Research report

SC1, A SPARC-related glycoprotein, exhibits features of an ECM component in the developing and adult brain

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Accepted 14 November 1995

Abstract

Although extracellular matrix (ECM) components have been shown to play important roles in the development of the CNS, expression generally decreases in the adult brain. This study examines the expression of the SPARC-related glycoprotein SC1 in the rat brain during postnatal development and in the adult. In situ hybridization analysis indicates that expression of SC1 mRNA increases in a caudal to rostral manner as postnatal neural development proceeds and is found at near maximal levels in the adult brain. SC1 mRNA is expressed in glial-enriched areas of the brain at postnatal day 1 (P1) and P5. Between P10 and P20, SC1 mRNA increases in neuron-enriched regions of the hippocampus, dentate gyrus, and cerebral cortex. Immunohistochemistry in the adult shows that SC1 protein is localized to neurons in these regions and to scattered glial cells. Subcellular fractionation demonstrates that the SC1 116/120 kDa doublet is associated with synaptosomes. SC1 is present in the aqueous phase following extraction of membranes with TX-114, suggesting that it is not a transmembrane protein, a property consistent with other adult brain ECM components. Furthermore, in cerebellar granule cells grown in culture, high levels of the 120 kDa component are secreted into the media. These results are consistent with the hypothesis that SC1 is an ECM glycoprotein expressed in both the developing and adult brain.

Keywords: Anti-adhesive glycoprotein; Extracellular matrix; Follistatin module

1. Introduction

Recent studies have shown that inhibitory phenomena are important in neural development. For example, inhibitory glycoproteins have been implicated in the formation of barriers to growth by repelling the extension of neurites [4,7,8,28,33,38,44]. During neural development, extracellular matrix (ECM) components influence events including cell migration, process outgrowth, and cell differentiation by modulating adhesive and anti-adhesive interactions [46]. Although levels of many ECM molecules decrease substantially following neural development [35,40,42], some anti-adhesive molecules such as SPARC, tenascin, and the NG2 chondroitin proteoglycan are associated with the extracellular matrix in the adult brain [3,8,30,32]. It has been suggested that matrix components in the adult brain regulate adhesive events at synaptic contacts as well as playing a role in the prevention of further neurite outgrowth [19].

SC1 is a putative ECM glycoprotein which is expressed during postnatal development and in the adult brain [23]. It shows partial sequence homology with the extracellular matrix glycoprotein SPARC/osteonectin [23]. SPARC has been shown to be anti-adhesive and modulate the expression and interaction of a wide range of matrix components and growth factors [25]. We have recently shown that SPARC is expressed during neural development and is present in regions high in synaptic contacts in the mature CNS [30,32]. Due to the homology between these two molecules, they may share common properties.

SC1 contains a module that is also present in follistatin, agrin, hevin and SPARC [15]. Follistatin has been shown to induce neural differentiation [15,16], while agrin has been implicated in the formation of neuromuscular junctions in the peripheral nervous system [6,18,29]. Hevin (the putative human homologue of SC1) has been cloned from endothelial cells with unique adhesive properties, and shown to be expressed at high levels in adult brain [12]. Expression of these related molecules in the developing and adult brain suggests that they may be members of a new class of ECM molecules which play roles in the
mature nervous system, possibly associated with synapse stability. It is noteworthy that SC1 was cloned by screening a library with an antibody against synaptic junctions [23].

The present investigation examines the pattern of expression of SC1 mRNA and protein during the postnatal development of the rat brain. We demonstrate that SC1 exhibits a glial-enriched pattern of expression early in development, but is later expressed predominantly in neurons. Interestingly, SC1 is strongly expressed in regions that are rich in synaptic contacts such as the molecular layers of the cerebellum and hippocampus. Our results show that SC1 is associated with synaptic membranes but is not a transmembrane glycoprotein. In vitro experiments demonstrate that SC1 is secreted, as is suggested by its primary structure [23]. These results indicate that SC1 exhibits properties of a neural ECM glycoprotein which is expressed in both the developing and adult brain.

2. Materials and methods

2.1. Preparation of tissue

Wistar Rats ranging in age from postnatal day 1 to 60 (P1 to P60) were anaesthetized with 50 mg/kg pentobarbitol and perfused intracardially with 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS. Brains were removed and mounted in OCT embedding medium. Preparation of tissue for in situ hybridization and immunohistochemistry were identical. Frozen sections (10 µm for in situ hybridization; 25 µm for immunohistochemistry) were cut, floated on distilled water, collected on gelatin coated slides, and allowed to air dry as described by Mendis et al. [31].

2.2. In situ hybridization

In situ hybridization was carried out as described by Mendis et al. [31]. Sections were probed with 35S-labelled antisense or sense riboprobes transcribed in vitro from a 295 bp HindIII-EcoRI fragment of the SC1 cDNA inserted into the vector pGEM-3Z [23]. Following hybridization and washing, slides were exposed to Kodak NTB2 liquid emulsion for autoradiography for 4 weeks. Sections were subsequently stained with cresyl violet and viewed using both lightfield and darkfield microscopy. Experiments were repeated six times.

2.3. Immunohistochemistry

Following cutting and drying, sections were rehydrated in 0.1 M PBS containing 0.2% v/v Triton X-100 and 0.1% BSA for 20 min. Blocking occurred in the same buffer containing 1.5% horse serum for 30 min. Sections were incubated for 16 h in anti-SC1 E/P II antibody [23] diluted 1:300 in blocking solution. Slides were washed for 3 × 10 min in PBS and incubated for 1 h in biotinylated, affinity purified, immunoglobulin, diluted 1:500 (Vectorstain). Following washing, endogenous peroxidase activity was blocked by incubation with 3% H2O2 for 3 min. Immunoreactivity was visualized using diaminobenzidine according to manufacturer's instructions (Vectorstain Labs). Results are representative of six repeats.

2.4. Preparation of samples for Western blot analysis

Rat hippocampal tissue from various ages was homogenized in 0.32 M sucrose containing 1 mM MgCl2 at 4°C, and protein quantitation of each sample was determined by the method of Lowry et al. [27]. Synaptosomes were prepared from 5-week-old rat forebrains as previously described [9,47]. Membrane fractions were isolated and partitioned into soluble and particulate fractions according to Ellis et al. [10]. Triton X-114 extractions were performed according to Paladino et al. [36]. Western blots were performed with the anti-SC1 E/P II antibody as previously described [31].

2.5. Cerebellar primary cultures

Granule cells were obtained from the cerebella of litters of 9-day-old rats, according to Thangnipon et al. [45] as modified by Paladino et al. [36]. Cells were counted using trypan blue exclusion and plated at a density of 3 × 105 cells in 2 ml basal Eagle's medium supplemented with 2 mM glutamine, penicillin (0.5 units/ml), streptomycin (0.05 µg/ml), 25 mM KCl, and 10% fetal calf serum. Time 0 days in vitro (0 DIV) was considered to be 3 h after plating at which point cells had adhered to the surface of the culture dish.

Culture media was collected by aspiration and centrifuged at 1000 × g for 10 min to remove any cells that might have been present. Cells from one well or media (25 µl from the 2 ml total volume) were processed for Western blotting with the anti-SC1 E/P II antibody and visualized as previously described [31]. Actin was detected with an anti-actin antibody (Amersham) at a 1:10,000 dilution.

Western blots were scanned using densitometry and the areas under the peaks corresponding to SC1 were determined. All samples analyzed were within the linear range of the colour development system. Levels of SC1 are expressed relative to the value for the cell fraction at time 0. Results presented are representative of six separate granule cell cultures.

3. Results

3.1. Distribution of SC1 mRNA during postnatal development of the rat brain

Sagittal brain sections were hybridized with an SC1 35S-labelled antisense riboprobe [31] and mRNA distribu-
tion visualized by darkfield microscopy at the indicated stages of postnatal development (Fig. 1). At postnatal day one (P1), SC1 mRNA expression was low in rostral regions such as the hippocampus (Hp) and cerebral cortex (Cc) with higher levels detectable in caudal regions such as the cerebellum (Cb), and brainstem (Bs). The overall pattern of mRNA expression at this age is similar to the related SPARC glycoprotein, which has been shown to be expressed by astrocytes [30,31]. Cells lining the ventricles (V) in germinal zones express SC1 mRNA at P1, P5 and P10 but this pattern was not detected at later ages. In the brainstem, expression increased to P15, after which SC1 mRNA became localized to discrete foci such as the trapezoid nucleus (Tz).

At P5, higher expression was noted in midbrain (Mb) as well as the hippocampus and cerebral cortex. Levels of

Fig. 1. In situ hybridization of SC1 mRNA distribution during postnatal development of the rat brain. Sagittal brain sections taken at postnatal day 1 (P1) through to postnatal day 20 (P20) and adult, were hybridized with 35S-labelled SC1 antisense riboprobe as described in Section 2. Photographic emulsion was exposed for 4 weeks and viewed using darkfield microscopy to visualize the distribution of SC1 mRNA. Bs, brain stem; Cb, cerebellum; Cc, cerebral cortex; Hp, hippocampus; Mb, midbrain; P1 to P20, postnatal age in days; Tz, trapezoid nucleus; V, ventricle. Bar = 1 mm.
SCI mRNA continued to increase in these rostral regions by P15 and reach maximal levels by P20. At this stage, expression was apparent in neural-enriched layers of the hippocampus and cerebral cortex. While levels in these regions decreased slightly in the adult brain, expression remained high in the cerebellum [23,31]. In contrast to the expression in neuronal-enriched regions of the forebrain, we have previously shown that the Bergmann glia expressed SCI at high levels in the adult cerebellum [31]. Fig. 1 demonstrated that levels of SCI increased in a caudal to rostral pattern as development proceeds, reaching maximal levels at P20.

### 3.2. SCI mRNA localization during hippocampal development

SCI mRNA levels were spatially and temporally regulated in the developing hippocampus as seen in Fig. 2 (left panels - cresyl violet staining for cellular detail, right panels - SCI mRNA distribution). At postnatal days 1 and 5 (P1 and P5), SCI mRNA was localized to giall-enriched regions such as the hippocampal fissure (HF) and ventricular surface (V), with low amounts detected over neuron-enriched regions of the dentate gyrus (DG) and CA1 to CA4 regions of the hippocampus. This pattern of expression is similar to that reported for the astrocyte marker GFAP [24]. As development proceeded, levels of SCI mRNA decreased along the gli-enriched ventricular surface (V) and hippocampal fissure (HF), and increased in the neuronal-enriched CA1 and CA2 regions of the hippocampus by P10 and P15 and then by P20 in the CA3, CA4, and dentate gyrus (DG). In the adult, low amounts of SCI mRNA were associated with the ventricular surface or the hippocampal fissure with higher amounts found over neuron-rich regions of the CA1 to CA4 and dentate gyrus. Low levels of SCI mRNA were detected in the molecular layer (ML), suggesting that SCI is also expressed by some glial cells within the adult hippocampus.

SCI was expressed in a glial-enriched pattern in the hippocampus at birth. As postnatal development proceeds, SCI mRNA decreased in gli-enriched regions and increased in neuronal enriched regions, a pattern which was retained in the adult.

### 3.3. Cellular distribution of SCI protein in the adult hippocampus and cerebral cortex

Immunohistochemical studies were undertaken to determine the adult expression of SCI protein (Fig. 3). In agreement with the in situ hybridization results (Fig. 2), SCI immunoreactivity was observed in pyramidal neurons of the hippocampus (H) and granule neurons of the dentate gyrus (DG) (Fig. 3A). At higher magnification in the dentate gyrus (Fig. 3B), SCI protein was seen in neuronal cell processes (indicated by longer arrows), as well as in scattered glial cells (G) in the molecular layer (ML). In the adult cerebral cortex (Fig. 3C), SCI protein was observed in neuronal cell bodies and processes (N) and in smaller scattered glial cells (G).

### 3.4. Developmental changes in SCI protein in the hippocampus

A Western blot analysis was undertaken to examine changes in SCI levels in the developing hippocampus between postnatal day 1 and 60 (Fig. 4). Maximal levels of SCI protein were reached at P10 and maintained until P30. Levels of the protein then decreased at postnatal day 60. The increased level of SCI protein between P10 and P30 coincides with the appearance of SCI mRNA in neuronal-enriched regions of the developing hippocampus which can be seen in Fig. 2. As previously shown, SCI is expressed as a 116/120 kDa doublet in many regions of the rat brain [31]. In that study, the 120 kDa component was found at higher levels in the hippocampus compared to the 116 kDa component.

### 3.5. Association of SCI protein with synaptic fractions

SCI was initially isolated by screening an expression library with a polyclonal antibody raised against synaptic junctions [23]. In order to determine if SCI was associated with synapses, its presence in synaptosomes isolated from total forebrain homogenates was assessed. These structures were then separated into particulate and soluble fractions, and examined using Western blotting (Fig. 5A). The 116/120 kDa SCI doublet was observed in total synaptosomes (lane 1). Both SCI components were also present in the particulate fraction (lane 2), while only the 120 kDa component was found in the soluble fraction (lane 3). The presence of SCI in the particulate fraction suggests that SCI is associated with synaptic membranes.

### 3.6. Phase partitioning of SCI protein in Triton X-114

Phase partitioning of a forebrain P3 microsomal fraction was used to determine if SCI is an integral membrane protein (Fig. 5B). SCI immunoreactivity was not detected in the lipid (detergent) extract phase (L) but was observed in the aqueous phase (A). This suggests that SCI, while

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Fig. 2. Distribution of SCI mRNA in the developing hippocampus. Tissue sections at the indicated stages of postnatal development were hybridized with 35S-labelled SCI antisense riboprobe. Emulsion was exposed for 4 weeks and viewed using darkfield microscopy to visualize SCI mRNA (right panels) while cresyl violet staining shows cellular detail (left panels). CA1 to CA4, regions of the hippocampus; DG, dentate gyrus; HF, hippocampal fissure; ML, molecular layer; P1 to P20, postnatal age in days; V, ventricle. Bar = 250 μm.
associated with neural membranes, is not an integral membrane protein. This property is consistent with other neural extracellular matrix components [17].

3.7. Expression of SC1 by cerebellar granule cells grown in primary culture

In order to determine if SC1 might be secreted, as would be expected if it is a component of the ECM, we examined levels of the SC1 protein in cell and media fractions collected from cultures of cerebellar granule cells (Fig. 6). Samples were collected at 3-day intervals after initial plating. As shown in Fig. 6A, the SC1 protein was secreted into the media and the amount present in the media increased over the 9-day period in culture (right panel). Levels of SC1 associated with the granule cells showed comparatively little change over the time-period examined (left panel). Both the 116 and 120 kDa SC1 protein components were present in the cell fraction, while only the 120 kDa component was secreted and accumulated in the media. Previous studies have shown that the two SC1 protein components have different carbohydrate moieties associated with them [23].

Fig. 4. Developmental changes in SC1 protein in the hippocampus. Homogenates were prepared from rat hippocampus at various stages of postnatal development. Aliquots (20 μg) were resolved on a 10% polyacrylamide gel and analyzed by Western blotting. Numbers beneath each lane refer to postnatal age in days. The scale on the left indicates the position of the molecular mass markers in kDa.

Fig. 5. Panel A: association of SC1 protein with synaptic fractions. Western blot analysis of SC1 protein distribution in forebrain synaptosomes (lane 1) and following partitioning into synaptosome particulate (lane 2) and soluble fractions (lane 3). Panel B: phase partitioning of SC1 protein in Triton X-114. A P3 microsomal fraction was isolated from total forebrain homogenates. SC1 immunoreactivity was detected in the P3 fraction (P3) and following partitioning in the aqueous phase (A) but not in the lipid (detergent) extract phase (L).

Fig. 3. Cellular localization of SC1 protein in the adult hippocampus and cerebral cortex. Sagittal cryostat sections (20 μm) were processed for immunohistochemistry as described in Section 2. Panel A: adult hippocampus, bar = 1 mm. Panel B: granule cell layers of the dentate gyrus in adult hippocampus, bar = 50 μm. Panel C: pyramidal cell layer of the adult cerebral cortex, bar = 50 μm. DG, dentate gyrus; G, glia; H, hippocampus; ML, molecular layer; N, neuron.
Densitometry was performed to determine relative levels of SC1 protein associated with the cell fraction versus that present in the media. As seen in Fig. 6B, after 9 days in culture, the amount of SC1 protein in the media was 40-times that associated with the cell fraction. The possibility that cell rupture contributed to the SC1 protein found in the media was examined by testing for the presence of actin (Fig. 6C). Actin is known to exist in equilibrium between soluble and polymerized states. Western blot analysis with an anti-actin antibody detected a single band in cerebellar homogenates (lane 1) and the cell fraction at nine days in culture (lane 2). Actin immunoreactivity was not detected in the media (lane 3), even when this fraction was concentrated ten times (lane 4). Thus, the presence of

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**Fig. 6.** Western blot analysis of SC1 protein levels in cell and media fractions of cerebellar granule cells grown in primary culture. Cultures were harvested at 3-day intervals after the initial plating and separated into cellular and media fractions. A: aliquots were processed for Western blot analysis on 8% polyacrylamide gel as described in Section 2. B: quantitation of the relative levels of SC1 protein in cell versus media fractions. Western blots, similar to that shown in panel A, were subjected to densitometry, and the area under the peak corresponding to SC1 calculated. Results are expressed relative to the SC1 levels present in the cell fraction at the initial plating. Measurements of the media fractions were multiplied by a factor of 40 in order to indicate the relative amount found in the total media relative to the cell fraction at 0 DIV (days in vitro) which had a value of 1. C: actin levels in cell versus media fractions. Aliquots were separated on a 10% polyacrylamide gel and Western blotting carried out using an anti-actin antibody (Amersham). Lane 1, total adult cerebellar tissue homogenate; lane 2, cell fraction from one dish of a 9 DIV granule cell culture; lane 3, 25 μl and lane 4, 250 μl of lyophilized media from a 9 DIV culture.
SC1 in the media cannot be attributed to cell rupture, indicating that the 120 kDa component was secreted by granule cells in culture.

4. Discussion

In many tissues, the extracellular matrix (ECM) is composed of collagens, proteoglycans, and non-collagenous glycoproteins that form complex and well-defined structures such as the basement membrane or basal lamina [1]. In the brain, however, collagens and matrix structures are absent, with the exception of barrier layers of the pial surface and surrounding blood vessels [41]. Despite the lack of these ECM hallmarks, the developing brain has been shown to express a number of matrix components [46]. The role these molecules play in the developing brain has been deduced from their pattern of expression in vivo, as well as their affects on cellular interactions in tissue culture [46]. ECM components have been shown to play important functions in directing early developmental events in the nervous system such as cell migration, apoptosis, differentiation, and cell division [46]. Once these events have ceased, the expression of the majority of these molecules, such as laminin, fibronectin, and thrombospondin, decreases [35,40,42]. Based on the pattern and timing of expression of these ECM molecules, it does not appear likely that they function in late developmental events, such as synaptogenesis and synaptic stabilization.

Recently, a small number of ECM components, predominantly proteoglycans, have been identified in the adult brain [17,19,20,22,26,34]. Little is known about these neural proteoglycans other than their existence in insoluble membrane-containing subcellular fractions. The adult brain ECM has been suggested to function as a barrier to inhibit further neurite outgrowth, acting to stabilize synaptic structures through interactions with various cell adhesion molecules [19]. The adult brain ECM may also help regulate the extracellular environment through the binding of calcium, as well as diffusible proteins such as growth factors and cytokines [19].

SC1 is a putative, calcium-binding ECM glycoprotein which is expressed at near maximal levels in the adult brain and is not detected in non-neural tissues such as liver and kidney [23]. Previous analysis of the SC1 cDNA and derived amino acid sequence indicates that the carboxyterminal 204 amino acids of the protein shows approximately 65% conservation with the final two-thirds of SPARC/osteonectin, a known anti-adhesive modulator of cell–matrix interactions [23,25]. Recent studies have focused on the ability of inhibitory glycoproteins to form barriers to growth by repelling the extension of neurites [4,7,8,28,32,38,44]. Anti-adhesive glycoproteins are associated with the extracellular matrix in the adult brain, including the related SPARC [4,8,30,31].

SPARC has been shown to interact with growth factors, and affect cell morphology in a wide range of systems [14,39]. This molecule is expressed by astrocytes in synaptically-enriched regions of the adult brain, suggesting a role in neural plasticity [32]. Similarly, SC1 is present along Bergmann glial fibers in the synaptic-rich molecular layer of the cerebellum [31]. Suggestions that SC1 may be involved in cell–matrix interactions are supported by the cloning and expression of the putative human homologue, hevin, from high endothelial venule cells [12]. These cells have altered morphologies and display unique cell–cell contact characteristics [2,11].

It is interesting to note that a number of components of the developing and adult ECM share a follistatin-like module. For example, SC1 contains 11 cysteine residues also found in the related SPARC, QR1, and hevin proteins [12,25]. This is similar to the cytokine-binding follistatin module that has been suggested to play a role in neural differentiation by accumulating, protecting, and regulating the activity of growth factors [13,37,43]. Agrin, which has been shown to be involved in the formation of synapses in the peripheral nervous system, also contains this sequence [6]. Recent reports have shown that SPARC, agrin, and SC1 are all expressed in the developing and adult brain, suggesting that these molecules represent a new class of adult brain ECM components [23,29–32].

This report demonstrates that levels of SC1 increase in a caudal to rostral manner during postnatal development of the rat brain. At postnatal day 1, expression of SC1 is low throughout the brain with the exception of caudal regions such as the brainstem, cerebellum, and midbrain and cells lining the ventricles. This pattern is similar to that reported for the related protein SPARC/osteonectin [30] and the hyaluronan-binding protein BEHAB [21,22]. These molecules have been suggested to play roles in glial cell proliferation and differentiation. While levels of SPARC and BEHAB exhibit a generally astrocytic pattern of expression throughout the adult brain [21,32], levels of SC1 increase in neuronal-enriched regions of the forebrain as postnatal development proceeds. The overall distribution in the adult brain is similar to agrin. For example, agrin also appears to be expressed by Bergmann glia of the Purkinje cellular layer in cerebellum, and by neurons of the forebrain [34].

Expression of SC1 mRNA in hippocampal neurons is not apparent at birth, but occurs after migratory events have ceased. This suggests an association with neuronal maturation. Furthermore, highest levels of hippocampal SC1 protein are observed between postnatal days 10 and 30, a period of synaptogenesis and synaptic redefinition and stabilization in the hippocampus. SC1 was originally isolated by screening an expression library with an antibody raised against synaptic junctions [23]. We now show that the SC1 protein is associated with rat forebrain synaptosomes, which contain both pre- and post-synaptic elements. SC1 is not specific to synapses, however, as it is also associated with glial cells and some non-neural tissues [23].
SC1 is enriched in neural membrane fractions [23] and both the 116/120 kDa components are associated with microsomal (P3) and synaptosomal membranes. The location of both proteins in the aqueous rather than the lipid (detergent) phase, following partitioning in TX-114, suggests that they are not transmembrane proteins. This is in general accord with the property of other adult brain ECM glycoproteins which have been identified, including the related SPARC glycoprotein [17,20,32]. Furthermore SC1 is secreted from cerebellar granule cells grown in primary culture. This agrees with the predicted amino acid sequence which suggests the presence of a signal sequence with no transmembrane domain [23]. Future studies will need to examine if SC1 interacts with other ECM components in the developing and adult brain.

In summary, our studies have shown that SC1 exhibits a generally glial pattern of expression neonatally, and may play a role in glial cell proliferation and differentiation as has been suggested for BEHAB and SPARC [22,30]. Unlike these proteins, SC1 is expressed in neurons later in postnatal development, suggesting an association with neuronal maturation. In the adult brain, SC1 displays general properties of an ECM component, and can be added to the list of other matrix components which include the proteoglycans hyaluronic acid [5], Cat-301 [19], T1 antigen [20], tenascin/cytotactin [3], and agrin [34]. SC1, hevin, and the anti-adhesive SPARC protein are related non-proteoglycan ECM components identified in the adult brain [12,23,30,32]. These glycoproteins may represent members of a new family of adult brain ECM components that contain a cytokine-binding follistatin module. This report adds further support to the contention that anti-adhesive molecules may contribute to the functioning of the adult brain in addition to playing a role during neural development.

Acknowledgements

We thank S. Woodrow for isolation of rat forebrain synaptosomes, Dr. G.O. Ivy for help in the interpretation of the in situ hybridization results, and Dr. I.G. Johnston and Ms. Sheila Rush for critical reading of this manuscript. These studies were supported by grants to I.R.B. from NSERC and J.W.G. from MRC.

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