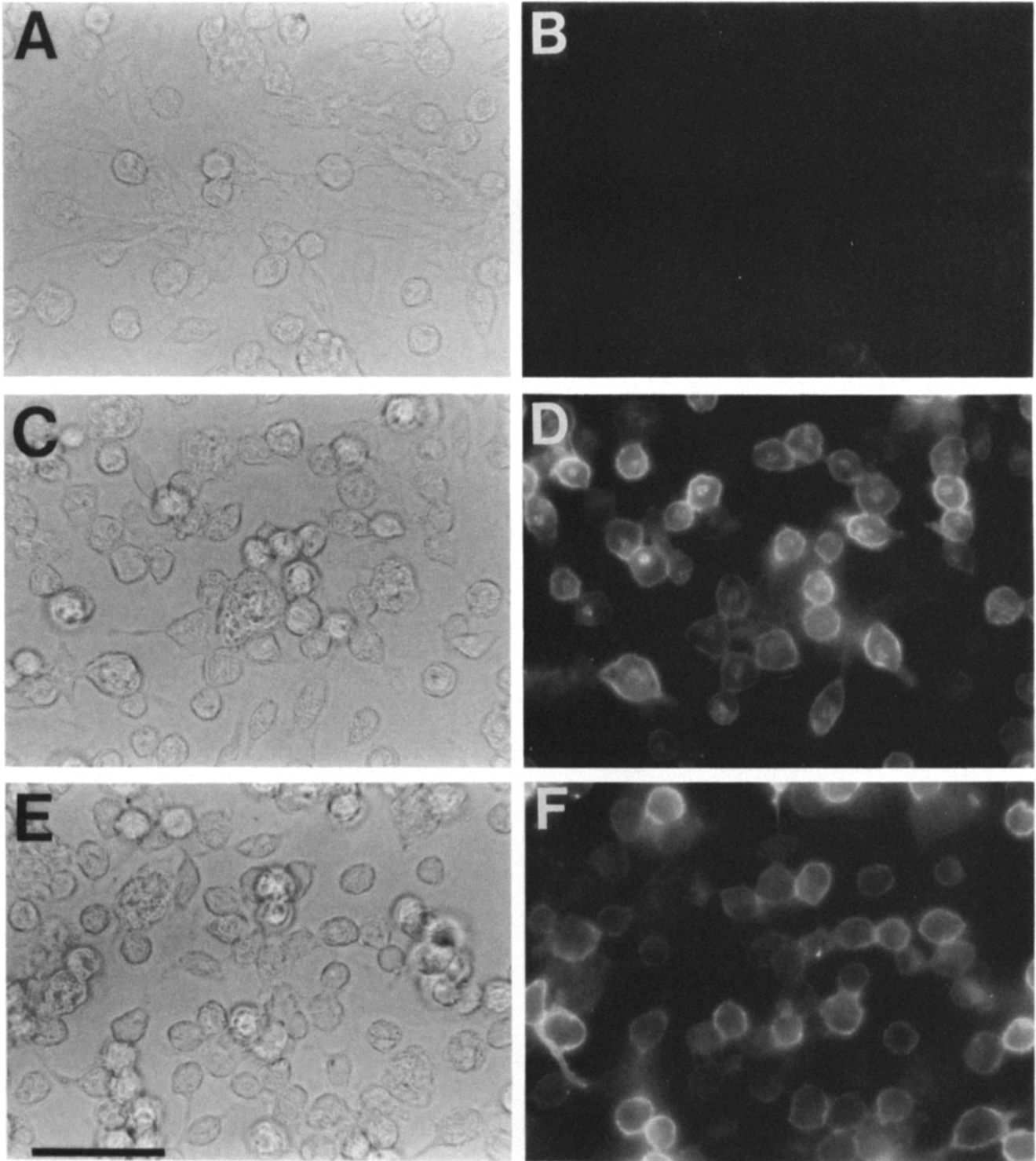
Figure 6. Immunolocalization of syndecan-1 with mAb 281.2. Thioglycollate-elicited mouse peritoneal macrophages were cultured for 9 h in the absence (*A*, *B*) or presence (*C–F*) of 1 mM dbcAMP. Cells were fixed in PBS containing 4% formaldehyde. In *A–D*, cells were permeabilized with 0.2% Triton X-100 for 5 min after fixation. Syndecan-1 was stained in *B*, *D*, and *F* by incubation of cells with 25 μg/ml mAb 281.2 followed by a 1:40 dilution of FITC-conjugated rabbit anti-rat IgG. Note that syndecan-1 stains intensely around the perimeter of dbcAMP-treated macrophages, but is also detected in an intracellular location in panel *d*, possibly reflecting proteoglycan in the Golgi apparatus. Controls incubated with 25 μg/ml non-specific rat IgG showed no staining (not shown). Corresponding bright field micrographs are shown in *A*, *C*, and *E*, respectively. Bar, 50 μm.

of 1 mM dbcAMP to the culture (Fig. 5 B). Macrophages exposed to dbcAMP for 12 h reproducibly show reduced expression of syndecan-1 compared with cells stimulated for 9 h (Fig. 5 B). Therefore, for routine induction of syndecan-1 expression, elicited peritoneal macrophages are cultured for 9 h in the presence of 1 mM dbcAMP.

To determine the distribution of syndecan-1 in the macrophage cultures, cells were incubated for 9 h in the absence or presence of 1 mM dbcAMP and immunostained with mAb 281.2 (Fig. 6). This verified that elicited macrophages fail to express the proteoglycan unless they are triggered with the cAMP analog (compare Fig. 6, B with D). Macrophages cultured in the presence of dbcAMP show intense staining for syndecan-1 which is distributed primarily around the perimeter of the cell (Fig. 6, D and F). Permeabilization with 0.2% Triton X-100 reveals additional syndecan-1 located in a perinuclear distribution, presumably reflecting the Golgi apparatus (Fig. 6 D). However, the bulk of the proteoglycan is visible in nonpermeabilized cells (Fig. 6 F), indicating that it is located at the cell surface. Furthermore, no cell is visible that shows intracellular staining that does not also show cell surface staining. This indicates that syndecan-1 is not stored intracellularly in the macrophages.

Dibutyryl cAMP promotes the expression of syndecan-1 in most, but not all of the macrophages in the culture (compare Fig. 6 C with D). Roughly 25% of the cells fail to express syndecan-1. Because cultures are typically 95% pure as assessed by Mac-1 staining, the cells which fail to express syndecan-1 likely reflect a sub-population of macrophages which does not respond to the inductive signal employed here. These may be resident macrophages which were present in the peritoneal cavity before the inflammatory recruitment or a subset of elicited macrophages that does not express syndecan-1 in response to dbcAMP.

Stimulation of syndecan-1 expression by dbcAMP is specific for elicited peritoneal macrophages. Resident peritoneal macrophages do not respond. As expected, these cells, which lack a substantial pool of syndecan-1 mRNA, fail to express detectable levels of this proteoglycan (Fig. 7 A). Following exposure to 1 mM dbcAMP for 9 h, these cells remain negative for syndecan-1 expression (Fig. 7 B). Prolonged exposure to dbcAMP for 18, 24, or 36 h also fails to induce syndecan-1 expression (data not shown). This suggests that dbcAMP affects primarily the utilization of stored mRNA in the elicited peritoneal macrophages, rather than stimulating transcription of novel mRNA.

Syndecan-1 Is Induced by Agents that Raise Intracellular cAMP Levels

The effect of dbcAMP suggests that agents that cause an elevation of intracellular cAMP specifically induce syndecan-1 expression. This was verified by culturing elicited peritoneal macrophages in the presence of cholera toxin and forskolin, which raise cAMP concentrations in the cell by different mechanisms. Both reagents induce syndecan-1 expression (Fig. 8). In contrast, dbcGMP, though chemically similar to dbcAMP, fails to promote syndecan-1 expression. Furthermore, stimulation of protein kinase C by the phorbol ester PMA also fails to trigger production of this proteoglycan (Fig. 8). Therefore, the expression of cell surface syndecan-1 appears tightly regulated by agents that raise intracellular cAMP levels.
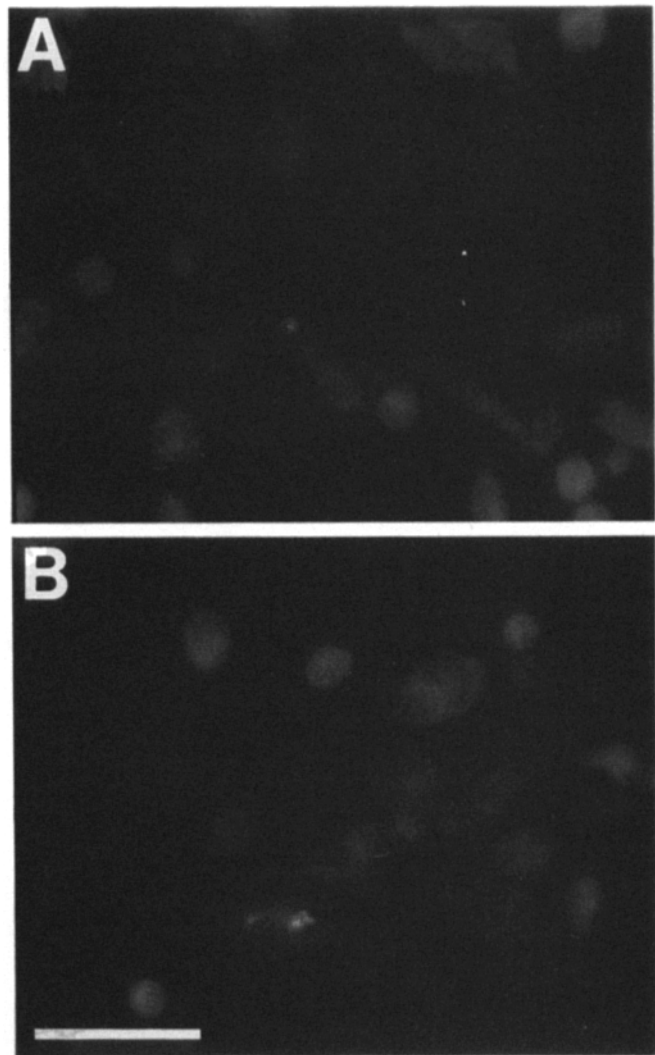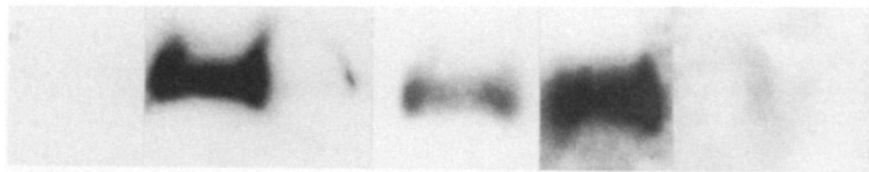

Figure 7. Resident peritoneal macrophages do not express syndecan-1 in response to dbcAMP. Resident mouse peritoneal macrophages were cultured for 9 h in the absence (A) or presence (B) of 1 mM dbcAMP. Cells were fixed in PBS containing 4% formaldehyde. Syndecan-1 expression was evaluated by incubation of nonpermeabilized cells with 25 μg/ml mAb 281.2 followed by a 1:40 dilution of FITC-conjugated rabbit anti-rat IgG. Note that resident peritoneal macrophages, which lack a large pool of syndecan-1 mRNA, fail to express syndecan-1 in response to dbcAMP. Controls incubated with 25 μg/ml nonspecific rat IgG appear similar to those incubated with mAb 281.2 (not shown). Bar, 50 μm.

Elicited Peritoneal Macrophages Express Syndecan-1 in Response to Prostaglandins

To determine whether syndecan-1 expression is induced by physiologically relevant agents, elicited peritoneal macrophages were cultured in the presence of E-type prostaglandins. Macrophages produce and respond to prostaglandins in vivo (Zwilling and Justement, 1988). Furthermore, the effects of E-type prostaglandins on macrophage activities are believed to be mediated through a cAMP signaling pathway (Zwilling and Justement, 1988). Therefore, it is not surprising that prostaglandin E2 stimulates syndecan-1 proteoglycan expression by elicited peritoneal macrophages in a dose-dependent manner (Fig. 9 A). The inductive effect of this

Figure 8. Syndecan-1 expression by elicited peritoneal macrophages is specifically induced by pharmacological agents that raise intracellular cAMP levels. Thioglycollate-elicited mouse peritoneal macrophages were cultured for 9 h in the absence or presence of 1 mM dbcAMP or 1 mM dbcGMP or for 12 h in the presence of cholera toxin (10 nM), forskolin (100 μM), or the phorbol ester PMA (100 nM). For analysis of syndecan-1 expression, proteoglycans were isolated from equal numbers of cells (2.5 × 10^6 cells/lane) and digested with heparinase III and chondroitinase ABC as described in Materials and Methods. Samples were fractionated by electrophoresis in 7% SDS-polyacrylamide gels and transferred to cationic nylon. Membranes were fixed, blocked, and probed for syndecan-1 with mAb 281.2 as described in Materials and Methods. A range of PMA concentrations (10–1000 nM) failed to stimulate syndecan-1 expression by these cells (not shown).

agent can be seen at concentrations as low as 10 nM, but increasing concentrations, up to 10 μM, promote increasingly higher levels of syndecan-1 expression. This effect is not restricted to prostaglandin E2. Treatment of macrophages with prostaglandin E1 (1 μM) induces syndecan-1 expression as effectively as the equivalent concentration of prostaglandin E2 (Fig. 9 *a*). In contrast, potent macrophage activation agents such as interferon-γ, added alone or in combination with LPS, fail to induce expression of syndecan-1 (Fig. 9 *B*). Therefore, macrophages are not stimulated to express this proteoglycan by all activating agents. Rather, the stimulation appears to be specific for a certain class of mediators, the E-type prostaglandins, that elevate intracellular cAMP levels.

Discussion

Syndecan-1 is expressed on a variety of cell types and has been proposed to be regulated at both the mRNA and protein levels (reviewed by Bernfield et al., 1992). In this study, maturing macrophages were employed as a model system to begin investigating the mechanisms controlling syndecan-1 expression. Macrophages responding to an inflammatory signal in the peritoneal cavity express abundant syndecan-1 mRNA compared to resident peritoneal macrophages and blood monocytes. This transcript is not utilized, however, suggesting the presence of a post-transcriptional block. Expression of this heparan sulfate proteoglycan is triggered by agents that increase intracellular cAMP levels. These include
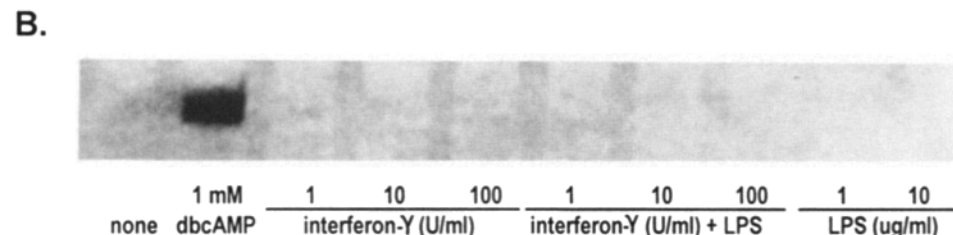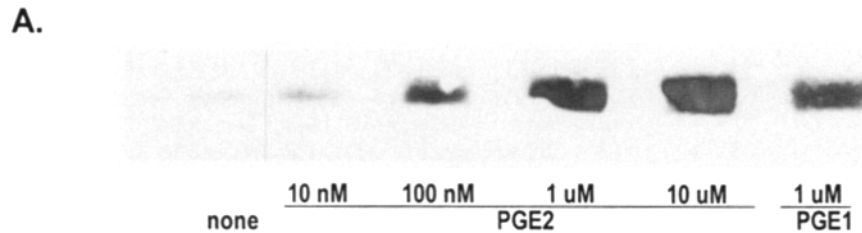
A.

B.

Figure 9. E-type prostaglandins induce syndecan-1 expression by elicited peritoneal macrophages. Thioglycollate-elicited mouse peritoneal macrophages were cultured for 12 h in the absence or presence of dbcAMP, prostaglandin E2 (PGE2), prostaglandin E1 (PGE1), LPS or interferon-γ at the concentrations indicated. Some cells were cultured in the presence of increasing concentrations of interferon-γ plus LPS (10 μg/ml). For analysis of syndecan-1 expression, proteoglycans were isolated from cell lysates and digested with heparinase III and chondroitinase ABC as described in Materials and Methods. Samples were fractionated by electrophoresis in 7% SDS-polyacrylamide gels and transferred to cationic nylon. Membranes were fixed, blocked, and probed as described in Materials and Methods.

pharmacological agents, such as dbcAMP, forskolin, and cholera toxin, and physiologically relevant agents, such as prostaglandins E1 and E2. The mechanism controlling syndecan-1 expression in the macrophage may serve to regulate the expression of this proteoglycan in other tissues, such as the embryonic kidney mesenchyme, where the accumulation of syndecan-1 mRNA and proteoglycan is uncoupled.

Syndecan-1 and Syndecan-4 mRNAs Accumulate Independently During Macrophage Activation

Syndecan-1 expression during peritoneal macrophage activation is regulated at two levels: (a) accumulation of mRNA and (b) synthesis of the proteoglycan core protein. The macrophages that invade the peritoneal cavity during inflammation express ninefold higher levels of syndecan-1 mRNA than the population of macrophages that resides in the cavity during homeostasis. Because blood monocytes contain only low levels of the transcript, it is likely that invading macrophages encounter a signal during their extravasation into the injured tissue which promotes the accumulation of this mRNA. The nature of such a signal, as well as the mechanism responsible for elevating syndecan-1 mRNA levels are presently unknown, but may involve regulation at the level of transcription or mRNA stability.

Although syndecan-1 mRNA levels in elicited macrophages are an order of magnitude higher than those in resident macrophages, we do not detect a corresponding increase in syndecan-4 mRNA after inflammatory macrophage recruitment. The levels of this transcript are roughly the same in monocytes, resident, and elicited peritoneal macrophages. Furthermore, treatment of the elicited macrophages with dbcAMP results in a ∼threefold increase in the abundance of syndecan-1 mRNA in elicited peritoneal macrophages, but has no effect on the level of syndecan-4 transcript in these cells (unpublished data). These results imply that (a) although the macrophages already express at least one heparan sulfate proteoglycan, they specifically induce syndecan-1, which likely serves an important role on these cells at a defined point in their development; and (b) different members of the syndecan family of proteoglycans are regulated by distinct mechanisms.

A Post-transcriptional Mechanism Regulates Syndecan-1 Expression in Peritoneal Macrophages

Despite the expression of abundant syndecan-1 mRNA, however, the proteoglycan is not detected in elicited macrophages. One explanation for this finding is that syndecan-1 may be rapidly metabolized, via shedding from the cell surface or internalization and degradation. However, syndecan-1 is not recovered in the conditioned medium of macrophages. If it were degraded, it would have to be extremely rapid. On NMuMG cells, syndecan-1 exhibits a half-life of ∼6 h (unpublished data). Because macrophages accumulate at least 100-fold less syndecan-1 than NMuMG cells, the proteoglycan would have to exhibit a turnover time of <5 min to account for its absence from these cultures if the translational efficiency of syndecan-1 mRNA were the same in these two cell types. Thus, it is likely that macrophages simply do not translate the syndecan-1 mRNA.

The syndecan-1 transcript that accumulates in macrophages during inflammatory recruitment appears to be a latent pool of mRNA awaiting a signal to trigger its utilization. In this respect, the macrophage is analogous to uninduced mesenchymal cells during kidney development, which appear to store syndecan-1 mRNA and utilize it for proteoglycan expression only after induction by the metanephric duct (Vainio et al., 1992). Elicited macrophages also store syndecan-1 mRNA. Comparison with NMuMG cells, which are noted for expressing high levels of syndecan-1, reveals a similar accumulation of this transcript in the two cell types, yet the macrophages fail to express the proteoglycan. Furthermore, the macrophages can be cultured for at least 48 h without detectable expression of syndecan-1 proteoglycan (unpublished data). However, within 3–6 h after exposure to dbcAMP, the proteoglycan accumulates in macrophages to a level approaching that in NMuMG cells. This suggests that the utilization of syndecan-1 mRNA for proteoglycan production is rapidly stimulated by elevation of intracellular cAMP levels.

The mechanism by which dbcAMP induces syndecan-1 expression is not known, but is likely to involve activation of cyclic AMP-dependent protein kinases (PKA) rather than transcriptional activation of syndecan-1. Northern analysis reveals a ∼threefold increase in syndecan-1 mRNA levels in macrophages following a 12 h exposure to dbcAMP (unpublished data). However, the abundance of the proteoglycan increases more than 100-fold in response to dbcAMP, suggesting that the analog acts primarily at a post-transcriptional level. Furthermore, resident peritoneal macrophages, which lack a pool of stored mRNA, do not respond to dbcAMP. It is tempting to speculate that the 5'-untranslated region of the syndecan-1 mRNA regulates the translation of this transcript. A similar mechanism has been proposed for the regulation of ornithine decarboxylase mRNA translation (Grens and Scheffler, 1990; Manzella and Blackshear, 1990). The 5'-untranslated region of syndecan-1 mRNA is 70% G/C-rich (Saunders et al., 1989) and is predicted to form stable secondary structures, which may impede scanning by the 40S ribosomal subunit (Kozak, 1991). Elevation of intracellular cAMP levels and subsequent stimulation of cAMP-dependent protein kinases may activate a "translation factor," which serves to melt secondary structures in the syndecan-1 mRNA, thus allowing the initiation of translation.

Syndecan-1 expression in other tissues also shows a high level of regulation. For instance, syndecan-1 is temporally regulated during differentiation of B lymphocytes (Sanderson et al., 1989). Stratifying keratinocytes change both the structure and abundance of the proteoglycan, although the level of syndecan-1 mRNA does not change (Sanderson et al., 1992a). Syndecan-1 exhibits temporal as well as cell-type specific regulation during epithelial-mesenchymal interactions in development (Solursh et al., 1990; Vainio et al., 1991, 1992). The proteoglycan is transiently lost from the epithelium as it changes shape, but regained when this tissue becomes morphologically stable. Syndecan-1 is also induced in the proliferating mesenchyme. In the developing kidney this appears to involve post-transcriptional activation of stored mRNA (Vainio et al., 1992). The regulation of syndecan-1 during macrophage development appears also to involve the accumulation and post-transcriptional activation of latent mRNA. Therefore, the molecular mechanisms responsible for syndecan-1 regulation in the macrophage may reflect a widespread process controlling the expression of this molecule in a variety of cell and tissue types.

Note Added in Proof: Kirjavainen et al. have recently shown that syndecan-1 expression is translationally suppressed in Ha-*ras*-transformed epithelial cells (Kirjavainen, J., S. Leppä, N. E. Hynes, and M. Jalkanen. 1993. *Mol. Biol. Cell.* In press).

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