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The Drosophila learning and memory gene *linotte* encodes a putative receptor tyrosine kinase homologous to the human RYK gene product

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Abstract **The *linotte* mutant was isolated on the basis of its learning and memory deficit. Interestingly, *linotte* individuals carrying a null mutation are viable, indicating that the *linotte* gene is not required for vital functions. We show here that the *linotte* gene encodes a putative receptor tyrosine kinase, homologous to the human protein RYK. These products are unique among receptor tyrosine kinases, since they possess a short extra cellular domain, and a modified intracellular catalytic domain. In particular, the subdomains directly involved in ATP binding and phosphotransfer reaction display remarkable variations. These results suggest that *linotte* is part of a novel signal transduction cascade involved in learning and memory.**

Key words: Central nervous system; *Drosophila melanogaster*; Learning and memory; Receptor tyrosine kinase; Signal transduction

1. Introduction

A fundamental property of the brain is its ability to store and process information and *Drosophila* displays a wide range of learned behaviors. For example, normal *Drosophila* can avoid an odor if it has been associated with electric shocks. By developing such olfactory conditioning protocols, retention intervals ranging from hours [1] to one day [2], and recently a week [3], were revealed (for a life span of a few weeks). Associative learning has been induced in many other situations – association between light wavelength and mechanical vibrations [4]; between an odor and the presence of food [5]; between geometrical patterns and heat [6] –, outlining that *Drosophila* can adapt its behavior based on experience. Several mutants with weak learning and memory capacities have been described (*dunce* [7]; *amnesiac* [8]; *dopa decarboxylase* [9]; *rutabaga* [10]; *mushroom-bodies-deranged* and *mushroom-body-miniature* [11]; *Shaker* [12]; *latheo* [13]; *Su-var(3)6* [14]; *DCO* [15]; *radish* [16]; *linotte* [17] and for a review see [18]). If some mutations alter brain development, other most likely affect brain functioning. For example, *dunce* encodes a phosphodiesterase [19,20], and *rutabaga* an adenylate cyclase [10,21,22]. These two enzymes are involved with the cAMP pathway, which has been implicated in synaptic plasticity in several species. Interestingly, the DUNCE and RUTABAGA proteins accumulate in the mushroom bodies [23–24], a symmetrical structure of insects involved in olfactory learning [25]. We report here that *linotte* (*lio*) encodes a putative receptor tyrosine kinase (RTK), which displays several unusual features, and is homologue of the human protein RYK. This report represents the initial framework necessary for a molecular genetic analysis of the role of the LINOTTE (LIO) protein in *Drosophila* learning and memory.

2. Materials and methods

2.1. Molecular biology

Genomic DNA carrying the *lio* gene was isolated by 'plasmid rescue'. DNA from the *lio*^{1} *P-lacW*-induced mutant was digested with *Sac*II, circularized, and used for bacteria transformation. Cells with the pMT21 plasmid carrying an adjacent genomic DNA were recovered. The Lorist 6 cosmid library [26] was then screened to isolate 63 kb from the *lio* region. 45 kb were subcloned into the bluescript plasmid. For Northern analysis, poly-A mRNA were extracted from larvae, pupae, and adults with RNA BTM system following instructions from the manufacturer (Bioprobe). For Northern blots, 5 to 15 μg of poly-A RNA were run per lane. The blots were hybridized with single stranded RNA probes. The *lio* RNA was detected with a 2.3 kb probe corresponding to the 5′ end of the 2.5 kb cDNA. The pigeon RNA was detected with a 1.3 kb *Xho*I-*Hin*dIII genomic fragment.

About 10^6 colonies from adult head cDNA libraries [27] were screened. Two apparently identical 2.5 kb *lio* cDNA clones were isolated. A third 3.4 kb cDNA was also found, which turned out to be identical to the 2.5 kb species, except for an internal artificial 0.9 kb duplication. These 2.5 kb of cDNA were sequenced on both strands (0.5 kb using the Sequenase kit (USB), and 2.0 kb by the Genomic company). Sequence comparisons with the EMBL sequence library were made with the GCG program (Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group, Madison, Wisconsin, USA). Six pigeon cDNA were isolated, with a size ranging from 2.5 kb to 3.5 kb.

2.2. Excision of the lio^{1} P element

Individuals were generated which carried simultaneously the *lio*^{1} *P* element over a large deficiency, together with the transposase source *Δ2.3* [28]. These w^{1118}/*Y; lio*^{1}*[PlacW(white*$^{+}$*)]/Sp,Df(2L)TW130 rdo pr cn; Sb Δ2.3*/+ males were crossed to w^{1118}/w^{1118}; $T(2,3)ap^{Xa}$/*TM3,Sb* females. Excisions of the *P* element were recovered from the progeny as w^{1118}; $T(2,3)ap^{Xa}/lio^{exc}$ individuals. A total of 18 independent lines were recovered. All of them are homozygous viable. Genomic DNA from these 18 lines was analyzed by Southern blot. The scheme used to generate these excision lines afforded the maintenance of the Canton-S background from the original *lio*^{1} mutation, which permitted direct behavioral analysis [17]. Strains were maintained on standard medium food at 25°C. Mutants are described in Lindsley and Zimm [29].

2.3. Behavioral analysis of the lio^{2} mutant

Flies were conditioned with the pavlovian procedure developed by Tully and Quinn [2], with a few adaptations [17]. Odor avoidance responses and the ability to sense electric shock and to escape from it was tested as described by Dura et al. [17] modified by de Belle and Heisenberg [25].

3. Results

In an olfactory conditioning protocol, the *lio*^{1} mutant displays a 30% reduced immediate retention [17]. The *lio*^{1} mutation is due to the insertion of a transposable *P* element. Dura et al.

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[17] have demonstrated that the mutant phenotype is indeed caused by this *P* element since complete phenotypic revertants following excisions of the *P* element have been obtained. This *P* element carries a pMT21 plasmid. We could therefore clone a DNA fragment adjacent to the *P* insertion site using the 'plasmid rescue' method. Cosmids spanning 63 kb of genomic DNA were then isolated (Fig. 1). Single stranded probes, spanning the vicinity of the *P* element insertion site, were used for Northern analysis. Two transcription units were detected in a wild-type strain, encoding respectively a 3.5 kb and a 3.2 kb mRNA (Fig. 1). In the *lio*^{1} mutant, expression of the 3.2 kb transcript is strongly decreased, whereas expression of the 3.5 kb is unaffected (Fig. 1). This result identifies the 3.2 kb mRNA as the *lio* messenger. The transposable element is inserted between these two transcription units; it lies 3′ of the 3.5 kb mRNA encoding unit – which was named *pigeon* (*pio*) –, and 5′ of the 3.2 kb mRNA encoding unit. The fact that only the expression of the 3.2 kb RNA is decreased in *lio*^{1} individuals is in accordance with the general observation that, in *P*-induced mutants, inserts lie in the 5′ region of genes, but not in the 3′ non-transcribed region. cDNA corresponding to these two RNA were isolated. No cross-hybridization was detected between these two units, suggesting that they correspond to independent genes.

Since the *P* element in the *lio*^{1} strain is inserted in the 5′ non-coding region of the *lio* gene, the possibility remained that the gene was still partially active in this mutant. To analyze precisely the role of *lio* it was necessary to study the effect of

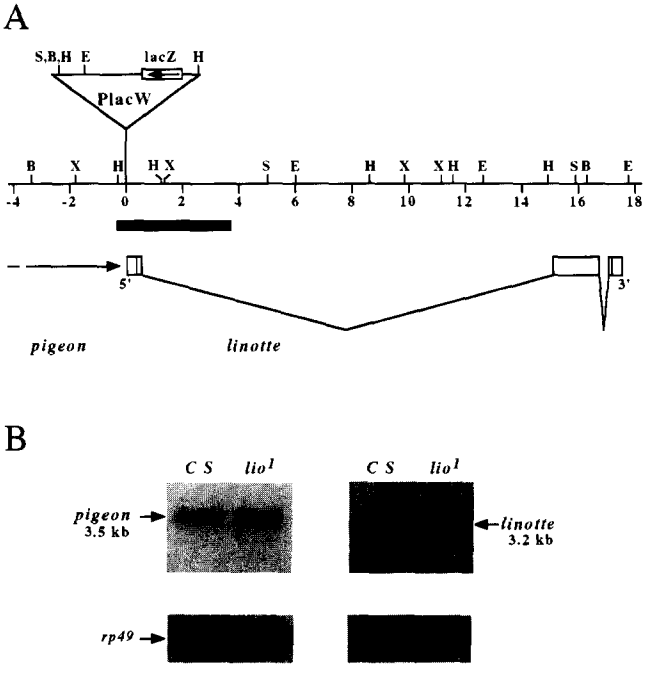

Fig. 1. Molecular organization of the *linotte* locus. (A) Genomic DNA with restriction sites (B: *Bam*HI; E: *Eco*RI; H: *Hin*dIII; S: *Sal*I; X: *Xho*I). The scale is in kb, and the *P-lacW* element is drawn at a 50% scale. The black bar represents the *lio*^{2} deficiency. Exons and introns of the *lio* mRNA are indicated, with the coding regions in gray. Only the 3′ end of the *pigeon* mRNA is represented (introns not shown). (B) Expression of the *pigeon* (adults) and *lio* (third instar larvae) mRNA in a wild-type *Canton-S* and mutant *lio*^{1} background. The *rp49* RNA was used as a control. Since the *lio* transcript is rare, the *rp49* RNA was revealed for a much shorter time than the *lio* transcript itself.

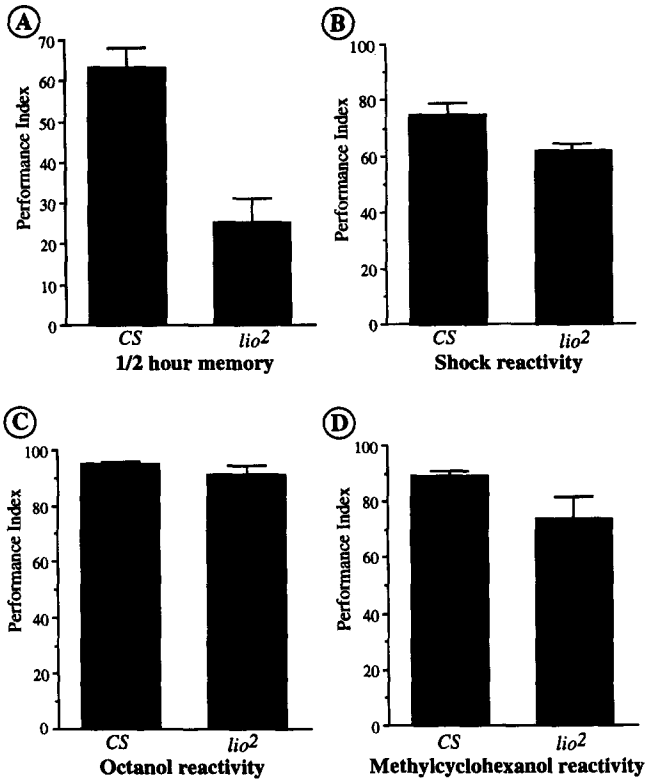

Fig. 2. Memory defect of the *lio*^{2} mutant. (A) The reduction in olfactory associative memory is highly significant (*t* test, $t = 6.17$, $P = 0.002$). (B) The reduction in reactivity to electric shock is marginally significant (*t* test, $t = 3.30$, $P = 0.022$). (C) Reactivity to octanol of the *lio*^{2} mutant is normal (*t* test, $t = 1.01$, $P = 0.357$). (D) Reactivity to methylcyclohexanol of the *lio*^{2} mutant is normal (*t* test, $t = 1.72$, $P = 0.146$). $N=6$ performance index for each point.

a complete lack of LIO product. Thus, a small deletion of this region was induced by jumping out the *P* element under the influence of the transposase (see section 2). Excisions were generated over a chromosome carrying a large deficiency of the *lio* region, to decrease the probability of a mutation being repaired from a wild-type homologous chromosome [30]. One deletion, *lio*^{2}, was recovered out of 18 excision lines. This mutation removes the 5′ regulatory sequences of the *lio* gene, together with the first exon (Fig. 1). Interestingly, *lio*^{2} individuals are viable, indicating that the *linotte* gene is not required for vital functions.

We assessed the memory of *lio*^{2} flies using a conditioning procedure in which flies learn to associate an odor with electric shocks [2]. *lio*^{2} mutant shows a strong 60% decrease of their 0.5 h memory (Fig. 2). We assessed the specificity of the learning/memory defect in *lio*^{2}, by testing its reactivity to the stimuli used for conditioning (Fig. 2). Reactivity to octanol and methylcyclohexanol was found to be normal, whereas reactivity to electric shock appears to be only slightly less than normal. All these controls were found to be normal for the *lio*^{1} mutant [17]. It is therefore very likely that the memory deficit of *linotte* mutants is not due to some peripheral (sensorial) impairment.

A 2.5 kb *lio* cDNA was isolated, which spans over nearly 18 kb of genomic DNA, as a large 14 kb intron resides in the 5′ coding region of the gene. This cDNA was sequenced. It includes a long open reading frame which starts with an ATG

initiation codon. This ATG matches the Kozak consensus [31], as it is preceded at position −3 by an A, and followed by a G (not shown). A long 5′ non-coding region precedes this ATG, which includes several 'stop' codons within all three reading frames.

The conceptual LIO protein is 610 amino acids long (Fig. 3). Sequences comparison to known proteins indicate that LIO is a putative transmembrane tyrosine kinase. At the aminoterminal end lies a putative peptide leader, made of a stretch of hydrophobic amino acids followed by an alanine, which probably marks the cleavage site [32]. The extracellular domain of the mature protein is short with only 218 amino acids. This putative receptor domain is unusual since it is devoid of the immuglobuline-like or cysteine rich motifs found in other RTK (reviewed in [33–34]). It contains three putative glycosilation sites (NXS/T), and a putative proteolysis site KRKK. The unique transmembrane domain is 25 amino acids long and appears clearly in a hydrophobicity plot (not shown). The intracellular juxtamembrane domain, with 75 amino acids, is about 50% bigger than that of most other RTK. It includes many serines and threonines, with 14 of them spanning over a stretch of 33 amino acids. Some of these residues might be the target of cytoplasmic serine/threonine kinase(s) which would regulate LIO activity, as shown for the epidermal growth factor receptor [35–36]. This juxtamembrane domain also includes four tyrosines, of which some might be phosphorylated by LIO itself, since many RTK are known to regulate their own activity [37–38]. LIO catalytic domain per se is 256 amino acids long.

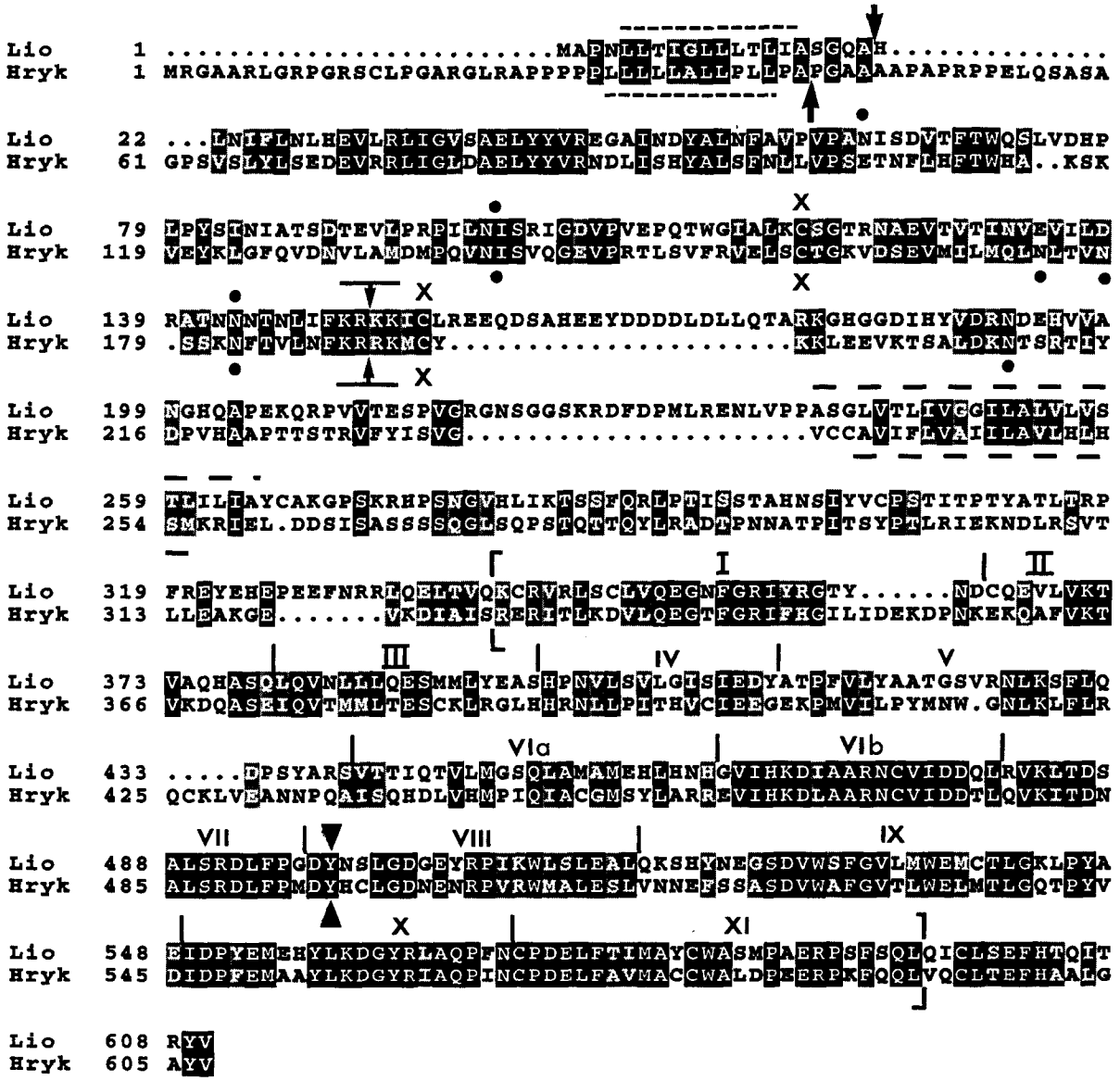

Fig. 3. *lio* encodes a putative receptor tyrosine kinase, homologous to the human RYK protein. Identical amino acids are delineated with black, and similar amino acids with gray. The extracellular domain lies at the amino terminal end. Several conserved features are indicated: the peptide leader (tight dotted line), with the cleavage sites (arrow); putative glycosilation sites (dots); two cysteines (X) possibly engaged in a disulfur bond, flanking a putative proteolysis site (bar with arrow); the transmembrane domain (loose dotted line); the catalytic domain with the twelve subdomains described by Hanks et al. [39]; Tyr(498) (triangle), is a fundamental autophosphorylated target [40]. The human RYK sequence is taken from Tamagnone et al. [44], except that these authors interpreted the amino terminal hydrophobic sequence as being a second transmembrane segment of the mature protein, rather than a peptide leader.

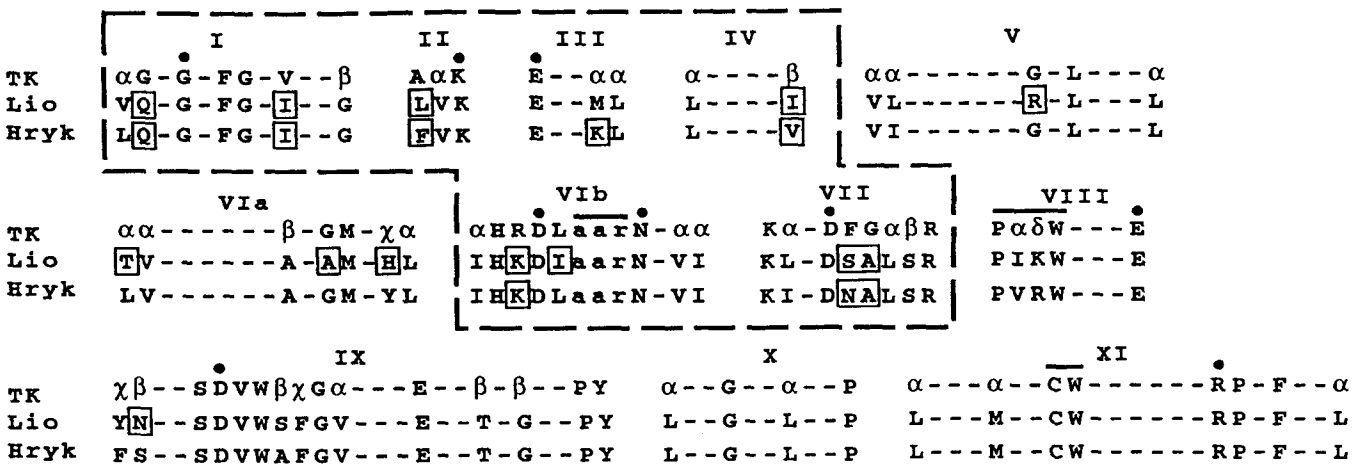

Fig. 4. Unusual aminoacids of LIO and human RYK catalytic domains. Conserved amino acids of tyrosine kinases catalytic domain were deduced from the 42 sequences aligned by Hanks and Quinn [48]. Positions with a single amino acid present in at least 93% of tyrosine kinases were taken into account, together with positions where aminoacids of a single class are present in at least 98% of tyrosine kinases (the following classes were considered: α = L, I, V, M; β = A, G, S, T; χ = F, Y; δ = K, R; no case corresponded to the D, E, Q, N class). Unusual amino acids present in LIO and human RYK are outlined by a square. The dotted lines mark the subdomains directly involved in Mg-ATP binding (I to IV) and catalysis (VIb and VII). Note that all human RYK unusual aminoacids belong to these subdomains, whereas LIO displays additional variations outside the dotted area. The 9 residues invariant in all kinases (including serine/threonine kinases) are marked with a dot. Residues E (VIII) and R (XI) probably interact together, and help maintaining the structure of the catalytic domain (Knighton et al. [46]). The three sequences which characterize tyrosine kinases are marked with a line. The 'aar' sequence (domain VIb) is written in small case letters as the alternative sequence 'raa' is found in some tyrosine kinases.

It shares about 30% of its amino acids with the catalytic domain of other RTK. Catalytic domains of protein kinases have been divided in 12 regions by Hanks et al. [39], and invariant or almost invariant amino acids have been identified within these regions. LIO possesses most of these generally conserved amino acids, and in particular Lys(II:371) involved in nucleotide binding, and Asp(VIb:468) required for phosphotransfer. LIO also bears the three motifs Ala(VIb:470)-Ala-Arg, Pro(VII:508)-Ile-Lys-Trp and Cys(XI:581)-Trp, characteristic of tyrosine kinases, as well as Tyr(VIII:498) which is the target of a autophosphorylation transactivating reaction [40]. However, LIO is unique as it lacks several otherwise conserved amino acids. Possible implications of these major changes on LIO's activity are discussed further.

LIO is most closely related to the vertebrate protein RYK [41–45], with 49% of common aminoacids between the catalytic domains of LIO and the human RYK (Fig. 3). These putative RTK display several unusual amino acids at identical positions in subdomains I, II, IV, VIb and VII (Fig. 4) . Interestingly, many of these unusual amino acids are either identical or similar in LIO and RYK. However, in subdomains V, VIa and IX, LIO displays unusual variations which are not found in RYK. The high degree of similarity between LIO and RYK extends throughout the entire proteic sequence, and in particular in the extracellular domain. Within this domain, two glycosilation sites are conserved, together with the putative proteolysis site. These results suggest strongly that *lio* and human *ryk* share a common ancestor gene existing prior to the arthropod-vertebrate divergence.

4. Discussion

We have shown here that the learning and memory gene *linotte* encodes a conceptual protein closely related to receptor tyrosine kinases. Moreover, *lio* is homologous to the vertebrate gene RYK, which was isolated during PCR screens designed to identify RTK encoding genes. The homology spans over the entire sequence. The role of RYK is not known. *Drosophila* molecular genetics thus offers a unique opportunity to analyze the function of these unusual proteins.

The general structure of kinase catalytic domains has been resolved [40,46], and the function of several invariant amino acids has been determined. The catalytic core of a protein kinase is made of two lobes, with a cleft between that can be occupied by the substrates. The smaller of the two lobes is involved in nucleotide binding. It corresponds to subdomains I to IV described by Hanks et al. [39]. Subdomains VIb and VII belong to the large lobe and are involved in catalysis. The remaining subdomains participate in proteic substrate recognition. The LIO catalytic domain displays the general organization found in other RTK. However, several aminoacids otherwise conserved in almost all RTK are either replaced or missing in LIO. Thus, among all the putative RTK which have been identified, LIO is by far the most variable within the region of usually conserved motifs. This observation raises questions about its activity. Hovens et al. [41] have studied the activity of the mouse RYK protein, which also displays remarkable variations in its catalytic domain. They could not reveal a bona fide tyrosine kinase activity. However, several arguments suggest that indeed these tyrosine kinase-related proteins are active. In particular, LIO and RYK possess the 9 invariant amino acids present in all kinases so far identified (75 serine/threonine kinases and 42 tyrosine kinases), despite the fact they lack several of the nearly invariant amino acids. More, the two proteins also possess all of the conserved motifs which distinguish tyrosine kinases from serine/threonine kinases. Interestingly, many of the non-conserved amino acids at common positions are identical in LIO and RYK. Thus, these unusual variations of the catalytic domain were most likely present in the ancestor gene, and they appear to have been conserved

during about 600 millions years of divergence. Therefore, these changes probably confer new important properties to the proteins. These common variations are not scattered randomly over the entire catalytic domain, but rather, they affect specifically the subdomains which are directly involved in ATP binding (subdomains I to IV) and phosphotransfer reaction (subdomains VIb and VII). These sequence variations might partially compensate for each other, and lead to new catalytic properties (for example, GTP might be used as a phosphate donor instead of ATP).

The extracellular domain of these proteins is also notable. It is short and devoid of the immuglobuline-like or cysteine rich motifs found in other RTK. A putative proteolytic cleavage site is present in both extracellular domains. This site is flanked by two conserved cysteins, which could be engaged in a disulfide bond that keeps the peptides linked after cleavage. Such a situation would be similar to that observed for the insulin receptor [47].

Taken together, these results suggest that LIO and RYK might be engaged in a new but as yet undetermined signal transduction pathway. Further dissection of the LIO pathway through molecular genetics offers an opportunity to tackle the problem of learning and memory in *Drosophila*. This approach should also help understanding the function of the vertebrate homologue gene RYK.

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