Published in final edited form as:

Biochem Soc Trans. 2013 August ; 41(4): 1029–1036. doi:10.1042/BST20130104.

Receptor Tyrosine Kinases with Intracellular Pseudokinase Domains

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Abstract

As with other groups of protein kinases, approximately 10% of the receptor tyrosine kinases (RTKs) in the human proteome contain intracellular pseudokinases that lack one or more conserved catalytically important residues. These include ErbB3, a member of the epidermal growth factor receptor (EGFR) family, and a series of unconventional Wnt receptors. We recently showed that, despite its reputation as a pseudokinase, the ErbB3 tyrosine kinase domain (TKD) does retain significant – albeit weak – kinase activity. This led us to suggest that a subgroup of RTKs may be able to signal even with very inefficient kinases. Recent work suggests that this is not the case, however. Other pseudokinase RTKs have not revealed significant kinase activity, and mutations that impair ErbB3's weak kinase activity have not so far been found to exhibit signaling defects. These findings therefore point to models in which the TKDs of pseudokinase RTKs participate in receptor signaling by allosterically regulating associated kinases (such as ErbB3 regulation of ErbB2) and/or function as regulated 'scaffolds' for other intermolecular interactions central to signal propagation. Further structural and functional studies – particularly of the pseudokinase RTKs involved in Wnt signaling – are required to shed new light on these intriguing signaling mechanisms.

Keywords

Receptor; tyrosine kinase; pseudokinase; Ror2; ErbB3; oncogene

INTRODUCTION

Receptor tyrosine kinases (RTKs) are key regulators of critical cellular processes, such as proliferation and differentiation, cell survival and metabolism, cell migration and cell cycle control [1]. Humans have 58 known RTKs, which fall into twenty subfamilies. All have a similar overall architecture (Figure 1). An extracellular ligand-binding region is connected through a single transmembrane α -helix to the cytoplasmic region that contains a protein tyrosine kinase domain (TKD) – or pseudokinase domain – plus additional C-terminal and juxtamembrane regulatory regions. 'Canonical' RTKs are normally activated by binding of their regulatory ligands to the extracellular region of the receptor, which results in the induction of RTK homo- or hetero-dimerization and/or stabilization of a specific relationship between individual receptor molecules within an 'active' RTK dimer or oligomer [1].

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Mutations in RTKs, and/or aberrant activation of their intracellular signaling pathways, have been causally linked to cancers, diabetes, inflammation, severe bone disorders, arteriosclerosis and angiogenesis. These connections have driven the development of a new generation of drugs that block or attenuate RTK activity [1–3], several of which are now FDA approved and play important roles in cancer therapy. These drugs fall into two categories: monoclonal antibodies [4] that interfere with RTK activation – and target RTK-expressing cells for destruction by the immune system – and small molecule tyrosine kinase inhibitors (TKIs) that target the ATP-binding site of the intracellular TKD and compete with ATP binding [2]. FDA-approved examples of the latter include imatinib (which targets Kit and PDGFR), erlotinib and gefitinib (which both inhibit EGFR), lapatinib (which targets ErbB2 and EGFR), and sunitinib (which targets 8 different RTKs). Together, these drugs target at least 12 of the 58 RTKs. Most other RTKs have now been implicated in cancer and other diseases by a host of studies, including the identification of mutations in recent sequencing efforts across a wide variety of tumors [5]. New agents to inhibit many of these recently-identified targets are currently under development. Broadening the ability to target RTKs therapeutically is important not only to cover the growing range of known oncogenic drivers in human disease, but also to overcome resistance to existing therapies. It is now clear that drug resistance almost invariably develops in cancer patients treated with TKIs or RTK-targeted monoclonal antibodies. Mechanisms include the emergence of drug-resistant variants in the targets themselves, or compensations in signaling networks that overcome the need for the targeted oncogenic driver to promote tumor cell proliferation [6, 7]. A particularly notable example is upregulation – through amplification of the *MET* gene [8] or other mechanisms [7] – of signaling by ErbB3 (an ‘RTK pseudokinase’), which mediates resistance to inhibitors of EGFR and ErbB2 in non-small cell lung cancer and breast cancer [9].

RTK Pseudokinases

Intriguingly, approximately 10% of the 518 protein kinases found in humans lack highly conserved residues thought to be crucial for catalytic activity, and are therefore widely assumed to be ‘kinase-dead’ [10–12] – with experimental support in several cases. Similarly, as shown in Figure 1, five of the 58 human RTKs (~10%) contain intracellular domains suggested by Manning *et al.* [11] to be inactive (ErbB3, PTK7/CCK4, EphB6, EphA10 and SuRTK106). Another three (Ror1, Ror2, and Ryk) have substitutions at other important residues and, although predicted to be active by Manning *et al.* [11], at least Ror1 [13] and Ryk [14] have been reported to lack kinase activity, and the structure of the Ror2 kinase domain suggests that it is also inactive [15]. Thus, 8 of the 58 human RTKs may in fact be ‘RTK pseudokinases’, raising the question as to how these unusual receptors might signal.

Figure 2 details the sequence changes in the eight RTK pseudokinases as defined here, which have one or more alterations in the key conserved features of protein kinases [16] summarized below, and marked on the active EGFR TKD structure shown in Figure 3A:

Glycine-rich loop (altered in PTK7/CCK4, Ror1, Ror2, Ryk, and SuRTK106)—

The glycine-rich (or phosphate-binding) loop, between strands 1 and 2, has the consensus sequence GxGxxG and associates closely with the phosphate groups of bound nucleotide through backbone interactions. Substitution with amino acids that have side-chains, especially at the first two G positions, is expected to distort the ATP binding site.

β3-lysine/αC-glutamate salt bridge (altered in ErbB3, EphB6, EphA10, SuRTK106)—A key lysine in strand 3 forms a salt bridge with a glutamate in the key C helix in the active configuration of protein kinases, and also interacts with the - and -

phosphates of the bound Mg-ATP to position it for phosphotransfer. Mutations at the 3 lysine are typically made in 'kinase dead' variants of canonical kinases.

HRD motif (altered in ErbB3, EphB6, EphA10, and SuRTK106)—This motif lies at the beginning of the catalytic loop, and the aspartate within it is thought to function as a catalytic base and/or to correctly orient the hydroxyl group of the residue to be phosphorylated. This residue is replaced with an asparagine in ErbB3, and with serine and glycine respectively in EphB6 and EphA10. The arginine of the HRD motif is conserved only in eukaryotic kinases, so its replacement with lysine in PTK7/CCK4, Ror1, Ror2, and Ryk is not necessarily thought to be inactivating.

β7-asparagine (altered in EphB6 and EphA10)—This residue plays a key role in orienting the HRD aspartate. The two RTKs that lack the 7-asparagine (EphB6 and EphA10) also lack the HRD aspartate, suggesting a different structural arrangement in this region.

DFG motif (altered in PTK7/CCK4, Ror1, Ror2, Ryk, EphB6, EphA10, and SuRTK106)—The DFG motif lies at the beginning of the activation loop. The aspartate that begins it is a crucial residue for divalent cation binding – and is arguably one of the most important conserved residues in kinase domains. PTK7/CCK4, EphB6, EphA10 and SuRTK106 lack this residue, suggesting impaired cation binding. The DFG phenylalanine plays an important role in positioning the catalytic aspartate and in several other key regulatory interactions as described below.

Interestingly, it has been reported – albeit with qualitative studies – that one of these pseudokinase RTKs (Ryk) does show kinase activity if its DFG motif (altered to DNA in wild-type Ryk) is restored [14]. By contrast, analogous experiments with ErbB3 (restoring the glutamate in C and the HRD aspartate) did not increase kinase activity [17, 18].

How Might Pseudokinase RTKs signal?

There are two obvious possibilities for how pseudokinase RTKs might signal – which are not mutually exclusive. In the first, they may be able to catalyze phosphotransfer sufficiently well to function like other RTKs – despite their unusual sequences. In the second (activity-independent) possibility, allosteric TKD regulation may be key – or the pseudokinases may function as 'scaffolds' that control downstream signaling through regulated protein-protein interactions [10, 12].

How weak kinases might be sufficient for RTKs to signal—In a simplified 'canonical' model for RTK signaling [1], ligand binding to RTKs induces the formation of receptor homo- and/or hetero-dimers. Within these dimers, one molecule phosphorylates its partner in *trans*, leading to kinase activation and recruitment of SH2 domain-containing downstream signaling molecules. Studies of the fibroblast growth factor (FGF) receptor in particular [19] have suggested three 'phases' of RTK autophosphorylation [1]. In the first phase, *trans*-autophosphorylation of one RTK within the dimer by its partner serves primarily to enhance catalytic activity (by 50–100 fold). In the second phase, *trans*-autophosphorylation on tyrosines creates the phosphotyrosine-containing binding sites for SH2 and PTB domains, driving the recruitment of key downstream signaling molecules to the receptor. The third phase – not seen for all RTKs – elevates the kinase activity a further ~10-fold to promote phosphorylation of downstream signaling molecules. In this three-phase process, the kinase activity of the TKD is increased by several hundred to 1000-fold (depending on the RTK [20, 21]). It is important to note that the initiating *trans*-autophosphorylation event is actually catalyzed by a TKD that, for all intents and purposes,

is considered to be inactive. Otherwise stated, the triggering *trans*-autophosphorylation event in RTK signaling is actually mediated by a TKD with a catalytic efficiency that may be 1000-fold weaker than is typically considered to be ‘sufficient’ for an effective tyrosine kinase. The ability of such a low level of kinase activity to play such an important signaling role does not seem wholly unreasonable at first thought, since a TKD within an RTK dimer need only phosphorylate one or two sites within a strongly bound dimerization partner over a period of several minutes in order to achieve the ‘phase one’ activation event (ignoring phosphatase activity).

How the TKD of an RTK may function without catalyzing phosphotransfer—An alternative view of pseudokinase RTKs would posit that their TKDs serve as allosteric activators or scaffolds for nucleating signaling complexes. The fact that many RTKs are thought to heterodimerize provides one clear possibility for allosteric activation if a pseudokinase RTK heterodimerizes with another RTK that harbors an active kinase domain – as in the ErbB and Eph families of RTKs (Figure 1). In these cases, the pseudokinase TKD may interact directly with the TKD of its dimerization partner and activate it allosterically [12]. One precedent for this is seen outside the RTKs, with the pseudokinase STRAD (for Ste20-related adaptor) [22]. STRAD, in its active-like conformation, associates with the LKB1 kinase (and the MO25 adaptor) and allosterically activates LKB1. Another precedent is seen in the ErbB family, where receptor activation involves the formation of an asymmetric TKD dimer (instead of *trans*-autophosphorylation) [23]. In this case, the C-lobe of an ‘activator’ TKD interacts with the N-lobe of a ‘receiver’ TKD and induces allosteric changes in the receiver that promote its activation. Importantly, the activator TKD needs no kinase activity in this model (serving solely as a cyclin-like allosteric activator), so even kinase-dead variants of ErbB receptor TKDs function efficiently as activators [24].

Such allosteric activation models require that one of the two TKDs in a receptor heterodimer has the capacity to be an active kinase. In dimers where both are true pseudokinases, therefore, one would need to appeal to regulated protein-protein interactions. In one possibility, allosteric alterations induced in the TKD might promote binding of downstream ‘effector’ signaling molecules in a model that shares similarities with activation of small G-proteins and interaction with their effectors. Alternatively, one might speculate that certain downstream signaling molecules bind specifically (and exclusively) to the dimeric form of a pseudokinase RTK intracellular region. Investigation of such possibilities is at a very early stage.

The Case of ErbB3

With these considerations in mind, we set out to ask whether the presumed pseudokinase ErbB3/HER3 has kinase activity. Consistent with the absence of a glutamate in the C helix and replacement of the HRD catalytic base aspartate with asparagine, early biochemical studies suggested that ErbB3 is kinase-inactive [25, 26]. These studies were not performed, however, under conditions expected to activate the TKD of this neuregulin receptor that depends on heterodimerization with ErbB2/HER2 for its signaling [27, 28]. In the course of our own studies of the ErbB family, we revisited the issue of ErbB3 tyrosine kinase activity, and showed that the TKD of this receptor binds ATP with a K_D of $\sim 1.1 \mu\text{M}$ and is in fact capable of *trans*-autophosphorylation [17]. Similar results were recently published for full-length ErbB3 [29]. Further investigation showed that *trans*-autophosphorylation occurs ~ 500 – 1000 fold more slowly in an ErbB3 homodimer than in a similarly generated EGFR homodimer [17], raising doubts about whether the activity that we reported is relevant for cell signaling.

Based on the argument presented above that *trans*-autophosphorylation of RTKs may not require strong kinase activity, and motivated by the increasing realization that ErbB3 might be an important cancer drug target [30], we sought to investigate the signaling role of ErbB3's tyrosine kinase activity. Approaches employing an analog-sensitive allele of ErbB3 and 'bumped' inhibitors [31] have failed because typical inhibitors do not inhibit the ErbB3 kinase activity. We therefore generated several mutated ErbB3 variants that show impaired (or absent) kinase activity in our *in vitro* studies. Interestingly, none of these mutations abolished the ability of ErbB3 to mediate neuregulin-induced activation in transfected CHO cells, and we have not detected any defect in neuregulin-dependent survival signaling in BaF3 cells co-transfected with ErbB2 and mutated ErbB3 in our studies to date. Although preliminary, our data so far do not support a signaling role for the weak ErbB3 kinase activity that we observed *in vitro* [17]. Moreover, recent analyses of phospho-turnover on RTKs at the cell surface have shown that it is very rapid, and that any RTK must work against a background of significant phosphatase activity in order to increase steady state levels of autophosphorylation [32]. It is therefore possible that ErbB3's kinase activity is vestigial – although it remains conceivable that it plays an important role in circumstances that we have not accessed experimentally.

In structural studies, the ErbB3 kinase domain crystallized in a conformation (Figure 3B) that very closely resembles the inactive conformation of EGFR and ErbB4 [17, 33]. Intriguingly, however, activation loop mutations that activate EGFR in non small cell lung cancer actually appear to inhibit *in vitro* ErbB3 kinase activity [17] by reducing ATP-binding affinity – although they do not appear to affect ErbB3 signaling (F. Shi and M.A. Lemmon, unpublished).

Other Pseudokinase RTKs

Prompted by our ability to detect phosphotransfer activity for ErbB3, and by reports of kinase activity for other pseudokinases such as WNK1 [34], CASK [35], KSR [36, 37], and JAK2's pseudokinase domain [38], we have investigated the TKDs of other pseudokinase RTKs. Our preliminary studies indicate an absence of phosphotransfer activity for the purified TKDs of PTK7/CCK4, Ryk, Ror1, Ror2, and EphB6 so far (J.M. Mendrola, F. Shi, J.H. Park and M.A. Lemmon, unpublished). An important caveat is that we do not know whether the conditions of our assay will adequately mimic the requirements for activation of these TKDs. We simply clustered them on an Ni-NTA membrane surface (as described for ErbB3 [17]) and assessed their ability to phosphorylate a poly(Glu-Tyr) substrate. Our efforts to detect ATP binding so far have also only given positive results for ErbB3, consistent with the altered glycine-rich loop in PTK7/CCK4, Ror1, Ror2, and Ryk, and the loss of the 3 lysine in EphB6 (Figure 2). Previous biochemical studies have reported an absence of detectable kinase activity for PTK7/CCK4 [39], Ror1 [13, 40], Ryk [14], and EphB6 [41]. By contrast, kinase activity and/or its importance in signaling has been reported for Ror2 [40, 42, 43] and SuRTK106 [44].

Determination of the crystal structures of the pseudokinase domains from these RTKs is likely to be very useful for understanding why they are inactive, why they do not bind ATP (if this is correct), and how their conformational state might be regulated. Indeed, similar studies of ROP2 from *Toxoplasma gondii* [45] and the vaccinia-related kinases (VRKs) 2 and 3 [46] have been very informative. So far, we have succeeded in determining the crystal structure of the Ror2 TKD [15], as shown in Figure 3C. The Ror2 TKD crystallized in a conformation that closely resembles the inactive insulin receptor TKD (IRK) [47], with the three tyrosines of the activation loop YxxxYY motif in very similar locations to that seen in IRK – suggesting a very similar mode of autoinhibition. The Ror2 TKD contains an aspartate in place of one of the conserved glycines in the glycine-rich loop (Figures 2 and 3C), which is likely to impair ATP binding. However, unlike the situation in inactive IRK

(Figure 3D), the phenylalanine of the DFG motif does not occlude the ATP binding site in Ror2. The DFG motif has the sequence DLG instead, and the leucine (L634) does not project into the ATP-binding pocket – in part because it is smaller than phenylalanine, but also because the position of this motif is altered. Intriguingly, another tyrosine from elsewhere in the protein – Y555, two residues away from the gatekeeper residue – projects into the ATP-binding pocket from the opposite side and fills up the cavity otherwise occupied by the adenine ring. These observations explain why Ror2's TKD does not bind ATP. It is intriguing, though, that this is a tyrosine. The possibility remains for Ror2 that, with phosphorylation of Y555 and the three tyrosines in the activation loop, this kinase could actually be active. This is currently under investigation, as are structures of other pseudokinase RTK TKDs.

Pseudokinase RTKs and Wnt Signaling

As noted on Figure 1, it is intriguing that PTK7/CCK4, Ryk, and the Ror RTKs are all implicated in Wnt signaling [48], as is the more canonical RTK MuSK. In other words, half of the pseudokinase RTKs appear to mediate Wnt signals – although mechanistic details are currently very scant. Whereas Wnt-Frizzled complexes engage LRP5/6 as co-receptors in -catenin-dependent Wnt signaling, it is thought that (for certain Wnts) they may engage a Ror family member, or PTK7 as an alternative co-receptor in the planar cell polarity (PCP) pathway. Ryk and Ror2 may be engaged in different ways in other Wnt responses, and details are currently being worked out [48]. The ligand for Ror1 remains unclear [13]. For Ror2, there remains some question as to whether its intracellular TKD can be activated as a kinase – and the structure presented in Figure 3C (as outlined above) suggests some possibilities for this. Interestingly, though, Wnt signaling by Ror2 appears to involve its engagement with other kinases including glycogen synthase kinase 3 (GSK3), TGK - activated protein kinase 1 (TAK1), Src, and casein kinase 1 (CK1) in different circumstances [42, 48]. Moreover, Ryk (or the derailed family in *D. melanogaster*) interacts directly with Src [49]. Thus, it is certainly possible in multicomponent Wnt receptor complexes containing these pseudokinase RTKs that members of the Ror, Ryk, and PTK7/CCK4 family might allosterically regulate other kinases through mechanisms similar to those mentioned for STRAD and ErbB3 above.

CONCLUSIONS

Eight of the 58 human RTKs lack one or more conserved residues in their intracellular TKD that are thought to be important for kinase activity. Most of these TKDs appear to be kinase-inactive, although ErbB3 clearly retains significant (but weak) tyrosine kinase activity, and it is possible that Ror2 might be activated as a kinase by phosphorylation of key tyrosines. Given recent studies demonstrating that plasma membrane protein tyrosine phosphatase activity is substantial – and counters RTK autophosphorylation in *de facto* futile cycles – it seems highly unlikely that weak kinase activity of the sort that we reported for ErbB3 is signaling-relevant. We therefore suggest that the primary role of intracellular TKDs in the pseudokinase RTKs is likely to involve allosteric activation of other kinases (as seen in ErbB3/ErbB2 heterodimers and possibly in Wnt signaling complexes) or regulated formation of scaffolds for other signaling interactions. Understanding how these events are regulated, and how they might be modulated therapeutically is an important challenge that will require parallel studies of structure and signaling function in a variety of contexts. As with other studies of pseudokinases, it seems highly likely that new signaling paradigms will emerge.

Acknowledgments

We thank Kevan Shokat and his laboratory for help with investigating the importance of ErbB3's kinase activity. Our work in this area was supported in part by grants from the National Institutes of Health (R01-GM099891, to M.A.L.) and the U.S. Department of Defense Breast Cancer Research Program (W81XWH-10-1-0362 to F.S. and W81XWH-10-1-0124 to M.A.L.), as well as a Fellowship from the Great Rivers Affiliate of the American Heart Association (11PRE7670020 to J.H.P.).

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Figure 1.

Schematic representation of the RTK complement in humans, showing 58 RTKs that fall into 20 different families [1]. The names of the pseudokinase RTKs are highlighted in red, and the RTK Wnt receptors are denoted in the upper part of the figure. The bottom part of the figure presents a key for domain type. Red rectangles are tyrosine kinase-homologous domains. Extracellular domains are described by Lemmon and Schlessinger [1].

Figure 2.

Sequence alignment of TKD domains from the 8 RTK pseudokinases alongside protein kinase A (PKA) and EGFR. Secondary structure elements are noted. Catalytic residues conserved in all known kinases are shaded gray, and are circled in black (bold text) when altered in the pseudokinases. Uncircled residues in bold text are 'unusual', but found in some active kinases [11]. The 'gatekeeper' residue – mutated to generate analog sensitive kinases [31] – is boxed in black.

Figure 3.

Structures of the TKDs from: **A.** EGFR (active: PDB ID 1M14 [50]); **B.** ErbB3 (PDB ID 3LMG [17]); and **C.** Ror2 (PDB ID 4GT4 [15]). Kinase structures are shown in cartoon representation. ANP-PNP is modeled into **A** based on insulin receptor structures, and was observed crystallographically for ErbB3 (**B**). No nucleotide is bound to the Ror2 TKD. The N-lobe, C-lobe, glycine-rich loop, α C helix, DFG motif and HRD motif (see text) are labeled. Labeled in red text are residues corresponding to glycines in the glycine-rich loop, the 3 lysine (3K), the α C helix glutamate (α CE), the 7 asparagine (α 7N), as well as the D, F, and G of the DFG motif and the H, R, and D of the HRD motif. Where elements of these motifs are altered, the residue letter is surrounded by a red-shaded circle (as in the HRN sequence of ErbB3, the DLG motif of Ror2, and the aspartate substitution in the Ror2 glycine-rich loop). In **D.**, closeups of the ATP-binding site are seen, focusing on the conformation of the DFG motif and the gatekeeper region. In EGFR (green) and ErbB3 (pale blue) it is clear that these conformations are consistent with ATP binding, with the DFG motif in a DFG-in conformation. By contrast, in Ror2 (inactive: magenta) and inactive IRK [47] shown for comparison, the backbone region close to the DFG motif (in DFG-out conformation) occludes the phosphate binding site. Whereas the DFG phenylalanine occludes ATP binding in IRK, the DLG leucine of Ror2 does not. Rather, Y555 – close to the gatekeeper – adopts a unique conformation in Ror2 that occludes ATP binding through a different mechanism.