Polyanions and the Proteome*

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The behavior of the proteome reflects spatial and temporal organization both within and without cells. We propose that various macromolecular entities possessing polyanionic character such as proteoglycans, lipid bilayer surfaces, microtubules, microfilaments, and polynucleotides may provide a functional network that mediates a variety of cellular phenomena. The interaction of proteins with this array of polyanions is characterized by a lower degree of specificity than seen with most commonly recognized macromolecular interactions. In this commentary, potential roles for this polyanion network in diverse functions such as protein/protein interactions, protein folding and stabilization, macromolecular transport, and various disease processes are all considered, as well as the use of polyanions as therapeutic agents. The role of small polyanions in the regulation of protein/polyanion interactions is also postulated. We provide preliminary experimental analysis of the extent to which proteins interact with polyanions inside cells using a combination of two-dimensional chromatographic and electrophoretic methods and antibody arrays. We conclude that many hundreds to thousands of such interactions are present in cells and argue that future understanding of the proteome will require that the "polyanion world" be taken into account. ***Molecular & Cellular Proteomics 3:746–769, 2004.***

Functional cellular behavior is thought to arise primarily from highly specific interactions between molecular components. These interactions are usually based on tight associations that display the ability to differentiate between remarkably subtle differences in structure. Most biochemical studies are conducted with an emphasis on the details of molecular structure that are responsible for this presumed specificity. Less specific interactions have been given considerably less attention. An exception involves gene regulation, which frequently appears to involve significant nonspecific interactions between proteins and their DNA substrates (1). We propose here, however, that at least one type of "nonspecific" interaction deserves more attention, especially with regard to the proteome. In this perspective, we postulate that less specific interactions between various cellular polyanions and proteins are of critical importance to our understanding of the proteomic world. The basis of these interactions is complementary electrostatic interactions between polyanionic and polycationic entities. The extreme polyanionic nature of several common cellular macromolecules and macromolecular complexes involved in such interactions (*e.g.* proteoglycans, DNA, RNA, ribosomes, actin microfilaments, and microtubules) is illustrated in Fig. 1, with the extensive red surfaces corresponding to regions of negative charge. The polyanions in Fig. 1 are only meant to be representative of this class of molecules, which also includes smaller polyanions such as the inositol phosphates (InP_n) as well as polysaccharides such as the glycosaminoglycans (*e.g.* heparin, chondroitin sulfates, dextran sulfate) and extended structural regions of many other proteins. The potential global cellular significance of such electronegative surfaces is clearly illustrated by recent cryo-electron micrographs that show just how highly concentrated two common polyanions (ribosomes and actin) actually are in the cytoplasm (Fig. 2) (2). Thus, as we will argue in more detail below, the interior and exterior of cells can be viewed as a dense network of polyanionic surfaces that are readily accessible to other cellular proteins and, in particular, those that possess regions of concentrated positive charge. Furthermore, we contend that what may at first appear to be a series of unrelated phenomena whose common feature is the involvement of certain polyanions actually reflects a broad role for polyanion/polycation interactions in diverse cellular functions.

Partial origin of the idea that electrostatic interactions of intermediate or low specificity may play vital roles in cellular function arises from the existence of so-called "heparin-binding proteins." Well over a hundred such proteins have so far been identified, and an entire book has been dedicated to this topic (3). It is, in fact, rare to find an issue of a major biochemical publication, such as *The Journal of Biological Chemistry*, that does not contain multiple papers devoted to "heparin-binding proteins." Although it is common to view heparin/protein interactions in terms of their specificity, in those (few) cases where interactions with multiple polyanions have been tested, interactions with other large (*e.g.* DNA, actin, tubulin, etc.) and small (*e.g.* inositol phosphates, phytic acid, etc.) polyanions are often observed (see below). This ability to bind to other polyanions suggests that such interactions are not highly specific, although they may be of high affinity. Thus, these proteins are more appropriately designated "polyanion-binding proteins" to emphasize that they may be able to bind to other glycosaminoglycans, nucleotides, actin, tubulin, and other cellular polyanions through interactions that may vary in affinity but are not highly specific in the sense that large

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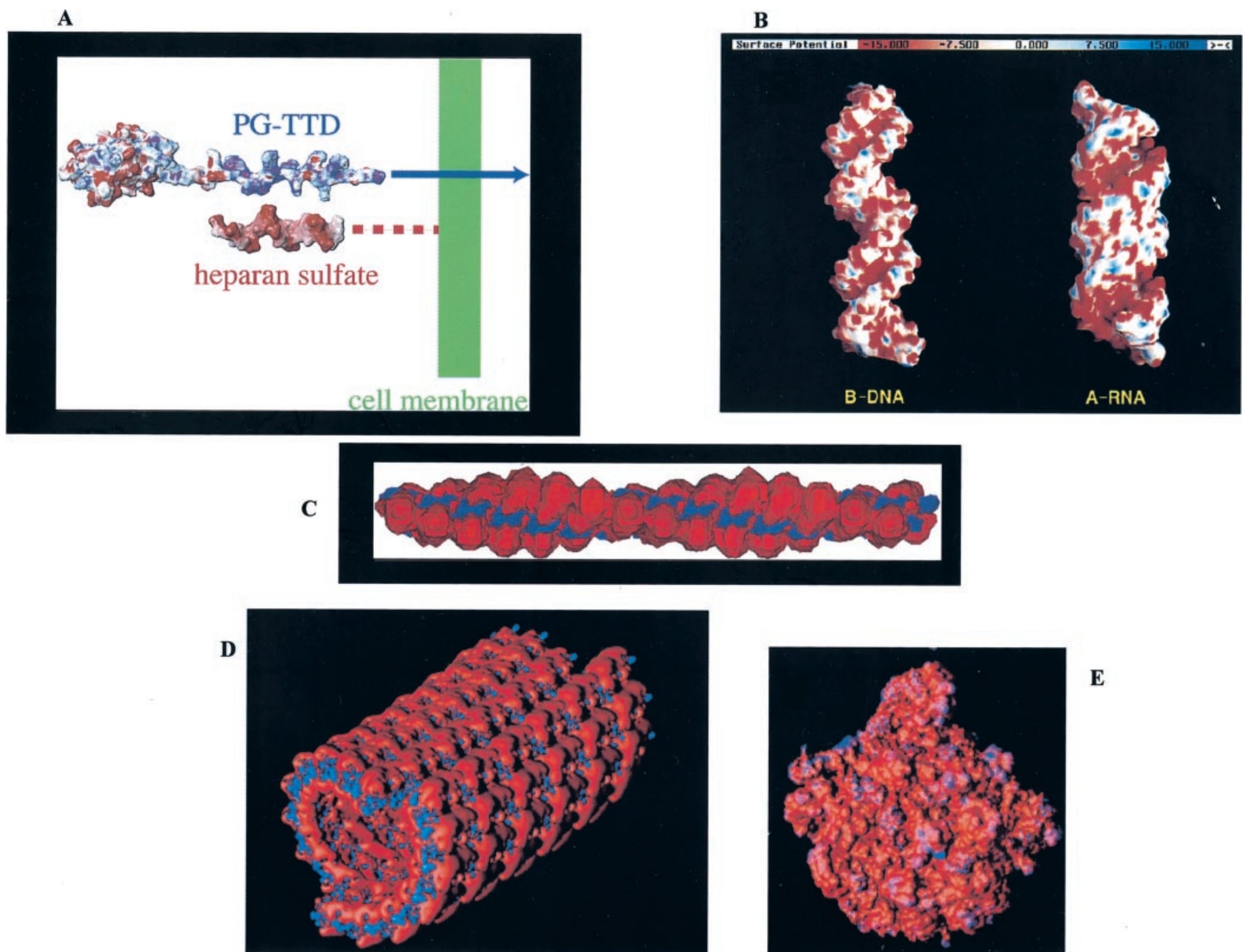

FIG. 1. **Electrostatic surfaces of representative cellular polyanions.** *Red* represents negatively charged surfaces, while *blue* surfaces are positively charged. *A*, heparan sulfate proteoglycan. Reprinted with permission from Hakansson, S., and Caffrey, M. (2003) Structural and dynamic properties of the HIV-1 tat transduction domain in the free and heparin-bound states. *Biochemistry* **42,** 8999–9006. Copyright 2003, American Chemical Society. *B*, DNA and RNA. Reprinted with permission from Chin, K. Sharp, K. A. Honig, B., and Pyle, A. M. (1999) Calculating the electrostatic properties of RNA provides new insights into molecular interactions and function. *Nat. Struct. Biol.* **6,** 1055–1061. Copyright 1999, Nature Publishing Group. *C*, actin. Reprinted with permission from Toward computational cell biology. NPACI and SDSC Online Newsletter **4** (18), www.npaci.edu/online/v4.18/baker.html. *D*, microtubule. Reprinted with permission from Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Electrostatics of nanosystems: Applications to microtubules and the ribosome. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 10037–10041. Copyright 2001, National Academy of Sciences, U.S.A. *E*, 50S ribosome. Reprinted with permission from Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Electrostatics of nanosystems: Applications to microtubules and the ribosome. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 10037–10041. Copyright 2001, National Academy of Sciences, U.S.A.

variations in ligand (polyanion) structure do not necessarily dramatically lower the affinity of such contacts.

A good example of a class of proteins that illustrates this phenomenon is the 23 known fibroblast growth factors (FGFs).[1] In 1984, Shing *et al.* reported the purification of a tumor angiogenesis factor (which was later designated basic FGF, FGF-2 (4)) from chondrosarcoma cells using a single heparin affinity column and stated that the successful purification resulted from a "specific affinity for heparin" (5). Thus, extensive studies began that continue to this day concerning the interactions between FGFs and heparin and related glycosaminoglycans (GAGs) and proteoglycans. Much of this effort has focused on the specificity of these interactions with little effort made to address the opposite question, namely the absence of such specificity. As is often the case, the ques-

[1] The abbreviations used are: FGF, fibroblast growth factor; GAG, glycosaminoglycan; FGFR, fibroblast growth factor receptor; HSP, heat shock protein; sHSP, small heat shock protein; PTD, protein transduction domain; Aβ, β-amyloid; PrP, prion protein; MWCO, molecular weight cut-off; HSPG, heparan sulfate proteoglycan.

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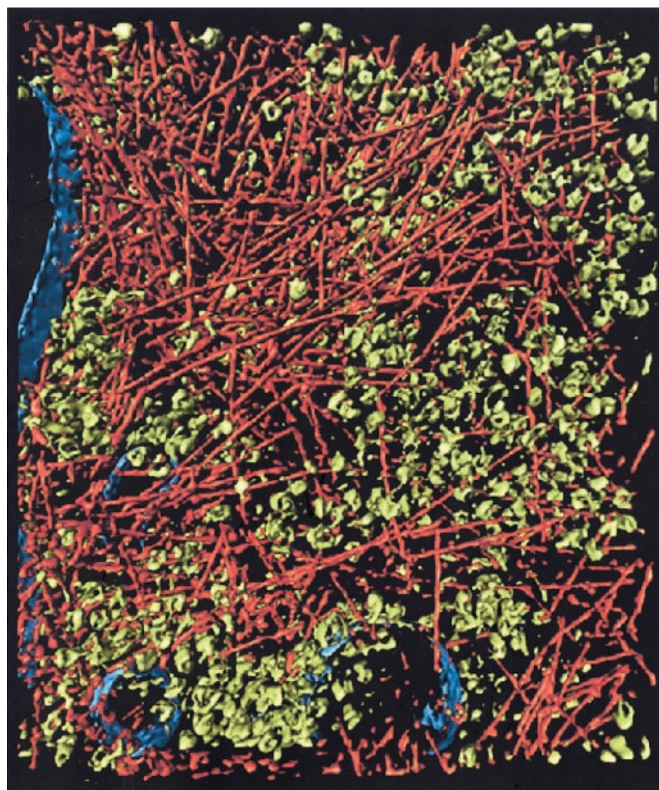

FIG. 2. **The very crowded environment of the cell.** Actin (*red*), macromolecules, primarily ribosomes (*green*), and membranes (*blue*) are represented. Reprinted with permission from Medalia, O., Weber, I., Frangakis, A. S., Nicastro, D., Gerisch, G. and Baumeister, W. (2002) Macromolecular architecture in eukaryotic cells visualized by cryoelectron topography. *Science* **298,** 1209–1213. Copyright 2002, AAAS.

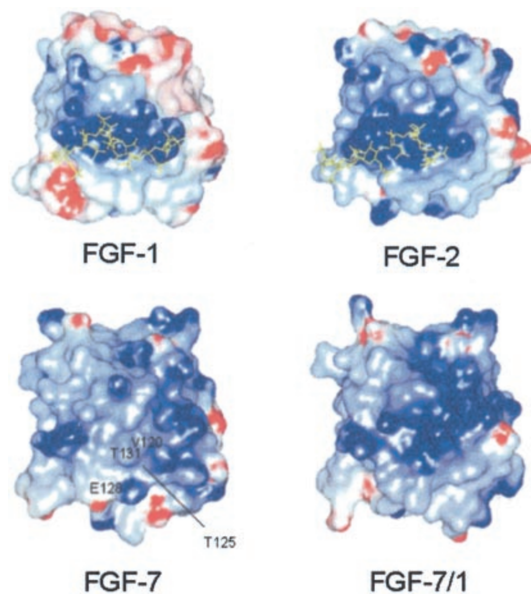

FIG. 3. **Electrostatic surfaces of FGFs and their heparin-binding domains.** Reprinted with permission from Ye, S., Luo Y., Lu., W., Jones, R. B., Linhardt, R. J., Capila I., Toida, T., Kan, M., Pelletier, H., and McKeehan, W. L. (2001) Structural basis for interaction of FGF-1, FGF-2, and FGF-7 with different heparan sulfate motifs. *Biochemistry* **40,** 14429–14439. Copyright 2001, American Chemical Society.

tions one asks very much determine the answers one receives. For example, there have been numerous studies aimed at identifying a link between the specific sulfation patterns in GAGs and their ability to bind to growth factors (6, 7). One such study looked for correlations between heparin sulfate oligosaccharide lengths (8–12 disaccharide units) and sulfation patterns (*N*-sulfate, 2-*O*-sulfate, 6-*O*-sulfate) and the activation of FGF-1 and FGF-2 (6). In general, increases in oligosaccharide length and 6-*O*-sulfation (even to a small extent) resulted in increases in FGF-1 mitogenic activity. The authors concluded that the data support the viewpoint that specific or precise positioning of the sulfate group is necessary for FGF-1 mitogenic activity. The data clearly demonstrates, however, that a range of sulfation patterns and oligosaccharide lengths can activate FGF-1 and FGF-2, albeit to differing extents. It is also noteworthy that the *N*-sulfation and 2-*O*-sulfation contents within the oligosaccharides were essentially constant. Thus, the apparent correlation between 6-*O*-sulfation and activity may actually reflect an increase in activity that is due to an increase in nonspecific electrostatic interactions, an alternative interpretation that the authors concede may be drawn. In this case and many others, apparent specificity can certainly be found, especially as one alters the charge on the interacting molecules, but it is equally likely that changes in affinity are being confused with alterations of specificity.

A large body of evidence now exists to support the view that interactions between the FGFs and diverse polyanions are highly nonspecific. In fact, much is known about the nature of the interactions between heparin and other polyanions and various FGFs because numerous nuclear magnetic resonance and crystal structures of such complexes are now available (*e.g.* Refs. 8–10). These studies clearly identify the primarily electrostatic (polycationic) nature of such sites and demonstrate such low specificity in terms of the many quite different polyanions that are able to occupy them with high affinity. Electrostatic potential surfaces of four members of the FGF family are shown in Fig. 3. Each molecule is oriented to show the "heparin-binding" face, and FGF-1 and FGF-2 are shown with fragments of bound heparin. The highly dispersed nature of this electrostatic surface is apparent from these representative images. Similar models are available for FGFs bound to polyanions as diverse as sucrose octasulfate (10, 11) and inositol hexaphosphate as well as simple sulfate anions from salts used in the crystallization process (*e.g.* ammonium sulfate) (12). Further evidence for this lack of specificity comes from the fact that photoaffinity labeling studies indicate that small polyanionic ligands can be bound in multiple orientations in the polyanion-binding site (13).

FGFs must bind to specific protein receptors (FGF receptors, or FGFRs) as well as proteoglycans to activate signaling

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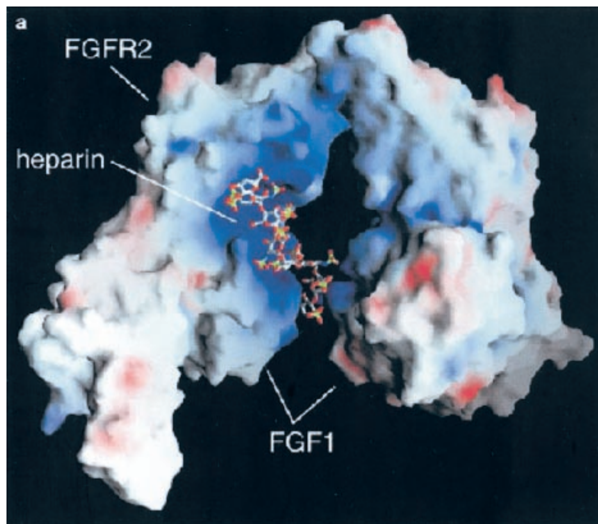

FIG. 4. **Electrostatic surfaces of FGF1:heparin:FGFR2 asymmetric complex.** Reprinted with permission from Pellegrini, L., Burke, D. F., von Delft, F., Mulloy, B., and Blundell, T. L. (2000) Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature* **407,** 1029–1034. Copyright 2000, Nature Publishing Group.

pathways that produce their various cellular responses. Crystal structures of FGF:heparin:FGFR complexes reveal that polyanions such as heparin interact with both FGF and FGFR (Figs. 4 and 5*B*). The crystal structure of the FGF-1:heparin: FGFR2 dimer finds a 2:1:2 complex that suggests that heparin facilitates dimerization of FGF-1, which in turn induces dimerization of the receptor, potentially a critical activation event (14). On the other hand, the 1:1:1 complex formed by FGF-2, heparin, and FGFR1 observed by others suggests that heparin can also interact with single FGF and FGFR molecules to form a stable complex (8, 10, 15). After the first stable complex is formed, each subunit is positioned to undergo a favorable interaction with another 1:1:1 FGF-2:heparin:FGFR1 complex to form a dimer (a 2:2:2 "two-end model"). In addition to its role in the formation of FGF/receptor complexes, heparin and other polyanions (see below) have also been argued to reduce the rate of dissociation of the protein from its receptor, thus increasing binding affinity (10). Physical effects of binding of polyanions to the FGFs themselves include stabilization of their structure (13, 16–22), stimulation of their folding (23, 24), and effects on their nonclassical transport into and out of cells (25–27) (see below).

Despite these high-resolution structural studies, which are usually interpreted in terms of a high degree of specificity of polyanion/FGF/receptor interactions, significant evidence exists to argue that such effects are actually relatively nonspecific in the generally understood use of the term. In fact, over 20 different polyanions of widely varying structure, both natural and synthetic, have been shown to stabilize FGF-1 by inhibiting the formation of molten-globule-like states of the protein, which lead to subsequent aggregation (13, 16–23, 25, 28–31). These polyanions include molecules as diverse as polysaccharides, polynucleotides, polypeptides, and synthetic polymers, with the only obvious common feature being their high density of negative charge (20). This nonspecificity is also illustrated by a comparison of the crystal structure of FGF-2 bound to a fragment of heparin and the very different, small molecule sucrose octasulfate (Fig. 5*A*). This disaccharide not only stabilizes and activates FGF-2, but the nature of the complexation of the macromolecules is reminiscent of the symmetric complex formed with heparin (10). We should note that throughout our discussion of "nonspecific" interactions, we do not mean to argue that there does not exist some degree of specificity between polyanions and their multitude of binding partners. More precisely, we argue that there is an intermediate specificity based on a gradient of electrostatic interactions that are a function of relative charge densities in contrast to high conformationally based structure specificity. We will argue below that such nonspecificity between various polyanions and cationic sites on proteins is not the exception but rather is typical of such interactions and may be of great functional consequence for cellular function and some disease states. Furthermore, we will present evidence that the distribution and behavior of proteins within cells may be modulated by such interactions.

EMERGING EVIDENCE FOR THE UBIQUITOUS NATURE OF POLYANION/PROTEIN INTERACTIONS

Polyanions as Storage Depots for Proteins

A commonly hypothesized role for proteoglycans is that of storage sites for various proteins in the extracellular matrix and basement membranes. Release from such complexes is proposed to modulate the activity of such proteins (*e.g.* growth factors). It is well established that FGFs as well as other growth factors can be isolated in large quantities from the polyanionic extracellular matrix (32–34). Competition studies with heparin, heparan sulfate, dermatan sulfate, as well as other polyanions and GAG-specific degrading enzymes implicate the involvement of multiple GAGs in protein storage within the extracellular matrix (34). High salt will also disrupt such interactions, providing additional evidence that the interactions are primarily electrostatic in nature (35). Such interactions appear to be of functional significance because the proteins are localized near sites of activation, alterations of the GAGs perturb activation processes, and bound proteins are protected from proteolytic and other forms of degradation.

Polyanions and Chaperone Function

Polyanions as Molecular Chaperones—Protein folding and transport chaperones are generally thought to be proteinaceous in nature (*e.g.* GRoEL and DnaK). It has been demonstrated, however, that only a small fraction of proteins actually

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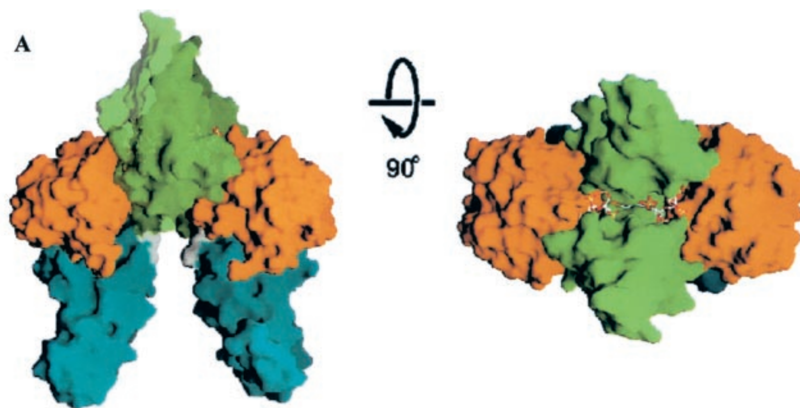

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FIG. 5. **Crystal structures of FGF2: GAG:FGFR1 symmetric complexes.** The color coding for the molecular surface rendering of the proteins is as follows: *green*, FGFR1 immunoglobulin-like D2 domain; *cyan*, FGFR1 immunoglobulin-like D3 domain; *gray*, D2:D3 linker; *orange*, FGF2. The GAGs are ball and stick rendered. *A*, FGF2:sucrose octasulfate:FGFR1 dimer. *B*, FGF2:heparin: FGFR1 dimer. Reprinted with permission from Yeh, B. K., Eliseenkova, A. V., Plotnikov, A. N., Green, D., Pinnell, J., Polat, T., Gritli-Linde, A., Linhardt, R. J., and Mohammadi, M. (2002) Structural basis for activation of fibroblast growth factor signaling by sucrose octasulfate. *Mol. Cell. Biol.* **22,** 7184–7192. Copyright 2002, American Society for Microbiology.

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require known chaperones (36). Some evidence, however, supports the idea that polyanions such as the GAGs and nucleic acids also have (at least) *in vitro* chaperone-like activity mediated by electrostatic interactions between these polyanions and target proteins. For example, heparin, polyvinylsulfate, polyglutamate, RNA, and single- and double-stranded DNA can accelerate the *in vitro* refolding of the Arc repressor protein (37). Polycations have no such effect. The refolding of FGF-1 can also be enhanced by a variety of polyanions (23). In the absence of polyanions, FGF-1 does not refold at temperatures above 30 °C. In contrast, the addition of heparin, inositol hexasulfate, or sodium sulfate permits the protein to be refolded at temperatures up to 50 °C, albeit at a slower rate. Tubulin, a protein with polyanionic character, has also been proposed to possess chaperone activity (see below) (38). Although these are *in vitro* studies, the ubiquitous nature of such polyanions inside the cell suggests that such activity could be of biological significance, especially with regard to the inhibition of protein aggregation during refolding through a simple sequestration mechanism.

The Interaction of Polyanions with Molecular Chaperones—The majority of proteins identified as having chaperone activity were initially identified as heat shock proteins (HSPs), proteins that are characteristically overexpressed in cells under various forms of stress (39–43). Many HSPs have been shown to associate with nucleic acids (44–46), GAGs (47), and proteinaceous polyanions (*e.g.* cytoskeletal proteins (39–43). For example, Hsp15 binds single- and double-stranded DNA, RNA, and the 50S ribosomal subunit (45, 46). In fact, Hsp15 possesses a highly conserved, positively charged RNA-binding motif that can be found in ribosomal proteins, tRNA synthases, and stress proteins (44). Korber *et al.* describe this binding as nonspecific but high affinity (45, 46). Although protein refolding activity has not been demonstrated with Hsp15, this protein appears to have a unique functional activity directed toward nucleic acids. Its effect on free 50S ribosomes with nascent peptide chains suggests the existence of a class of HSPs directed toward nucleic acids instead of proteins. In this regard, Hsp15 appears to be involved in the recycling of the 50S ribosome by binding to ribosomal RNA with an associated nascent chain and aid in its release (45). Evidence that many HSPs can also bind to GAGs comes from the observation that various HSPs can be purified from tumor cell protein extracts using heparin affinity chromatography and require high salt for elution (47). This may be of further significance *in vivo* if affinity for cellular GAGs plays a role in HSP function. Members of both the large (40–42) and small (43) HSP families interact with the polyanion tubulin/microtubules, with varying effects on the structure and stability of

these cytoskeletal proteins. In fact, some Hsp70 proteins were first identified as microtubule-associated proteins (42, 48–50). In addition, the small HSPs (sHSPs), which are themselves polyanions (see below), are thought to be chaperones that are intimately involved in the control of microfilament assembly and activity (42, 51).

Protein Chaperones as Polyanions—Small HSPs have flexible C-terminal extensions that, although variable in length and sequence, are rich in acidic amino acids (52). Although their polyanionic character alone is not sufficient for sHSP function, mutants with C-terminal truncations have reduced function (Ref. 52 and references therein). The primary role of this domain is hypothesized to be the solubilization of the sHSP/protein complex, with unfolded proteins presumably bound to apolar domains of the sHSP. Edwards *et al.*, however, have presented evidence that the sHSP α-crystallin has chaperone activity with FGF-1 mediated by electrostatic interactions between the basic regions of the growth factor (Figs. 3 and 4) and acidic regions of α-crystallin (24). Given the previously mentioned studies of FGF-1 folding in the presence of diverse polyanions, this result is not surprising. Nonspecific interactions between a sHSP and polyanion-binding proteins was also recently elucidated from identifying proteins that bind to *Synechocystis* Hsp16.6 following heat shock of the cyanobacterium (53). Of the 13 proteins whose identities were determined, several also bind to other polyanions. These proteins include an RNA polymerase, a ribosomal protein, and tRNA/rRNA methyltransferase. Additionally, α-synuclein has also been described as similar to sHSPs based on its small size, overexpression at elevated temperatures, acidic C terminus, and *in vitro* chaperone activity (54). Like sHSPs, the polyanion α-synuclein (which also binds to other polyanions; see below) requires the acidic domain for its chaperone function. The importance of this domain to its chaperone function was further demonstrated when the acidic C terminus of α-synuclein (aa 96–140) was fused to the C terminus of glutathione *S*-transferase and dihydrofolate reductase. Both fusion proteins prevented the heat-induced precipitation of aldolase, while neither wild-type glutathione *S*-transferase nor dihydrofolate reductase could prevent aggregation (54).

HSPs are not alone in possessing chaperone function mediated by acidic domains. Nucleolar protein B23 has been hypothesized to be a nucleolar chaperone (55–57). B23 has two acidic regions that are necessary for its chaperone-like activity, and removal of one or both these domains reduces its ability to inhibit bovine liver rhodanse activity by 43 and 70%, respectively (55). As indicated previously, tubulin also displays chaperone-like activity and has been used to suppress the aggregation of soluble lens proteins, equine liver alcohol dehydrogenase, and insulin, but only when tubulin's acidic C terminus was intact (58). Finally, polyanionic propeptides in a number of proteins appear to act as intramolecular chaperones to aid their folding (59–61).

Polyanions and Post-translational Modifications

Zymogens and Propeptides—Many enzymes and hormones are expressed as zymogens, precursor proteins with a peptide (usually N-terminal) that must be cleaved for activation. The highly charged nature of these propeptides is highly conserved, despite little sequence homology among the propeptides even within the same class of enzymes (*e.g.* the propeptide regions of the serine proteases trypsinogen and chymotrypsinogen have no sequence homology) (62). In some proenzymes, prohormones, and prodefensins, acidic homopolymeric Asp regions are present in the propeptides and seem to have multiple functions (63). For example, trypsinogen, the precursor to trypsin, has a DDDDK sequence highly conserved across species that is necessary for recognition by its propeptide cleavage enzyme enterokinase (62). In addition to inhibiting the premature activation of the enzyme defensin or certain hormones by neutralizing the positive charges of the parent protein, the cleaved Asp-rich propeptides also possess antimicrobial activity (63). Thus, the anionic peptide fragments of some zymogens are also hypothesized to be a part of the innate immune system.

Protein Phosphorylation—Protein phosphorylation is the most widely studied post-translational modification that results in protein acidification. Each such covalent modification results in a gain of two negative charges, rendering the protein significantly more anionic. In some cases, the degree of phosphorylation can be quite extensive (64–66). The addition of negatively charged phosphate groups often acts as allosteric regulatory devices by inducing conformational changes within the protein based on the formation or release of electrostatic contacts. The altered conformations permit the phosphorylated proteins to have unique activities relative to their dephosphorylated or less phosphorylated counterparts. Unlike the modification of zymogens, phosphorylated proteins can be turned off by phosphatase dephosphorylation. Several diseases have been linked to the improper induction of kinases and phosphatases. Besides the well-known example of overactive kinases, which are found in cancer cells (67), hyperphosphorylation of the microtubule associated protein tau has been linked to tau fibrillation, which is associated with Alzheimer's disease (64, 68, 69) (see below). The protein stathmin, which also regulates microtubule function, is known to be phosphorylated in up to four positions. The addition of negative charge negates the inhibitory effect of the protein by apparently reducing its interactions with tubulin (70). Numerous other proteins, especially in the kinase and transcription factor family, are subject to hyperphosphorylation, but the role of these modifications remains obscure. Furthermore, we note that intermediate filaments, which are highly polycationic, are subject to hyperphosphorylation imparting partial polyanion properties to them. This phosphorylation appears to be intimately involved in the apoptotic, mechanical, and cell signaling activities of intermediate filaments (71). Other well-

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documented modifications with the potential to introduce extensive negatively charged modifications into proteins include sulfation and sialyation.

Polyanions and Nonclassical Macromolecular Transport

Nonspecific electrostatic interactions appear to be very important in the cellular uptake and compartmentalization of proteins that translocate through what have been referred to as nonclassical routes (26, 27, 72, 73). Examples include proteins that have been modified with positive charges to cross the blood brain barrier as well as proteins that contain naturally basic regions that are responsible for such transport. Synthetic basic peptides and cationic lipids also display such activity and have been used as delivery vehicles for both DNA and non-cell-permeable proteins. Although the exact mechanisms of entry of these proteins are not well-understood (and somewhat controversial), domains of positively charged amino acids that interact with cell-surface polyanions (primarily proteoglycans) are generally agreed to be involved in the transport process.

Cationized Proteins—Cationization (the addition of basic charge) to proteins to enhance their delivery has been practiced for decades. Over 20 years ago, several groups demonstrated that the renal and blood brain barriers are susceptible to penetration by cationized proteins. Even in the early days of cationization, it was postulated that cationized albumin exhibited enhanced uptake both *in vivo* and *in vitro* due to nonspecific, high-affinity interactions with anionic cell-surface moieties (74, 75). Reports of permeabilization by cationized albumin led to the investigation of cationized albumin-peptide chimeras to facilitate the uptake of otherwise impermeable peptides by the blood brain barrier (74, 76, 77). Hexamethylenediamine-cationized immunoglobulins also show enhanced uptake (78). The ability of these proteins to enter cells appears to be generally related to their degree of cationization, with the more basic proteins most effective.

Protein Transduction Domains—Many proteins with naturally occurring regions of high positive charge density appear to cross biological membranes by nonclassical routes. These regions, often designated as protein transduction domains (PTDs), are usually less than 30 amino acids in length and acquire their basic character from high arginine and to a lesser extent lysine contents. Although the mechanism of transport of these domains is still uncertain (with some evidence suggesting that endocytosis is involved), there now seems to be a general consensus of a role for polyanions. Evidence for the involvement of polyanions in this form of transport is discussed below for the uptake of three representative proteins with PTDs (HIV-1 tat, *Drosophila antennepedia*, and herpes virus protein VP22).

HIV-1 tat PTD (localized to the sequence YGRKKRRQRRR) interacts with GAG segments of cell-surface proteoglycans as an early step in internalization (79). The hypothesis that these domains are involved arises in part from the observations that HIV-1 tat PTD binds heparin and that the biological activity of extracellular HIV-1 tat can be inhibited with soluble heparin as well as other GAGs and small polyanions (79–85). HIV-1 tat PTD has also been complexed with various cellular polyanions (*i.e.* heparan sulfate, dextran sulfate, and DNA) with a resultant enhancement in their the cellular uptake (86). The diversity of types of polyanions that can be delivered to cells using the HIV-1 tat PTD and that appear to be involved in the uptake of the protein itself lend additional support to the idea that the interactions themselves are relatively nonspecific although of fairly high affinity.

Drosophila antennepedia PTD is also highly basic, and heparan sulfate proteoglycans have been implicated in its internalization as well (87–89). It has also been reported that cell-surface polysialic acids enhance the uptake of *Drosophila antennepedia* PTD (88, 89). This again suggests the nonspecific nature of the electrostatic interactions involved in this process.

Herpesvirus protein VP22, which has the remarkable ability to both exit and enter cells in a facile manner, also interacts with multiple polyanions (26, 27, 90). Kueltzo *et al.* report that oligonucleotides and heparin both bind to VP22 and stabilize the protein (90). The authors hypothesize that a molten globule-like state of VP22 is necessary in the transport process but that polyanions modulate the presence of this state and in some way mediate transport. In this regard, FGF-1 conjugated to the B-subunit of diphtheria toxin has its entry into cells blocked by the binding of polyanions, possibly through inhibition of the appearance of transport competent partially unfolded states (91).

In attempting to utilize this technology for drug delivery purposes, it has been found that homopolymers of lysine, arginine, histidine, and ornithine can all be internalized (92–94). These peptides differ in their propensity to cross biological membranes depending on their length and the identity of their side chains. In general, polyarginines of 6–11 amino acids have been found to more efficiently enter cells than other basic synthetic homopeptides of similar length. This finding has been attributed to the ability of the arginine guanidinium group to form uniquely strong electrostatic interactions with a variety of negatively charged groups (92). Futaki *et al.* report that a search of the Swiss-Prot database for proteins with seven or more arginines in a 15-aa stretch results in 591 hits, suggesting that many hundreds of proteins may have motifs that might enable the protein to transverse cell membranes or at a minimum bind cellular polyanions electrostatically (95). To illustrate the diversity of polyanions that can interact with polyarginine, a commonly used model for such peptides, the diffusion of fluorescently labeled polyarginine was examined in the presence of various polyanions. We used a 9-aa polypeptide consisting of eight arginine residues and a C terminus tryptophan (RRRRRRRRW or R_8W) to investigate the ability of a polyarginine peptide to be differentially sequestered across a semi-permeable 10,000 mo-

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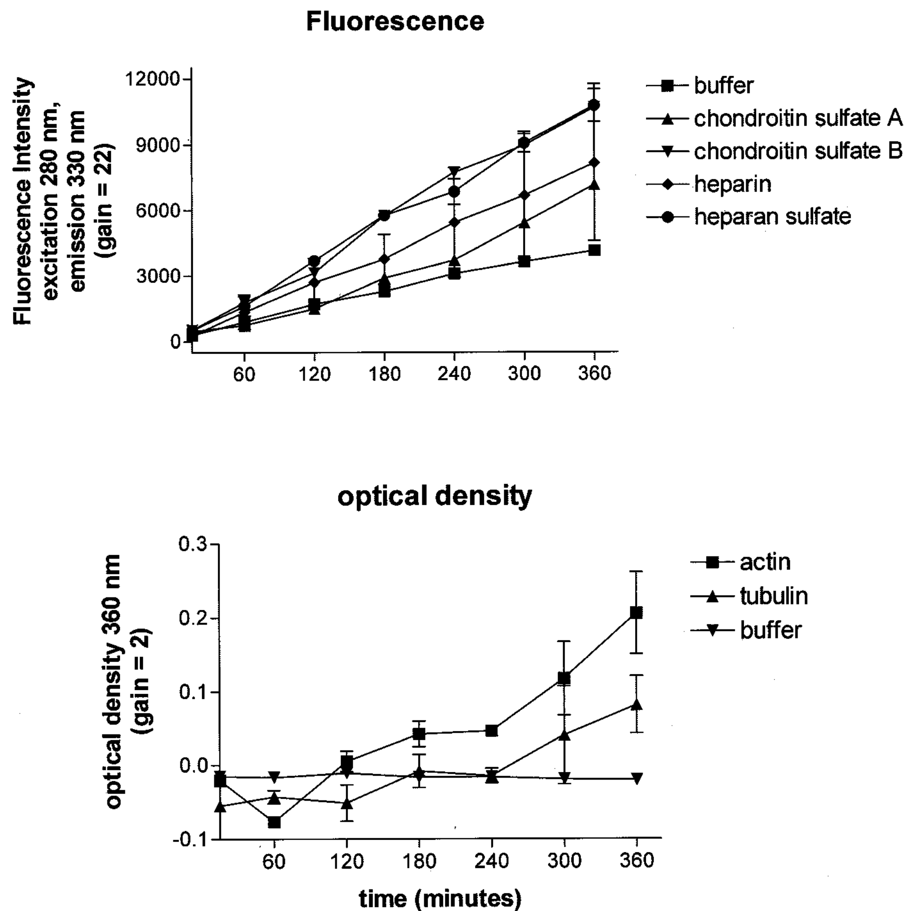

FIG. 6. **These figures illustrate the affinity of a basic peptide (RRRRRRRRW, R_8W) for various polyanions.** Each polyanion (heparin and heparan sulfate, 0.47 mg/ml (3 mM (–) charge); chondroitin sulfate A and chondroitin sulfate B, 1.38 mg/ml (3 mM (-) charge); tubulin, 0.6 mg/ml; and actin, 0.5 mg/ml) occupied a single well in a 10-well microdialysis chamber. The peptide solution (0.1 mg/ml, 335 μM (+) charge) was separated from the wells by a 10,000 MWCO cellulose ester membrane (Spectrum Chemical Company, Gardena, CA). The membrane was only permeable to the peptide. For the polyanions shown, the peptide fluorescence intensities of solutions from the well are greater than that for the well-lacking polyanion (buffer alone). Peptide interactions with actin and tubulin were monitored by actin and tubulin aggregation. Because the beef brain tubulin is susceptible to aggregation at ambient temperature, the experiment was conducted at 4 °C. Aliquots of the actin and tubulin stock solutions were assayed in parallel with the microdialysis samples to confirm that the observed increase in OD of the sample in the microdialysis unit was due to the presence of the basic peptide. (It should be noted that dextran sulfate, DNA, and RNA occupied the remaining wells of the dialysis unit. Membrane fouling (dextran sulfate) and increasing volumes of the polynucleotide wells complicated their analysis. Extensive aggregation of dextran sulfate was observed by monitoring the OD_{350} of a dextran sulfate solution following the addition of the peptide in a conventional UV/Vis spectrophotometer (data not shown). In a separate microdialysis experiment in which the wells only contained either DNA, RNA, or buffer, there is a slight increase in the fluorescence intensity of the polynucleotide-containing wells (data not shown).)

lecular weight cut-off (MWCO) synthetic membrane into various polyanion-containing sample wells of a multi-well microdialysis unit. The peptide in 10 mM sodium phosphate buffer (pH 7.2) was loaded into a common dialysate chamber of the microdialysis unit, and solutions of different cell-surface polyanions (*i.e.* heparin, heparan sulfate, chondroitin sulfate A, chondroitin sulfate B, actin, tubulin) were loaded into each of the sample wells, with one well containing buffer alone as a control. Over a course of 6 h, the solutions in the wells were assayed for evidence of interactions between the peptide and the polyanions by transferring aliquots from the wells to a microtiter plate for analysis. The tryptophan residue permitted fluorescence intensity detection of the peptide in the sample wells in most cases, although OD measurements (reflecting aggregation) were used to assay the wells containing actin and tubulin, which have strong interfering fluorescence intensities. The wells containing chondroitin sulfate A, chondroitin sulfate B, heparin, and heparan sulfate all had higher fluorescence intensities than the buffer well by the conclusion of the study (Fig. 6*A*). This higher intensity indicates the presence of nonspecific interactions between the R_8W peptide and GAGs. OD increases at 360 nm indicate this same peptide interacts with actin and tubulin and induces aggregation (Fig. 6*B*). An interesting consequence of the results of these experiments is

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TABLE I
Neurodegenerative diseases characterized by abnormal proteins and deposits
The information in table is a partial extraction from Ref. 172.

Disease	Protein deposits	Toxic protein	Toxic protein binds polyanions?
Alzheimer's disease	Extracellular plaques	Aβ	Yes
	Intracellular tangles	Tau	Yes
Familial amyotrophic lateral sclerosis	Bunina bodies	Superoxide dismutase 1 (SOD1)	Yes
Parkinson's disease	Lewy bodies	α-Synuclein	Yes
Prion disease	Prion plaque	PrP^{Sc}	Yes
Polyglutamine disease	Nuclear and cytoplasmic inclusions	Polyglutamine-containing protein	Yes
Tauopathy	Cytoplasmic tangles	Tau	Yes

the proposal that transport of proteins containing polycationic regions from one polyanion to another appears feasible. The interactions between the R_8W peptide and the various polyanions were suppressed when the buffer contained 150 mM sodium chloride, providing additional support for the nonspecific, electrostatic nature of the binding.

Basic peptides are not the only agents for which electrostatic interactions with GAGs are proposed to be important. Delivery of complexes of DNA with cationic liposomes has also been proposed to involve cell-surface proteoglycans, and a wide variety of experimental evidence has been presented to support this hypothesis. For example, the diversity in the cell types transfected using cationic lipid/DNA complexes implies that the underlying mechanism is of low specificity (96). Moreover, adding GAGs or heparan sulfate proteoglycans (HSPGs) to the culture media during *in vitro* transfection of cells using cationic lipid/DNA complexes has been shown to lower the transfection efficiency (97, 98). Reduced transfection efficiency using cationic lipid/DNA complexes has also been observed when the level of cell-surface GAGs has been metabolically or enzymatically reduced (99). GAGs may not only be significant in the interaction of the liposomes with the cell surface, but free polyanions may also have roles in releasing the DNA from the complexes (96, 100, 101). Although there is less direct experimental evidence, gene delivery vehicles containing polyethyleneimines, amino dendrimers, and other basic polymers may also utilize this pathway (102).

Polyanions and Diseases

There is considerable support for the involvement of polyanions in that class of diseases that are most often defined in terms of the *in vivo* deposition of certain proteins ("protein solubility diseases"). A partial summary of relevant observations is shown in Table I. As can be seen by inspection of this information, the presence of polyanions (primarily proteoglycans) appears to be ubiquitous in such disorders. In fact, based on this observation, it does not seem entirely fanciful to suggest the idea that the term "protein solubility disease" could be replaced by "polyanion-related disorder."

Neurodegenerative Diseases—Alzheimer's disease is a neurodegenerative disease in which β-amyloid (Aβ) peptide plaques and tangles of tau, a microtubule-associated protein involved in regulating neuronal microtubules stability and orientation, are characteristic markers. Examination of diseased brain tissues has revealed that the polyanions heparan sulfate, keratan sulfate, dextran sulfate, and chondroitin sulfate proteoglycans as well as DNA can all be found associated with the abnormal protein deposits (103–105). Aβ interactions with heparan sulfate and other proteoglycans have been the most extensively studied (103–108). Although the interactions are clearly not highly specific, there are differences in affinities that are related to both sulfation patterns and iduronic acid contents (105). Moreover, soluble heparin, heparan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan sulfate have been shown to induce *in vitro* fibrillation (106, 107). The significance, if any, of differences in affinity of Aβ for the various cellular polyanions with respect to the severity of disease remains to be established.

Tau, the other Alzheimer's disease-related protein that forms characteristic aggregates, is a microtubule-binding protein capable of both reversible and essentially irreversible associations with microtubules (109). Heparin can disrupt the interactions between tau and microtubules. To varying extents, heparin and other GAGs, DNA, RNA, and tubulin can stimulate the phosphorylation of tau by a several kinases (68). *In vivo*, the post-translational acidification of tau is thought to inhibit its binding to the polyanionic microtubule (through charge repulsion?) and is the form found in neurofibrillary tangles. In fact, hyperphosphorylation of tau has been suggested to be responsible for its fibrillation. Dou *et al.* have presented evidence that tau association with microtubules can be enhanced in cultured cells by the up-regulation of two other polyanion-binding proteins, Hsp70 and Hsp90 (110). Moreover, the down-regulation of these same HSPs results in decreased association of tau with microtubules and an increase in tau phosphorylation. These finding correlate with the observed levels of Hsp70 and Hsp90 in the hippocampus of an Alzheimer's disease mouse model and that of an Alzheimer's disease patient relative to normal hippocampus levels (110). Thus, there appears to be a correlation between disease and the levels of certain polyanion-binding proteins. Dou

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et al. suggest that the mechanism of decreased hyperphosphorylation may involve the direct interaction between tau and the HSPs to facilitate the correct folding of tau (110). While this mechanism is quite plausible, we would suggest that competitive interactions with cellular GAGs might also play a role. The binding of polyanions to tau alters its conformation to a form that is similar to the enzymatically hyperphosphorylated form (111). The Dou *et al.* study shows that if the level of one class of polyanion-binding protein (*e.g.* the HSPs) is low, tau becomes hyperphosphorylated and cannot bind to neuronal microtubules. Because HSPs and tau are both polyanion-binding proteins, at normal levels the HSPs may act to sequester cellular polyanions that would otherwise facilitate tau's hyperphosphorylation. Thus, it is possible that competitive interactions between polyanions and various polyanion-binding proteins play a role in regulating the function of tau. There is also evidence for the involvement of other protein/polyanion interactions in the progression of Alzheimer's disease (*e.g.* heparin sulfate regulation of BACE1 (112)), and it follows that yet to be defined roles for polyanions in the progression of Alzheimer's disease seem quite possible.

Prion plaques, which characterize several neurodegenerative diseases in humans and other mammals, are formed when native prion protein (PrP) is conformationally altered to a new form (PrP^{Sc}). Like the protein plaques of Alzheimer's disease, heparan sulfate proteoglycans are associated with these protein deposits and have been implicated in the disease itself (113–115). Studies of the conversion of the wild-type PrP to its pathogenic conformation in both cellular and cell-free environments implicate heparan sulfate in the conversion and subsequent disease progression (113–119). The interaction between PrPs and heparan sulfate proteoglycans is, however, relatively nonspecific. For example, the administration of a variety of other polyanions has been shown to reduce levels of cell-associated PrP (PrP^{c}) at cell surfaces, delay scrapie infection, and reduce PrP^{Sc} formation in infected cells (116, 117, 120). The extent of reduction seems to correlate with the relative binding strengths of the polyanions (117, 120). It has been proposed that the reduction in deposition results from either the stabilization of nonpathological forms of PrP^{Sc} or successful competition with endogenous heparan sulfate proteoglycans for PrP binding (116, 117, 120). Endogenous levels of soluble GAGs or other polyanions may have some role in the disease process as well (116).

The brains of Parkinson's patients contain deposits of the protein α-synuclein. *In vitro* fibrillation of this protein can also be induced by electrostatic interactions with polyanions (121). Basic regions of α-synuclein bind cellular polyanions, including heparin, heparan sulfate, dextran sulfate, chondroitin sulfates A and B, and polyglutamic acid. The net charge of α-synuclein is negative, however, reflecting a high concentration of acidic residues in its C terminus. Thus, α-synuclein itself also possesses significant polyanionic character. One consequence of this is that fibrillation has also been induced *in vitro* with a variety of cellular (putresine, spermine, spermidine, histone) and synthetic (polyarginine, polylysine, polyethyleneimine) polycations (122, 123). The structural diversity of these compounds strongly suggests that fibril induction can be induced by a range of nonspecific electrostatic interactions.

In the neurodegenerative diseases discussed above, it is generally assumed that protein aggregation leads directly to the observed pathology although this view has frequently been challenged. For example, changes in cellular polyanion content or structure could potentially result in alterations of protein folding processes (see above). It is also possible that changes in polyanions could perturb protein/protein interactions. Whatever the actual situation, a direct role for polyanions in these diverse disease processes seems worthy of further consideration.

Bacterial and Viral Infections—To propagate infection, viruses and bacteria must usually gain entry into host cells. To initiate this event, the invaders must be able to interact with naturally occurring cell-surface components, whatever the mechanism of entry, be it through membrane fusion, receptor-mediated endocytosis, pore formation, membrane disruption, or phagocytosis. For many viruses and bacteria, cell-surface proteoglycans as well as other polyanions (*e.g.* polysialic acid) have been shown to serve as receptors or co-receptors facilitating their entry. The entry of influenza virus is known to require the presence of sialic acid moieties at the cell surface (124–127). Recently, this long-established view was challenged by Stray *et al.* who have suggested that the virus could enter desialylated cells via a sialic acid-independent receptor (128). One possibility is that other cell-surface polyanions substitute for sialic acid. There is also evidence that multiple HIV-1 proteins involved in viral entry and infectivity (*e.g.* GP120, vpr, etc.) can interact with extracellular polyanions, cell-surface proteoglycans, and DNA (129, 130). Other viruses known to employ polyanions in their entry pathway include herpes simplex virus 1 (26, 27), human papillomavirus type 16 (HPV-16) and type 33 (HPV-33) (131), classical swine fever virus (132), and hepatitis B virus (133), among others. Many viruses also use microtubules to traffic to the nucleus of cells, although it is currently unclear whether this apparently dynein-dependent process depends on the polyanionic nature of the microtubule.

Pathogenic bacteria also possess surface proteins that bind polyanions that may, in turn, be involved in infectivity. Analysis of the amino acid sequences of the bacterial cell wall proteins of the pathogenic bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* reveal that both have fibronectin-binding sequences (134). Fibronectin is a heparin-binding protein. Fibronectin-binding proteins/fibronectin interactions are important for bacterial entry into host cells and evasion of the host immune system. If bacterial fibronectin-binding proteins and heparin or other polyanions compete for electrostatic interactions with fibronectin, then the pathogen's ability to avoid the immune system may depend, at least in part, on the success

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TABLE II
Functions and diversity of intrinsically disordered proteins (data from Ref. 157)
The proteins in bold type are known polyanion-binding proteins.

Function	No. of examples given	Function	No. of examples given
Protein-tRNA binding	**1**	Fatty acylation	4
Protein-mRNA binding	**1**	Polymerization	4
DNA unwinding	**1**	**Protein-rRNA binding**	**5**
DNA bending	**1**	Protein-lipid interaction	6
Cofactor/heme binding	1	Substrate/ligand binding	6
Methylation	1	Disordered region not essential to function	6
ADP ribosylation	1	Autoregulatory	7
Entropic bristle	1	Regulation of proteolysis in vivo	7
Entropic clock	1	Flexible linkers/spacers	7
Entropic spring	2	Metal binding	9
Protein-genomic RNA binding	**3**	Structural mortar	>10
Glycosylation	3	Unknown	16
Self-transport through channel	**3**	Phosphorylation	16
Protein detergent	3	**Protein-DNA binding**	**19**
Acetylation	4	Protein-protein binding	54

of the fibronectin-binding proteins in the competition. Thus, disease states caused by viral and bacterial infections may also be viewed in terms of alterations in protein/polyanion interactions in the cell and interventions in such pathologies could potentially benefit from therapy at this level (see below).

Polyanions as Drugs

One hint that polyanions may be of some general significance in cellular systems came from the observation that many polyanions have been used as drugs for decades. Best known of these are the anticoagulants such as heparin. Less well-known are polyanions such as suramin, a polysulphonated napthlurea compound that has been used for treatment of early stage trypanosomiasis (African sleeping sickness) since the 1920s (135). Suramin binds to plasma proteins, which are then internalized by the trypanosomes with the result that high concentrations of the polyanion inhibit vital enzymes, a number of which have been identified to bind the drug (136). Suramin has also been administered to combat onchocerciasis (river blindness) caused by the *Onchocerca volvulus* neamotode, although the use of the drug for this indication is tempered by serious systemic toxicity (137). Suramin was one of the earliest drugs found to have anti-HIV activity (138, 139). More recently, this polyanion has been examined as an anti-cancer therapeutic for patients with advanced stages of various cancers (140). Suramin binds to many growth factors containing heparin-binding sites, and it has been suggested that many of its various effects are due to this phenomenon (18). The aluminum salt of sucrose octasulfate (sucralfate) is used to treat and prevent gastrointestinal ulcers. The efficacy of this drug has also been attributed to the activation (through stabilization) of the heparin-binding FGFs, which then stimulate wound healing (141). Human, canine, and equine forms of osteoarthritis are often treated with polysulfated GAGs, most commonly the chondroitin sulfates. Oral administration has resulted in significant relief from pain-related symptoms (142). An intramuscularly injected product for veterinary usage, Adequan® (Luitpold Pharmaceuticals, Inc., Shirley, NY), is employed to not only relieve pain and inflammation but to stimulate repair of damaged cartilage (143). A wide variety of other polyanionic compounds has also been used for various therapeutic applications. These include pentosan polysulfate for proposed anti-tumor activities and inositol hexasulfate (IHP_6), a dietary supplement suggested to have T cell stimulatory effects that leads to immune system enhancements.

Polyanionic therapeutics directed toward the protein-solubility diseases discussed above are also in development. The Canadian-based company Neurochem (Laral, Quebec, Canada) has two "GAG mimetic" drug candidates [Fibrillex™ (phase II/III) and Azhemed™ (phase II completed, extended phase II underway)] in advanced clinical trials. Fibrillex™ is directed toward AA amyloidosis, a fatal disorder with characteristic amyloid deposits in the liver, spleen, and kidney. Similar to Alzheimer's disease, GAGs have also been isolated in the amyloid deposits of AA amyloidsis patients (144, 145). The rationale for using GAG mimetics in the treatment of both conditions is that these small GAG analogues can competitively inhibit the binding of endogenous GAGs to AA or Aβ proteins, thereby preventing fibrillation. The company's drug candidate for the treatment of Alzheimer's disease similarly is designed to prevent the formation of fibrils by binding to soluble Aβ and to inhibit the deposition of brain Aβ fibrils. The success of these approaches in humans remains to be determined, but studies in animal models are promising.

The tentative conclusion we propose from this brief discussion is that the existence of a diverse array of polyanionic

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Table III
Electrostatic patch features analyzed by Stawiski et al. for predicting DNA-binding proteins

The first seven are listed in order of most relevant inputs of the neural networks based on neural net validation. The features below the dotted line are in no particular order (data from Ref. 162).

Electrostatic patch features
Average surface area per residue
Percent patch/cleft overlap
Molecular weight/residue
Number of lysine isostere in Lys_{off} patches
Patch size
Percent of conserved positive residues
Hydrogen-bonding potential—average number of acceptors
....
Percent helix in patch
Hydrogen-bonding potential—average number of donors
Number of polar residues in Lys_{off} patches
Percent conserved arginines
Percent conserved aromatics

drugs with a range of therapeutic activities argues for systemic (or nonspecific) effects on cellular systems in many cases. Although the therapeutic effects of compounds such as heparin, sucrose octasulfate, suramin, the chondroitin sulfates, pentosan polysulfate, and the polyphosphorylated inositols have generally been interpreted in terms of conventional, highly specific interactions with proteins, it seems more likely to us that many of their effects may well be explained by their perturbation of the network of polyanion/protein interactions that exists within and without cells, as we now discuss.

A NETWORK OF POLYANIONS?

There is strong evidence that protein/protein interactions in cells are highly spatially organized. One recent view is that such interactions are well described as scale-free or hierarchical networks (146–149). In random network models, nodes (sites of connection/interaction) are not highly connected. In such a random system of k nodes, all have approximately the same number of links, $<k>$, where $<k>$ is the peak in a Poisson distribution describing the connectivity of the nodes. Thus, in a complex system with a large number of nodes, the probability of a high degree of connectivity among the nodes is low due to the exponential decay dependence of random network behavior. In scale-free models, the connectivity of the nodes possesses power law behavior, and the probability that a node is highly connected to other nodes in a system of k nodes is described by $P(k) \sim k^{\gamma}$. Hierarchical models are modified scale-free systems that permit some degree of modularity or boundaries, providing compartmentalization, within the scale-free model. For both large scale-free and hierarchical networks, the distance or number of links between any two nodes is on average significantly smaller than that of nodes of random networks. In contrast to random networks, scale-free and hierarchical networks have a high degree of redundancy, which from a biological and evolutionary perspective may serve to minimize the occurrence of adverse events upon failure of individual components within the system. Scale-free networks also have the property of being both robust and sensitive, capable of small responses to large stimuli and large responses to small stimuli, respectively (150). Scale-free and hierarchical behavior has been used to describe *Caenorhabditis elegans* nervous systems (151), protein domain networks (148), physical interactions between yeast nuclear proteins (152), and *Escherichia coli* metabolic networks (147, 153), among others. Moreover, networks of the polyanion RNA (154) and genetic systems (which are partially controlled by noncoding RNA) (155) also appear to display scale-free behavior. We find attractive the possibility that an integrated system of polyanions may also be described in terms of network behavior, with the polyanions providing organizational and perhaps other roles in the constitution of biologically functional (*e.g.* protein/protein interaction) networks. In a recent special section in the journal *Science* concerning biological networks, Alon compares biological to engineering networks, emphasizing the common use of modularity, robustness, and the presence of recurring elements in both (156). Using Alon's engineering analogy, we suggest that polyanions may serve as wires, roads, and interaction matrices within biological networks. That is to say, polyanions could serve as links between the interacting nodes and by organizing such interactions, regulate the behavior of the various elements (*e.g.* proteins) of the networks. Alon also argues that "The cell can be viewed as an overlay of at least three types of networks, protein-protein, protein-DNA and protein-metabolite interactions" (156). To this list, we propose the existence of a protein-polyanion network in which polyanions act as nodes and/or connecting branches. When protein-polyanion interactions are considered as entities onto themselves, connectivity emerges that should be both specific and possess the properties of robustness, modularity, and other properties present in other proposed biological networks. It is tempting to predict that such polyanion networks will also be found to be scale-free.

POLYANIONS AND THE PROTEOME

It is in terms of proteomics that the global role of polyanions both within and without cells might have its greatest immediate consequences. What at first appears to be a series of unrelated phenomena whose only common features are the involvement of polyanionic substances, may actually reflect a broad underlying role for highly negatively charged macromolecules in diverse cellular functions and environments. The elucidation of these roles will require that polyanions be taken into account as they relate to the spatial, structural, and temporal behavior of the proteome. Tools now exist to begin to ask to what extent protein/polyanion interactions are present in cells and to probe their biological roles.

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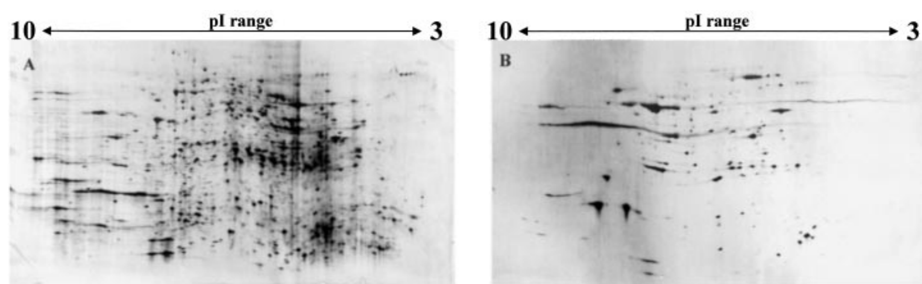

FIG. 7. **These gels illustrate the binding of COS 7 cell proteins to heparin.** Total COS 7 cell protein extract is shown in gel A. Gel B represents proteins remaining in solution after incubation with immobilized heparin for 2 h at 4 °C from the same extract shown in gel A, that is proteins that do not bind to the immobilized heparin. There were 807 fewer proteins detected in gel B than in gel A. Proteins were extracted from the COS 7 cells by transferring frozen cells (7 -9 $\times$ 10^6 cells) to a mortar containing liquid nitrogen and 1- 2 ml of 10 mM sodium phosphate buffer (pH 7.2) with 50 μg of DNase I (Sigma Chemical Co., St. Louis, MO), 2.5 μg of RNase A (Sigma Chemical Co.), and 5 μl of protease inhibitor mixture for mammalian cells (Sigma Chemical Co.) and pulverizing the frozen material with a pestle. The crushed sample was allowed to thaw on ice in 1.7 ml of microcentrifuge tubes. The cell debris was separated from the soluble protein by centrifugation at 14,000 $\times$ *g* at 4 °C. The starting material in each figure for the proteins in the control gel and the gel of the supernatant proteins following incubation with the immobilized polyanion(s) are from a single-cell extract preparation (7 -9 $\times$ 10^6 cells). The immobilized heparin (heparin immobilized on 4% beaded agarose, 10 mg heparin/ml gel; Sigma Chemical Co.) was prepared by rinsing with a 5-volume excess of 10 mM sodium phosphate buffer (pH 7.2) prior to the addition of the protein. For this experiment, 200 μl of heparin agarose gel was washed, and 100 μl of COS 7 cell protein extract was added. All electrophoresis was conducted using a Multiphor II Flatbed electrophoresis system (Amersham Biosciences, Piscataway, NJ), and gels were obtained from Amersham Biosciences. First-dimensional separation of the proteins in the control sample as well as the supernatant following incubation with the immobilized polyanion was conducted simultaneously. Proteins were separated in the first dimension using Immobiline Drystrips (pH 3–10, 24 cm) which were rehydrated according to the manufacturer's instruction in the Drystrip Reswelling Tray. The proteins were loaded onto the gel during rehydration by adding 100 μl of either the whole-cell extract or the supernatant from the incubation with immobilized heparin to 350 μl of rehydration buffer (8 M urea, 2% Triton X-100, 0.5% IPG buffer, pH 3–10, 2.8 mg/ml DTT, and trace bromophenol blue) and then immersing the strip in the rehydration solution. Following rehydration for at least 10 h at room temperature, separation was performed with a gradient program (500 V 2 mA 5W, 1 min; 3,500 V 2 mA 5W, 1 h 30 min; 3,500 V 2 mA 5 W, 10 h 40 min; total time, 12 h 11 min). The temperature of the gels was maintained at 20 °C throughout the first-dimension electrophoresis by using a circulating water bath to cool the electrophoresis unit. As specified in the gel manufacturer's protocol, following the first-dimension separation, the gel strips were stored at −80 °C until the second-dimension separation could be performed. ExcelGel GradientXL,12–14% was used for second-dimension separation, and again the experiment was carried out according to the manufacturer's specifications. Briefly, the first-dimension strips were first equilibrated for 15 min in 15 ml of second-dimension equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, trace bromophenol blue) containing 10 DTT (10 mg/ml). Next, the strip was equilibrated for an additional 15 min in second-dimension equilibration buffer (15 ml) with iodoactamide (25 mg/ml). The equilibrated strip was positioned gel-side down on the ExcelGel, and electrophoresis was begun. The second-dimension electrophoresis conditions were as follows: 200 V 20 mA 20 W, 40 min; and 800 V 40 mA 40 W, 2 h 40 min. After 35 min, electrophoresis was interrupted briefly to remove the first-dimension gel strip. The temperature of the gel was maintained at 15 °C using a recirculating bath to cool the Multiphor Unit. Proteins were visualized by silver stain (PlusOne Silver Staining Kit; Amersham Biosciences). The stained gels were dried and then scanned for analysis using an FX scanner (Bio-Rad, Hercules, CA). The data was analyzed using the Compugen Z3 software package (Compugen, St. Louis, MO).

Computational Approaches—The availability of extensive protein and nucleic acid databases and advances in computational power now provide a mechanism to probe large biological datasets for structural correlations. Such approaches provide some preliminary hints for significant roles of nonspecific electrostatic interactions in various cellular phenomena. For example, using a series of predictors of natural disordered regions (PONDRs) that are based on various neural networks, Dunker *et al.* have predicted the existence of thousands of yet to be identified intrinsically disordered proteins (the depository for intrinsically disordered proteins is available at www.ist.temple.edu/disprot) (157). Until fairly recently, so-called "natively disordered proteins" have received limited attention. This reflects the previous view that the typically highly ordered stereoscopically precise forms of most proteins are necessary for their biological function. This idea appears to be altering, however, to one in which a three (or more)-state "protein trinity" model of protein function is beginning to replace the simple two-state [native (active)/unfolded (inactive)] dichotomy (157–160). In the more complex model, the "native" functional state can be highly ordered (in multiple forms), partially disordered (again in multiple forms), or extensively disordered (also in multiple forms), with each of these conformational states (distributions) possessing some biological activity. One role of polyanions could be to modulate the relative distribution of these states. To illustrate this possibility, Table II lists identified functions of some known disordered proteins (157). From this information, it is immediately clear that approximately a third (29%) of these same proteins are already known to interact with polyanions. This is very much a minimum estimate because the majority of these proteins have never been tested for their polyanion-binding activity. As will be illustrated experimentally below, it may well be that the majority of proteins in a cell fall into

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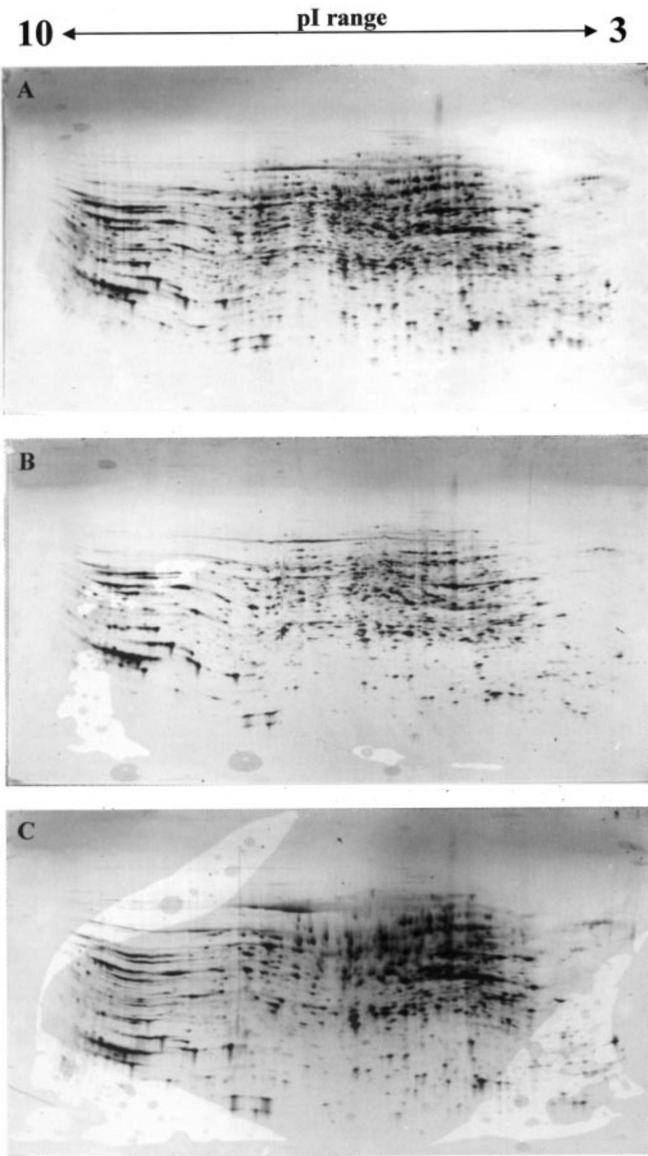

FIG. 8. **These gels indicate that hundreds of COS 7 cell proteins interact with actin and tubulin.** In comparison to the control (*A*), there are fewer spots in the gels of proteins remaining in the supernatant following incubation with immobilized actin (413 fewer proteins) (*B*) or tubulin (681 fewer proteins) (*C*). The experimental protocol is identical to that given in the legend to Fig. 7 with the exception of the preparation of the immobilized polyanions and that twice the amount of cells was used. Immobilized actin and tubulin were prepared by crosslinking biotinylated actin (Cytoskeleton, Denver, CO) or tubulin (Cytoskeleton) to streptavidin (ImmunoPure™ Immobilized Streptavidin; Pierce Chemical Co., Rockford, IL). Briefly, 200 μg of each protein (*i.e.* actin and tubulin) was reconstituted according to the directions on the manufacturer (2 μl of Millipore (Bedford, MA) water was added to each 20-μg vial of biotinylated actin and 4 μl of G-PEM (1 mM GTP, 80 mM Na-PIPES, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA) buffer purchased from Cytoskeleton was added to each 20-μg vial of biotinylated tubulin). The reconstituted proteins were transferred to appropriately labeled microcentrifuge tubes. Following reconstitution, PBS (180 μl for actin and 160 μl for tubulin) was added to each tube to bring the final biotinylated protein concentration to 1 mg/ml. Immobilized streptavidin gel (140 μl) was added to each tube. The beads and proteins were gently mixed for 2 h at 4 °C. The beads were then washed by first adding 1 ml of PBS to each tube and centrifuging at 2,500 × *g* for 2 min and removing the supernatant. Four additional washes were then performed, adding 1 ml of 10 mM sodium phosphate (pH 7.2) to each tube instead of the PBS. The pull-down assays, electrophoresis, staining, and scanning were conducted as described above, except 50 μl of COS 7 cell protein solutions was included in the gel rehydration solutions. The gels were analyzed using the PDquest (Bio-Rad) software package.

the polyanion-binding category. One interesting possibility is that the presence of polyanions acting as molecular chaperones may result in many "disordered" proteins actually being folded inside cells.

Dunker *et al.* have also recently developed a predictor of protein phosphorylation sites, disorder-enhanced phosphorylation (DISPHOS, www.ist.temple.edu/DISPHOS) and found that including disorder prediction in the algorithm yields an optimized phosphorylation predictor with an accuracy that exceeds two other widely used predictors, Scansite (scansite.mit.edu) and NetPhos (www.cbs.dtu.dk/services/NetPhos/) (161). Results of the former approach argue that protein phosphorylation occurs primarily in more disordered regions providing another link between polyanionicity (phosphorylation) and disordered proteins.

Protein databases combined with computational methods have been used to predict proteins that bind to nucleic acids and to identify nucleic acid-binding sites within proteins (162, 163). For example, Stawiski *et al.* selected 304 proteins (54 dsDNA-binding proteins and 250 non-dsDNA-binding proteins) to analyze 12 features (Table III) of positive electrostatic protein patches within these proteins to train neural networks to predict nucleic acid-binding proteins (162). Validation of the parameters included in the predictor reveal that the number of lysine isosteres in Lys_{off} patches (electrostatic patches in which lysine residues have been computationally mutated to a hydrophobic lysine with a net charge of 0) and the percentage of conserved positive residues within a given electrostatic patch across species were among the most critical features necessary for accurate prediction. Using their algorithm, they were able to correctly classify 44 dsDNA-binding proteins and 236 non-dsDNA-binding proteins, reflecting accuracies of 81 and 94%, respectively (162). Jones *et al.* have demonstrated that electrostatic potentials alone can be used to predict DNA-binding sites of DNA-binding proteins, and using this method they were able to correctly identify the DNA-binding sites in 38 of 56 (68%) known DNA-binding proteins (163). This new method outperforms their previously published method, which is based on three-dimensional motif templates, by reducing the number of false-positives (163, 164). Extension of such approaches to other polyanions should be of significant interest (95).

Experimental Approaches—More direct approaches to as-

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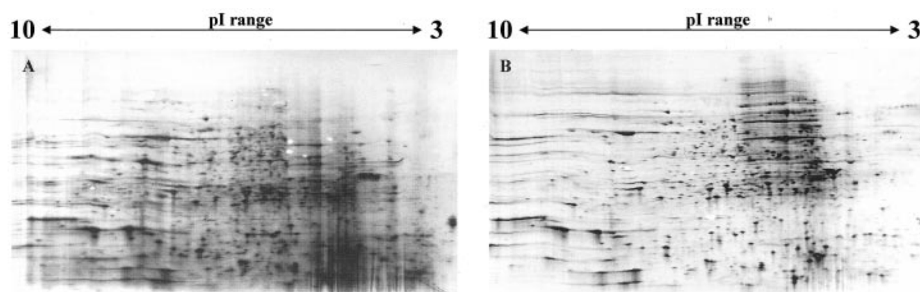

FIG. 9. **When the control gel (*A*) and the gel revealing the proteins remaining in solution following protein extract incubation with immobilized DNA (*B*) are compared, only a small fraction of proteins appear to interact with the DNA.** Two-dimensional gel analysis (PDQuest; Bio-Rad) indicates there are ~100 fewer proteins in gel B. The experimental protocol is essentially as given in the legend for Fig. 7, with the following exceptions. DNase I was not included in the protein extract buffer. Immobilized DNA (DNA cellulose: 4.8 mg dsDNA/g; Sigma Chemical Co.) replaced the immobilized heparin. Prior to incubation with the cell extract, DNA cellulose (10 mg) was suspended in 1 ml of 10 mM sodium phosphate buffer (pH 7.2) and gently mixed for 30 min at 4 °C. The DNA/cellulose gel was centrifuged at 9,000 × *g* and washed two additional times with the sample buffer. The two-dimensional gels were analyzed as above.

certaining the extent of potential protein/polyanion interactions are experimental in nature. For example, Saito *et al.* devised a technique they designate "glyco-Western blotting" (165). This modified Western blotting technique has been used as an "in-gel" probe for proteins that interact with dermatan sulfate, a GAG. This technique was applied to 3LL and A549 cellular extracts (mouse and human lung cells, respectively), and over 30 bands appeared on each blot. Confirmation that the bands resulted from electrostatic interactions between the proteins and dermatan sulfate was evident from the diminished blotting efficiency seen at increased salt concentrations and the ability of unlabelled dermatan sulfate to displace the labeled GAG. The authors report that "chondroitin sulfate A and heparan sulfate were not effective [competitors] under similar conditions" (165), but the extent of this apparent specificity was not examined with a diverse panel of polyanions.

Two-dimensional gel electrophoresis can also be used probe the degree to which proteins have the potential to interact with various cellular polyanions. To demonstrate the utility of this approach, we used "pull down" studies employing immobilized polyanions to deplete COS 7 cell protein extracts of proteins that bind to the various polyanions (Figs. 7-9). Despite significant experimental variability in the maximum number of proteins detectable in each gel set (*i.e.* the number of detected proteins in control gels range from 1,145 to 2,648), comparisons of gels of the proteins remaining in solution following incubation with matrix-bound polyanions *versus* the total proteins extracted during an individual extraction preparation reveal that hundreds to thousands of COS 7 proteins interact with cellular polyanions under these conditions. The greatest extent of interaction (944 proteins out of a total of 1,751) was observed when heparin, a polyanion with a high negative charge density, was employed (Fig. 7). Proteins are clearly missing from both basic and acidic regions of the gel, confirming that the net protein charge is not necessarily an indicator of the propensity to bind polyanions. This again presumably reflects the presence of well-established localized polyanion binding regions in such proteins. Immobilized actin and tubulin depleted the cell extract similarly, with 413 proteins missing from the actin gel and 681 proteins missing from the tubulin gel with respect to the 2,648 proteins seen in the control gel (Fig. 8). Immobilized DNA, which somewhat surprisingly was least effective in depleting the sample, resulted in a loss of ~100 proteins (1,046 protein spots remaining *versus* 1,145 in the control) (Fig. 9). Similar results were obtained when bound proteins were eluted from matrices by high salt and examined by two-dimensional electrophoresis. Careful comparison of all of the experiments suggest that many, if not most, of the proteins bind to several or all of the polyanions, consistent with the low selectivity of the protein/polyanion interactions. Unique interactions as well as differences in the extent of interaction (affinity) are clearly present, however. The identities of the proteins that interact with the various polyanions are currently being established by two-dimensional liquid chromatographic/mass spectrometric methods.

To gain a more intuitive, visual idea of the extent to which polyanion-binding proteins are present in cells, a high-resolution, two-dimensional chromatographic method was used. Levels of individual proteins in COS 7 cell extracts before and after incubation with heparinized beads were examined using a Beckman Coulter ProteomeLab PF2D protein fractionation system (Beckman Coulter, Fullerton, CA). Analogous to two-dimensional gel electrophoresis, this method first separates proteins by chromatofocusing. The proteins are then further separated using reversed-phase chromatography. After performing such operations on both whole-cell extracts (control) and the supernatant from the extract incubated with heparinized beads, the intensities of the chromatograms' peaks can be compared and differences plotted. In Fig. 10, the red bars indicate proteins in the whole-cell extract that are present in greater amounts than in the supernatant from the heparin bead complexes, while the green bars designate greater

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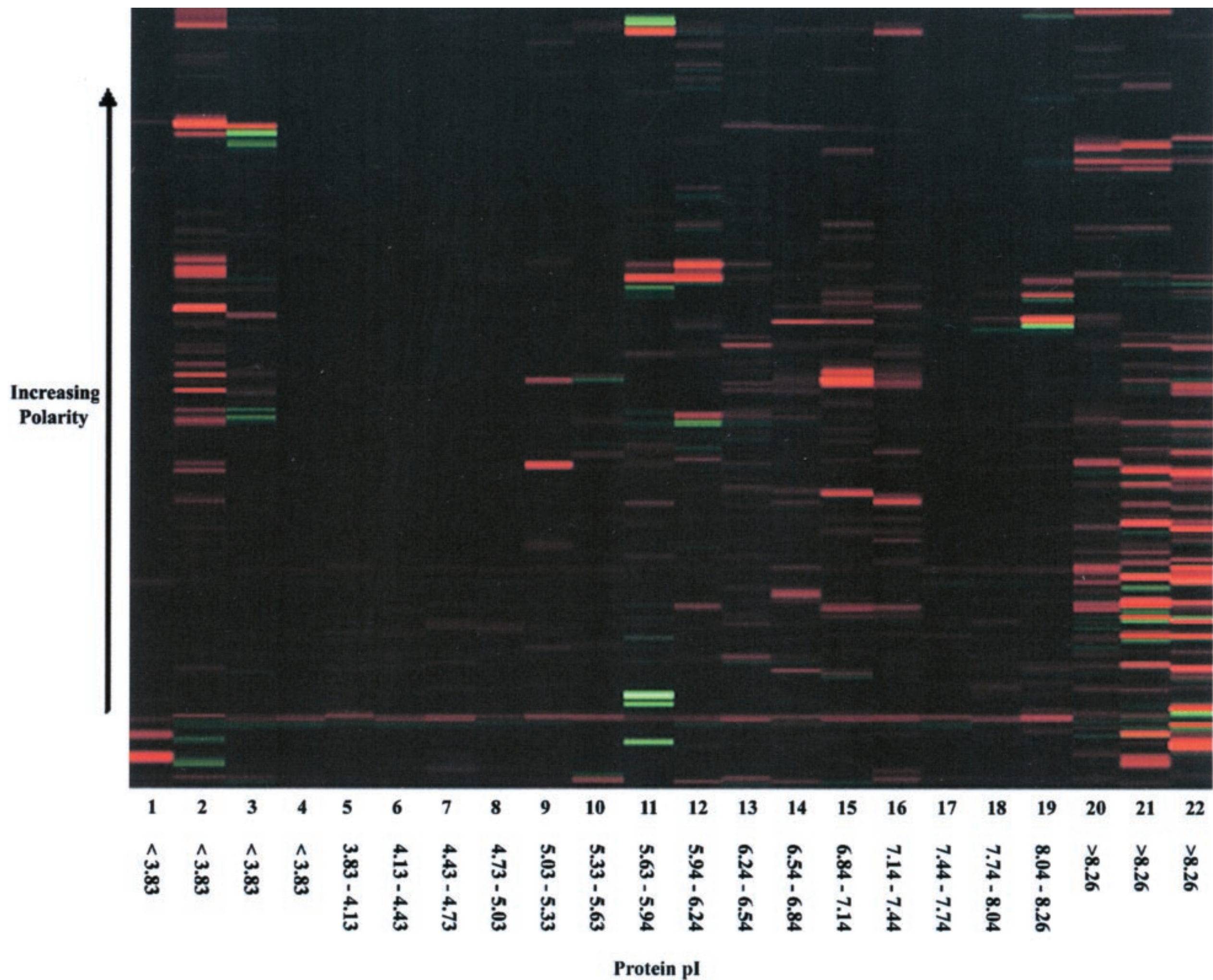

FIG. 10. **Comparison map of protein levels in COS 7 cell extract before (*red*) and after (*green*) incubation with heparinized beads.** The intensity of the color is proportional to the difference in protein levels. The COS 7 cell protein was extracted using liquid nitrogen as described in the legend for Fig. 7. Again, 10 mM sodium phosphate (pH 7.2) is the buffer. As in the two-dimensional gel studies, a single extract preparation was used for the control sample, and the sample was incubated with the immobilized heparin for 2 h at 4 °C. Prior to injection into the ProteomeLab PF2D system, the proteins were exchanged into start buffer (pH 8.3) using a PD-10 column, as specified by Beckman Coulter. The chromatography was performed using a Beckman Coulter protocol. For the first dimension, 1 ml of the protein sample in start buffer was injected. The flow rate was 0.2 ml/min and the column (HPCF 1D Column; Beckman Coulter) was at ambient temperature. Prior to the start of the run, the chromatofocusing column was pre-equilibrated with 100% start buffer (pH 8.3). For the first 20 min, 100% start buffer was used to remove proteins that don't bind to the column. The flow was then switched to 100% eluent buffer (pH 3.78), and the pH began to decrease ~45 min into the run. The pH continued to decrease for ~45 min. At 95 min, the flow was changed to 100% 1 M sodium chloride to wash off any proteins that remained associated with the column after the pH gradient. Fractions were collected as 0.5-ml sample volumes or at 0.3 pH intervals, whichever came first. An in-line pH meter was used to monitor the pH, and in-line UV absorbance at 280 nm was used to detect the proteins. Each second-dimension run used a 200-μl injection of a single fraction collected during the chromatofocusing step (the lanes in this figure represent 22 injections or second-dimension chromatography runs). All second-dimension chromatography was conducted at a column temperature of 50 °C and a buffer flow rate of 0.75 ml/min. A Beckman Coulter HPRP 2D column was used. The reversed-phase column (preheated to 50 °C) was pre-equilibrated with 0.08% TFA in water (buffer A) before the start of each run. At the beginning of the chromatography, the flow was 75% buffer A and 25% buffer B (0.1% TFA in ACN). After 2 min, a gradient from 25–45% buffer B was run for 20 min. This was followed by a 6-min gradient from 45–75% buffer B, which was followed by a 75–100% buffer B 1-min gradient. The protein was detected using an in-line UV detector set at 214 nm. The individual chromatograms were baseline corrected using a GRAMS AI/Razor Tools software package. The ProteomeLab PF2D software suite was used for all other data analysis (*i.e.* peak picking, peak quantification, peak matching, and graphing).

peak intensities in the supernatant sample. The relative intensities of the colors are directly proportional to the differences in protein concentrations. Regions of black indicate no measurable difference in protein levels. As observed using two-dimensional gel electrophoresis, the protein map in Fig. 10 indicates that hundreds of proteins bind to heparin. In fact,

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Table IV
Antibodies on the Becton Dickinson microarray with differential binding between control sample and the supernatant of the same extract following incubation with heparinized agarose beads for 1 h

Antibody	Antigen	M_r pI length	Potential polyanion-binding regions	Comments
Acetylcholinesterase	Acetylcholinesterase precursor	67796.06 5.87 **614**	**RRR** 275–277	
Adaptin g	Adapter-related protein complex 1 γ-1 subunit	91260.29 6.41 **821**	**RKK** 153–155 **RHR** 344–346 **KRR** 361–363	
AMPK b	5′-AMP-activated protein kinase, β-1 subunit	30251.12 5.95 **269**	**RH**GG**HK**TP**RR** 12–21 **HR**Y**KKK** 254–259	The longest consecutive stretch is near the C-term
Androgen receptor	Androgen receptor	98988.63 6.00 **919**	**K**F**RRK** 605–609 **RK**L**KK** 629–633	
ARF3	ADP-ribosylation factor 3	20469.57 7.04 **180**	**H**SL**RHR** 145–150 **K**N**KK** 177–180	
Cadherin-5	Vascular endothelial-cadherin precursor	87516.36 5.22 **784**	**HRR**Q**KR** 42–47 **RRR** 621–623	
CDC27	Protein CDC27Hs	91867.33 6.59 **824**	**KK**L**K**M**K** 394–399	
CDC42GAP	Rho-GTPase-activating protein 1	50435.76 5.85 **439**	**RK**Y**KK**NI**K** 149–156	
CRIK	Citron protein	146506.37 6.49 **1286**	**KKH** 400–402 **KKKK** 509–512 **HRK** 554–556 **RRK** 598–600 **HHR** 1169–1171	There are 20 pairs of positively charged amino acids in the sequence, not including those amino acids sequences at the left. This information is for the fragment
Cyclin D2	G1/S-specific cyclin D2	33067.23 5.06 **289**	**RR**AV**R**D**R** 12–18 **RKH** 178–180	
DLP1	Disks large-associated protein 1	108873.36 6.66 **977**	**R**S**HHH** 8–12 **HH** S.D.**RK** 25–30 **R**S**K**S**K**E**RR** 181–188 **RKK**D**H**F**KKR** 639–648 **KK**E**RR** 907–911	
FKBP 51	51-kDa FK506-binding protein	51212.16 5.71 **457**	**RR**T**KRK** 150–155 **KK**A**K**E**H** 396–401	
FMS (CD115)	Macrophage colony stimulating factor I receptor precursor	107983.76 5.93 **972**	**R**V**R**G**R**PLM**RH** 142–151 **K**Y**K**Q**K**P**K** 539–545 **RRK** 676–678 **KK**YV**RR** 706–711	
FXR2	Fragile X mental retardation syndrome-related protein 2	74128.16 5.85 **673**	**RRRR**S**RRRR** 544–552 **R**PQ**RR**N**R**S**RRRR**N**R**GN**R** 581–597	RNA-binding protein
GAGE	GAGE-8	12763.60 4.23 **116**	**R**P**R**P**RR** 10–15	

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Table IV—*continued*

Antibody	Antigen	M_r pI length	Potential polyanion-binding regions	Comments
Max	Max protein	18274.83 5.88 **160**	**KR**A**HH** 24–28 **RKRR** 33–36 **RRK**N**H** 75–79 **RKK**L**R** 152–156	DNA binding (basic domain) 24–36
MKK7	Dual specificity mitogen-activated protein kinase kinase 7	47484.90 9.26 **419**	**R**EA**RRR** 22–27 **K**D**HRKKR**P**K** 363–370	ATP-binding region
Neurogenin3	Neurogenin 1	25747.77 6.90 **237**	**RRRRRR**G**R**T**R**V**R** 71–81 **H**SL**RR**S**RR**V**K** 87–96 **R**E**R**N**R**M**H** 100–106	Basic domain 93–104 (DNA Bind)
Nur77	Orphan nuclear receptor HMR	64463.33 6.82 **598**	**KRRR**N**R** 313–318 **K**G**RR**G**R** 343–348	267–332 DNA-binding region
p140mDia	Diaphanous protein homolog 1	138978.58 5.31 **1248**	**KKK**G**R** 17–21 **KKK**V**K** 819–823 **RRKR** 1189–1192	Binds to GTP-bound RHO and profiling to promote actin polymerization
p300	E1A-associated protein p300	264143.51 8.78 **2414**	**KR**P**K** 14–17 **KHK** 77–79 **KRK** 334–336 **KRR**T**R** 647–651 **KKK** 1045–1047 **KRK** 1103–1105 **KRK** 1233–1235 **RKR** 1527–1529 **KKK**NN**KK** 1549–1555 **KKK** 1568–1570 **KHK** 1590–1592 **KHH** 1674–1676 **KRK** 1772–1774 **RRR** 1829–1831	Nuclear localization signal (pot.) 11–17 There are 31 pairs of basic amino acids in the sequence
Per2	Period circadian protein 2	136579.47 6.04 **1255**	**KK**A**K**G**K** 126–131 **HKK** 382–384 **RRRR** 510–513 **KKK**537–539 **KRK** 621–623 **KK**TG**K**N**RK**L**K**S**KR**V**K**P**R** 789–805	Nuclear localization signal 789–806
PU.1(Spi-1)	31-kDa transforming protein	30408.13 6.20 **264**	**KKK**I**R** 161–165 **KHK** 198–200 **RKK** 214–216 **KK**V**KKK** 236–241 **RRH**PP**H** 259–264	DNA binding (ETS-domain) 164–248
Rb2	Retinoblastoma-like protein 2	128356.61 7.27 **1139**	**RRR** 679–681 **RKK** 865–867 **K**G**KRKRR** 933–939 **KKR** 1100–1102	There are 18 pairs of basic amino acids
SRP54	Signal recognition particle 54-kDa protein	55704.71 8.87 **504**	**KRK** 71–73 **RHK** 194–196	
Synaptotagmin	Synaptotagmin I	47573.11 8.26 **422**	**KK**CLF**KKK**N**KKK**G**K**E**K**GG**K** 81–99 **KKKK** 190–193 **KR**L**KKKK**TTI**KK** 322–333 **KK** 421–422	C2B domain is highly basic and binds polynucleotides, inositol phosphates, and lipids

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TABLE IV—*continued*

Antibody	Antigen	M_r pI length	Potential polyanion-binding regions	Comments
TEF-1	Transcriptional enhancer factor TEF-1	47945.54 8.33 **426**	**RRK** 55–57 **K**T**R**T**RK** 83–88 **RRK**S**R**DF**H** 99–106 **HK**L**KH** 377–371	DNA-binding domain 30–97 R/H/K-RICH REGION 55–121
VHL	Von Hippel-Lindau disease tumor suppressor	24152.78 4.7 **213**	**RR** 3–4 **RR**I**H** 107–110 **RR** 176–177	The 107–110 region is among those that vary in VHL disease
ZO-1	Tight junction protein ZO-1	194722.56 6.32 **1736**	**RRKKK** 95–99 **KKK** 490–492	

out of a total of 1,473 peaks resolved, 799 represented proteins with a concentration in the control (whole-cell extract) of at least twice that in the supernatant from the heparin bead extract. If this range is extended to include proteins with peak intensities that are at least 1.5 times that in the control, this number is increased to 937. Conversely, only 123 proteins had peak intensities that were at least two times as high in the supernatant sample than the control.

To further characterize the complement of polyanion-binding proteins in cells, we employed antibody microarrays. Examination of the levels of proteins remaining in the supernatant following incubation of COS 7 cell protein extract with heparinized beads in comparison to whole-cell lysates identifies 29 out of 378 proteins defined by the array as polyanion-binding proteins (Table IV). A wide variety of different types of proteins are identified by this method. Again, the net charge does not necessarily correlate with a protein's ability to interact with polyanions. In contrast, all of these polyanion-binding proteins have long runs of basic amino acids (Table IV). Several are also nucleic acid-binding proteins (*i.e.* GAGE-8, Max protein, orphan nuclear receptor protein, 31-kDa transforming protein, and transcriptional enhancer factor TEF-1) or possess ATP-binding domains (*i.e.* dual-specificity mitogen-activated protein kinase kinase 7). At least one is known to have a role in actin polymerization (diaphanous protein homolog 1), and several have basic nuclear localization sequences (*i.e.* period circadian protein 2 and E1A-associated protein p300). Synaptotagmin I, which possess a highly basic C2B domain that is known to bind polynucleotides, inositol phosphates, and lipid (166), was also identified as a heparin-binding protein using the microarray. More extensive microarrays should be useful in further global analysis of cellular polyanion-binding proteins.

CONCLUSIONS

We propose that a matrix (or more formally, a network) of polyanions including GAGs, lipid-bilayer head groups, microtubules, microfilaments, and polynucleotides, among others, exists on the surface and inside cells that is of functional significance. This network may even extend into the systemic circulation with serum albumin serving as the major polyanion. This extended polyanionic surface may provide a spatially organizing entity that brings together proteins for specific interactions, a guide for transport and a chaperone-like activity to support protein folding and stabilization. Although we have not considered it at any detail here, it seems reasonable to hypothesize that small polyanions such as the inositol phosphates (InP_n, $n = 4-6$) and the more highly phosphorylated nucleotides (*e.g.* ATP, Ap_nA, etc.) might modulate binding of proteins to the polyanion network through competitive interactions thereby regulating various activities. This might account for the diverse activities of many polyanions inside cells that have yet to be clearly defined (167–170). We also postulate that alterations in this polyanionic network may be involved in certain disease processes. Preliminary proteomic analysis presented here suggests that a large number of proteins are involved in interactions with this matrix primarily through charge/charge interactions. The specificity of these protein/polyanion interactions appears to be significantly less than the high-affinity interactions that dominate our current understanding of biochemical systems, but some degree of specificity must certainly be present although yet to be clearly defined. Although the skeptical reader may not unfairly accuse the authors of simply rediscovering cation exchange-based chromatography, we are simply arguing for a functional role for interactions that must certainly be present in cells. Recent prebiotic studies of clay montmorillonites indicate that negatively charged clays can become encapsulated into anionic fatty acid micelles and catalyze polymerization of RNA. Thus, a global role for polyanions might be postulated, traceable back to the early precellular world (171) and provide a potential explanation for the ubiquitous role of polyanions in present day cellular activity. An additional conclusion suggested from the preceding considerations is that caution needs to be exercised in terms of the frequent observation that a particular protein binds to polyanions whether they be heparin, proteoglycans, polynucleotides, microtubules, or microfilaments. This interaction may well reflect a nonspecific association that, while in one context be of functional signif-

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icance as discussed above, may not be properly attributable to the more specific functional alteration tested. If we are to understand the proteome, it is our belief that interactions between proteins will, in many cases, need to be understood in the context of such polyanionic-based interactions. It seems quite likely that many more examples of polyanion/protein interactions of potential functional significance will eventually be elucidated. Therefore, definition of the "polyanion/protein world" should become an important new area of inquiry.

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REFERENCES

1. Ptashne, M., and Gann, A. (2002) *Genes and Signals*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Medalia, O., Weber, I., Frangakis, A. S., Nicastro, D., Gerisch, G., and Baumeister, W. (2002) Macromolecular architecture in eukaryotic cells visualized by cryoelectron tomography. *Science* **298,** 1209–1213
3. Conrad, H. E. (1998) *Heparin-binding proteins*, Academic Press, San Diego, CA
4. Ribatti, D., Vacca, A., and Presta, M. (2000) The discovery of angiogenic factors: A historical review. *Gen. Pharmacol.* **35,** 227–231
5. Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., and Klagsbrun, M. (1984) Heparin affinity: Purification of a tumor-derived capillary endothelial cell growth factor. *Science* **223,** 1296–1299
6. Pye, D. A., Vives, R. R., Hyde, P., and Gallagher, J. T. (2000) Regulation of FGF-1 mitogenic activity by heparan sulfate oligosaccharides is dependent on specific structural features: Differential requirements for the modulation of FGF-1 and FGF-2. *Glycobiology* **10,** 1183–1192
7. Catlow, K., Deakin, J. A., Delehedde, M., Fernig, D. G., Gallagher, J. T., Pavao, M. S., and Lyon, M. (2003) Hepatocyte growth factor/scatter factor and its interaction with heparan sulphate and dermatan sulphate. *Biochem. Soc. Trans.* **31,** 352–353
8. Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., Linhardt, R. J., and Mohammadi, M. (2000) Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell* **6,** 743–750
9. Ye, S., Luo, Y., Lu, W., Jones, R. B., Linhardt, R. J., Capila, I., Toida, T., Kan, M., Pelletier, H., and McKeehan, W. L. (2001) Structural basis for interaction of FGF-1, FGF-2, and FGF-7 with different heparan sulfate motifs. *Biochemistry* **40,** 14429–14439
10. Yeh, B. K., Eliseenkova, A. V., Plotnikov, A. N., Green, D., Pinnell, J., Polat, T., Gritli-Linde, A., Linhardt, R. J., and Mohammadi, M. (2002) Structural basis for activation of fibroblast growth factor signaling by sucrose octasulfate. *Mol. Cell. Biol.* **22,** 7184–7192
11. Zhu, X., Hsu, B. T., and Rees, D. C. (1993) Structural studies of the binding of the anti-ulcer drug sucrose octasulfate to acidic fibroblast growth factor. *Structure* **1,** 27–34
12. Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., and Rees, D. C. (1991) Three-dimensional structures of acidic and basic fibroblast growth factors. *Science* **251,** 90–93
13. Chavan, A. J., Haley, B. E., Volkin, D. B., Marfia, K. E., Verticelli, A. M., Bruner, M. W., Draper, J. P., Burke, C. J., and Middaugh, C. R. (1994) Interaction of nucleotides with acidic fibroblast growth factor (FGF-1). *Biochemistry* **33,** 7193–7202
14. Pellegrini, L., Burke, D. F., von Delft, F., Mulloy, B., and Blundell, T. L. (2000) Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature* **407,** 1029–1034
15. Plotnikov, A. N., Schlessinger, J., Hubbard, S. R., and Mohammadi, M. (1999) Structural basis for FGF receptor dimerization and activation. *Cell* **98,** 641–650
16. Burke, C. J., Volkin, D. B., Mach, H., and Middaugh, C. R. (1993) Effect of polyanions on the unfolding of acidic fibroblast growth factor. *Biochemistry* **32,** 6419–6426
17. Mach, H., Ryan, J. A., Burke, C. J., Volkin, D. B., and Middaugh, C. R. (1993) Partially structured self-associating states of acidic fibroblast growth factor. *Biochemistry* **32,** 7703–7711
18. Middaugh, C. R., Mach, H., Burke, C. J., Volkin, D. B., Dabora, J. M., Tsai, P. K., Bruner, M. W., Ryan, J. A., and Marfia, K. E. (1992) Nature of the interaction of growth factors with suramin. *Biochemistry* **31,** 9016–9024
19. Tsai, P. K., Volkin, D. B., Dabora, J. M., Thompson, K. C., Bruner, M. W., Gress, J. O., Matuszewska, B., Keogan, M., Bondi, J. V., and Middaugh, C. R. (1993) Formulation design of acidic fibroblast growth factor. *Pharm. Res.* **10,** 649–659
20. Volkin, D. B., Tsai, P. K., Dabora, J. M., Gress, J. O., Burke, C. J., Linhardt, R. J., and Middaugh, C. R. (1993) Physical stabilization of acidic fibroblast growth factor by polyanions. *Arch. Biochem. Biophys.* **300,** 30–41
21. Volkin, D. B., Verticelli, A. M., Marfia, K. E., Burke, C. J., Mach, H., and Middaugh, C. R. (1993) Sucralfate and soluble sucrose octasulfate bind and stabilize acidic fibroblast growth factor. *Biochim. Biophys. Acta* **1203,** 18–26
22. Volkin, D. B., Verticelli, A. M., Bruner, M. W., Marfia, K. E., Tsai, P. K., Sardana, M. K., and Middaugh, C. R. (1995) Deamidation of polyanion-stabilized acidic fibroblast growth factor. *J. Pharm. Sci.* **84,** 7–11
23. Dabora, J. M., Sanyal, G., and Middaugh, C. R. (1991) Effect of polyanions on the refolding of human acidic fibroblast growth factor. *J. Biol. Chem.* **266,** 23637–23640
24. Edwards, K. L., Kueltzo, L. A., Fisher, M. T., and Middaugh, C. R. (2001) Complex effects of molecular chaperones on the aggregation and refolding of fibroblast growth factor-1. *Arch. Biochem. Biophys.* **393,** 14–21
25. Mach, H., and Middaugh, C. R. (1995) Interaction of partially structured states of acidic fibroblast growth factor with phospholipid membranes. *Biochemistry* **34,** 9913–9920
26. Kueltzo, L. A., and Middaugh, C. R. (2003) Nonclassical transport proteins and peptides: An alternative to classical macromolecule delivery systems. *J. Pharm. Sci.* **92,** 1754–1772
27. Kueltzo, L. A., and Middaugh, C. R. (2000) Potential use of non-classical pathways for the transport of macromolecular drugs. *Expert Opin. Investig. Drugs* **9,** 2039–2050
28. Copeland, R. A., Ji, H., Halfpenny, A. J., Williams, R. W., Thompson, K. C., Herber, W. K., Thomas, K. A., Bruner, M. W., Ryan, J. A., Marquis-Omer, D., et al. (1991) The structure of human acidic fibroblast growth factor and its interaction with heparin. *Arch. Biochem. Biophys.* **289,** 53–61
29. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) Heparin structure and interactions with basic fibroblast growth factor. *Science* **271,** 1116–1120
30. Mach, H., Volkin, D. B., Burke, C. J., Middaugh, C. R., Linhardt, R. J., Fromm, J. R., Loganathan, D., and Mattsson, L. (1993) Nature of the interaction of heparin with acidic fibroblast growth factor. *Biochemistry* **32,** 5480–5489
31. Mach, H., and Middaugh, C. R. (1994) Probing the affinity of polyanions for acidic fibroblast growth factor by unfolding kinetics. *Arch. Biochem. Biophys.* **309,** 36–42
32. Weiner, H. L., and Swain, J. L. (1989) Acidic fibroblast growth factor mRNA is expressed by cardiac myocytes in culture and the protein is localized to the extracellular matrix. *Proc. Natl. Acad. Sci. U. S. A.* **86,**

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2683–2687
33. Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., and Klagsbrun, M. (1987) Endothelial cell-derived basic fibroblast growth factor: Synthesis and deposition into subendothelial extracellular matrix. *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2292–2296
34. Bashkin, P., Doctrow, S., Klagsbrun, M., Svahn, C. M., Folkman, J., and Vlodavsky, I. (1989) Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules. *Biochemistry* **28,** 1737–1743
35. Baird, A., and Ling, N. (1987) Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: Implications for a role of heparinase-like enzymes in the neovascular response. *Biochem. Biophys. Res. Commun.* **142,** 428–435
36. Van Dyk, T. K., Gatenby, A. A., and LaRossa, R. A. (1989) Demonstration by genetic suppression of interaction of GroE products with many proteins. *Nature* **342,** 451–453
37. Rentzeperis, D., Jonsson, T., and Sauer, R. T. (1999) Acceleration of the refolding of Arc repressor by nucleic acids and other polyanions. *Nat. Struct. Biol.* **6,** 569–573
38. Manna, T., Sarkar, T., Poddar, A., Roychowdhury, M., Das, K. P., and Bhattacharyya, B. (2001) Chaperone-like activity of tubulin. binding and reactivation of unfolded substrate enzymes. *J. Biol. Chem.* **276,** 39742–39747
39. Csermely, P., and Kahn, C. R. (1991) The 90-kDa heat shock protein (hsp-90) possesses an ATP binding site and autophosphorylating activity. *J. Biol. Chem.* **266,** 4943–4950
40. Nishida, E., Koyasu, S., Sakai, H., and Yahara, I. (1986) Calmodulin-regulated binding of the 90-kDa heat shock protein to actin filaments. *J. Biol. Chem.* **261,** 16033–16036
41. Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H., and Yahara, I. (1986) Two mammalian heat shock proteins, HSP90 and HSP100, are actin-binding proteins. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8054–8058
42. Liang, P., and MacRae, T. H. (1997) Molecular chaperones and the cytoskeleton. *J. Cell Sci.* **110,** 1431–1440
43. Hino, M., Kurogi, K., Okubo, M. A., Murata-Hori, M., and Hosoya, H. (2000) Small heat shock protein 27 (HSP27) associates with tubulin/microtubules in HeLa cells. *Biochem. Biophys. Res. Commun.* **271,** 164–169
44. Staker, B. L., Korber, P., Bardwell, J. C., and Saper, M. A. (2000) Structure of Hsp15 reveals a novel RNA-binding motif. *EMBO J.* **19,** 749–757
45. Korber, P., Stahl, J. M., Nierhaus, K. H., and Bardwell, J. C. (2000) Hsp15: A ribosome-associated heat shock protein. *EMBO J.* **19,** 741–748
46. Korber, P., Zander, T., Herschlag, D., and Bardwell, J. C. (1999) A new heat shock protein that binds nucleic acids. *J. Biol. Chem.* **274,** 249–256
47. Menoret, A., and Bell, G. (2000) Purification of multiple heat shock proteins from a single tumor sample. *J. Immunol. Methods* **237,** 119–130
48. Weller, N. K. (1988) A 70 kDa microtubule-associated protein in NIL8 cells comigrates with the 70 kDa heat shock protein. *Biol. Cell* **63,** 307–317
49. Green, L. A., and Liem, R. K. (1989) Beta-internexin is a microtubule-associated protein identical to the 70-kDa heat-shock cognate protein and the clathrin uncoating ATPase. *J. Biol. Chem.* **264,** 15210–15215
50. Bloch, M. A., and Johnson, K. A. (1995) Identification of a molecular chaperone in the eukaryotic flagellum and its localization to the site of microtubule assembly. *J. Cell Sci.* **108,** 3541–3545
51. Mounier, N., and Arrigo, A. P. (2002) Actin cytoskeleton and small heat shock proteins: how do they interact? *Cell Stress Chaperones* **7,** 167–176
52. Narberhaus, F. (2002) Alpha-crystallin-type heat shock proteins: Socializing minichaperones in the context of a multichaperone network. *Microbiol. Mol. Biol. Rev.* **66,** 64–93
53. Basha, E., Lee, G. J., Breci, L. A., Hausrath, A. C., Buan, N. R., Giese, K. C., and Vierling, E. (2004) The identity of proteins associated with a small heat shock protein during heat stress in vivo indicates that these chaperones protect a wide range of cellular functions. *J. Biol. Chem.* **279,** 7566–7575
54. Park, S. M., Jung, H. Y., Kim, T. D., Park, J. H., Yang, C. H., and Kim, J. (2002) Distinct roles of the N-terminal-binding domain and the C-terminal-solubilizing domain of alpha-synuclein, a molecular chaperone. *J. Biol. Chem.* **277,** 28512–28520
55. Hingorani, K., Szebeni, A., and Olson, M. O. (2000) Mapping the functional domains of nucleolar protein B23. *J. Biol. Chem.* **275,** 24451–24457
56. Szebeni, A., and Olson, M. O. (1999) Nucleolar protein B23 has molecular chaperone activities. *Protein Sci.* **8,** 905–912
57. Szebeni, A., Hingorani, K., Negi, S., and Olson, M. O. (2003) Role of protein kinase CK2 phosphorylation in the molecular chaperone activity of nucleolar protein b23. *J. Biol. Chem.* **278,** 9107–9115
58. Guha, S., Manna, T. K., Das, K. P., and Bhattacharyya, B. (1998) Chaperone-like activity of tubulin. *J. Biol. Chem.* **273,** 30077–30080
59. Wu, Z., Powell, R., and Lu, W. (2003) Productive folding of human neutrophil alpha-defensins in vitro without the pro-peptide. *J. Am. Chem. Soc.* **125,** 2402–2403
60. Zhu, X. L., Ohta, Y., Jordan, F., and Inouye, M. (1989) Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. *Nature* **339,** 483–484
61. Silen, J. L., and Agard, D. A. (1989) The alpha-lytic protease pro-region does not require a physical linkage to activate the protease domain in vivo. *Nature* **341,** 462–464
62. Venekei, I., Graf, L., and Rutter, W. J. (1996) Expression of rat chymotrypsinogen in yeast: A study on the structural and functional significance of the chymotrypsinogen propeptide. *FEBS Lett.* **379,** 139–142
63. Brogden, K. A., Ackermann, M., and Huttner, K. M. (1997) Small, anionic, and charge-neutralizing propeptide fragments of zymogens are antimicrobial. *Antimicrob. Agents Chemother.* **41,** 1615–1617
64. Liu, F., Zaidi, T., Iqbal, K., Grundke-Iqbal, I., and Gong, C. X. (2002) Aberrant glycosylation modulates phosphorylation of tau by protein kinase A and dephosphorylation of tau by protein phosphatase 2A and 5. *Neuroscience* **115,** 829–837
65. Bodwell, J. E., Hu, L. M., Hu, J. M., Orti, E., and Munck, A. (1993) Glucocorticoid receptors: ATP-dependent cycling and hormone-dependent hyperphosphorylation. *J. Steroid Biochem. Mol. Biol.* **47,** 31–38
66. Orti, E., Hu, L. M., and Munck, A. (1993) Kinetics of glucocorticoid receptor phosphorylation in intact cells. Evidence for hormone-induced hyperphosphorylation after activation and recycling of hyperphosphorylated receptors. *J. Biol. Chem.* **268,** 7779–7784
67. Vivanco, I., and Sawyers, C. L. (2002) The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat. Rev. Cancer* **2,** 489–501
68. Goedert, M., Jakes, R., Spillantini, M. G., Hasegawa, M., Smith, M. J., and Crowther, R. A. (1996) Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. *Nature* **383,** 550–553
69. Gong, C. X., Liu, F., Wu, G., Rossie, S., Wegiel, J., Li, L., Grundke-Iqbal, I., and Iqbal, K. (2004) Dephosphorylation of microtubule-associated protein tau by protein phosphatase 5. *J. Neurochem.* **88,** 298–310
70. Niethammer, P., Bastiaens, P., and Karsenti, E. (2004) Stathmin-tubulin interaction gradients in motile and mitotic cells. *Science* **303,** 1862–1866
71. Kirfel, J., Magin, T. M., and Reichelt, J. (2003) Keratins: A structural scaffold with emerging functions. *Cell Mol. Life Sci.* **60,** 56–71
72. Leifert, J. A., and Whitton, J. L. (2003) “Translocatory proteins” and “protein transduction domains”: A critical analysis of their biological effects and the underlying mechanisms. *Mol. Ther.* **8,** 13–20
73. Kabouridis, P. S. (2003) Biological applications of protein transduction technology. *Trends Biotechnol.* **21,** 498–503
74. Kumagai, A. K., Eisenberg, J. B., and Pardridge, W. M. (1987) Absorptive-mediated endocytosis of cationized albumin and a beta-endorphin-cationized albumin chimeric peptide by isolated brain capillaries. Model system of blood-brain barrier transport. *J. Biol. Chem.* **262,** 15214–15219
75. Smith, K. R., and Borchardt, R. T. (1989) Permeability and mechanism of albumin, cationized albumin, and glycosylated albumin transcellular transport across monolayers of cultured bovine brain capillary endothelial cells. *Pharm. Res.* **6,** 466–473
76. Pardridge, W. M., Kumagai, A. K., and Eisenberg, J. B. (1987) Chimeric peptides as a vehicle for peptide pharmaceutical delivery through the blood-brain barrier. *Biochem. Biophys. Res. Commun.* **146,** 307–313
77. Chuang, V. T., Kragh-Hansen, U., and Otagiri, M. (2002) Pharmaceutical strategies utilizing recombinant human serum albumin. *Pharm. Res.* **19,** 569–577
78. Triguero, D., Buciak, J. B., Yang, J., and Pardridge, W. M. (1989) Blood-brain barrier transport of cationized immunoglobulin G: Enhanced de-

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livery compared to native protein. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 4761–4765
79. Tyagi, M., Rusnati, M., Presta, M., and Giacca, M. (2001) Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **276,** 3254–3261
80. Hakansson, S., Jacobs, A., and Caffrey, M. (2001) Heparin binding by the HIV-1 tat protein transduction domain. *Protein Sci.* **10,** 2138–2139
81. Hakansson, S., and Caffrey, M. (2003) Structural and dynamic properties of the HIV-1 tat transduction domain in the free and heparin-bound states. *Biochemistry* **42,** 8999–9006
82. Rusnati, M., Urbinati, C., Caputo, A., Possati, L., Lortat-Jacob, H., Giacca, M., Ribatti, D., and Presta, M. (2001) Pentosan polysulfate as an inhibitor of extracellular HIV-1 Tat. *J. Biol. Chem.* **276,** 22420–22425
83. Rusnati, M., Tulipano, G., Spillmann, D., Tanghetti, E., Oreste, P., Zoppetti, G., Giacca, M., and Presta, M. (1999) Multiple interactions of HIV-I Tat protein with size-defined heparin oligosaccharides. *J. Biol. Chem.* **274,** 28198–28205
84. Rusnati, M., Tulipano, G., Urbinati, C., Tanghetti, E., Giuliani, R., Giacca, M., Ciomei, M., Corallini, A., and Presta, M. (1998) The basic domain in HIV-1 Tat protein as a target for polysulfonated heparin-mimicking extracellular Tat antagonists. *J. Biol. Chem.* **273,** 16027–16037
85. Rusnati, M., Coltrini, D., Oreste, P., Zoppetti, G., Albini, A., Noonan, D., d'Adda di Fagagna, F., Giacca, M., and Presta, M. (1997) Interaction of HIV-1 Tat protein with heparin. Role of the backbone structure, sulfation, and size. *J. Biol. Chem.* **272,** 11313–11320
86. Sandgren, S., Cheng, F., and Belting, M. (2002) Nuclear targeting of macromolecular polyanions by an HIV-Tat derived peptide. Role for cell-surface proteoglycans. *J. Biol. Chem.* **277,** 38877–38883
87. Console, S., Marty, C., Garcia-Echeverria, C., Schwendener, R., and Ballmer-Hofer, K. (2003) Antennapedia and HIV transactivator of transcription (TAT) "protein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J. Biol. Chem.* **278,** 35109–35114
88. Joliot, A. H., Triller, A., Volovitch, M., Pernelle, C., and Prochiantz, A. (1991) Alpha-2,8-polysialic acid is the neuronal surface receptor of antennapedia homeobox peptide. *New Biol.* **3,** 1121–1134
89. Schwartz, J. J., and Zhang, S. (2000) Peptide-mediated cellular delivery. *Curr. Opin. Mol. Ther.* **2,** 162–167
90. Kueltzo, L. A., Normand, N., O'Hare, P., and Middaugh, C. R. (2000) Conformational lability of herpesvirus protein VP22. *J. Biol. Chem.* **275,** 33213–33221
91. Wiedlocha, A., Madshus, I. H., Mach, H., Middaugh, C. R., and Olsnes, S. (1992) Tight folding of acidic fibroblast growth factor prevents its translocation to the cytosol with diphtheria toxin as vector. *EMBO J.* **11,** 4835–4842
92. Mitchell, D. J., Kim, D. T., Steinman, L., Fathman, C. G., and Rothbard, J. B. (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. *J. Pept. Res.* **56,** 318–325
93. Futaki, S. (2002) Arginine-rich peptides: potential for intracellular delivery of macromolecules and the mystery of the translocation mechanisms. *Int. J. Pharm.* **245,** 1–7
94. Rudolph, C., Plank, C., Lausier, J., Schillinger, U., Muller, R. H., and Rosenecker, J. (2003) Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells. *J. Biol. Chem.* **278,** 11411–11418
95. Futaki, S., Goto, S., Suzuki, T., Nakase, I., and Sugiura, Y. (2003) Structural variety of membrane permeable peptides. *Curr. Protein Pept. Sci.* **4,** 87–96
96. Wiethoff, C. M., Smith, J. G., Koe, G. S., and Middaugh, C. R. (2001) The potential role of proteoglycans in cationic lipid-mediated gene delivery. Studies of the interaction of cationic lipid-DNA complexes with model glycosaminoglycans. *J. Biol. Chem.* **276,** 32806–32813
97. Ruponen, M., Yla-Herttuala, S., and Urtti, A. (1999) Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: Physicochemical and transfection studies. *Biochim. Biophys. Acta* **1415,** 331–341
98. Belting, M., and Petersson, P. (1999) Intracellular accumulation of secreted proteoglycans inhibits cationic lipid-mediated gene transfer. Cotransfer of glycosaminoglycans to the nucleus. *J. Biol. Chem.* **274,** 19375–19382
99. Mislick, K. A., and Baldeschwieler, J. D. (1996) Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 12349–12354
100. Zelphati, O., Uyechi, L. S., Barron, L. G., and Szoka, F. C., Jr. (1998) Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. *Biochim. Biophys. Acta* **1390,** 119–133
101. Xu, Y., and Szoka, F. C., Jr. (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35,** 5616–5623
102. Wiethoff, C. M., and Middaugh, C. R. (2003) Barriers to nonviral gene delivery. *J. Pharm. Sci.* **92,** 203–217
103. Cotman, S. L., Halfter, W., and Cole, G. J. (2000) Agrin binds to beta-amyloid (Aβ), accelerates Aβ fibril formation, and is localized to Aβ deposits in Alzheimer's disease brain. *Mol. Cell Neurosci.* **15,** 183–198
104. Donahue, J. E., Berzin, T. M., Rafii, M. S., Glass, D. J., Yancopoulos, G. D., Fallon, J. R., and Stopa, E. G. (1999) Agrin in Alzheimer's disease: Altered solubility and abnormal distribution within microvasculature and brain parenchyma. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6468–6472
105. Verbeek, M. M., Otte-Holler, I., van den Born, J., van den Heuvel, L. P., David, G., Wesseling, P., and de Waal, R. M. (1999) Agrin is a major heparan sulfate proteoglycan accumulating in Alzheimer's disease brain. *Am. J. Pathol.* **155,** 2115–2125
106. Castillo, G. M., Lukito, W., Wight, T. N., and Snow, A. D. (1999) The sulfate moieties of glycosaminoglycans are critical for the enhancement of beta-amyloid protein fibril formation. *J. Neurochem.* **72,** 1681–1687
107. McLaurin, J., Franklin, T., Zhang, X., Deng, J., and Fraser, P. E. (1999) Interactions of Alzheimer amyloid-beta peptides with glycosaminoglycans effects on fibril nucleation and growth. *Eur. J. Biochem.* **266,** 1101–1110
108. Watson, D. J., Lander, A. D., and Selkoe, D. J. (1997) Heparin-binding properties of the amyloidogenic peptides Abeta and amylin. Dependence on aggregation state and inhibition by Congo red. *J. Biol. Chem.* **272,** 31617–31624
109. Makrides, V., Massie, M. R., Feinstein, S. C., and Lew, J. (2004) Evidence for two distinct binding sites for tau on microtubules. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6746–6751
110. Dou, F., Netzer, W. J., Tanemura, K., Li, F., Hartl, F. U., Takashima, A., Gouras, G. K., Greengard, P., and Xu, H. (2003) Chaperones increase association of tau protein with microtubules. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 721–726
111. Hasegawa, M., Crowther, R. A., Jakes, R., and Goedert, M. (1997) Alzheimer-like changes in microtubule-associated protein Tau induced by sulfated glycosaminoglycans. Inhibition of microtubule binding, stimulation of phosphorylation, and filament assembly depend on the degree of sulfation. *J. Biol. Chem.* **272,** 33118–33124
112. Scholefield, Z., Yates, E. A., Wayne, G., Amour, A., McDowell, W., and Turnbull, J. E. (2003) Heparan sulfate regulates amyloid precursor protein processing by BACE1, the Alzheimer's beta-secretase. *J. Cell Biol.* **163,** 97–107
113. Brimacombe, D. B., Bennett, A. D., Wusteman, F. S., Gill, A. C., Dann, J. C., and Bostock, C. J. (1999) Characterization and polyanion-binding properties of purified recombinant prion protein. *Biochem. J.* **342,** 605–613
114. Gonzalez-Iglesias, R., Pajares, M. A., Ocal, C., Espinosa, J. C., Oesch, B., and Gasset, M. (2002) Prion protein interaction with glycosaminoglycan occurs with the formation of oligomeric complexes stabilized by Cu(II) bridges. *J. Mol. Biol.* **319,** 527–540
115. Warner, R. G., Hundt, C., Weiss, S., and Turnbull, J. E. (2002) Identification of the heparan sulfate binding sites in the cellular prion protein. *J. Biol. Chem.* **277,** 18421–18430
116. Ben-Zaken, O., Tzaban, S., Tal, Y., Horonchik, L., Esko, J. D., Vlodavsky, I., and Taraboulos, A. (2003) Cellular heparan sulfate participates in the metabolism of prions. *J. Biol. Chem.* **278,** 40041–40049
117. Caughey, B., and Raymond, G. J. (1993) Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J. Virol.* **67,** 643–650
118. Gabizon, R., Meiner, Z., Halimi, M., and Ben-Sasson, S. A. (1993) Heparin-like molecules bind differentially to prion-proteins and change their intracellular metabolic fate. *J. Cell. Physiol.* **157,** 319–325
119. Lasmezas, C. I. (2003) Putative functions of PrP(C). *Br. Med. Bull.* **66,** 61–70

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120. Caughey, B., Brown, K., Raymond, G. J., Katzenstein, G. E., and Thresher, W. (1994) Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and congo red [corrected]. *J. Virol.* **68,** 2135–2141
121. Cohlberg, J. A., Li, J., Uversky, V. N., and Fink, A. L. (2002) Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from alpha-synuclein in vitro. *Biochemistry* **41,** 1502–1511
122. Antony, T., Hoyer, W., Cherny, D., Heim, G., Jovin, T. M., and Subramaniam, V. (2003) Cellular polyamines promote the aggregation of alpha-synuclein. *J. Biol. Chem.* **278,** 3235–3240
123. Goers, J., Uversky, V. N., and Fink, A. L. (2003) Polycation-induced oligomerization and accelerated fibrillation of human alpha-synuclein in vitro. *Protein Sci.* **12,** 702–707
124. Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., and Wiley, D. C. (1988) Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* **333,** 426–431
125. Suzuki, Y., Nagao, Y., Kato, H., Matsumoto, M., Nerome, K., Nakajima, K., and Nobusawa, E. (1986) Human influenza A virus hemagglutinin distinguishes sialyloligosaccharides in membrane-associated gangliosides as its receptor which mediates the adsorption and fusion processes of virus infection. Specificity for oligosaccharides and sialic acids and the sequence to which sialic acid is attached. *J. Biol. Chem.* **261,** 17057–17061
126. Varghese, J. N., McKimm-Breschkin, J. L., Caldwell, J. B., Kortt, A. A., and Colman, P. M. (1992) The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. *Proteins* **14,** 327–332
127. Sauter, N. K., Hanson, J. E., Glick, G. D., Brown, J. H., Crowther, R. L., Park, S. J., Skehel, J. J., and Wiley, D. C. (1992) Binding of influenza virus hemagglutinin to analogs of its cell-surface receptor, sialic acid: Analysis by proton nuclear magnetic resonance spectroscopy and X-ray crystallography. *Biochemistry* **31,** 9609–9621
128. Stray, S. J., Cummings, R. D., and Air, G. M. (2000) Influenza virus infection of desialylated cells. *Glycobiology* **10,** 649–658
129. Batinic, D., and Robey, F. A. (1992) The V3 region of the envelope glycoprotein of human immunodeficiency virus type 1 binds sulfated polysaccharides and CD4-derived synthetic peptides. *J. Biol. Chem.* **267,** 6664–6671
130. Coeytaux, E., Coulaud, D., Le Cam, E., Danos, O., and Kichler, A. (2003) The cationic amphipathic alpha-helix of HIV-1 viral protein R (Vpr) binds to nucleic acids, permeabilizes membranes, and efficiently transfects cells. *J. Biol. Chem.* **278,** 18110–18116
131. Giroglou, T., Florin, L., Schafer, F., Streeck, R. E., and Sapp, M. (2001) Human papillomavirus infection requires cell surface heparan sulfate. *J. Virol.* **75,** 1565–1570
132. Langedijk, J. P. (2002) Translocation activity of C-terminal domain of pestivirus Erns and ribotoxin L3 loop. *J. Biol. Chem.* **277,** 5308–5314
133. Oess, S., and Hildt, E. (2000) Novel cell permeable motif derived from the PreS2-domain of hepatitis-B virus surface antigens. *Gene Ther.* **7,** 750–758
134. Schwarz-Linek, U., Werner, J. M., Pickford, A. R., Gurusiddappa, S., Kim, J. H., Pilka, E. S., Briggs, J. A., Gough, T. S., Hook, M., Campbell, I. D., and Potts, J. R. (2003) Pathogenic bacteria attach to human fibronectin through a tandem beta-zipper. *Nature* **423,** 177–181
135. Hawking, F. (1978) Suramin: with special reference to onchocerciasis. *Adv. Pharmacol. Chemother.* **15,** 289–322
136. Kirchhoff, L. V., Bacchi, C. J., Wittner, M., and Tanowitz, H. B. (2000) African trypanosomiasis. *Curr. Treatment Options Infect. Dis.* **2,** 66–69
137. Cooper, P. J., and Nutman, T. B. (2002) Onchocerciasis. *Curr. Treatment Options Infect. Dis.* **4,** 327–335
138. Kaplan, L. D., Wolfe, P. R., Volberding, P. A., Feorino, P., Levy, J. A., Abrams, D. I., Kiprov, D., Wong, R., Kaufman, L., and Gottlieb, M. S. (1987) Lack of response to suramin in patients with AIDS and AIDS-related complex. *Am. J. Med.* **82,** 615–620
139. Levine, A. M., Gill, P. S., Cohen, J., Hawkins, J. G., Formenti, S. C., Aguilar, S., Meyer, P. R., Krailo, M., Parker, J., and Rasheed, S. (1986) Suramin antiviral therapy in the acquired immunodeficiency syndrome. Clinical, immunological, and virologic results. *Ann. Intern. Med.* **105,** 32–37
140. Vogelzang, N. J., Karrison, T., Stadler, W. M., Garcia, J., Cohn, H., Kugler, J., Troeger, T., Giannone, L., Arrieta, R., Ratain, M. J., and Vokes, E. E. (2004) A Phase II trial of suramin monthly x 3 for hormone-refractory prostate carcinoma. *Cancer* **100,** 65–71
141. Szabo, S., Vattay, P., Scarbrough, E., and Folkman, J. (1991) Role of vascular factors, including angiogenesis, in the mechanisms of action of sucralfate. *Am. J. Med.* **91,** 158S-160S
142. Curtis, C. L., Harwood, J. L., Dent, C. M., and Caterson, B. (2004) Biological basis for the benefit of nutraceutical supplementation in arthritis. *Drug Discov. Today* **9,** 165–172
143. Lust, G., Williams, A. J., Burton-Wurster, N., Beck, K. A., and Rubin, G. (1992) Effects of intramuscular administration of glycosaminoglycan polysulfates on signs of incipient hip dysplasia in growing pups. *Am. J. Vet. Res.* **53,** 1836–1843
144. Baskin, E., Saatci, U., Ciliv, G., Bakkaloglu, A., Besbas, N., Topaloglu, R., and Ozen, S. (2003) Urinary glycosaminoglycans in the course of familial Mediterranean fever. *Eur. J. Pediatr.* **162,** 305–308
145. Lindahl, B., and Lindahl, U. (1997) Amyloid-specific heparan sulfate from human liver and spleen. *J. Biol. Chem.* **272,** 26091–26094
146. Barabasi, A. L., and Albert, R. (1999) Emergence of scaling in random networks. *Science* **286,** 509–512
147. Jeong, H., Tombor, B., Albert, R., Oltvai, Z. N., and Barabasi, A. L. (2000) The large-scale organization of metabolic networks. *Nature* **407,** 651–654
148. Wuchty, S. (2001) Scale-free behavior in protein domain networks. *Mol. Biol. Evol.* **18,** 1694–1702
149. Batada, N. N., Shepp, L. A., and Siegmund, D. O. (2004) Stochastic model of protein-protein interaction: Why signaling proteins need to be colocalized. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6445–6449
150. Bar-Yam, Y., and Epstein, I. R. (2004) Response of complex networks to stimuli. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 4341–4345
151. Watts, D. J., and Strogatz, S. H. (1998) Collective dynamics of 'small-world' networks. *Nature* **393,** 440–442
152. Maslov, S., and Sneppen, K. (2002) Specificity and stability in topology of protein networks. *Science* **296,** 910–913
153. Ravasz, E., Somera, A. L., Mongru, D. A., Oltvai, Z. N., and Barabasi, A. L. (2002) Hierarchical organization of modularity in metabolic networks. *Science* **297,** 1551–1555
154. Wuchty, S. (2003) Small worlds in RNA structures. *Nucleic Acids Res.* **31,** 1108–1117
155. Mattick, J. S., and Gagen, M. J. (2001) The evolution of controlled multitasked gene networks: The role of introns and other noncoding RNAs in the development of complex organisms. *Mol. Biol. Evol.* **18,** 1611–1630
156. Alon, U. (2003) Biological networks: The tinkerer as an engineer. *Science* **301,** 1866–1867
157. Dunker, A. K., Brown, C. J., Lawson, J. D., Iakoucheva, L. M., and Obradovic, Z. (2002) Intrinsic disorder and protein function. *Biochemistry* **41,** 6573–6582
158. Romero, P., Obradovic, Z., Kissinger, C. R., Villafranca, J. E., Garner, E., Guilliot, S., and Dunker, A. K. (1998) Thousands of proteins likely to have long disordered regions. *Pac. Symp. Biocomput*, 437–448
159. Dunker, A. K., and Obradovic, Z. (2001) The protein trinity-linking function and disorder. *Nat. Biotechnol.* **19,** 805–806
160. Dunker, A. K., Lawson, J. D., Brown, C. J., Williams, R. M., Romero, P., Oh, J. S., Oldfield, C. J., Campen, A. M., Ratliff, C. M., Hipps, K. W., Ausio, J., Nissen, M. S., Reeves, R., Kang, C., Kissinger, C. R., Bailey, R. W., Griswold, M. D., Chiu, W., Garner, E. C., and Obradovic, Z. (2001) Intrinsically disordered protein. *J. Mol. Graph. Model.* **19,** 26–59
161. Iakoucheva, L. M., Radivojac, P., Brown, C. J., O'Connor, T. R., Sikes, J. G., Obradovic, Z., and Dunker, A. K. (2004) The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res.* **32,** 1037–1049. Print 2004
162. Stawiski, E. W., Gregoret, L. M., and Mandel-Gutfreund, Y. (2003) Annotating nucleic acid-binding function based on protein structure. *J. Mol. Biol.* **326,** 1065–1079
163. Jones, S., Shanahan, H. P., Berman, H. M., and Thornton, J. M. (2003) Using electrostatic potentials to predict DNA-binding sites on DNA-binding proteins. *Nucleic Acids Res.* **31,** 7189–7198
164. Jones, S., Barker, J. A., Nobeli, I., and Thornton, J. M. (2003) Using structural motif templates to identify proteins with DNA binding function. *Nucleic Acids Res.* **31,** 2811–2823
165. Saito, A., Munakata, H., and Satoh, K. (2002) Glyco-western blotting: biotinylated dermatan sulfate as a probe for the detection of dermatan

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sulfate binding proteins using Western blotting. *Connect Tissue Res.* **43,** 1–7
166. Sudhof, T. C. (2002) Synaptotagmins: Why so many? *J. Biol. Chem.* **277,** 7629–7632
167. Steger, D. J., Haswell, E. S., Miller, A. L., Wente, S. R., and O'Shea, E. K. (2003) Regulation of chromatin remodeling by inositol polyphosphates. *Science* **299,** 114–116
168. Couchman, J. R., Vogt, S., Lim, S. T., Lim, Y., Oh, E. S., Prestwich, G. D., Theibert, A., Lee, W., and Woods, A. (2002) Regulation of inositol phos-pholipid binding and signaling through syndecan-4. *J. Biol. Chem.* **277,**49296–49303
169. Verspohl, E. J., and Johannwille, B. (1998) Diadenosine polyphosphates in insulin-secreting cells: Interaction with specific receptors and degradation. *Diabetes* **47,** 1727–1734
170. Kisselev, L. L., Justesen, J., Wolfson, A. D., and Frolova, L. Y. (1998) Diadenosine oligophosphates (Ap(n)A), a novel class of signalling molecules? *FEBS Lett.* **427,** 157–163
171. Hanczyc, M. M., Fujikawa, S. M., and Szostak, J. W. (2003) Experimental models of primitive cellular compartments: encapsulation, growth, and division. *Science* **302,** 618–622
172. Taylor, J. P., Hardy, J., and Fischbeck, K. H. (2002) Toxic proteins in neurodegenerative disease. *Science* **296,** 1991–1995

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