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Essay

Proteins in Unexpected Locations

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Members of all classes of proteins—cytoskeletal components, secreted growth factors, glycolytic enzymes, kinases, transcription factors, chaperones, transmembrane proteins, and extracellular matrix proteins—have been identified in cellular compartments other than their conventional sites of action. Some of these proteins are expressed as distinct compartment-specific isoforms, have novel mechanisms for intercompartmental translocation, have distinct endogenous biological actions within each compartment, and are regulated in a compartment-specific manner as a function of physiologic state. The possibility that many, if not most, proteins have distinct roles in more than one cellular compartment has implications for the evolution of cell organization and may be important for understanding pathological conditions such as Alzheimer's disease and cancer.

INTRODUCTION

The early concept of "one gene, one enzyme" has given way to a more sophisticated view of protein structure and function in which proteins are comprised of multiple distinct modules. A single protein may interact with a bewildering number of other moieties in the course of its everyday function. For example, as actin forms cytoskeletal filaments in the cytoplasm, actin monomers exhibit binding to themselves, bind nucleotides and ions, have ATPase activity, and interact with a score of other proteins, which regulate its polymerization, length, and bundling (Pollard and Cooper, 1986). Similarly, laminin binds not only to itself but to half-a-dozen other matrix proteins and glycoconjugates within the extracellular matrix, as well as to a variety of cell-surface proteins. Both actin and laminin are expressed as a family of isoforms, allowing specialized functions to be played or regulated differentially in different contexts.

In contrast to this view of proteins as highly multifunctional entities, our current understanding of their cellular compartmentalization remains unitary: After its biosynthesis and processing, each protein is generally envisioned as having a single cellular compartment in which it primarily resides and acts, notwithstanding certain proteins (e.g., glucocorticoid receptors) which operate by virtue of shuttling across compartments. Actin is thought to act in the cytoplasm, and laminin in the extracellular matrix. "One protein, one compartment" has become an implicit expectation in cell biology, as evidenced by the fact that whenever a protein has been reported to reside in a compartment other than the expected one or is reported to bind to ligands in vitro that reside in a different compartment, there has been a natural tendency in the field to regard the findings as artifactual, or as indicating a certain degree of "leaky" routing of proteins to wrong locations. In a few cases (e.g., fibroblast growth factor (FGF)-2, see below), it is accepted that the same protein plays distinct roles at the cell surface and within the nucleus, but such proteins have been regarded as exceptions to the rule.

In fact, a surprisingly large number of diverse proteins and glycoconjugates have independently (and credibly) been reported to reside in unexpected cellular compartments. The purpose of this essay is to point out the ubiquity of this phenomenon and to consider some of its implications.

Actin and Other Cytoskeletal Proteins

At least eight research groups have reported the existence of actin at the external face of the cell surface in lymphocytes (Owen *et al.*, 1978; Bachvaroff *et al.*, 1980; Rubin *et al.*, 1982; Sanders and Craig, 1983), monocytes (Por *et al.*, 1991), endothelial cells (Pardridge *et al.*, 1989; Moroianu *et al.*, 1993), and L cells (Rosenblatt *et al.*, 1985). (Evidence and control experiments will be discussed only for this first example, but may be taken as representative of the other proteins and locations mentioned below.) Actin was localized by three methods:

1) Surface labeling of proteins in live cells was carried out with biotin (Moroianu *et al.*, 1993) or iodina-

tion using lactoperoxidase or other methods (Bachvaroff *et al.*, 1980; Rubin *et al.*, 1982). Controls confirmed that iodination was not mediated by endogenous peroxidases and that it did not cause cell permeabilization. Abundant cytoplasmic proteins such as vimentin were not labeled. Labeled actin was identified by comigration with authentic actin on two-dimensional polyacrylamide gel electrophoresis gels (Bachvaroff *et al.*, 1980; Rubin *et al.*, 1982).

2) Release of actin from the cell surface of viable cells was achieved by treatment with trypsin (Owen *et al.*, 1978). In endothelial cells, surface-associated actin could be displaced by heparin or angiogenin (Moroianu *et al.*, 1993). Controls indicated that lymphocytes did not bind actin added exogenously, either in purified form or when incubated in cell-conditioned medium (Bachvaroff *et al.*, 1980; Sanders and Craig, 1983). Although actin is present in fetal calf serum, the form of actin on endothelial cells is smooth muscle α-actin, *not* the same type as found in fetal calf serum (Moroianu *et al.*, 1993).

3) Binding of anti-actin antibodies to live cells: Various monoclonal and polyclonal anti-actin antibodies have been utilized with similar results. Flow cytometry and confocal microscopy have been used to confirm that labeled actin is present as punctate dots at the cell surface and that intracellular structures are not labeled except when the cells are deliberately permeabilized (Por *et al.*, 1991). Immunoelectron microscopy has also been carried out on endothelial cells (Pardridge *et al.*, 1989). Immunostaining was diminished by absorption with purified actin; antibodies against other antigens (e.g., vimentin) did not stain cells. Use of $F(ab')_2$ fragments or preblocking with other antibodies have confirmed that the anti-actin antibodies are not simply recognizing cell-surface Fc receptors. Western blotting of cellular extracts revealed the expected 43 kDa actin band with no evidence of other cross-reactive proteins (Sanders and Craig, 1983). Although one early report suggested that anti-actin antibodies might recognize cell-surface Ig (Owen *et al.*, 1978), this was not confirmed by another group (Sanders and Craig, 1983).

The presence of cell-surface actin is regulated by the physiologic state of cells: Transformed or mitogen-stimulated lymphocytes express substantially more actin than their normal counterparts (Owen *et al.*, 1978; Bachvaroff *et al.*, 1980; Rubin *et al.*, 1982; Sanders and Craig, 1983). In contrast, activating U937 monocytes led to a decrease in cell-surface actin (Por *et al.*, 1991). Potential functions have been proposed for cell-surface actin: 1) binding angiogenin (Moroianu *et al.*, 1993), 2) stimulating mitogenesis (Rosenblatt *et al.*, 1985), and 3) binding extracellular fibronectin (cf. Chen *et al.*, 1978). G-actin has been shown to have neurite-promoting activity for embryonic chick telencephalic neurons (Nobusada and Taguchi, 1992). It may be also be relevant to recall that the actin-sequestering protein thymosin β4 was originally isolated as a putative hormone having potent biological effects when added exogenously to cells (Safer *et al.*, 1991).

Although the evidence is strong that actin is expressed on the cell surface under some conditions, further research is necessary before its significance can be fully accepted: 1) It still must be determined how actin moves to the cell surface. Is actin routed into the endoplasmic reticulum (ER)–Golgi pathway, exported via novel translocation pathways (see below), or placed into endosomes and shuttled from there to the cell surface? Is it anchored to the plasma membrane directly or is it bound to other membrane proteins? 2) Biosynthetic pulse-chase studies of metabolically labeled actin need to be carried out to confirm this pathway, to measure rates of synthesis and turnover separately, and to characterize the basis for changes in abundance of cell-surface actin as a function of the cell's physiologic state. 3) The precise structure of cell-surface actin needs to be elucidated. 4) Most importantly, a plausible function for cell-surface actin needs to be better established. What happens to cellular functions, for example, when this pool of actin is selectively knocked out?

A number of studies have also detected α- and β-tubulin on the cell surface using methods similar to those described for actin (Estridge, 1977; Bachvaroff *et al.*, 1980; Rubin *et al.*, 1982; Por *et al.*, 1991). Interestingly, double-labeling of monocytes with anti-actin and anti-tubulin antibodies indicated that each was expressed in a punctate distribution, but the two proteins did not colocalize (Por *et al.*, 1991). Actin has been found repeatedly in the nucleus of many cell types as well and may have a functional role in this cellular compartment (Parfenov *et al.* [1995] and references cited therein).

The cytoskeletal protein tau has also been localized to the nucleus of certain neural and nonneural cell types in primates, where it can either be present diffusely throughout the nucleus or can colocalize with nucleolar-organizing regions during mitosis and with the nucleolus in interphase (Loomis *et al.*, 1990; Wang *et al.*, 1993; Brady *et al.*, 1995). Intriguingly, nuclear tau protein in human brain neurons is resistant to sodium dodecyl sulfate and requires formic acid for its solubilization; the same unusual solubility properties are observed for tau-containing paired helical filaments seen in the cell bodies of neurons in Alzheimer's disease (Brady *et al.*, 1995). However, it is not known whether tau normally forms filaments within the nucleus or whether nuclear tau contributes to paired helical filaments in Alzheimer's disease. The nuclear routing signal for tau remains uncertain; although recombinant tau fails to enter the nucleus when expressed in CHO cells (Wang *et al.*, 1993), microinjected purified native tau protein rapidly localizes to the

nucleolus (in addition to microtubule-containing sites in the cytoplasm) (Lu and Wood, 1993). Possibly tau needs to be phosphorylated to enter the nucleus, but it is not known definitely if nuclear tau is phosphorylated, either in a constitutive or regulated manner.

Growth Factors

Many growth factors, including FGF-1, FGF-2, Schwannoma-derived growth factor, ciliary neurotrophic factor, and IL-1β are soluble cytoplasmic proteins lacking signal sequences. These provide an interesting counterpoint to the case of actin: For example, FGF-2 has a well-established role as an extracellular signaling molecule acting on specific cell-surface receptors (Partanen *et al.*, 1993) and is known to be specifically transported into the nucleus (Bugler *et al.*, 1991; Woodward *et al.*, 1992; Rifkin *et al.*, 1994, Stachowiak *et al.*, 1994; Prochiantz and Theodore, 1995) where it may be involved independently in regulating cell proliferation. What is *not* accepted is a role for FGF-2 in its primary, cytoplasmic distribution! It is clear that these growth factors are released from cells in a regulated manner extracellularly, although the underlying mechanisms are poorly understood (Rubartelli *et al.*, 1990, 1992; Colman, 1991; Muthukrishnan *et al.*, 1991; Mignatti *et al.*, 1992).

Transmembrane cell-surface receptors have been identified in the nucleus for a number of polypeptide growth factors, secreted by both conventional and novel pathways (e.g., EGF receptor: Jiang and Schindler, 1990; insulin receptor: Podlecki *et al.*, 1987, Wong *et al.*, 1988; FGF receptor type-1: E. Stachowiak *et al.*, 1995, M. Stachowiak *et al.*, 1995). These receptors appear to be involved in translocating the growth factor from the cell surface to the nucleus, although they may not provide the only means of nuclear translocation for the growth factors (Lin *et al.*, 1996), and it remains uncertain whether the receptors continue to play functionally active roles once within the nucleus.

Similarly, the *Notch* gene product is a transmembrane protein that is involved in regulating cell fate in both invertebrates and vertebrates and has features suggesting that it may be a signal-transducing cell-surface receptor for ligands such as Delta gene product (Lyman and Young, 1993). Yet the isolated cytoplasmic domain of Notch is a constitutively active repressor of myogenesis and neurogenesis, contains nuclear localization sequences, and is targeted to the nucleus (Lieber *et al.*, 1993; Kopan *et al.*, 1994). Altering or removing the nuclear localization sequences prevents nuclear accumulation and diminishes the repressive effects on myogenesis, while attachment of exogenous nuclear localization sequences restores the repressive activity (Kopan *et al.*, 1994). On the other hand, wild-type Notch protein and some functionally active Notch constructs appear to reside at the plasma membrane and cannot be detected within the nucleus (Lieber *et al.*, 1993). Further research is needed to establish whether ligand activation of Notch in living cells causes its cytoplasmic domain to be liberated and to learn whether a nuclear pool of Notch protein (perhaps transient or present at low levels) can explain its functional effects.

Annexins, S100, and soluble galactose-binding lectins are three other families of abundant cytoplasmic proteins lacking signal sequences whose members appear to play multifaceted roles as secreted proteins, cell-surface receptors, cytoskeletal components, and nuclear proteins (Donato, 1991; Harrison, 1991; Barger *et al.*, 1992; Raynal and Pollard, 1994). For example, the lectin galectin-3 (Mac-2) is not only a major cell-surface protein of activated macrophages; the same protein also is expressed in the nucleus of differentiated (but not neoplastic) colonic epithelial cells (Lotz *et al.*, 1993). Nuclear galectin-3 appears to participate in pre-mRNA splicing in HeLa cells (Dagher *et al.*, 1995).

Finally, Prochiantz and Theodore (1995) have reviewed evidence supporting their proposal that certain nuclear transcription factors, in particular homeodomain proteins, may be secreted extracellularly, become taken up by neighboring cells, and accumulate within the nucleus to affect gene transcription in a paracrine manner.

Glycolytic Enzymes

Because the biological significance of FGFs, annexins, and lectins are already accepted, workers have been willing to explore their multicompartmental roles. In contrast, reports that glycolytic enzymes have growth factor-like and nuclear functions have not received much attention. For example, neuroleukin was isolated as a growth factor for cells in both neural and immune systems (Gurney *et al.*, 1986). Once neuroleukin was found to be identical to glucophosphoisomerase, research on it was largely abandoned—even though the trophic effects were shown to be specifically restricted to the enzymatically inactive monomeric form (Mizrachi, 1989). Another survival factor, for cultured cortical neurons, was identified as neuron-specific enolase; only the neuronal-specific isoform showed survival activity, the other tissue-specific isoforms of enolase being inactive (Takei *et al.*, 1991). Several glycolytic enzymes are known to have nonenzymic functions in specific tissues as crystallins (Piatigorsky and Wistow, 1989) and appear to have nuclear roles as well (Ronai, 1993; Angelov *et al.*, 1994). Levels of glyceraldehyde-3-phosphate dehydrogenase rise in cerebellar granule cells undergoing apoptosis, and antisense oligonucleotides that block this rise in expression also inhibit neuronal death (Ishitani *et al.*, 1996).

Glycosyltransferases and Kinases

The proposal that glycosyltransferases (Roseman, 1970) and kinases are present at the external face of the cell surface is no longer controversial. The best-documented example of an ecto-glycosyltransferase is probably β1,4-galactosyltransferase, which exists in two forms—a shorter form residing in the Golgi apparatus and a longer form arising from the same gene, which is unique to the plasma membrane (Evans *et al.*, 1995). (It is still not clear whether the enzymatic activity of the glycosyl transferases is critical for their roles on the cell surface or indeed whether the uridine diphosphate sugar substrates are present extracellularly.) Ecto-protein kinases have also been extensively characterized, and in some cases appear to be distinct from their intracellular counterparts (Hogan *et al.*, 1995). The molecular cloning of a transmembrane kinase C isoform (Johannes *et al.*, 1994) has corroborated earlier biochemical and cellular evidence supporting an ecto-location for kinases. Numerous substrates for ecto-kinases also have been identified (Vilgrain and Baird, 1991; Kubler *et al.*, 1992; Jordan *et al.*, 1994).

Calmodulin, calmodulin kinase II, casein kinase-1, cyclic 3′,5′-cyclic monophosphate-dependent protein kinase, protein kinase C, and other kinases have been reported to be translocated into the nucleus along with specific binding proteins (Bachs *et al.*, 1992; Kearney *et al.*, 1994; Portoles *et al.*, 1994; Srinivasan *et al.*, 1994; Cho-Chung *et al.*, 1995). There is strong evidence that nuclear kinases regulate the cell cycle via exerting permissive or modulatory actions on transcription factors and other nuclear proteins.

For example, casein kinase II is localized predominantly within the nucleus in interphase cells, although it is released into the cytoplasm during S phase (Yu *et al.*, 1991; Krek *et al.*, 1992; Pepperbok *et al.*, 1994 [Yu *et al.* found a nuclear localization only for the α′ isoform]). Casein kinase II phosphorylates a large number of transcription factors, oncoproteins, and other nuclear proteins in vitro (Meisner and Czech, 1991) at sites that are phosphorylated in vivo (Luscher *et al.*, 1989) and that are important for their nuclear translocation, DNA binding, and other functions (Berberich and Cole, 1992; Bousset *et al.*, 1993; Oelgeschlager *et al.*, 1995; Vancurova *et al.*, 1995). Lin *et al.* (1992) suggested that casein kinase II-mediated phosphorylation of c-*jun* inhibits AP-1 activation in vivo, because microinjecting competitive peptide inhibitors of casein kinase II into the nucleus of cultured cells induces AP-1 activation, whereas injecting casein kinase II directly has a suppressive effect. Pepperbok *et al.* (1994) showed further that casein kinase II is likely to exert at least some of its effects directly within the nucleus by demonstrating differential effects of antibodies against casein kinase II on the cell cycle when microinjected into the nucleus versus the cytoplasm of mitogenically stimulated primary fibroblasts.

Luby-Phelps *et al.* (1995) have directly visualized the translocation of calmodulin into the nuclei of living smooth muscle cells stimulated at micromolar levels of intracellular Ca^{2+}. Recently, in a particularly elegant study, Wang *et al.* (1995) selectively abrogated the function of calmodulin in the nucleus by expressing an inhibitor peptide that blocks its interaction with nuclear-binding proteins; by expressing this peptide in the lungs of transgenic mice, they demonstrated that nuclear calmodulin was required for normal tissue development and cell survival.

Proteins Involved in Protein Synthesis or Protein Folding

Many putative cell-surface receptors have turned out to be identical to abundant intracellular multifunctional proteins. For example: 1) A lipoprotein- and laminin-binding protein was identified as nucleolin (Semenkovich *et al.*, 1990; Kleinman *et al.*, 1991; Kibbey *et al.*, 1995); 2) A 67-kDa laminin-binding protein was identified as a ribosomal subunit protein (Mecham *et al.*, 1989; Auth and Brawerman, 1992); 3) A collagen II-binding protein proved to be identical to annexin V (Pfaffle *et al.*, 1988; Hoffmann *et al.*, 1992; Raynal and Pollard, 1994); 4) A protein binding to high-mannose saccharide residues on laminin was identified as calreticulin (White *et al.*, 1995); 5) The retinal cell aggregation factor cognin was identified as protein disulfide isomerase (Krishna Rao and Hausman, 1993); 6) At least one well-defined cell-surface receptor shows homology to a heat–shock protein (Foltz *et al.*, 1993); and 7) A well-characterized mitochondrial heat–shock protein, Hsp60, has been identified in many nonmitochondrial locations in mammalian cells, including a localization directly on the cell surface (Soltys and Gupta, 1996).

A skeptic would point out rightly that such proteins are the most promiscuous at binding other proteins and therefore are the most prone to be picked up artifactually using in vitro assays. Indeed, some of these putative receptors may still prove to be artifacts. However, these proteins appear to be truly expressed at the cell surface and exhibit developmentally regulated patterns of expression; experimental studies (e.g., antibody perturbation experiments in living cells) also favor a functional role at this location (references cited above and Jordan *et al.*, 1994).

Extracellular Matrix Proteins

Astrocytes are the major source of extracellular matrix proteins in the vertebrate brain. However, several matrix components have also been found diffusely in the cytoplasm of neurons in the mature brain:

Proteoglycans. Histochemical studies had suggested that glycosaminoglycans were present in neuronal cytoplasm (Alvarado and Castejon, 1984). Margolis and colleagues have confirmed and extended this finding in a long series of studies, which have used polyclonal and monoclonal antibodies against soluble chondroitin sulfate proteoglycan core proteins or glycosaminoglycan saccharide sequences (Aquino *et al.*, 1984a,b; Ripellino *et al.*, 1989; Flaccus *et al.*, 1991; Rauch *et al.*, 1991, 1992). Immunoelectron microscopy has demonstrated that these antibodies decorate extracellular structures in developing brain; however, in the mature brain they stain diffusely within the cytoplasm (i.e., not delimited within endosomes or other membrane-bound structures), as well as in the nuclei of neurons. Moreover, using biotinylated link protein as a probe, the polysaccharide hyaluronic acid has also been found to have a cytoplasmic localization within mature neurons (Ripellino *et al.*, 1988).

It remains unclear whether these proteins and glycoconjugates are synthesized by neurons or are taken up from the outside. The latter possibility has a precedent in that mild damage to neurons has been shown to facilitate transient diffuse cytoplasmic uptake of extracellular proteins such as horseradish peroxidase and IgG (Loberg and Torvik, 1991, 1992; Aihara *et al.*, 1994).

Apolipoprotein E. Apolipoprotein E (apo E) is of great interest as a participant in the pathogenesis of Alzheimer's disease (Strittmatter *et al.*, 1994). Although apo E is generally thought to be synthesized and secreted primarily by astrocytes and other nonneuronal cells, light microscopic and electron microscopic immunocytochemical studies have recently indicated that apo E is present widely at low levels within the cytoplasm of some rat and human neurons, particularly within the aged brain (Han *et al.*, 1994a,b; Metzger *et al.*, 1996). Moreover, in apo E-deficient mice, neurodegenerative changes are observed within neurons (Masliah *et al.*, 1995). Low levels of cytoplasmic apo E, not associated with membrane-delimited organelles, also have been described in several nonneuronal cell types (hepatocytes: Hamilton *et al.*, 1990; adrenocortical cells: Williams *et al.*, 1995).

Although both apo E3 and E4 transport similar amounts of β-very-low-density-lipoprotein into fibroblasts and cultured Neuro-2A cells (Nathan *et al.*, 1995), the two isoforms have opposite effects on neurite outgrowth: Apo E3 stimulates neurites in a manner dependent on its uptake into neurons via the low-density lipoprotein-related receptor, whereas apo E4 is inhibitory (Holtzman *et al.*, 1995). Neuro-2A cells treated with equal concentrations of apo E3 versus E4 show a dramatic difference in diffuse intracellular (apparently cytoplasmic) accumulation of the two isoforms, with much more E3 than E4 residing in both cell body and neurites (Nathan *et al.*, 1995). Cytoplasmic accumulation did not occur in fibroblasts treated with apo E isoforms (Nathan *et al.*, 1995). Apo E3 isoform binds better in vitro than the E4 isoform to the cytoskeletal proteins tau (Strittmatter *et al.*, 1994) and MAP2c (Huang *et al.*, 1994), and to intact microtubules (Nathan *et al.*, 1995). It has been proposed that the differential binding of E3 versus E4 to cytoskeletal structures may underlie the neurite-promoting effects of apo E3 (Nathan *et al.*, 1995) and the relevance of apo E isoforms to Alzheimer's disease (Strittmatter *et al.*, 1994).

Laminin. A laminin-related protein has been observed within CNS neurons. Although the isoforms expressed are still incompletely identified, this protein is recognized by many different polyclonal and monoclonal antibodies to laminin; immunostaining is abolished by absorption with purified laminin, and Western blotting of brain extracts reveals bands with mobilities typical of laminins (Yamamato *et al.*, 1988; Hagg *et al.*, 1989; Zhou, 1990; Jucker *et al.*, 1992). Laminin is expressed as a mixture of diffuse and punctate staining patterns within different subsets of neuronal perikarya in the mature, but not in the developing, brain (Yamamato *et al.*, 1988; Hagg *et al.*, 1989; Zhou, 1990; Jucker *et al.*, 1992). Neurons appear to synthesize laminin because an in situ hybridization study has confirmed that retinal ganglion cells synthesize laminin B1 chain mRNA (Sarthy and Fu, 1990) and because immunoelectron microscopy has demonstrated the presence of laminin associated with rough ER and lysosomes (Yamamoto *et al.*, 1988). However, published studies have not clearly resolved whether the diffuse perikaryal staining seen at the light microscopic level indeed reflects, at least in part, laminin present diffusely in the cytoplasm.

Acetylcholinesterase. Like apo E and laminin, acetylcholinesterase (AChE) is a neurite-promoting extracellular matrix protein (Karpel *et al.*, 1996 and references cited therein). Soreq and colleagues have recently suggested that cytoplasmic AChE may exert functional effects within cells: Microinjecting constructs for human AChE isoforms into cultured C6 glioma cells resulted in diffuse cytoplasmic AChE and cell body enlargement and process formation, which is characteristic of differentiated astrocytes. However, neighboring noninjected cells showed no changes, suggesting that the effects were due to intracellular protein rather than a secreted pool (Karpel *et al.*, 1996).

To summarize, four classical extracellular matrix components—neurocan, hyaluronic acid, apo E, and laminin—have been described as unexpectedly residing within mature neurons of the CNS. Apo E stimulates neurite outgrowth in an isoform-specific manner that correlates with isoform-specific intracellular accumulation in living cells and with isoform-specific binding of cytoskeletal proteins in vitro. A fifth matrix component, AChE, can induce morphological changes

in glial cells when overproduced endogenously in the cytoplasm. Although information is still relatively sketchy for this class of proteins, the findings suggest that matrix proteins are present diffusely within the cytoplasm in some situations in which they might have physiologically (or at least pathologically) significant interactions with cytoplasmic proteins or with each other (cf. Huang *et al.*, 1995).

Glycosaminoglycans are also present in the nucleus of a variety of cell types. Cell-surface heparan sulfate can become internalized and transported to the nucleus as free saccharide chains in a manner that correlates with the state of cell proliferation (Fedarko and Conrad, 1986). Hyaluronic acid, chondroitin sulfate, and dermatan sulfate are also associated with the nucleus in amounts that cannot be explained by adventitious association during cell fractionation (Margolis *et al.*, 1975; Furukawa and Terayama, 1977; Hiscock *et al.*, 1994). The basic DNA-binding sequences of several transcription factors resemble high-affinity heparin-binding consensus sequences, and many transcription factors are retained on heparin affinity columns (Jackson *et al.*, 1991); endogenous nuclear glycosaminoglycans have been implicated in regulating the action of Fos and Jun/AP-1 (Busch *et al.*, 1992), although it is not clear whether they act by inhibiting DNA binding.

Mechanisms of Protein Translocation

Proteins can be routed from cell surface to cytoplasm and vice versa by a number of mechanisms aside from the conventional ER–Golgi secretory pathways and the conventional vesicular endocytic pathways:

Soluble proteins can pass bidirectionally across "leaky" plasma membranes in transiently damaged cells (Loberg and Torvik, 1991, 1992; Muthukrishnan *et al.*, 1991; Mignatti *et al.*, 1992; McNeil, 1993; Aihara *et al.*, 1994). This may be a major means by which FGF and other cytoplasmic growth factors act as "wound" hormones (McNeil, 1993) and appears to contribute to the pathogenesis of injury-related diseases such as atherosclerosis. Moreover, in mechanically active cell types such as gut epithelium, aortic endothelium, and skeletal and cardiac muscle, "wounding" has been found to occur in a fraction of cells during normal physiological activity and may contribute to normal exercise-induced tissue growth and repair (McNeil and Khakee, 1992; Yu and McNeil, 1992; Clarke *et al.*, 1993, 1995).

Certain proteins such as yeast mating factor, interleukin-1β, and thioredoxin are secreted from cells in a signal peptide-independent manner (Rubartelli *et al.*, 1990, 1992; Colman, 1991). The mechanism(s) underlying signal peptide-independent translocation across plasma and organellar membranes remain poorly characterized in mammalian cells; however, it is possible that ATP-dependent membrane transporter proteins (Kuchler and Thorner, 1990) or pore complexes (Goncz and Rothman, 1995 and references cited therein) may be involved.

A portion of proteins that are initially internalized into multivesicular bodies or endosomes can also eventually enter the cytoplasm (folate–protein conjugates: Turek *et al.*, 1993; Tat protein and heterologous conjugates: Fawell *et al.*, 1994).

The *Antennapedia* homeodomain peptide can translocate across the plasma membrane in an energy-independent, apparently endocytosis-independent manner to accumulate in the nucleus and cytoplasm (Derossi *et al.*, 1994). The ability to translocate is due to peptide sequences within the third helix of the homeodomain; both general hydrophobic and specific conformational aspects of these sequences appear to be important (Derossi *et al.*, 1994). Although no receptor or transporter protein is thought to be involved, translocation is facilitated by binding to highly negative polysialic acid residues on the cell surface (Joliot *et al.*, 1991). Because other homeodomain proteins contain similar peptide sequences (Prochiantz and Theodore, 1995) and because other peptides linked to these sequences can translocate membranes as well (Perez *et al.*, 1992), it is possible that this may represent an example of a more general and biologically significant means of translocation across membranes.

DISCUSSION

All classes of proteins, including cytoskeletal components, secreted growth factors, glycolytic enzymes, kinases, transcription factors, chaperones, transmembrane proteins, and extracellular matrix proteins, have been reported to reside in multiple cellular compartments. Although many authors have pointed out that a particular protein fails to conform to a single conventional compartment or role, I do not believe that the ubiquitous nature of this finding has been previously reviewed.

Multicompartmentalization of proteins appears to be a real phenomenon and is not explainable, in general, by artifacts such as movement of proteins during isolation, contamination by proteins released from dead or wounded cells, or adventitious cross-reactions of antibodies with unrelated proteins. After years of controversy, the evidence has become compelling in support of several classes of proteins, notably ecto-glycosyltransferases and ecto-kinases. Yet, in almost every case reviewed here, one or more important gaps need to be filled before the study of the protein in its unexpected location can be pursued with confidence: How is the protein routed to and from its unconventional compartment? What is the precise structure of the protein found in this compartment? What functions are served by the protein in this compart-

ment, and how is it regulated under physiological conditions?

Despite the gaps in current knowledge, a strong motivator for studying this phenomenon further is that several diseases have been identified that may involve multicompartmentalization. Apo E is undergoing intensive scrutiny not only as a classical extracellular lipid transport protein but also in its unconventional cytoplasmic locations in the study of Alzheimer's disease (Strittmatter *et al.*, 1994; Nathan *et al.*, 1995; Metzger *et al.*, 1996). Interestingly, apo E is one of a larger set of amyloid plaque-associated proteins in this disease, including S100, acetylcholinesterase, and laminin (Barger *et al.*, 1992; Murtomaki *et al.*, 1992), which were also reviewed here as possibly having dual extracellular and cytoplasmic sites of action. The possible role of nuclear tau in the formation of paired helical filaments is still an open issue (Lu and Wood, 1993; Wang *et al.*, 1993; Brady *et al.*, 1995).

Several forms of cancer are being correlated with deficits in nuclear translocation of specific proteins (Mac-2 in colon cancer: Lotz *et al.*, 1993; BRCA 1 in breast cancer: Chen *et al.*, 1995; cf. Jensen *et al.*, 1996); conversely, the presence of nuclear FGF-2 correlates with dysregulated autocrine proliferation of gliomas (M. Stachowiak *et al.*, 1995). Another cancer-related protein, the von Hippel–Lindau tumor suppressor gene product, is normally transported between nucleus and cytoplasm in cultured cells as a function of cell density (Lee *et al.*, 1996).

The Null Hypothesis

Given that a surprising variety of proteins do reside in multiple compartments, what might this mean? At one extreme, many proteins might arrive at unexpected compartments in small amounts solely via misrouting and diffusion, thus representing a certain level of "noise" that cells might tolerate but not use for specific functions. Such a null hypothesis needs to be taken seriously, particularly in those cases (e.g., secreted glycolytic enzymes) in which the functional significance is least certain. However, the null hypothesis cannot account for cases that exhibit: 1) compartment-specific protein isoforms; 2) novel mechanisms for compartmental translocation; 3) compartment-specific regulation; 4) lack of promiscuity (for example, cell-surface actin is not found in all lymphocytes or under all conditions); and 5) functional complexes of proteins within unexpected locations (e.g., nuclear calmodulin is present as a complex with other binding proteins [Portoles *et al.*, 1994; Wang *et al.*, 1995]).

The Misrouting Hypothesis: Misrouting as a Source of Variation for Natural Selection

Perhaps most importantly, the null hypothesis fails to take into account the effects of natural selection over time. Mutations are rare, random, purposeless events that are known to be important over an evolutionary time scale; so, too, might misrouting events be expected to affect the evolution of cellular organization by providing an important source of phenotypic variation.

There is ample reason to expect that misrouted proteins would be able to interact directly with other proteins residing in different compartments given the view of proteins as highly multifunctional entities (see INTRODUCTION) and considering the in vitro binding assays and functional assays reviewed above. The frequency of protein misrouting is unknown (Pugsley, 1990), but suppose that 99.8% of an abundant cytoplasmic protein X (1% of the total) is retained in the cytoplasm and 0.2% is misrouted to the cell surface. The cell surface abundance of protein X will be 0.002%, which is still in the range of the natural abundance of specific cell-surface proteins. If protein X is fortuitously capable of binding to other proteins in its new environment and thus influences the cell's phenotype, natural selection may favor cell variants that express altered forms of protein X or that have an altered degree of routing of protein X to the cell surface (see also Pugsley, 1990).

In this fashion what was originally an accident of misrouting may come to acquire a physiological significance for the cell:

> Leopards break into the temple and drink to the dregs what is in the sacrificial pitchers; this is repeated over and over again; finally it can be calculated in advance, and it becomes a part of the ceremony (Kafka, 1961).

The notion of proteins acquiring new functions in new compartments is similar in spirit to "gene sharing," in which genes encode a single protein that acquires entirely new functions in different *tissues* (Piatigorsky and Wistow, 1989). However, the underlying mechanisms are different: "Gene sharing" per se refers to changes in tissue-specific control over transcription without changes in the protein-coding region. In contrast, to regulate compartmentalization of a protein within a cell, other mechanisms must be involved such as gene families, differential RNA splicing, posttranslational modifications, or recognition sequences for specific routing/transport proteins. Note also that misrouting can be subject to selection, not only by affecting the fitness of entire organisms, but also by affecting cell populations differentially as they proliferate within an organism (Michaelson, 1993).

Linking Roles across Compartments

The misrouting hypothesis does not imply that the roles of a given protein will necessarily be linked in two different compartments. Yet many proteins (e.g.,

glucocorticoid receptors) are well-established as moving from nucleus to cytoplasm and back as part of their normal functions—so well-established, in fact, that they were not included in this review of "unexpected" locations. It has been proposed that some of the nuclear growth factors and kinases discussed above also move from cell surface or cytoplasm to nucleus in modulating the cell cycle. As multicompartment proteins are investigated further, it remains to be seen to what extent their functions will prove to be linked in an organized fashion across different compartments. For example, it is presently unclear whether the extracellular and cytoplasmic functions of multifunctional proteins such as annexins and S100β are ever linked (Barger *et al.*, 1992; Raynal and Pollard, 1994) as might occur if protein released from one cell entered the cytoplasm of other cells to exert its effects.

The Stress Hypothesis

Cells have elaborate mechanisms (e.g., DNA repair, heat–shock response, and DNA amplification) by which cells respond and adapt to environmental stresses. These stress responses are associated with cellular events (e.g., delaying cell cycle progression, inhibiting most protein synthesis, and amplifying stretches of genes) that would be considered aberrant or detrimental under many normal conditions. Is it possible that passage of proteins to unexpected locations may also be specifically regulated and actively induced in some cases, as part of a cell's adaptive response to stress?

"Wounding" provides the clearest example to date of how an intercompartmental event, normally forbidden, can be viewed not only as a manifestation of cellular stress, but as an active, adaptive response to it (McNeil, 1993). The expression of neuron-specific enolase in the nucleus is also stress-related: The proportion of facial and hypoglossal neurons exhibiting detectable nuclear neuron-specific enolase increases dramatically after their axons are severed and reverts to baseline after the nerves have successfully regenerated (Angelov *et al.*, 1994). It has been suggested, although not demonstrated, that nuclear glycolytic enzymes might provide a local supply of ATP to support the additional macromolecular syntheses needed for nerve regeneration. The percentage of cells exhibiting nuclear neuron-specific enolase is also greatly increased in neural tumor cells treated with hydrogen peroxide (Angelov *et al.*, 1994).

Physiological responses can be stressful, too: For example, submaximal exercise in rats has been shown to activate heat shock factor leading to an increase in Hsp70 mRNA levels in cardiac muscle (Locke *et al.*, 1995), an elevation that has been shown to be independent of heating effects (Skidmore *et al.*, 1995). In this context, the recent work of Clarke *et al.* (1995), linking contraction-induced "wounding" in cardiac muscle cells to the release of cytoplasmic growth factors that stimulate hypertrophy in response to exercise, suggests that "wounding" is important at levels of stress found in normal cell physiology. Insofar as mitogenic stimuli have been shown to induce increases of mRNA and protein levels for several heat–shock proteins in normal human lymphocytes (Hansen *et al.*, 1991), the increased expression of cell-surface actin in proliferating lymphocytes might arguably be said to correlate with a state of cellular stress as well.

Conclusion

Proteins in unexpected locations need not be regarded as anomalies, puzzles, or paradoxes. I have identified several possible scenarios to explain how and why proteins may arrive in unexpected locations: Some may have become misrouted, acquiring new functions in the course of evolution. Others may move among compartments as part of physiological control systems that have not yet been fully elucidated. Still others may be induced as part of cellular stress responses. Before these proteins can serve as examples to alter current concepts of cellular organization, further experimental evidence is needed on their functions and the mechanisms controlling their compartmentalization—but this depends, in turn, on the willingness of cell biologists to approach these as potentially important problems for study.

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