Dependence receptors : Mechanisms of an Announced Death

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ABSTRACT

Dependence receptors form a family of functionally related receptors which are all able to induce two completely opposite intracellular signals depending on the availability of their ligand. Indeed, in its presence, they mediate a positive, classical signal transduction of survival, differentiation or migration but without it, they trigger a negative signal which leads to cell death. The molecular mechanisms involved in triggering cell death in the absence of ligand are starting to be unravelled: dependence receptors are recruited at well-defined domains at the plasma membrane, they trigger cell death through a monomeric form, they are cleaved by caspases and they recruit a caspase activating complex.

Introduction

Receptors are usually considered to be inactive in the absence of their ligand. This generally accepted idea has been shown to be untrue for a family of unconventional receptors. Indeed certain receptors trigger “positive” signaling on survival, differentiation, and migration in the presence of their ligands, while they transduce a “negative” signal of cell death induction in the absence of their ligands (Fig.1). Thus, cells expressing these receptors are in a state of dependence on their respective ligands and that is why these receptors have been named “dependence receptors”. To date, 15 receptors have been shown to display these two opposite activities : $p75^{NTR}$, DCC, Neogenin, UNC5H receptors family, Androgen Receptor, Patched, RET, MET, TrkC, ALK, EphB4. These receptors are all involved both in embryonic development and in the regulation of tumor progression (for a full review, see [1]). One hypothesis is that the coexistence of these two signaling pathways (a positive one for migration, differentiation and a negative one for death) is probably crucial for the proper control of development processes (e.g. neural tube formation, …) and represents a safeguard mechanism to limit tumor expansion (see for reviews [1-3]).

Several recent reports have described the molecular mechanisms of death induced by some of the dependence receptors in the absence of their ligands. So far, these

mechanisms appear to require the localization in lipid rafts and monomerization of the receptors. Furthermore, dependence receptors can both activate caspases (by recruiting a caspase-activating complex) and be their substrates. We will review here the current knowledge on how dependence receptors could trigger apoptosis.

The dependence receptors here discussed are $p75^{NTR}$ (a receptor for NGF), DCC (Deleted in Colorectal Cancer) and UNC5H, (both are netrin-1 receptors), the Androgen receptor (AR), Patched (Ptc, the receptor of Sonic hedgehog Shh), integrins, Neogenin (a close homologue of DCC, receptor for RGM) and several tyrosine kinase receptors RET (REarranged during Transfection), MET, ALK, TrkC, EphA4, (receptors for GDNF, HGF/SF, pleiotrophin/midkine, BDNF and EphB3 respectively) (for a detailed description of dependence receptors and their ligands, see [1]).

Localization in lipid rafts

Lipid rafts are dynamic membrane domains, rich in cholesterol and sphingolipids and characterized by their resistance to solubilization by mild detergents such as Triton X-100 [4]. These subdomains of the plasma membrane are involved in clustering macromolecules. It has been proposed that lipid rafts serve as signaling platforms allowing binding/coupling between different molecules such as receptors with adaptors or effectors of signaling pathways such as kinases. Several studies report association of dependence receptors with lipid rafts, either to control the positive pathway (e.g., cell survival and thus differentiation), or to control the negative signaling pathway (cell death) (Fig 2). Concerning cell death induction, the dependence receptor localization to lipid rafts has been shown for DCC in both immortalized cells and primary neurons [5] and for UNC5H2 [6]. Indeed, after disruption of lipid rafts, (e.g., by cholesterol depletion), dependence receptors lose their ability to interact with proteins involved in the death signaling pathway, such as caspase-9 for DCC, or DAPK for UNC5H2 and thus their proapoptotic activity.

The mechanisms regulating receptor localization to lipid rafts are still unclear. For instance, DCC needs to be palmitoylated to translocate into lipid rafts [7] [5]. Palmitoylation of the $p75^{NTR}$ receptor has also been described [8] and seems to reduce

the receptor association with lipid rafts [9], although conclusive evidence supporting these findings is yet to be provided. However, $p75^{NTR}$ phosphorylation by PKA is a step required to allow accumulation of $p75^{NTR}$ in lipid rafts [10]. UNC5H2 palmitoylation is required for its proapoptotic activity but not for its lipid rafts localization [6].

The presence of the ligand has been shown to be important for some dependence receptors to be integrated within lipid rafts. Indeed both $p75^{NTR}$ and RET massively relocate within rafts after ligand binding [10 11] and at least in RET case, this represents a necessary step to control cell survival. However, this does not apply to the dependence receptors DCC and UNC5H2 whose localization on lipid rafts appears to be ligand independent [7 12 6] Lipid raft localization is thus crucial for the proapoptotic activity of some of the dependence receptors. Of note, clustering of the dependence receptor integrins has also been observed at the surface of dying cells [13], suggesting a possible localization of integrins in membrane subdomains. Moreover, Ptc has been described in specific membrane subdomains called caveolae, corresponding to non-clathrin coated invaginations [14]. Moreover, Ptc ligand Hedgehog also localizes to lipid rafts in *Drosophila* [15]. This is in line with previous results obtained with DCC, $p75^{NTR}$, RET and Met demonstrating that the localization to lipid rafts is not restricted to death signaling. Thus, dependence receptors requirement to localize to lipid rafts before triggering cell death could be a conserved mechanism.

Monomerization of dependence receptors

It has recently been shown that dependence receptor monomerization is a critical step for their pro-apoptotic activity, with multimerization being inhibitory for cell death (Fig 2). Indeed, the proapoptotic effect of $p75^{NTR}$ may be blocked by the addition of dimeric neurotrophins [16] or dimeric NGF-derived peptides [17] but not monomeric NGF-derived peptides [17], suggesting that $p75^{NTR}$-induced apoptosis requires the monomeric form of the receptor. Using an artificial system of dimerization, Wang *et al.,* have shown that monomeric $p75^{NTR}$ is indeed required for apoptosis induction whereas dimerization and higher orders of oligomerization block this effect [18]. The authors identified the last 19

amino-acids at the carboxy-terminus as the region required for the receptor dimerization. However, Rabizadeh *et al* showed that the deletion of these 19 amino-acids did not abolish the receptor proapoptotic activity and defined an extended sequence of 29 amino-acids at the carboxy-terminus (stretching from amino-acids 349 to 377) as the domain mediating the proapoptotic activity of $p75^{NTR}$ [19].

DCC has also been shown to homomultimerize in the presence of netrin-1, an important feature for the positive signaling of the receptor during axon guidance [20]. Two domains were found to be involved in DCC oligomerization: one is the extracellular domain which aggregates upon netrin-1 binding ; the second one is in the cytoplasmic domain (C-Terminal part of the receptor named P3) which is responsible for self-association leading to constitutive multimerization independently of netrin-1 binding [20]. The authors conclude that the cytoplasmic domain of DCC is able to oligomerize constitutively but that this association is normally inhibited in the full-length receptor. The hypothesis is that netrin-1 stimulates the formation of a receptor complex through association of the extracellular domains, then inducing a conformational change allowing DCC cytoplasmic domain multimerization. Knowing that DCC is able to oligomerize following netrin-1 binding, we investigated whether netrin-1-induced DCC and/or UNC5H2 multimerization is a critical step for DCC and/or UNC5H-mediated pro-apoptotic activity. We recently showed that DCC and/or UNC5H2 multimerize in response to netrin-1, and that this event is sufficient to inhibit apoptosis [21]. We also demonstrated that a domain of DCC, DCC-5Fbn, that triggers cell death *in vitro* and tumor regression in animal models [22] [23] does so by interfering with netrin-1-induced multimerization of DCC and/or UNC5H2 [21].

Other dependence receptors that have been shown to oligomerize in the presence of their ligand include all tyrosine kinase receptors: RET, TrkC, MET, ALK, EphB4 (for a review see [24]) and also Ptc [25]. Interestingly, several data had long suggested that Ptc could act as a multisubunit protein. First, Ptc is a multipass membran

protein structurally similar to a RND family of channels and transporters (such as NPC1), several of which are known to have an oligomeric structure [26]. Second, interallelic complementation has been observed during genetic analyses of *Drosophila ptc* suggesting direct interaction between partially impaired subunits [27]. Third, Lu et al. recently showed that *Drosophila* Ptc is able to multimerize[25]. Indeed, they demonstrated that the last intracellular domain (the C-Terminal domain = CTD; Ptc is a multipass membrane protein) is able to trimerize by self-association. However, similarly to DCC [28], Ptc is able to trimerize even if deleted of CTD [25]. The process of oligomerization is essential to Ptc survival-inducing function and regulates its turnover in part *via* binding to Nedd4, an E3 ubiquitin ligase which could target Ptc to lysosomal degradation [25].

So far, the importance of the oligomerization in blocking proapoptotic activity has been proved for the receptors $p75^{NTR}$, DCC and Unc5H2. Experiments on other dependence receptors forced to oligomerize will help to understand whether the proapoptotic activity of this entire class of receptors depends on their ability to be on a monomeric state.

Recruitment of a caspase activating complex

Dependence receptors are able to activate initiator and effector caspases in the absence of their ligand (for a review, see [29]) (Fig 2). DCC [30], TrkC [31] and Ptc [32] activate caspase-9 and -3, while integrin-induced death depends on caspase-8 and -3 activation [13]. The death of sympathetic neurons deprived of NGF is mediated by $p75^{NTR}$ *via* the recruitment of caspase-2 [33]. The deletion of caspase-2, however, does not rescue neurons deprived of NGF because of the activation of an alternative pathway dependent on caspase-9 [34]. Along a similar line, GDNF-deprived neurons die by caspase-2 and -7 activation [35]. Finally, the initiator caspases activated by the recently described dependence receptors such as ALK, MET, EphA4 still remain to be identified.

Several proapoptotic partners of dependence receptors have been identified (Table 1), but so far none of them emerge as a universal proapoptotic adaptor for dependence receptor-induced death.

DIP13α, a candidate partner of DCC identified by a two-hybrid screen, has been characterized as a mediator of DCC-induced cell death [36]. Interestingly, the domain that allows DCC to interact with DIP13α closely resembles the domain that is responsible for DCC proapoptotic activity [37]. Indeed, the overexpression of DIP13α enhances DCC-induced cell death, while its down regulation promotes survival [36]. It remains to be elucidated how DIP13α is linked link to caspase activation. DCC triggers caspase activation directly and in particular it interacts directly with caspase-3 and indirectly with caspase-9 [30]. Furthermore, the DCC intracellular domain is able to activate caspase-3 in a cell free system, suggesting that DCC interacts through an adaptor protein with caspase-9, allowing caspase-3 activation. It is thus tempting to speculate that DIP13α is the missing link between DCC and caspase-9.

NRAGE, a member of the MAGE family, is known to interact with UNC5H1 in the ZU-5 domain [38]. This region of UNC5H1 (with an adjacent PEST sequence) is required for UNC5H1-mediated apoptosis, which is prevented in a model of down-regulation of NRAGE.

DAPK (for death-associated protein kinase) was identified as the proapoptotic partner of the dependence receptor UNC5H2 [39]. DAPK is a crucial protein mediating cell death induction *via* its serine/threonine kinase activity and *via* its death domain. This protein was first identified in an *in silico* search as the one having the death domain closest to that of UNC5H2. Llambi *et al* (2005) showed that DAPK is required for a large part of UNC5H2-induced apoptosis and that UNC5H2 triggers cell death by activating DAPK in the absence of its ligand, netrin-1. More recently, DAPK has also been shown to be involved in the death process induced by another dependence receptor, Neogenin, which is not structurally related to the UNC5H2 receptor [12]. Indeed, Neogenin lacks a death domain but contains domains of high homology to DCC in its extracellular part and domains of less extended homology to DCC in its cytoplasmic part. However, DAPK is not an interactor of DCC [12]. These data suggest that even if DAPK is involved

in death signaling induced by several dependence receptors, the mechanisms of DAPK recruitment by the single receptors are different from one another. However, at the functional level, the role of DAPK in dependence receptors induced cell death is similar to that of UNC5H2 and Neogenin. Indeed, the DAPK kinase activity of is evident only in the absence of ligands.

For the RET dependence receptor, another death signaling pathway has been described in somatotrophic cells, the pituitary cells deputed to secrete growth hormones. In the absence of RET's ligand GDNF, the intracellular part of RET forms a complex with caspase-3 and PKCδ in which all three proteins become activated by cleavage [40]. This activated complex leads to binding of c/EBPα to the promoter of Pit-1, a pituitary specific transcription factor depending on JNK and CREB signaling. The up-regulation of Pit-1 expression then leads to increased p53 expression and apoptosis. Importantly, *in vivo* observations showing larger adenopituitary glands in knock-out RET animals strengthened the *in vitro* data.

Recent data from our laboratory shown that, in the absence of its ligand, Ptc interacts with the adaptor protein DRAL/FHL2 to trigger cell death. DRAL was identified by a two-hybrid screen taimed at finding proteins involved in cell death control that would interact with the propaoptotic domain of Ptc (i.e., its 7th intracellular domain). We demonstrated that, in the absence of Shh, Ptc actually recruits a protein complex that includes DRAL, one of the caspase recruitment domain-CARD containing proteins TUCAN or NALP1, and the apical caspase-9 [32]. All these proteins were identified as the partners required by Ptc to induce apoptosis both in immortalized cells and during neural tube development in chick embryos. Moreover, Ptc triggers caspase-9 activation and enhances cell death through a caspase-9-dependent mechanism. In view of these findings, we proposed that in the absence of its ligand Shh, the dependence receptor Ptc serves as the anchor for a caspase-activating complex that includes DRAL and caspase-9.

In summary, no proapoptotic partners common to all dependence receptors have been so far characterized. Future studies should tell us if each dependence receptor triggers

cell death by means of a unique signaling pathway or if a common proapoptotic effector indeed exists. However, as shown for DCC and UNC5H2 [5] [6] the interaction of dependence receptors with their proapoptotic partners seems to require their localization to lipid rafts. Indeed, when a mutant of receptor palmitoylation is used or when the lipid rafts organization is perturbed, no interaction with the proapoptotic partner was observed. Similarly, clustering of integrins results in caspase-8 recruitment [13] suggesting the necessity to group dependence receptors in a restricted domain at the membrane before the caspase-activating complex can be recruited.

Cleavage of dependence receptors by caspases

In the absence of their ligand, dependence receptors can be cleaved by caspases. This has been shown *in vitro* for most dependence receptors with the exception of integrins and $p75^{NTR}$ which is processed proteolytically by an unknown protease [41]. $p75^{NTR}$, however, can be cleaved *in vitro* by caspases but it is still unclear whether this cleavage is required for $p75^{NTR}$ proapoptotic activity (S. Rabizadeh, personal communication).

The caspase found to be responsible for the cleavage of most dependence receptors *in vitro* is caspase-3. This finding, of course, does not exclude the requirement of other caspases *in vivo*. The cleavage sites of caspases have been mapped (Table 1) and their mutations abolish the proapoptotic activity of the corresponding dependence receptor, suggesting that the cleavage of these receptors is required to promote apoptosis. Dependence receptors can be classed in two categories depending on the number and position of caspase cleavage sites. In the presence of one cleavage site located upstream of the proapoptotic domain, the proapoptotic activity of the receptor is anchored to the plasma membrane. The caspase cleavage then allows for the exposure of a proapoptotic domain which is normally masked by the C-terminal domain of the receptor. This mechanism has been described for DCC, Ptc, Neogenin, ALK and EphA4. In the other hand, in the presence of two caspase cleavage sites, an intracellular fragment containing the proapoptotic activity of the receptor is then released from the membrane to the cytoplasm; this seems to occur for RET, MET and TrkC.

Futhermore, it was shown that the cleavage of MET occurs in a sequential manner, with the site located at the C-Terminal region promoting a cleavage at the juxtamembrane region of the protein [42]. The C-terminal cleavage generates a fragment of the last 5 amino acids of MET which is undetectable. However, Foveau et al. studied this cleavage thanks to a tagged version of the receptor having a V5 epitope at its carboxy-terminus[42]. Although not yet demonstrated, a cryptic cleavage site might also exist for other dependence receptors.

However the cleavage occurs, it is generally thought that it induces a conformational change of the dependence receptor, then allowing the exposition/release of an addiction/dependence domain (ADD) responsible for triggering cell death.

Cleavage of dependence receptors has been observed *in cellulo* for AR [43] and all the receptors belonging to the Receptor Tyrosine Kinase family : RET [44], MET [45], ALK [46], TrkC [31] and EphA4 [47]. Such a cleavage still remains to be observed *in cellulo* or *in vivo* for other dependence receptors in order to better understand its biological meaning. Tools to detect cleaved receptors *in vivo* are under development and their use should shed new light on this cleavage step.

The receptor need to be cleaved before exerting their proapoptotic activity raises the question as to wether they can be considered promoters of apoptosis rather than a mere substrate of caspases already activated by other stimuli. This apparent contradiction could be explained by the notion that caspases are not completely dormant in non-apoptotic cells, but possess a residual activity [48]; [49]; thus the receptors could be cleaved by little active caspase zymogens. Indeed, local activation, not resulting in cell death, has been documented in several systems ([50]; for a review see [51]) suggesting that apoptosis could be a consequence of the amplification rather than of the initiation of caspase activity (Fig 2). It is possible that dependence receptors trigger cell death after ligand withdrawal by first recruiting a caspase-activating complex; activated initiator caspases could then cleave and thus activate effector caspases. These caspases would then cleave dependence receptors, induce a change of conformation, expose/release the ADD then recruiting more caspase-activating complex or other partners in order to trigger more caspase activation. The cleavage of dependence

receptors could thus represents a threshold of caspase activation beyond which apoptosis is unleashed. However, we cannot dismiss the alternative possibility that caspase cleavage is a necessary step that will allow the interaction with the caspase-activating complex. Along this line, it was shown that DCC interaction with caspase-9 occurs only when the receptor is cleaved and is not observed when DCC is mutated in its caspase cleavage site [30]. The development of new tools, including specific antibodies raised against cleaved dependence receptors, should help to determine the precise sequence of events leading to an irreversible cell demise.

Conclusions

Recent studies have highlighted a number of the molecular mechanisms by which dependence receptors initiate cell death in the absence of their ligands. This death pathway is only activated when dependence receptors localize to lipid rafts and are in a monomeric state. Ligand-free dependence receptors could recruit a caspase-activating signal mediated by means of adpator molecules acting as linking agents. The activated initiator caspase (mainly caspase-9 but sometimes caspase-8) could then activate effector caspases such as caspase-3. Whether this recruitment and the ensuing caspase activation occur before or after the cleavage of these receptors by active caspases, a necessary step toward the amplification of their caspase activity, remains to be seen.

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Figure and Table Legends

Table 1: Summary of known death adaptors of dependence receptors, caspases involved and caspase cleavage sites

The fourth steps discussed in the text are highlight in this table. Lipid raft association of the dependence receptors-induced cell death is noticed. Monomerization of the receptor as a crucial step to trigger cell death upon ligand removal is mentioned. The state-of - the-art about the existence of a caspase-activating complex is broken down in (i) the known proapoptotic partner recruited, (ii) the caspase involved and (iii) the eventually description of cytochome c release associated with dependence receptor-cell death in order to characterized a mitochondrial death pathway. Caspase cleavage sites are indicated. * : reported *in cellulo* cleavage. ND : not determined.

Figure 1: Dependence receptors as two-sided receptors.

It is usually said that receptors function only in response to ligand binding. However, dependence receptors are not inactive in the absence of ligand, but rather display two opposing signaling properties: in the presence of ligands, they induce a positive signal of survival and then differentiation or migration; in their absence, they trigger apoptosis. Thus, the survival of cells expressing such dependence receptors depends on ligand availability.

Figure 2: Hypothetical model of dependence receptors-induced apoptosis.

In the absence of a ligand, dependence receptors may trigger apoptosis if receptors are localized into lipid rafts, are in a monomeric state and the recruitment of a complex leading to caspases activation and cleavage of the receptor by caspases that enhances the cell death signal.

		Caspase-activating complex		
Survival inducing ligand	**Dependence Receptor**	**Proapoptotic partners**	**Initiator caspase involved**	**Dependence receptor caspase cleavage site**
NGF	P75	NRAGE ?	caspase-2 ?	Uncharacterized
netrin-1	DCC	DIP13-α	caspase-9	D1290
netrin-1	UNC5H1	NRAGE	ND	ND
netrin-1	UNC5H2	DAPK	not caspase-8	D412
RGM	Neogenin	DAPK	ND	D1323
androgen	AR	ND	ND	D146 and around D660
EML (ex : laminin)	Integrin	ND	caspase-8	No cleavage
Shh	Patched Ptc	DRAL/FHL2, TUCAN/NALP1	caspase-9	D1392
GDNF	RET	Pit1/P53	caspase-2 ?	D707, D1017
HGF/SF	MET	ND	ND	D1000, D1374
Pleiotrophin/ midkine	ALK	ND	ND	D1160
NT3	TrkC	ND	caspase-9	D495, D641
EphB3	EphA4	ND	ND	D773/774

Table 1 -

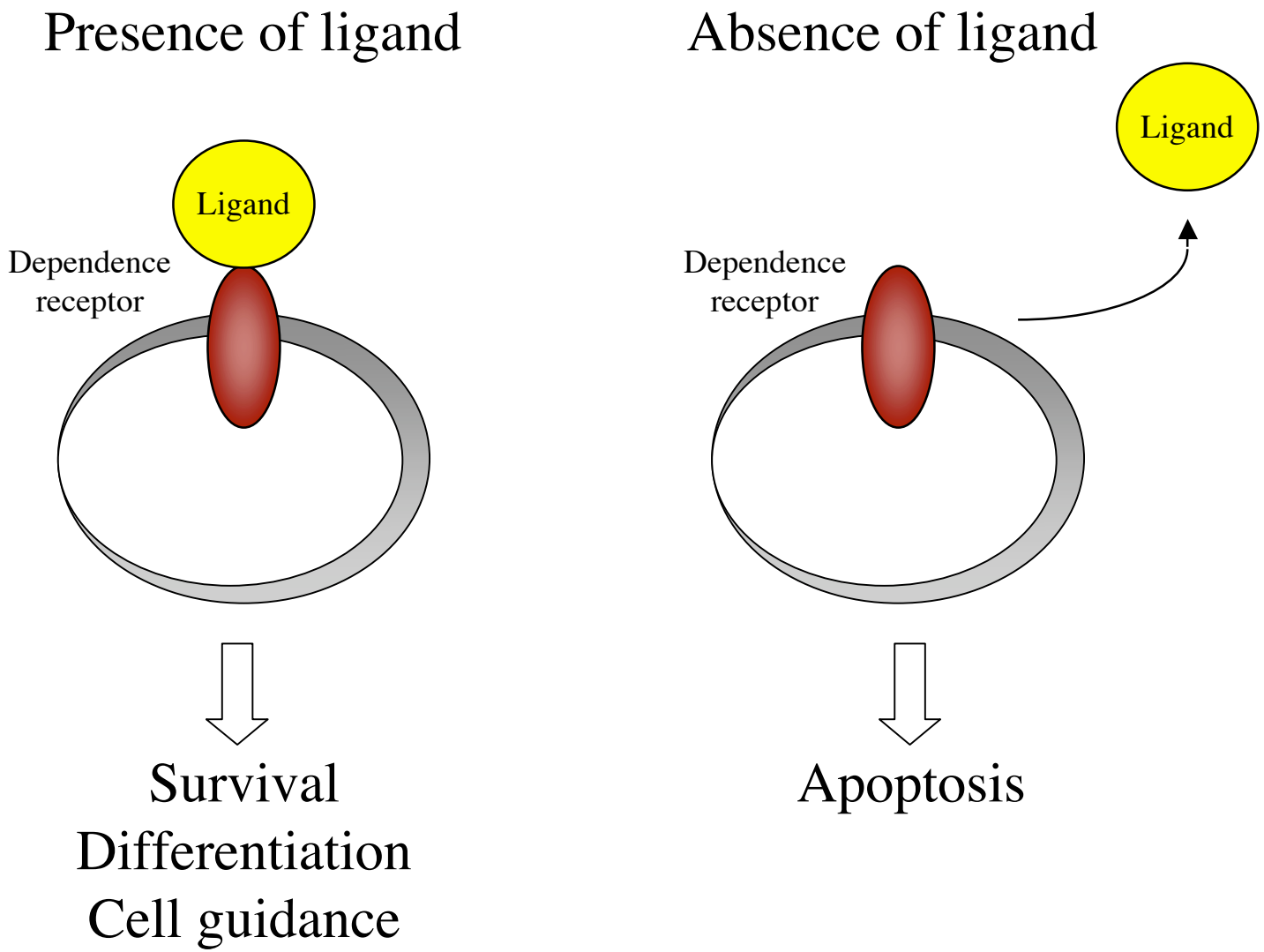
Presence of ligand
Ligand
Dependence receptor
Survival
Differentiation
Cell guidance
Absence of ligand
Ligand
Dependence receptor
Apoptosis

Fig 1 -

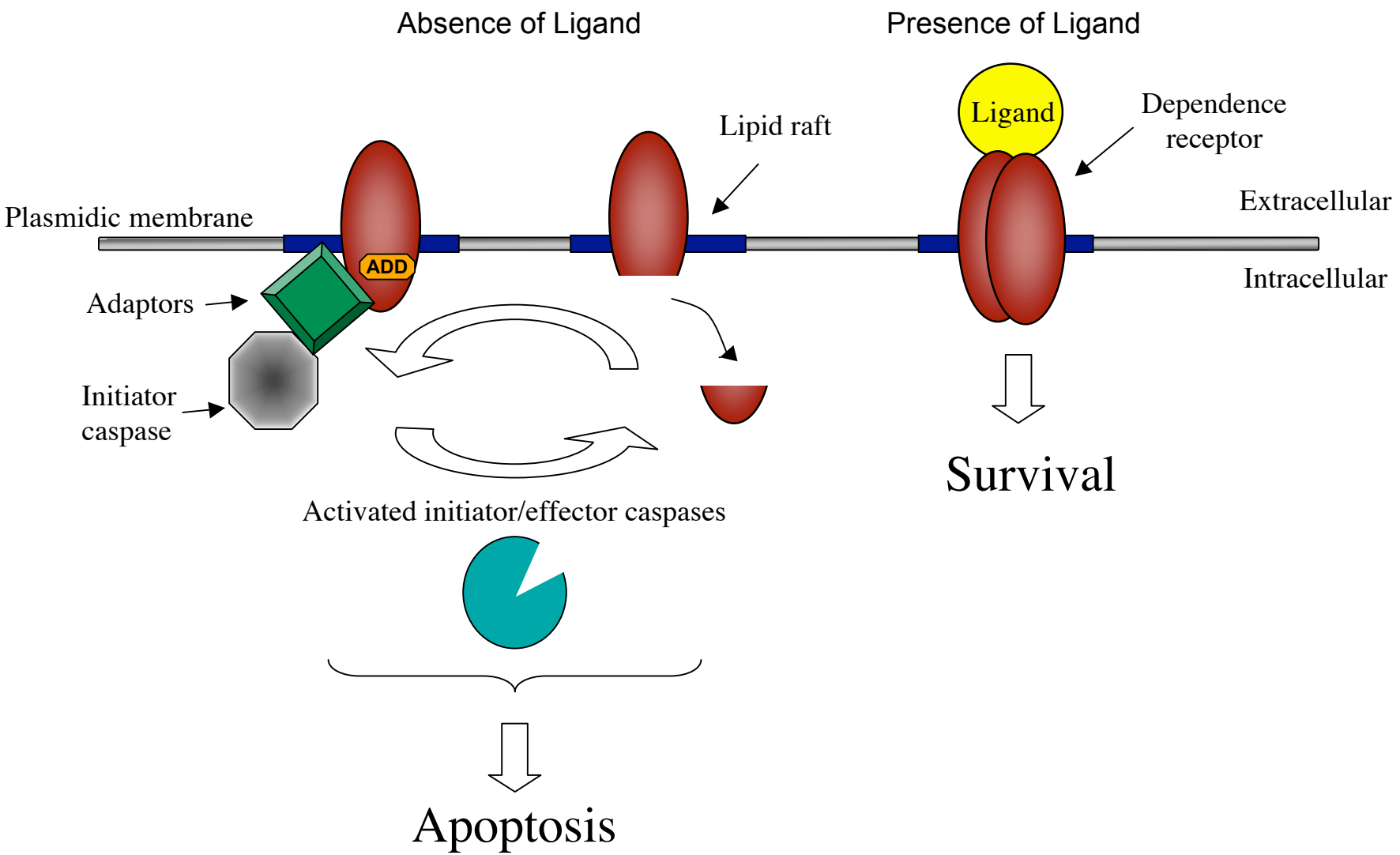
Absence of Ligand
Presence of Ligand
Ligand
Dependence receptor
Lipid raft
Plasmidic membrane
Extracellular
Intracellular
ADD
Adaptors
Initiator caspase
Survival
Activated initiator/effector caspases
Apoptosis

Fig 2 -