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# CONCLUDING REMARKS

## INTERNALIZATION OF POLYPEPTIDE GROWTH FACTOR RECEPTORS AND THE REGULATION OF TRANSCRIPTION

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In the last 2 years considerable progress has been made in our understanding of how the signals carried by peptide and polypeptide growth factors are transduced and bring about changes in gene expression [1–8]. Although the second messenger systems involved are multifaceted and often stimulate events in parallel pathways, the essential steps in all of the systems outlined to date consist of (1) a polypeptide ligand binding to its cognate receptor (an integral membrane protein of the plasma membrane), (2) transduction of the signal across the membrane and the stimulation of a cascade of events in the cytoplasm in the vicinity of the membrane bound receptor which culminate in (3) the translocation of second messenger molecules into the nucleus where, after a period of some hours (4) they alter the pattern of gene transcription. For EGF* the whole of this classical pathway is now almost fully documented [4, 5, 9–11], and there is good reason to believe that similar pathways (in some instances involving identical cytoplasmic messenger molecules) serve the other peptide and polypeptide growth factor and cytokine families which have been identified.

*Internalization of the EGFR*

In theory, receptors for peptide and polypeptide ligands can have their signals fully transduced at the plasma membrane. Since ligand binding is an equilibrium reaction the signal can be attenuated by ligand dissociation or by desensitization within the transduction mechanism [12] while the receptor remains on the cell surface. In practice, however, most growth factor receptors internalize once they bind ligand. The internalization process has been studied most thoroughly for EGFR where, at saturation levels of ligand binding, <80% of surface located-receptors are removed into the cell within 5 min [13, 14]. Signal transduction has been shown to begin while the receptor is on the cell surface [15] when its endogenous kinase is activated and it has not yet been possible to show unequivocally that any of the various signal transduction events which follow EGF binding requires internalization.

After being internalized EGF–EGFR complexes travel through the endosome to the lysosome. For the first 15–20 min of their intracellular processing EGFRs reside as integral membrane proteins within the perimeter membrane of the endosome [15]. This compartment, which is widely distributed throughout the cytoplasm, consists of a network of tubules and vacuoles [16] and as the receptor trafficks through its membrane boundaries the EGF–EGFR complex remains intact and the kinase of the receptor remains active [17]. Eventually, within the vacuoles of the endosome, segments of perimeter membrane containing the EGF–EGFR complexes pinch off as small vesicles into the lumen and multivesicular endosomes are formed. This process has been described in detail by several laboratories [14, 16, 18] and is thought to provide a means of removing EGFR and other integral membrane proteins from the perimeter membrane of the endosome. It delivers EGF–EGFR complexes to the lumen of the endocytotic pathway, where, along with other lumenal contents, they can be transferred to the lysosome and degraded. Taken together these observations suggest that the most straightforward explanation for the process of receptor internalization is that it attenuates further stimulation at the surface and widely distributes the actively transducing receptor throughout the cytoplasm. It is of interest that the transport of internalized ligands along the endocytic pathway requires an intact microtubular cytoskeleton [19] and that colchicine treatment which depolymerizes microtubules significantly enhances the mitogenic potency of EGF [20].

Ligand binding and uptake is not, of course, always associated with signal transduction. The process of receptor mediated internalization is, for example, widely employed in scavenger systems for extracellular materials such as asialoglycoproteins, $\alpha$2-macroglobulin–protease complexes, and antigen–antibody complexes. In the liver similar scavenger mechanisms [21] remove signal-bearing ligands like EGF from the circulation. A hallmark of these scavenger systems is the way they depend upon the internalized receptor recycling in order to provide for high capacity uptake. Where receptors for signal bearing ligands recycle, and if, as is often the case, they only bind ligand with low affinity, their primary responsibility is probably to reduce the extracellular concentration of their cognate ligand in the extracellular environment.

Evidence from a wide spectrum of studies has

* Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; NLS, nuclear localization sequence; NGF, nerve growth factors; bFGF, basic fibroblast growth factor; IL, interleukin.

questioned whether the transduction scheme outlined above which depends entirely upon cytoplasmic second messengers can account for the transduction processes of all peptide and polypeptide growth factors. There is, in particular, the question of whether it can provide a full account for processes like EGFR transduction which, when they lead to DNA replication, take many hours to exert their final affects.

*Signal transduction and the presence of ligand–receptor complexes in nuclei*

The most compelling reasons for considering that signal transduction involves more than second messenger cascade are the presence of polypeptide ligands and proteins identical to surface receptors within nuclei. The published studies which have identified intranuclear ligands and receptors fall into two main groups: (1) those which demonstrate the ligand and/or the receptor within the nuclei of intact cells and (2) those which demonstrate ligand and/or receptor in nuclei isolated from stimulated cells (or in isolated nuclei to which labelled ligand has been added).

(1) *Studies on intact cells.* (a) The demonstration of bFGF in the nucleus in a cell cycle dependent manner is well documented [22] and this ligand has also been shown to bear [23] an amino acid sequence which enables it to be transported through nuclear pores, into the nuclear matrix. It has also been shown that this polypeptide can interact directly with DNA and regulate transcription. Receptors for FGFs are present on the plasma membrane and their ability to transduce a signal on FGF binding is well characterized [23]. Thus bFGF is able to both bind and have its signal transduced at the cell surface and is found in the nucleus directly regulating transcription. However, these two processes need not necessarily be connected and to establish a direct, causal relationship between them it is clearly necessary to show that the FGF molecules which bind to cell surface receptors also enter the nucleus. This rigorous requirement is especially pertinent for bFGF because it is known that the polypeptide lacks an endoplasmic reticulum translocation signal and it is thus available as a soluble pool in the cytosol of any cell in which it is synthesized. Also because it bears an NLS, it is free to diffuse into the nucleus.

(b) Similar evidence is available for IL1, a ligand also likely to be involved in transduction events inside the cell because it is internalized bound to its receptor and remains, without significant degradation, within the cell for prolonged periods (up to 8 hr) [24–26]. Several studies have endeavoured to prove that the internalized IL1 is transported into the nucleus, the most convincing observations being made using morphological localization by autoradiography [27]. However, although nuclear accumulations of internalized labelled ligand accounting for up to 30% of the total cellular label have been reported, the general level of signal (giving 2–3 grains per nucleus) is very low. These studies require confirmation by techniques which allow intranuclear localizations to be achieved with equal certainty but which are more sensitive. As with bFGF, IL1 probably contains functional nuclear localization signals [26] and since some forms also lack an endoplasmic reticulum translocation signal sequence the peptide probably has important, additional functions to play within the cytoplasmic/nuclear matrix.

While evidence that IL1, which binds to receptors on the plasma membrane, is transported into the nucleus awaits a definitive confirmation it is supported by the knowledge that the internalized ligand is exceptionally resistant to intracellular degradation. Most of the reports for other polypeptide ligands in the nucleus (EGF, NGF) cannot exclude the possibility that the intranuclear signal which is observed is due to small molecular weight degradation products because all of these ligands are extensively degraded following their internalization.

Evidence to show that internalized surface receptors (rather than their ligand) are transported into the nucleus is very difficult to obtain and depends almost entirely upon localizations achieved with labelled cognate ligand. Since, as discussed above localizations of the ligands themselves are often open to question attempts to show that these labelled ligands also remain complexed to their receptors when they transfer to the nucleus must be viewed with circumspection.

(2) *Studies on isolated nuclei.* Evidence for nuclear localization which depends primarily upon identifying ligands or receptors within isolated nuclei is notoriously difficult to evaluate. Nuclei are difficult to purify and because they readily bind exogenous materials non-specifically, characterization procedures for nuclei isolation have to be extremely rigorous. Equating nuclear localization with a detergent insoluble fraction is clearly a weak assumption especially in view of the accumulating evidence which suggests that the cytoskeleton plays a central role in signal transduction. Controls in which labelled exogenous ligand is added to the lysate and its recovery compared with that of the endogenous ligand being assayed, should, in these circumstances, be an essential prerequisite.

Detailed estimates of specificity and affinity of the kind obtained for ligand–receptor interactions on the cell surface are very difficult to obtain for receptors in well characterized nuclear fractions. Non-specific binding within the nucleus also interferes with immunocytochemical localizations to an extent which frequently renders them unconvincing. Thus, although changes in the level of signal such as those recorded by light microscopy with bFGF are unequivocal [22], localizations reported for other ligands and their receptors, especially when carried out at the electron microscope level, need to be interpreted with extreme caution. Indeed, it is difficult to understand why so many published studies in this area have chosen to use electron microscope techniques since whether or not the label is located in the nucleus can be readily established in tissue sections with the resolution provided by light microscopy. Moreover, the labelling methods required for electron microscopy are usually an order of magnitude less sensitive than those used for light microscopy.

*The problem of transferring an integral membrane protein of the plasma membrane into the nuclear matrix*

Conceptually the greatest difficulty with proposing

that an intact cell surface receptor is transported into the nucleus is that it is necessary to explain how, once inserted, an integral membrane protein can be removed from the lipid bilayer of the membrane. Currently no mechanisms have been described which would allow an integral membrane protein to be withdrawn from a lipid bilayer and there is no evidence that the intracellular membrane boundaries along which internalized receptors move can disintegrate or otherwise lose their continuity. Fragmentation of the endocytic membrane boundary which could provide a means of releasing integral membrane proteins into the cytosol has yet to be described and although the polypeptide chains of toxins such as *Diphtheria* and *ricin* are known to translocate across lipid bilayer boundaries the requirements for transferring a free, soluble peptide across a membrane are very different from those required for removing an integral membrane protein.

From a cell biological point of view it is much easier to imagine that fragments derived from the cytoplasmic domains of internalized receptors might be transferred to the nucleus. There is, however, little evidence to support this proposition since most immunocytochemical localizations of receptors in the nucleus use antibodies directed against their external, extracytoplasmic domains rather than internal, cytoplasmic regions. There have been a number of studies [28, 29] which have suggested that the degradation of receptors (such as EGFR) yields fragments of intermediate size but the weight of the published evidence suggests that degradation within the endocytic pathway is carried out entirely within the lumen of the pathway and proceeds down to the level of single amino acid residues.

*Conclusion*

From the growing volume of published work on nuclear localizations the evidence suggesting that regulatory mechanisms exist which involve ligands which can both bind to cell surface receptors and also act within the nucleus is very persuasive. However, at the present time, the unequivocal demonstration that a ligand together with its cognate receptor, can be transferred intact from the plasma membrane to the nucleoplasmic matrix has yet to be achieved. If, with further work, this process is indeed shown to occur it will require the participation of membrane phenomena which have not yet been observed elsewhere in the cell.

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