Review

Nuclear localization and function of polypeptide ligands and their receptors: a new paradigm for hormone specificity within the mammary gland?

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Abstract

The specific effects triggered by polypeptide hormone/growth factor stimulation of mammary cells were considered mediated solely by receptor-associated signaling networks. A compelling body of new data, however, clearly indicates that polypeptide ligands and/or their receptors are transported into the nucleus, where they function directly to regulate the expression of specific transcription factors and gene loci. The intranuclear function of these complexes may contribute to the explicit functions associated with a given ligand, and may serve as new targets for pharmacologic intervention.

Keywords: cyclophilin, retrotransport, signal transduction, Stat5, transcription

Introduction

Peptide hormones and their cognate receptors are required for the development and differentiation of the mammary gland. The traditional paradigm of peptide hormone/receptor action in mammary tissues is best summarized as one of 'action-at-a-distance'. In short, a temporal and spatial activation of receptor-associated transduction/transcription factors is thought to mediate a specific signal triggered by ligand binding. These networks undoubtedly induce the necessary post-translational modifications (phosphorylation/dephosphorylation, prolyl isomerization, proteolytic cleavage, etc.) required for transcription factor/co-activator function. On careful examination of the signaling networks emerging from functionally disparate peptide hormone receptors, however, a molecular basis for specificity remains unclear, as the transduction cascades utilized by these receptors are widely shared.

A potential solution to the conundrum of polypeptide hormone specificity has recently emerged in a series of articles detailing the intranuclear translocation and action of peptide ligands and their receptors, or fragments thereof. As contrasted later, striking parallels in the intranuclear actions of peptide hormone/receptors exist with steroid and Notch receptor complexes. These findings taken together lead to the novel hypothesis that peptide hormone specificity is generated by the conjoint activation of receptor-associated signaling networks (i.e. 'nongenomic action') and by the direct intranuclear function of ligand and/or receptor complexes (i.e. 'genomic action').

Translocation and function of polypeptide receptors within the nucleus

Several transmembrane polypeptide receptors have been reported within the nucleus, including epidermal growth factor receptor (EGFr) [1], erbB-3 [2], erbB-4 [3], fibroblast growth factor receptor (FGFr) [4,5], nerve growth factor receptor [6], IL-5 [7], prolactin receptor (PRLr) [8], and growth hormone receptor (GHr)/growth hormone receptor binding protein [9,10]. Aside from delineating the nuclear localization of these peptides, recent studies have begun to elucidate their potential intranuclear functions and mechanisms of nuclear internalization (also referred to as 'retrotrans-

CK2 = casein kinase 2; CypB = cyclophilin B; EGF = = epidermal growth factor; EGFr = epidermal growth factor receptor; ER = endoplasmic reticulum; FGF = fibroblast growth factor; FGFr = fibroblast growth factor receptor; GHBP = growth hormone binding protein; GHr = growth hormone receptor; IFN = interferon; IL = interleukin; MHC = major histocompatibility complex; PIAS3 = peptide inhibitor of activated Stat3; PRL = prolactin; PRLr = prolactin receptor; PPI = peptidyl prolyl isomerase.

port'). The present review will focus on those receptors with acknowledged function within the mammary gland.

Epidermal growth factor receptor

While immunohistochemical and biochemical studies have localized the EGFr within the nucleus, the potential function of this receptor at this site has only been recently elaborated [1]. Through the use of cross-linked ^{125}I-epidermal growth factor (EGF), Kwong and colleagues revealed that phosphorylated, full-length EGFr is rapidly transported into the nucleus within 1 min of ligand stimulation. They also showed that the C-terminal (intracytoplasmic) domain of the EGFr potently activated the expression of a luciferase reporter construct when fused to a GAL DNA binding domain. Using the approach of cyclic amplification and selection of targets ('CASTing'), Kwong and colleagues demonstrated that the EGFr preferentially bound to an AT-rich sequence. Stimulation of cells transfected with a luciferase reporter construct containing this AT-rich sequence resulted in EGF-induced expression of the reporter. Furthermore, when a similar sequence was identified in the cyclin D_1 (an EGF-inducible gene) promoter region and subsequently mutated in a reporter construct, EGF-induced expression was lost. Additional *in vivo* confirmation of a physical association of the EGFr with the endogenous promoter region of cyclin D_1 was demonstrated by chromatin immunoprecipitation analysis.

These findings taken together suggest that the nuclear retrotransport and promoter binding of the EGFr following ligand stimulation may contribute to the expression of specific EGF-induced genes. While this study represents a groundbreaking analysis of intranuclear EGFr function [1], several points remain to be addressed: how does the cell surface EGFr extricate itself from the plasma membrane? How does the EGFr cross the nuclear membrane? How does the intranuclear EGFr modulate endogenous gene expression from a mechanistic perspective? What gene loci are transactivated by the intranuclear EGFr? Indeed, the lack of a defined mechanism for EGFr extrication from the cell membrane and retrotransport to the nucleus has been challenged [11]. The facts remain, however, that multiple independent studies have now demonstrated EGFr within the nucleus [1,12]. The inability of some groups to reproduce these findings [11] may be related to the difficulty of some anti-EGFr antibodies to recognize the EGF in certain intracellular environments.

erbB-3

A recent study [2] has elegantly demonstrated, by confocal immunomicroscopy, immunogold electron microscopy, and biochemistry, the existence of full-length erbB-3 within the nucleus of several cultured malignant human breast cancer lines. Unlike the functional studies already detailed for the EGFr, Offterdinger and colleagues [2] examined the basis for the nuclear transport of erbB-3. Structure/function mutagenesis revealed a sequence in erbB-3 (termed NLS-2; RRRRHSP) that functioned as a nuclear internalization signal. As such, their study is noteworthy for its direct demonstration of this motif; however, the study needs independent confirmation and does not address any potential intranuclear function of erbB-3, nor has intranuclear erbB-3 been demonstrated in malignant or normal tissues. In addition, the mechanisms through which erbB-3 can remove itself from the cell membrane prior to nuclear translocation remain unresolved.

erbB-4

The presence of a soluble fragment of erbB-4 within the nucleus has been recently reported [3], following reports of the erbB-4 receptor within the nuclei of primary human breast cancers [13]. This fragment spanning the intracytoplasmic domain of this receptor appears to be the result of a sequential cleavage of erbB-4; first by an unidentified metalloprotease (resulting in cleavage of a soluble extracellular domain and a membrane-bound intracytoplasmic domain), followed by cleavage of the intracytoplasmic domain proper (into a cytoplasmic-soluble form), by a γ-secretase-like activity. This was shown through the use of pharmacologic inhibitors or dominant-negative precursors of γ-secretase that effectively blocked the formation of the soluble erbB-4 fragment. Use of immunofluorescence-based studies revealed that this fragment was capable of nuclear retrotransport, and that the motif mediating this event was present in the C-terminus of the intracytoplasmic domain. Fusion of the erbB-4 intracytoplasmic domain to the GAL4 DNA binding domain revealed a modest transactivation of a GAL4 reporter construct, suggesting that the erbB-4 fragment may be capable of modulating endogenous gene expression.

The study by Ni and colleagues [3] is remarkable because it demonstrated parallels between erbB-4 and Notch signaling. However, whether the erbB-4 fragment is associated with active chromatin and which gene loci this fragment activates remain to be determined. In addition, formal biochemical proof of the existence of this fragment within the nucleus remains to be demonstrated, as well as the precise motifs involved in the nuclear retrotransport and transactivation.

Fibroblast growth factor receptor

Stimulation of FGFr-expressing cells with fibroblast growth factor (FGF) 2 results in the nuclear translocation of the complex, despite the absence of a classic nuclear localization signal sequence in either the receptor or the ligand [14]. Continued work on this system has revealed that the nuclear transport of full-length FGFr is dependent upon importin β [5], as a FGFr/importin β complex is generated following ligand stimulation and as the immunodepletion of importin β blocks FGFr uptake in an isolated nuclear transport system. Targeting of FGFr directly into the

nucleus (by replacement of its endoplasmic reticulum [ER] leader signal with a nuclear localization signal sequence from SV40 T antigen) resulted in enhanced c-Jun and cyclin D_1 expression.

As with the members of the EGFr/erbB family, the mechanisms of transport of the FGFr from the cell membrane remain to be determined. In addition, the motifs required for receptor-induced gene expression have not been identified, and the physical association of the FGFr with the transcriptional apparatus on the endogenous c-Jun and cyclin D_1 loci requires demonstration.

Growth hormone receptor

The presence of both growth hormone and the GHr within the nucleus has been previously demonstrated [15]. Recent data from Lobie and colleagues have suggested that the extracellular domain of the GHr, also known as the growth hormone binding protein (GHBP), exists and functions within the nucleus in a manner independent of the ligand [9]. These studies have demonstrated that a modest potentiation of the expression of a Spi2.1 reporter construct occurred upon transfection of the GHBP. The potentiation of the expression of this reporter construct was increased if a nuclear localization signal was added to the GHBP via a recombinant technique [9]. These studies suggest that the extracellular domain of the GHBP may stimulate Spi2.1 transcription. These studies stand in contrast to other reports that suggest the GHBP acts in a dominant negative manner [16]. In addition, the manner of nuclear transport of the GHBP from either the ER or extracellular space, the effect of GHBP on endogenous gene expression, the gene loci activated by the GHBP, and the mechanisms utilized during transcriptional activation remain to be determined.

Prolactin receptor

The nuclear retrotransport of the PRLr has been noted [8,17] and associated with the activation of nuclear protein kinase C. Little else is know of the mechanisms of its nuclear internalization or intranuclear function. Indeed, whether the intranuclear PRLr represents a full-length receptor or a fragment thereof has been debated.

Translocation and function of polypeptide ligands within the nucleus

The nuclear retrotransport of numerous polypeptide ligands, including prolactin (PRL) [18–21], growth hormone [15], EGF [22], IFN-γ [23], nerve growth factor [6,22], platelet-derived growth factor [22], FGF [24–26], IL-5 [7], and insulin [27–29], has been reported within the nucleus following their addition into the extracellular medium. While the intranuclear transport and action of many of these ligands are not known, a considerable understanding of such mechanisms for FGF and PRL has been newly forthcoming, and is now detailed.

Fibroblast growth factor

A growing body of evidence has indicated a function for FGF internalized within the cytoplasm, and possibly within the nucleus. Precedent reports from Olsnes and colleagues have demonstrated that FGF is retrotransported into the cytoplasm and nucleus [26]. This transport required several hours and was dependent on the cytoplasmic domain of the FGFr, but not on its kinase activity [25]. Stimulation of NIH3T3 cells by the external application of a mutant FGF ligand containing a CAAX motif resulted in the demonstrable intracellular and intranuclear farnesylation of this mutant after several hours [30], revealing that the intracellular FGF was capable of crossing cellular membranes. Following receptor-mediated endocytosis, FGF could be found in the Golgi/ER. Transport from this vesicular compartment required the presence of an active proton pump; when blocked with the specific inhibitor bafilomycin A_1, entry into the cytoplasm was blocked [31]. In the absence of FGFr, a fusion protein of FGF and diphtheria toxin was capable of stimulating the proliferation of NIH3T3 cells expressing diphtheria toxin receptor [26]. These data suggest that the primary function of the FGFr, with respect to FGF-induced mitogenesis, was for the endocytosis of FGF. Phosphorylation of FGF appeared to contribute to its mitogenicity, as a specific mutation (K132E) to FGF resulted in a ligand demonstrating comparable levels of receptor binding and activation, but demonstrating decreased phosphorylation and ligand-induced proliferation [32].

What is the function of intracellular FGF? Several proteins have been found to interact with FGF intracellularly, including FGF intracellular binding protein, mortalin, synaptotagmin, and S100A13. A recent study has also demonstrated an interaction between FGF and casein kinase 2 (CK2) [33]. The interaction between these proteins, as determined by surface plasmon resonance, was of moderate affinity (in the order of 1 μM). Despite this, *in vitro* FGF was phosphorylated by CK2 and, in turn, the presence of FGF stimulated the autophosphorylation of CK2 [33]. CK2, like FGF, exists in both the cytoplasm and the nucleus. The ability of FGF mutants to bind CK2 was found to correlate with their mitogenic potential. Additional studies have indicated that intranuclear FGF2 can bind to the CK2 and can stimulate its activity towards the nucleolar substrate nucleolin [34]. These findings suggest, but obviously do not prove, a potential function for CK2 in the intracellular actions of FGF.

These data raise several interesting questions regarding the intracellular transport and function of FGF: what is the precise mechanism of FGF exit from the vesicular compartment following endocytosis? Is the nuclear localization of FGF truly of functional significance, or are its intracytoplasmic actions more relevant? What happens in

cell/animal models where CK2 is either knocked down or knocked out (i.e. one would predict a decrease in FGF action)? What are the relevant substrates for CK2 in both the cytoplasm and nucleus? These points are discussed at length in two recent reviews on the intranuclear function of FGF [35,36].

Prolactin

PRL has been reported in the nucleus of T cells and human breast cancer cell lines following co-mitogenic stimulation [19,37]. The nuclear uptake of PRL appears to be widespread, as previous biochemical and/or immunohistochemical studies have demonstrated this ligand in tissues of the breast, the immune system, the liver, the ovary, and the adrenal [38–40]. Nuclear retrotransport occurs within 1–2 hours of extracellular ligand stimulation and is dependent upon the PRLr. Immunogold electron microscopy studies using anti-PRL antibodies and colloidal gold-labeled PRL have revealed that, following endocytosis, the ligand is retrotransported from the endosome into the multivesicular body, where the ligand is either targeted into the lysosome (presumably for degradation) or into the Golgi/ER [20]. Shortly after this, ligand can be noted within the nucleus. When transfected into T-cell lines neither the secreted wild-type PRL (containing an ER leader sequence) nor a mutant of PRL lacking its leader (which accumulated in the cytoplasm) had a significant effect on T-cell growth. However, mutant PRL containing the SV40 large T nuclear signal sequence in place of the ER leader induced robust T-cell proliferation [21].

The mechanisms for PRL nuclear retrotransport and action were not fully appreciated until followup studies were performed to identify the binding partners of internalized PRL capable of mediating such functions. A yeast two-hybrid analysis was performed with PRL as bait to identify PRL-binding partners. This analysis, confirmed by *in vitro* and *in vivo* binding studies, revealed that a significant binding partner for PRL was the peptidyl prolyl isomerase (PPI) cyclophilin B (CypB). A ubiquitously expressed 22 kDa protein secreted from most cells, CypB is found in serum and milk, and also within cell nuclei. Exogenously applied CypB was found to dramatically potentiate PRL and growth hormone-driven proliferation and to potentiate gene expression (i.e. β-casein or Spi2.1), but only in cell types expressing either the PRLr or the GHr [37].

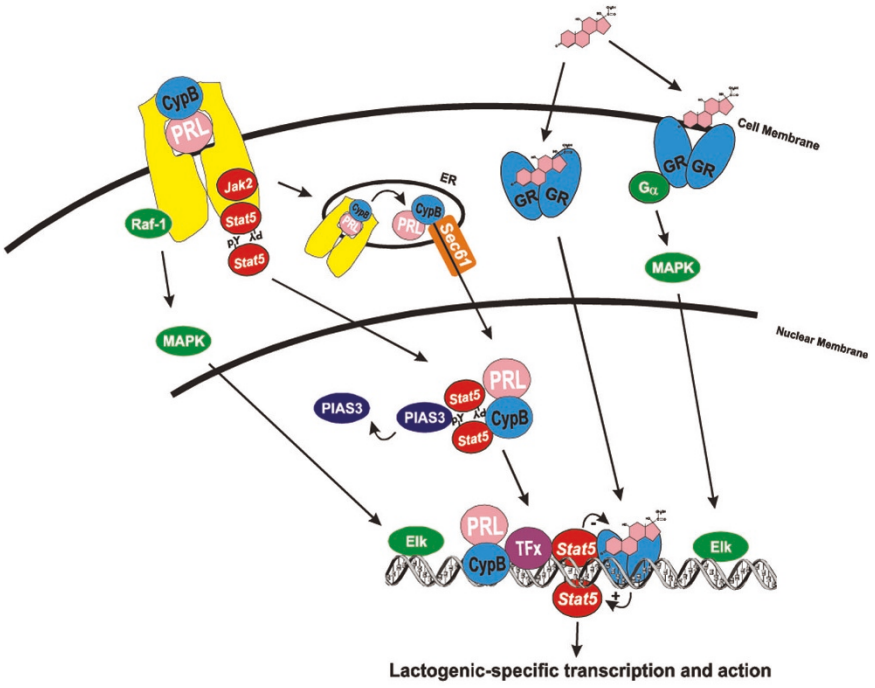
The observation of an interaction between CypB and PRL has enabled further insights into the mechanisms of PRL retrotransport and intranuclear function. Following endocytosis, PRL is presumably released from its receptor as a consequence of vesicular acidification, thus enabling transport from the multivesicular body to the Golgi/ER. Retrotransport of many bacterial toxins and MHC-targeted peptides has been demonstrated to occur via the Sec61 apparatus [41,42]. Of note, the PRL/CypB complex can also be co-immunoprecipitated with the Sec61 pore following endocytosis of this complex, an event that parallels the activation of PRLr-affiliated signaling pathways [19,20]. Nuclear transport of the PRL/CypB complex is facilitated by a newly recognized nuclear translocation signal in the N-terminus of CypB [37]. CypB mutants lacking this signal, while enzymatically active and fully capable of binding PRL, did not potentiate PRL-driven proliferation or the intranuclear accumulation of PRL. Taken together, these findings have revealed that CypB serves as a 'reverse' chaperone, facilitating the transport of PRL from the vesicular compartment into the nucleus.

Within the nucleus, the PRL/CypB complex has been found to interact with Stat5 in a PPI-dependent manner and to potentiate the activity of this transcription factor. Stat5 is a well-recognized mediator of PRL action within the mammary gland, as Stat5a knockout mice fail to lactate [43]. Following tyrosine phosphorylation by receptor-activated Jak2, Stat5 is known to dimerize, to translocate into the nucleus, and to bind to cognate DNA binding sites. Electrophoretic mobility shift analysis revealed that the PRL/CypB complex facilitated Stat5 DNA binding, in a manner that indicated the PRL/CypB complex was removing a repressor of Stat5 function. Co-immunoprecipitation analysis demonstrated this repressor to be the peptide inhibitor of activated Stat3 [44]. The exogenous addition of PRL/CypB released the peptide inhibitor of activated Stat3 from intranuclear Stat5, thereby enhancing Stat5 DNA binding [45]. The PRL/CypB complex directly interacts with the N-terminus of Stat5 and appears to induce a conformational change in this transcription factor, enabling the release of peptide inhibitor of activated Stat3 (PIAS3), thereby facilitating its association with additional elements of the transcriptional apparatus with Stat5 [45].

The functions of the PRL/CypB complex are dependent upon the PPI activity of CypB. While perfectly capable of binding PRL and undergoing nuclear retrotransport, CypB mutants deficient in this enzymatic activity are incapable of stimulating Stat5-mediated gene expression or proliferation. Indeed, the PPI-deficient CypB mutants are able to block the proliferation of breast cancer cell lines at concentrations two to three orders of magnitude less than other described mutant PRL antagonists [45].

These studies raise several interesting questions regarding the PRL/CypB complex: how does CypB facilitate the transport of PRL across the Sec61 pore? What are the other intranuclear targets of PRL/CypB? How important is CypB to PRL function *in vivo*? Answers to these questions will provide additional details regarding the function and relevance of this ligand/isomerase complex.

Figure 1



Parallels between prolactin and steroid-induced signal transduction. Classic theory has the prolactin (PRL)/prolactin receptor effecting gene expression 'at-a-distance' through actions of receptor-associated signaling networks, such as the Jak2/Stat5 and Raf/mitogen-activated protein kinase (MAPK) pathways. A growing body of evidence indicates, however, that following receptor-mediated endocytosis a complex between PRL and cyclophilin B (CypB) undergoes nuclear retrotranslocation, possibly through the Sec61 pore. Within the nucleus, the PRL/CypB complex binds to repressed Stat5 complexes, inducing the release of peptide inhibitor of activated Stat3 (PIAS3) enabling Stat5 to engage DNA. In addition, the PRL/CypB complex interacts with other elements of the transcriptional apparatus (Tfx). Like PRL/CypB, steroid/steroid receptors (GR) are also able to translocate into the nucleus, where they act as transcription factors. In contrast to these genomic actions of steroid receptors, the nongenomic actions of steroid/steroid receptor complexes occur at the cell surface, are rapid, and utilize some of the same signaling networks utilized by the polypeptide ligand/receptor complexes (i.e. MAPK). ER, endoplasmic reticulum.

Conclusions and perspective

The intranuclear transport and function of polypeptide ligands and their receptors have been a matter of considerable scientific debate over the past decade. Some of this controversy was due to a lack of mechanistic grounding inherent in the initial descriptions of intranuclear ligand and/or receptor. As outlined in the present review, however, the recent demonstration of pathways for the nuclear translocation of peptide ligands and receptors (i.e. receptor cleavage, ligand association with binding proteins/chaperones, etc.) from the cell surface/vesicular compartment and *bona fide* targets for intranuclear action (i.e. DNA, transcription factor, or kinase binding) should lay to rest many of these mechanistic concerns. Indeed, these studies provide an intelligent basis for speculation regarding how a ligand/receptor complex may make its way into the nucleus and exert functional activity at this site [46].

Acceptance of an intranuclear function for polypeptide ligands and their receptors, however, is not universal. This may be due to a lack of a larger perspective on the intranuclear action of all ligand/receptor complexes. Many of the concerns regarding the function of intranuclear polypeptide ligand/receptors can be addressed by examining the parallels that exist with other acknowledged signaling pathways. For instance, the ability of polypeptide receptors and, in particular, receptor fragments (such as the soluble erbB-4 intracytoplasmic domain) to function within the nucleus as a transcription factor/co-activator is highly analogous to Notch-associated signaling within the mammary gland [47]. Indeed, cleavage of the erbB-4 intracytoplasmic domain may use similar, if not identical, proteases required for the cleavage of the transcriptionally active Notch intracellular domain. Additional mechanistic parallels can also be drawn between the PRL/CypB complex and the actions of steroid/steroid receptor complexes (see Fig. 1). While the intranuclear actions of steroid receptor complexes have long been appreciated, the function of these complexes at the cell surface has only been recently recognized [48]. Indeed, the rapid 'nongenomic' functions of steroid receptor complexes

appear to utilize many of the signaling pathways associated with polypeptide receptors.

Many questions remain regarding the transport and actions of intranuclear polypeptide ligands and receptors. First, the mechanisms surrounding the extrication of membrane-inserted, full-length receptors need to be established. Second, the molecular basis for retrotransport of ligand into the cytoplasm requires further detailing. Third, the targets of intranuclear ligands and receptors (i.e. gene loci, transcription factors, kinases, etc.) are largely unidentified. Finally, detailing of the larger *in vivo* context for the actions of intranuclear ligand/receptor complexes at the tissue and organismal level will provide a final level of proof necessary to establish the biologic relevance of this process.

Several theories currently exist regarding how signaling specificity is achieved in a given cell by ligand/receptor complexes that share several common transduction pathways [49,50]. While some data support the notion that rare signaling pathways may be uniquely associated with a given receptor, most evidence now supports the notion that a combination of signals emanating from a given receptor results in signaling specificity. These signals may be modified in unique ways by the duration of receptor action, by compartmentalization of the receptor or its associated signaling networks, and by the proteome of a given cell. However, a rigorous demonstration of how combinatorial signaling alone works to achieve specificity in the context of peptide hormones remains to be demonstrated.

The identification of the intranuclear transport and action of polypeptide ligands and receptors may therefore provide an additional key to the conundrum of hormone/growth factor specificity. As such, the hypothesis emerging from these data would suggest that it is the function of ligand/receptor complexes in the nucleus that provides an additional level of direct regulation requisite for the specificity. Given this, the advances made in our understanding of the intranuclear actions of polypeptide ligands and receptors hold considerable pharmacologic promise. As evinced by the PPI-deficient CypB mutants, the development of pharmacophores capable of interrupting the intranuclear transport and/or function of these polypeptides may provide highly effective agents capable of inhibiting the hormone/growth factor-driven proliferation and progression of breast malignancies.

Competing interests

None declared.

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