Minireview

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Proteoglycans: Master Regulators of Molecular Encounter?

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Introduction

The past decade has seen an explosion of information about proteoglycans (PGs). We now know that there are multiple classes of core proteins that carry cell surface heparan sulfate, others that carry cell surface chondroitin sulfate, and still others that carry heparan, chondroitin, dermatan and keratan sulfate in extracellular matrices of myriad types. We also know that there is great diversity in the structures of glycosaminoglycan (GAG) species, and that this diversity can give rise to varying degrees of specificity in interactions with proteins. We further know that the expression patterns of many PGs are highly tissue-specific, and are often dramatically regulated during development or in disease.

Presumably, all this diversity of structure and expression tells us that the functions of PGs are many and varied. Yet with all the recent focus on the unique features of individual PGs, it is easy to forget that one of the factors that has long attracted researchers to the PG field is the sense – perhaps better articulated in earlier days – that the structural commonalities among PGs underlie some shared aspects of function. We will examine here the hypothesis that the common properties of PGs render them, as a class, particularly well suited to act as catalysts in the regulation of molecular interactions. To explain this idea, it is helpful first to review some of the fundamentals of binding theory.

Kinetics vs. thermodynamics in macromolecular binding

Mathematically, macromolecular binding can be described by two kinds of parameters: kinetic, the rates at which binding and un-binding happen; and thermodynamic, the free energy change between free ligands and bound complexes. At equilibrium, i.e., when rates of association and dissociation balance each other, proportions of free and bound ligands are entirely determined by thermodynamics, and are usually expressed by K_d, the equilibrium binding (or affinity) constant, which reflects and is determined by the free energy change of binding.

For simple, one-step binding, the value of K_d is equal to the ratio of a reverse and a forward rate constant (i.e., k_{off}/k_{on}); thus, complete knowledge of kinetics determines the thermodynamics, but not vice versa. Put another way, a value of K_d never describes a binding system as completely as the values of k_{off} and k_{on} together do. However, within the range of what goes on in biological systems, there may be cases when values of K_d alone are good enough to describe those features of binding that are of practical value. This is equivalent to asking whether some biological systems may be approximated by models in which binding events achieve equilibrium. Of course, no biological system is itself at true chemical equilibrium (one can argue that the goal of life is the constructive avoidance of this fate!). However, many molecular interactions in biology probably come sufficiently close to justify the approximation. The determining factor is whether or not molecular binding is biologically *rate-limiting*, i.e., whether binding is faster or slower than rates of subsequent events. When binding is rate-limiting, it follows that an alteration in the kinetic

Abbreviations used: AT, antithrombin; BMP, bone morphogenetic protein; GAG, glycosaminoglycan; HS, heparan sulfate; PG, proteoglycan.

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parameters of binding (i.e. k_{on} and k_{off}) can have substantial biological impact, even if there is no change in the value of K_d. Here we will consider the possibility that a common function of PGs is to have just such an effect.

Roles of PGs in growth factor signaling

A good place to begin exploring this hypothesis is in the area of growth factor signaling. Many classes of polypeptide growth factors bind tightly to heparin and heparan sulfate (HS) (Lander, 1994). Among these, some may be considered "HS-dependent", since *in vitro* manipulations that remove, or prevent the sulfation of, cellular HS result in greatly decreased growth factor binding to, and activation of, signaling receptors. The best characterized HS-dependent growth factors are FGF-1 and FGF-2, but other FGFs, HB-EGF, wingless (a member of the Wnt family) and probably several other growth factors belong to this group (Rapraeger et al., 1991; Yayon et al., 1991; Aviezer and Yayon, 1994; Tessler et al., 1994; Zioncheck et al., 1995; Reichsman et al., 1996). Models that have been put forth to explain HS-dependence include ones in which binding of HS to growth factor, receptor, or both directly alters affinity (e.g., through cross-linking, dimerization, or induction of a conformational change [Kan et al., 1993; Pantoliano et al., 1994; Spivak-Kroizman et al., 1994]). Thus, HS dependence is thought to reflect a contribution of HSPGs to binding affinity, i.e., to the thermodynamics of binding.

In addition to the HS-dependent growth factors, there are numerous others that bind to heparin and HS but don't seem to require the presence of HS for biological activity, at least in *in vitro* assays. Examples include TGF-βs, BMPs, PDGF and probably most other heparin-binding growth factors. Does this mean the ability of these growth factors to bind HS is coincidental and of no functional significance? Perhaps, but a more likely explanation is that the biological significance of the interactions of these growth factors with HS is simply not captured by the kinds of *in vitro* assays that are typically used to study growth factor signaling. Support for this idea has come from genetic analysis of PG core protein mutants *in vivo*, in which HSPGs appear to regulate signaling by growth factors, such as members of the BMP family, and components of the insulin-like growth factor pathway (Pilia et al., 1996; Jackson et al., 1997), which *in vitro* assays have suggested are not HS-dependent (e.g., Ruppert et al., 1996).

One reason why *in vitro* assays may fail to capture important aspects of growth factor signaling *in vivo* is that *in vitro* growth factor assays are often deliberately designed to be sensitive only to thermodynamic, and not kinetic, aspects of growth factor-receptor binding. Typically, this is accomplished by keeping growth factor concentrations high (to maximize the rate of approach to equilibrium), keeping binding volumes large (to avoid ligand depletion), and measuring downstream events that occur more slowly than the rate at which receptors become occupied by ligand (so receptor binding is not a rate-limiting step).

Thus, if HSPGs play a large role in controlling the kinetics of growth factor binding to receptors, but do not affect the thermodynamics of binding, the contribution of HSPGs to the binding of many growth factors might easily have gone unnoticed by investigators working only *in vitro*. Yet, the idea of molecules that affect kinetics but not thermodynamics is hardly foreign to biology – this is precisely what enzymes (and catalysts, in general) do. Normally, however, enzymes catalyze chemical reactions (in which the products are chemically different from the reactants), whereas here we are suggesting that HSPGs catalyze physical reactions, the product simply being the bound reactants. We may refer to this phenomenon as catalysis of molecular interaction. Just as with chemical catalysis, in which the catalyst is not consumed by the reaction, a catalyst of molecular interactions cannot be a permanent part of the binding complex (lest it affect the thermodynamics of binding).

Is the idea that HSPGs catalyze growth-factor receptor interaction biologically reasonable? To address this, we need first to consider whether, mechanistically, such a phenomenon is plausible. Next, we must consider whether growth factor-receptor interactions are the sorts of interactions where a change in kinetics would have much biological significance (i.e., is growth factor binding ever rate limiting?).

Catalytic roles of HSPGs are precedented in the control of blood coagulation

To see how PGs could catalyze growth factor-receptor interactions, it is instructive to look first to an area of physiology in which the functions of GAGs are better understood at a mechanistic level: blood coagulation. Although much of the research in this area has focused on the pharmacologically administered anticoagulant GAG, heparin, the conclusions have been extended to the HS that normally confronts the circulation, namely

that carried by the PGs of vascular endothelial cells (Marcum et al., 1987).

Briefly, heparin and HS accelerate, by several orders of magnitude, the rate at which the anti-protease antithrombin (AT) inactivates serine proteases, particularly thrombin and factor Xa, that are responsible for triggering the coagulation cascade. In both AT-thrombin and AT-factor Xa inhibition, the effect is essentially catalytic: i.e., the GAG increases the rate of AT-protease binding but is not a necessary and permanent part of the binding complex. Interestingly, the mechanism underlying this effect is different for these two proteases, and it is best explained by considering the process of molecular binding as occurring in two steps, *encounter* and *reaction* (Eigen, 1973). Molecular encounter literally means the bumping of one molecule into another; accordingly, it depends only on the physics of diffusion, i.e., the speeds at which molecules move around in solution and the paths they take. After an encounter, however, macromolecules may proceed to bind or not, depending on all sorts of parameters such as how the molecules are oriented when they meet, whether conformational adjustments occur, and so on. These events comprise the "reactional" stage of binding.

In the case of the inactivation of factor Xa by AT, the increased rate of binding in the presence of heparin or HS mainly reflects an increase in the reactional part of binding (Olson and Björk, 1992). This is because the GAG affects the conformation of AT such that encounters with factor Xa are more likely to lead to stable binding. On the other hand, the effect of GAGs on the inactivation of thrombin by AT is primarily due to a marked increase in the rate of molecular encounter (Olson and Björk, 1992). It may at first seem counterintuitive that GAGs can increase rates of AT-thrombin encounter, since encounter is supposed to reflect only the physics of diffusion, but it is in fact precisely at the level of diffusion that the change takes place. What happens in the presence of heparin or HS is that both thrombin and AT bind rapidly to the GAG, both molecules often binding to the same GAG chain. Because they do so, they find themselves in very close proximity for long periods of time. Accordingly, they bump into each other much more often than they would in free solution. This type of catalysis of molecular encounter has been referred to variously as an "approximation effect", or as "reduction in dimensionality", the latter term reflecting the fact that when thrombin and AT leave three dimensions (free solution) and become confined to one dimension (the length of a GAG chain), their probability of collision increases dramatically.

Rate enhancement due to reduced dimensionality has been studied in other biological systems, and the general phenomenon has been treated mathematically (Adam and Delbrück, 1968; Berg et al., 1981; Weigel and DeLisi, 1982; Berg, 1985). A well known example involves the binding of transcription factors to DNA, as illustrated by the Lac repressor of *E. coli*. There, the entire chromosome acts as a one-dimensional surface to which Lac repressor attaches and remains weakly bound while it "explores" the DNA until it ultimately encounters its high affinity binding site. The consequence is an enhancement of the rate of binding to that site by several orders of magnitude (Winter et al., 1981).

What kinds of molecules have the ability to accelerate molecular encounter in this way? First, they must be large and/or highly extended, in order to create a surface along which molecules can move. Second, they need to have a high rate of capture of ligands from free solution. On first principles, one can infer that the ideal structures for such an effect are long, thin, repeating polymers that bind ligands through ionic interactions of moderate (but not too strong) affinity (Pontius, 1993). The length provides the surface along which ligands "explore", the thinness optimizes (per unit mass) the frequency with which ligands are encountered by the polymer (Eigen, 1973), the ionic nature of binding can somewhat increase the diffusion-limited rate of collision with ligands (due to electrostatic attraction at short distances), the repetitiveness of the polymer is required to ensure that ligands can bind and stay bound along the entire length of the polymer, and the moderate affinity of binding is required to allow ligands to move along the polymer rather than simply stay put at a single binding site.

GAGs are essentially long, thin, repeating polymers that bind ligands through ionic interactions of moderate affinity. It is this remarkable concordance of features which strongly suggests that a role for PGs as catalysts of molecular encounter applies not just in the blood coagulation system, but in other places and involving other kinds of molecules.

Which brings us back to growth factors. As mentioned earlier, there are quite a few heparin-binding growth factors for which no strong thermodynamic (affinity-enhancing) role for GAGs has been found. Might cell surface GAGs be playing a catalytic role, increasing the rates at which such growth factors bind their receptors? By analogy to the blood coagulation system, we may imagine that cell surface HSPGs use a reduction-in-dimensionality strategy to increase the rate of encounter between growth factors and their receptors. Indeed, such a possibility has been raised explicitly (Schlessinger et

al., 1995). It is important to recognize, however, that the mechanism worked out for GAG catalysis of the encounter of AT and thrombin will most likely not be applicable in the growth factor-receptor case. This is because, with AT and thrombin, both proteins bind to a single GAG chain, along which at least one of the two can freely move until it encounters the other. In contrast, binding of a growth factor to a cell surface HSPG would confine the growth factor to the same two-dimensional surface on which receptors reside, but there would be no single molecule along which the growth factor could move until it encounters its receptor. Instead, to encounter receptors, the growth factor would have to rely on the (relatively slow) lateral mobility within the plasma membrane of the PG to which it is bound or, alternatively, on repeatedly dissociating from one HSPG and reassociating with a nearby HSPG. If the rates of these events are not sufficiently fast, one would predict that cell surface HSPGs should actually *decrease*, rather than increase, the over-all rate of growth factor-receptor encounter.

As a general problem, the question of whether "weak" cell surface binding sites are more likely to enhance or retard rates of ligand-receptor encounter has been treated mathematically by others (e.g., Weigel and DeLisi, 1982; Axelrod and Wang, 1994). Unfortunately, none of these theoreticians had PGs in mind when making their calculations, and, clearly, many key characteristics of cell surface HSPGs (their abundance, their ligand affinities) often lie outside the parameter ranges covered in these analyses. However, using similar approaches (details of which will be published elsewhere), it is possible to show that cell surface HSPGs can either be strong accelerants or strong retardants of growth factor receptor encounter, depending on parameter choice.

An especially important parameter is the transfer rate of growth factor from one HS chain to another. At minimum, this rate is determined by the rate of dissociation from one chain, the rate of association to another, and the concentration of the chains. However, as has been shown for the binding of molecules to DNA, at high polymer concentrations bound ligands can sometimes "jump" from one polymer chain to another without ever fully dissociating (Berg, 1986). Thus, many association-dissociation events can occur within the time that, ordinarily, a ligand would have remained bound to a single chain. As a result, ligand can diffuse farther during that time than if it had stayed attached to any single polymer molecule. If this sort of thing goes on for HS-bound ligands at the cell surface, one can imagine growth factors swinging rapidly from GAG to GAG like molecular Tarzans swinging from vine to vine. Even without invoking such behavior, however, one can build models using reasonable choices of parameters that demonstrate HSPGs acting as strong accelerants of growth factor-receptor encounter. Interestingly, most of the important parameters that are needed to build such models are readily measurable. They include values such as k_{on} and k_{off} for growth factor-GAG binding and growth factor-receptor binding (in the absence of PGs), as well as the concentrations of PGs and receptors in the plasma membrane. For heparin-binding growth factors, unfortunately, there are few if any instances in which all of these values have yet been determined together.

Before concluding the discussion of mechanisms by which PGs might catalyze growth factor-receptor interaction, it is worth briefly revisiting those growth factors for which PGs are clearly thought to have an affinity-enhancing (i.e., thermodynamic) effect on binding (e.g., FGFs). It should be obvious that, even when PGs do affect equilibrium growth factor-receptor binding, there is no reason why they can't also affect the kinetics of binding. In other words, the rate-enhancement mechanisms described above don't have to be limited to those growth factors generally thought of as non-heparin/HS-dependent.

A less obvious, but intriguing, possibility is that even the presumably thermodynamic effects of PGs for certain growth factor-receptor interactions may actually be kinetic effects after all. This could occur if the interactions of some growth factors with their receptors are so slow in the absence of PGs that equilibrium is never approached under normal conditions (if it could be approached, however, one would find binding to be as high affinity as when PGs are present). PGs would then appear to increase growth factor-receptor affinity, but in fact would be acting only as catalysts, to speed up what would eventually happen on its own (after a very long time). This type of effect could be revealed experimentally by examining whether the high affinity growth factor-receptor complexes that require PGs to form actually contain stoichiometric amounts of PGs at equilibrium. If they do not, it would strongly suggest that PGs are acting catalytically.

Would catalysis of growth factor-receptor binding matter in vivo?

Let us now turn to an important question that was deferred earlier, that of the biological relevance of rate control. It should be pointed out that the common practice of focusing only on the equilibrium aspects of growth

factor-receptor binding (rather than kinetic parameters) is not entirely unjustified. Simple math tells us that, at very low concentrations of a ligand, the time it can take for the occupancy of receptors by that ligand to reach 63% of its equilibrium value is equal to $1/k_{off}$, where k_{off} is the dissociation rate constant for the bound complex. At ligand concentrations equal to K_d, it takes only half as long to reach equilibrium, and this time continues to decrease as ligand concentration rises; for many polypeptide growth factor receptors, $1/k_{off}$ is in the range of only a few minutes (Lauffenburger and Linderman, 1993). Yet some of what are thought to be the major downstream consequences of growth factor receptor activation – e.g., induction of new gene expression – simply cannot happen on time scales much faster than a few minutes. It would seem that, for such events at least, the response to growth factors – not binding – is likely to be rate-limiting. Speeding up growth factor binding should have little biological effect.

Under what circumstances would acceleration of growth factor-receptor binding really matter? It is possible to conceive of many examples, and it is instructive to consider five of them here, since they provide a blueprint for where to look for catalytic effects of PGs.

When growth factor-receptor interactions are unusually slow

Not all growth factors bind receptors quickly. Indeed, the perception that fast binding is the rule has been based on rather few examples. Certainly, not many GAG-binding growth factors have even been evaluated with respect to binding kinetics, but at least two of these, BMP-2 and BMP-4 (members of the TGF-β superfamily), have an extraordinarily slow k_{on} and k_{off}, such that equilibrium occupancy of receptors can take hours after addition of ligand (Iwasaki et al., 1995; Natsume et al., 1997). Interestingly, similarly slow rates have been seen with other members of the TGF-β superfamily (Dyson and Gurdon, 1998).

When cell surface receptors turn over rapidly

The simplest forms of receptor binding theory assume that cell surface receptors are static, being neither produced nor removed. When receptor turnover is taken into account, the number of receptors on the surface is determined by a steady state, where the number of receptors destroyed balances the number of new ones put back on the surface. Receptors being destroyed may include those that have ligand bound as well as those that don't, whereas receptors being replaced never have ligand bound. For this reason, the process of receptor turnover lowers the fractional occupancy of receptors (the fraction of receptors at the surface that have ligand bound) below what it would be if receptors were static. Whether this effect is significant or not depends on whether the rate of receptor destruction is close to or faster than the rate at which ligands occupy new receptors. This can be seen by imagining binding from the perspective of a new receptor molecule emerging at the cell surface. If the average lifetime of the receptor on the surface is short compared with how long it is likely to have to wait before it becomes occupied by ligand, then there is a good chance it will disappear before ever being bound. When the wait is long there will be large numbers of "frustrated" receptors at the cell surface despite the presence of adequate levels of ligand. Whether conditions such as these prevail for actual receptors is unknown, since receptor turnover rates are not frequently measured. In the case of the EGF receptor, however, the rate constant for turnover has been estimated as 5×10^{-4} sec^{-1} (Lauffenburger and Linderman, 1993), which places it in the range of k_{off} for slowly-binding growth factors such as BMP-2 (see above).

When cell surface receptors undergo desensitization

Many kinds of receptors act only for brief periods before mechanisms such as cytoplasmic domain phosphorylation stop them from signaling. After ligand dissociation, resensitization mechanisms restore signaling ability. Due to this cycle, the amount of signal produced by such receptors on a cell surface is not simply proportional to the number of receptors that are occupied. Instead, it becomes a complicated function that takes into account how long a receptor has been occupied, and how long it was unoccupied before a ligand bound to it. Both of these values, in turn, depend on the rates of receptor binding and dissociation. With appropriate choices of parameters, it is easy to model systems in which changes in ligand binding kinetics dramatically improve signaling, without ever changing fractional receptor occupancy in the steady state. Do such situations obtain in real cells? At present, too little is known about the kinetics of growth factor receptor desensitization in most systems, so it is difficult to judge.

When ligand concentrations change rapidly

All of the above scenarios involve ways in which specific characteristics of receptors render them particularly

sensitive to changes in binding kinetics. Another class of scenarios comprises those in which ligand concentrations are not stable. Imagine that a collection of growth factor molecules is suddenly released near a cell. At the plasma membrane, ligand concentration will suddenly rise, and then fall again as ligand diffuses away (or is captured by other cells, bound to extracellular matrix molecules, or degraded). The cell has only a brief interval in which to "capture" the growth factor, and this will depend exquisitely on the kinetics of growth factor-receptor binding. If this interval is followed by another "pulse" of growth factor, and then another, one can develop a model system in which kinetics, rather than affinity, plays the predominant role in determining time-averaged receptor occupancy (related models have been used to describe neurotransmission at synapses [Wathey et al., 1979]). It is not known whether growth factors are released in such a pulsatile fashion, but with heparin-binding growth factors, it is likely that binding to the extracellular matrix would accelerate the removal of growth factor from solution, effectively narrowing the duration of any pulse and further enhancing the dependence of binding on kinetic, rather than thermodynamic, parameters.

When amounts of free growth factor are extremely low

An intriguing property of GAG-binding growth factors is that they would not be expected to spend much time in "free" solution *in vivo* (due to the high concentrations of extracellular matrix and cell surface GAGs to which they would be expected to bind). Under such conditions, amounts of free ligand may be so low that statistical variations in the ligand concentration become large. Under these circumstances, growth factor-receptor binding must be treated probabilistically, rather than deterministically (Lauffenburger and Linderman, 1993). In this regime, kinetic constants can matter considerably, even to the response of the cell over long periods of time.

Beyond catalysis of growth factor-receptor encounter: generalizing the diffusive effects of PGs

In the above discussion, we began with a description of how PGs act as catalysts in the inhibition of proteases by protease inhibitors, and then speculated that a general function of cell surface PGs is to increase the rates at which GAG-binding growth factors interact with their receptors. This generalization was motivated by the fact that PGs, and in particular their GAG chains, have just the characteristics needed to provide "surfaces" upon which other molecules can find each other. In even more general terms, we may say that PGs have the ability to control molecular diffusion by providing alternate diffusive paths for GAG-binding ligands. Put in this way, we can see that there is no inherent reason why the ideas developed here should be limited to growth factor-receptor interactions. It is known, for example, that cell surface PGs, via their GAG chains, bind a variety of extracellular matrix proteins, yet do not appear to act as independent receptors for such proteins. Instead, these PGs somehow collaborate with receptors such as integrins to modify cellular responses (Woods et al., this volume). Could it be that one of the roles of cell surface PGs is to regulate the rate of encounter of integrins with matrix proteins? Alas, modeling the interactions of cells with matrices is not nearly as straightforward as modeling growth factor-receptor binding (Lauffenburger and Linderman, 1993). However, the relatively slow kinetics of integrin-ligand interactions (e.g., Hu et al., 1996) and the likely cooperativity required to form stable cell-matrix contacts (Lauffenburger and Linderman, 1993) suggest that cell-matrix interaction could be quite sensitive to changes in the kinetics of integrin-ligand binding.

Finally, it should be pointed out that a role for PGs in the *acceleration* of molecular encounter is not the only diffusive role that is potentially biologically useful. It is easy to see how PGs, particularly at high concentrations, can act as "sinks" for molecules and thereby *slow* their rate of encounter with other molecules. It was already mentioned that, depending on the values of critical parameters, this could occur for growth factors on cell surfaces. One situation in which the retardation of diffusion by PGs may be of particular biological value is in the establishment of gradients of signaling molecules within PG-rich extracellular matrices. During development, important gradients of several GAG-binding growth factors, such as BMPs, hedgehogs and Wnts are employed (Nellen et al., 1996; Zecca et al., 1996), and during both development and inflammation, gradients of GAG-binding chemotactic factors, such as netrins, semaphorins, hepatocyte growth factor and chemokines, seem to be important (Witt and Lander, 1994; Messersmith et al., 1995; Ebens et al., 1996; Serafini et al., 1996). Reductions in molecular diffusivity – such as would be brought about by the reversible binding of these molecules to PGs – can have interesting consequences for the shapes of the gradients that these molecules will form by simple diffusion. In particular, the steepness of diffusion gradients as they are forming

(i.e., before a steady state is established) can actually be enhanced by decreased diffusivity (Berg, 1993). Since, for some molecules, cellular responses are very much controlled by gradient steepness (Devreotes and Zigmond, 1988), it follows that inhibition of molecular diffusion can sometimes – in theory at least – enhance the biological activities and range of action of diffusible molecules.

Conclusions

In the preceding paragraphs, we have explored the idea that PGs, by virtue of the unique biochemical and biophysical characteristics of their GAG chains, act as regulators of molecular encounter for a wide variety of GAG-binding proteins. With the exception of the effects of heparin and HS on inhibition of thrombin by AT, all of the mechanisms described above are speculative and need to be tested in the laboratory. Nevertheless, one often needs a hypothesis before identifying the experiments that are needed, and it is hoped that this article may help in this regard. Furthermore, it is hoped that the issues raised here will encourage readers to think more about rates of molecular binding, and when those rates are and are not potential targets for biological regulation. Even if PGs haven't always been selected by evolution to carry out such regulation, it will no doubt be fascinating to investigate the common features that tie together all these molecules that have.

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