IL1 receptor accessory protein like, a protein involved in X-linked mental retardation, interacts with Neuronal Calcium Sensor-1 and regulates exocytosis

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Previously, human genetics-based approaches allowed us to show that mutations in the IL-1 receptor accessory protein-like gene (IL1RAPL) are responsible for a non-specific form of X-linked mental retardation. This gene encodes a predicted protein of 696 amino acids that belongs to a novel class of the IL-1/Toll receptor family. In addition to the extracellular portion consisting of three Ig-like domains and the intracellular TIR domain characteristic of the IL-1/Toll receptor family, IL1RAPL contains a specific 150 amino acid carboxy terminus that has no significant homology with any protein of known function. In order to begin to elucidate the function of this IL-1/Toll receptor-like protein, we have assessed the effect of recombinant IL1RAPL on the binding affinity of type I IL-1R for its ligands IL-1α and β and searched for proteins interacting with the specific carboxy terminus domain of IL1RAPL. Our results show that IL1RAPL is not a protein receptor for IL-1. In addition we present here the identification of Neuronal Calcium Sensor-1 (NCS-1) as an IL1RAPL interactor. Remarkably, although NCS-1 and its non-mammalian homologue, frequenin, are members of a highly conserved EF-hand Ca2+ binding protein family, our data show that IL1RAPL interacts only with NCS-1 through its specific C-terminal domain. The functional relevance of IL1RAPL activity was further supported by the inhibitory effect on exocytosis in PC12 cells overexpressing IL1RAPL. Taken together, our data suggest that IL1RAPL may regulate calcium-dependent exocytosis and provide insight into the understanding of physiopathological mechanisms underlying cognitive impairment resulting from IL1RAPL dysfunction.

INTRODUCTION

Mental retardation (MR) is defined as an overall ‘intelligence quotient’ lower than 70 associated with functional deficits in adaptive behaviour (such as daily-living skills, social skills and communication), with an onset before 18 years (1,2). Moderate to severe MR (IQ < 50) is estimated to affect 0.4–0.8% of the population and the prevalence increases to 2% if mild MR (0 < IQ < 70) is included, although these estimates vary widely between epidemiological studies (2). The underlying causes of MR are extremely heterogeneous, they include non-genetic factors that act prenatally or during early infancy and cause brain injury, as well as established genetic causes, many of which are X-linked conditions (XLMR). In recent years, significant progress has been made in the delineation of genes and causative mutations underlying XLMR conditions, especially those defined as ‘non-specific’ forms of XLMR (MRX), for which cognitive impairment is the only defining clinical feature [for a review see (3)]. Although these recent findings are crucial for medical purposes, further investigations are required to elucidate the neurobiological role of these MRX genes, and the poorly understood cellular bases and pathophysiological mechanisms underlying mental retardation.

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Previously, through positional cloning approaches, we showed that the *IL1RAPL* (IL-1-Receptor Accessory Protein Like) gene is responsible for a non-specific form of X-linked mental retardation. Non-overlapping deletions and a nonsense mutation, resulting most likely in a loss of protein function, were identified in patients with cognitive impairment (4). In addition to its high level of expression in the CNS during development and in postnatal and adult stages, *IL1RAPL* gene is also expressed in fetal kidney and adult heart (4). The predicted amino acid sequence of *IL1RAPL* shares ~52% homology with the known human IL-1 receptor accessory proteins (IL-1RaCps) (5,6), and indicates the presence of a 359 amino-acid extracellular domain, a 17 amino-acid transmembrane segment and 319 amino-acid cytoplasmic domain. The homology with IL-1RaCp is evenly distributed through the protein with the exception of the terminal 150 amino-acid domain, which is present only in *IL1RAPL*. Further BLAST analysis suggested that *IL1RAPL* belongs to a novel class of the interleukin-1/Toll receptor family characterized by the presence of the 150 amino acid carboxy terminus that has no significant homology with any protein of known function (4,7). So far, only two members of this novel class of IL-1/Toll-like receptors have been identified (Fig. 1). These are *IL1RAPL*, also called *TIGIRR-2* and *IL1RAPL2*, localized in Xq22, also called *TIGIRR-1* (7). Their murine homologues, localized in syntenic regions of the mouse X chromosome (7,8), and a rat gene (unpublished data) similar to *IL1RAPL* have also been identified (Fig. 1). Since both IL-1R (IL-1 receptor) and IL-1RaCp (accessory protein) share a high degree of homology, it was hypothesized that these novel IL-1/Toll-like receptors may play a role similar to that of IL-1R, i.e. binding to IL-1 and initiating signalling events, including activation of c-Jun amino-terminal kinase (JNK) and NF-kB, which regulates the expression of many proinflammatory genes in the nucleus (9). Signalling experiment indicated that neither IL1RAPL nor IL1RAPL2 could mediate transcriptional activation of NF-kB in response to IL-1α, IL-1β or IL-18 (7). These data suggested that IL1RAPL and IL1RAPL2 could form a novel class of IL-1/Toll receptors-like for which almost all physiological and biological features remain to be defined, including their ligand(s), their downstream partners and their role in cognition.

In the present study, we have begun to explore the function of *IL1RAPL* in the central nervous system and search for relevant clues to understand the pathophysiology of mental retardation associated with *IL1RAPL* mutations. We show that *IL1RAPL* is not a receptor for IL-1α and IL-1β and its overexpression in mammalian cells does not increase the binding affinity of the recombinant IL-1R (type 1) for IL-1. We also searched for proteins interacting with *IL1RAPL* by screening a human fetal kidney library using the yeast two-hybrid system and *IL1RAPL*-intracellular domain as bait. Our results indicate that *IL1RAPL* interacts with the Neuronal Calcium Sensor-1 protein (NCS-1), which is a member of a large Ca<sup>2+</sup> binding protein family. We also provide functional data indicating that the *IL1RAPL* might be involved in the regulation of exocytosis.

**RESULTS**

**Cellular localization of *IL1RAPL* and 125I-IL-1 binding assay**

Prior to performing binding assay experiments, we confirmed the predicted cellular localization at the membrane of *IL1RAPL*. GFP-tagged *IL1RAPL* and different polyclonal antibody preparations were thus used to detect *IL1RAPL* and study the subcellular distribution of *IL1RAPL*. Figure 2A shows the schematic representation of *IL1RAPL* and the position of the sub-cloned segments used to produce GST-fusion polypeptides (amino acids 569–608 and 607–644) for the generation of polyclonal antisera. Western blots of cultured *IL1RAPL*-transfected CHO and PC12 cells were probed in order to test the specificity of the affinity-purified antisera. Only polyclonal antibodies raised against the peptide amino acids 569–608 (that we named anti-IL1RAPL) specifically detected the *IL1RAPL* protein. As shown in Figure 2B, the anti-IL1RAPL antibody revealed in CHO and PC12 cells stably transfected with *IL1RAPL* a band of ~83 kDa, which is the predicted molecular weight of *IL1RAPL*. In protein extracts prepared from adult mouse total brain and olfactory bulb, hippocampus and striatum extracts, a diffuse band at the expected size was detected (Fig. 2B and data not shown). This diffuse band of 83 kDa is likely to be specific since pre-incubation of antiserum with the purified recombinant peptide (GST-amino acids 569–608) completely blocked its detection, and pre-immune serum gave no signal at the same size on western blots (data not shown).
IL1RAPL interacts with NCS-1 (Neuronal Calcium Sensor-1)

To identify proteins that interact with IL1RAPL, we carried out a yeast two-hybrid screen of a human fetal brain cDNA library using the intracellular portion of IL1RAPL (amino acids 398–696), that includes the carboxy terminus-specific domain, as a bait. Six clones, out of 10^6 screened, scored positive for both reporter genes as they grew on selective medium without histidine, and were positive in the lacZ test. A BLAST search was performed using CHO cells that does not bind to IL1, but is known to increase the binding affinity of IL-1 to its receptor (5), and CHO cells stably expressing IL1RAPL. Despite high levels of expression of IL1RAPL in the generated stably transfected CHO cell lines, the presence of significant differences in the binding activities of CHO cells expressing either IL-1RAcP alone or IL-1RAcP and IL1RAPL suggest no specificity of binding of IL-1β and β with IL1RAPL occurs. Figure 3 shows the results obtained with IL-1β, but similar data were also obtained with IL-1α (data not shown). This result was confirmed by competitive binding assays using 2 x 10^{-10} M of labelled 125I-IL-1α in the presence of unlabelled IL-1α at different concentrations varying from 6 x 10^{-12} to 6 x 10^{-7} M (data not shown). These data indicate that IL1RAPL cannot bind directly to IL-1α or binds with very low affinity, as shown for IL-1RAcP (5). These 125I-IL-1β binding assays were also performed with CHO cells stably expressing IL1RAPL coexpressing both type 1IL-1R and IL-1RAcP or with CHO cells stably expressing type 1IL-1R and IL-1RAcP. While CHO cells expressing type 1 IL-1R and its accessory protein (IL-1RAcP) showed the expected results, i.e. binding of IL-1α to and β with its receptor, these experiments suggest that the presence of IL1RAPL leads to a reduction of the binding activity between IL-1β and its receptor (Fig. 3). Although a negative effect on the expression at the membrane of IL-1R and/ or IL-1RAcP resulting from IL1RAPL overexpression could not be excluded, the reduction of the binding could be explained by a competition between IL-1RAcP and IL1RAPL to interact with IL-1R protein. These results suggesting that IL1RAPL is not a receptor for IL-1 are in line with the previously reported data showing the inability of IL1RAPL to mediate transcriptional activation of the NF-κB pathway in response to IL-1α IL-1β or IL-18 (5,10).
specificity of these interactions was assessed by two-hybrid assays using SNF1 and SNF4 as controls (Fig. 4A). These results in combination with additional experiments using a construct spanning amino acids 1–175 of NCS-1 (data not shown) indicate that NCS-1 interacts specifically with IL1RAPL via its C-terminal domain (amino acids 174–190).

NCS-1 is an EF-hand-containing Ca\textsuperscript{2+}-binding protein. It is a member of a subfamily, the neuronal calcium sensor proteins, that are expressed predominantly or solely in retinal photoreceptor cells or neurons, suggesting that they have specialized roles in these cell types [for a review see (11)]. NCS-1 and its Drosophila orthologue, frequenin, have been shown to regulate a variety of Ca\textsuperscript{2+}-dependent processes including exocytosis in neurons (12) and neuroendocrine cells (13,14).

In order to further determine which part of IL1RAPL interacts with NCS-1, we conducted yeast two-hybrid experiments using different fusion proteins containing either the Toll/IL-1R homology domain (TIR domain, amino acids 398–569) or truncated variants of the conserved specific 150 amino acid carboxy terminus of IL1RAPL (Fig. 4B). These experiments suggested that the interacting domain resides downstream of the TIR domain, within a region spanning amino acids 549–644 of IL1RAPL (Fig. 4B). This portion of IL1RAPL contains the last 20 amino acids of the TIR domain and 75 amino acids of the specific domain.

The interaction between IL1RAPL and the carboxy terminus part of NCS-1 and the similarity between NCS-1 and other EF-hand-containing Ca\textsuperscript{2+}-binding proteins prompted us to search for a possible interaction between IL1RAPL and other members of this large family (11). Two-hybrid assays using fusion proteins containing C-terminal parts either of Hippocalcin or Calmodulin (18 and 59.9% identity with NCS-1, respectively) proteins were carried out. As shown in Figure 4C, no interaction was detected between IL1RAPL and the C-terminal part of these two proteins. These data strongly suggest a specific interaction between NCS-1 and IL1RAPL occurring between the C-terminal end of NCS-1 and the specific 150 amino acid C-terminal domain of IL1RAPL.

**Assessment of the interaction by in vitro and in vivo assays**

To confirm the interaction between IL1RAPL and NCS-1 observed in the yeast two-hybrid system, we performed GST pull-down experiments. Purified recombinant GST-tagged fusion proteins containing different segments of IL1RAPL (Fig. 5A) were incubated with protein extracts of PC12 cells which express NCS-1 (13). After extensive washing of the beads, bound NCS-1 was detected by western blotting with NCS-1-specific antibodies. As shown in Figure 5B and C, these experiments showed that NCS-1 interacts with GST–IC (IL1RAPL-intracellular domain) and GST-Specific Delta1 fusion proteins, while weaker interaction was detected with GST–TIR and GST-Specific Delta2 fusion proteins and no interaction with GST. As many NCS-1 activities are known to be Ca\textsuperscript{2+}-dependent, we also used this assay to assess the effect of Ca\textsuperscript{2+} on this interaction. Results shown in Figure 5C suggest that the interaction of IL1RAPL and NCS-1 does not require Ca\textsuperscript{2+}, as similar amounts of pulled down NCS-1 were obtained after incubation at different free Ca\textsuperscript{2+} concentrations (0, 1 and 10 \mu M). These results were confirmed by an in vitro binding assay, in which NCS-1 protein was translated and labelled with \[^{35}S\]methionine using a rabbit reticulocyte lysate system. The labelled protein was incubated with GST–TIR and GST-Specific Delta fusion proteins and bound NCS-1 was purified using Glutathione-Sepharose 4B. The radio-labelled NCS-1 was shown to bind specifically to the GST-Specific Delta1 and not to TIR–GST fusion protein nor to GST alone used as a control (data not shown).

While these studies indicated that IL1RAPL and NCS-1 can interact in yeast-based and in vitro assays, we attempted to confirm these observations in mammalian cells using a co-immunoprecipitation approach. Because of the poor efficacy of the available polyclonal anti-IL1RAPL antibodies to immunoprecipitate native IL1RAPL protein in brain extracts, we transfected HELA cells with IL1RAPL-GFP or NCS-1 constructs, or with a combination of both constructs, and used monoclonal anti-GFP antibody to immunoprecipitate the expressed fusion proteins. By western blot analysis, we showed that NCS-1 was present only in the immunoprecipitated complex corresponding to HELA cells cotransfected with IL1RAPL-GFP and NCS-1 (Fig. 5D). In order to confirm in vivo the specific interaction between the intracellular domain (IC) of IL1RAPL and NCS-1, we carried out similar experiments using IC-GFP and NCS-1 constructs and showed the presence of an IC-IL1RAPL–NCS-1 complex (Fig. 5D), while no NCS-1 was detected in immunoprecipitates of cells cotransfected with GFP and NCS-1. Similar results were obtained at different free calcium concentrations (0, 1 and 10 \mu M), suggesting therefore that this interaction does not require calcium ions (data not shown).

As signal transduction involving protein receptors containing TIR domains was shown to require receptor oligomerization, induced either by ligand binding or overexpression (15–19), we addressed the question of whether homotypic protein–protein
interactions can occur for IL1RAPL. Two constructs corresponding to IL1RAPL (IC–GFP and IC–HA) were cotransfected in PC12 cells and expressed proteins were immunoprecipitated with anti-GFP antibodies. The presence of two tagged versions of IL1RAPL in the immunoprecipitate was detected on western blots either by anti-GFP or with anti-HA monoclonal antibodies. These experiments showed the presence of homotypic interactions (data not shown), very likely via the TIR domain as previously suggested for Toll/IL1-R receptor family (20,21). On the basis of these results we can hypothesize that competitive interactions through TIR domains with either IL-1RacP or IL-1R could explain the suggested inhibitory effect on the binding of IL-1α and β to its receptor (Fig. 3).

Sub-cellular localization of NCS-1 and IL1RAPL

The level and the sub-cellular distribution of NCS-1, in wild-type or NCS-1-transfected cells, have been extensively investigated and multiple pools of NCS-1 with a complex distribution were reported (22–24). In addition to a partial
Figure 5. Pull-down and co-immunoprecipitation assays. (A) Representation of the different GST-IL1RAPL constructs tested in the GST pull-down assays. (B) Western blot analysis of bound proteins with fusion proteins. Bacterially expressed GST, GST–TIR, GST-Specific Δ1 and GST-Specific Δ2 were bound to Glutathione-Sepharose and then incubated with lysates of PC12 cells which express endogenously NCS-1. Bound NCS-1 was detected with NCS-1 antibody (1:1000). Coomassie blue stained gel showing the produced fusion proteins: GST, GST–TIR and GST-Specific Δ1 and GST-Specific Δ2 used in this assay, at expected sizes. Note the low amount of Δ1-GST fusion protein when compared to those of TIR-GST, Δ2-GST and GST proteins. (C) Western blot analysis showing the calcium independent binding of NCS-1 (normally expressed by PC12 cells) with the intracellular domain of IL1RAPL. (D) Coimmunoprecipitation experiments. Hela cells were transfected with various combinations of expression plasmids for IL1RAPL FL-GFP, intracellular portion of IL1RAPL (IC-GFP) and NCS-1. Following transfection, the lysates were immunoprecipitated with an anti-GFP antibody and bound proteins were detected with anti-GFP (1:1000) and anti-NCS1 (1:1000). Anti-GFP antibody specifically immunoprecipitates the complex IL1RAPL-NCS-1. Similar results were observed with the intracellular domain of IL1RAPL and NCS-1.
distribution in the cytosol, compelling evidence suggests the presence of a certain amount of NCS-1 that is bound at the plasma membrane. This latter localization was shown to be Ca\(^{2+}\)-independent (14, 25). In order to confirm the potential co-localization at the membrane of IL1RAPL and NCS-1, we transiently transfected PC12 cells that endogenously express NCS-1 with GFP-tagged-IL1RAPL and performed immuno-staining experiments. In addition to the detection of both proteins in the cytosol, the transfected cells depict a significant overlap in the staining at the periphery of the transfected cells, suggesting a co-localization at the plasma membrane. Similar results were also obtained with COS 7 cells co-transfected with IL1RAPL-GFP and NCS-1 (data not shown). Although these cellular results do not demonstrate that specific interacting domains are involved in the presence of both proteins at the plasma membrane, altogether, data reported in this study which are based on the two-hybrid system and on \textit{in vitro} and \textit{in vivo} assays suggest that a Ca\(^{2+}\)-independent interaction between IL1RAPL and NCS-1 occurs \textit{in vivo}.

**Functional relevance of IL1RAPL for exocytosis**

NCS-1 was shown to be expressed in chromaffin and PC12 cells and involved in the regulation of dense-core granule exocytosis. This suggested the possibility that IL1RAPL might also regulate exocytosis. The functional role of IL1RAPL in exocytosis was assessed using the reliable GH (growth hormone)-transient co-transfection in PC12 cells assay in which GH acts as a reporter of exocytosis (26). In this assay, overexpression of NCS-1 increased evoked GH release in intact cells in response to ATP (13). PC12 cells were transfected with control (pcDNA3 or pIRES hgy) or with IL1RAPL plasmids, along with a plasmid encoding GH, and exocytosis was examined in response to stimulation with ATP to activate purinergic receptors. In this assay, in contrast to NCS-1, IL1RAPL overexpression inhibited GH release (Fig. 6). No significant effect was seen on basal release but exocytosis evoked by ATP was specifically inhibited by around 50% with the stimulation by ATP above basal being reduced from 9-fold in pcDNA3-transfected cells to 6-fold in IL1RAPL-expressing cells (Fig. 6). This decrease was seen in three replicates for each condition, the data from which are pooled in Figure 6. These results indicate that IL1RAPL may have a functional relevance as a negative regulator of the pathway, leading to dense-core granule exocytosis in neuroendocrine cells.

**DISCUSSION**

We previously identified a novel gene with significant similarities to the Interleukin-1 receptor superfamily (IL-1Rs) and showed its involvement in mental retardation (4). IL-1Rs share a conserved structure consisting of an extracellular region with three Ig-like domains, a transmembrane segment and an intracellular TIR (Toll/IL-1R homology domain) domain similar to that of the \textit{Drosophila} Toll family receptors and mammalian Toll-like receptors (TLR) (27, 28). In addition to the TIR domain, IL1RAPL has a 150 amino acid C-terminal extension which is not present in any other mammalian IL-1Rs or TLR families. This C-terminal domain is reminiscent of—but not similar to—the non-homologous extension described in \textit{Drosophila} Toll family members, such as 18-Wheeler, Toll-6, Toll-7 and Toll-8 (29). As shown in Figure 1, homology searches in sequence data bases revealed the presence of a second human gene similar to \textit{IL1RAPL}, \textit{IL1RAPL2} (also known as \textit{TIGIRR-1}), localized in Xq22.3 (7, 8), and mouse homologous genes, \textit{Il1rapl} and \textit{Il1rapl2}, and a rat gene likely to be an orthologue (unpublished data). With these novel proteins, the mammalian IL-1R/Toll superfamily receptors can be tentatively grouped into three families (Fig. 7). Protein architectures of mammalian Toll/IL-1R superfamily members and their comparison to \textit{Drosophila} Toll receptors suggest that each family of proteins has evolved separately and resulted from the combination of different modules. These modules correspond to the extracellular leucine-rich or Ig-like domains (29), the intracellular TIR domain, and the 150 C-terminal domain that has, apparently, evolved more recently, as it is not conserved in lower species. The evolutionary relationship between the novel class of proteins (that includes so far \textit{IL1RAPL} and \textit{IL1RAPL2}) and the Toll/IL-1R receptor family known to be involved in both innate and adaptive immunity contrasts with the compelling evidence reported in this work and in previous studies (7) suggesting a divergent role for IL1RAPL and its homologue that is distinct from an involvement in the IL-1 inflammatory response.

The importance of this novel class of protein in brain function is supported by the data that showed an involvement of \textit{IL1RAPL} in X-linked mental retardation without any major developmental brain abnormality (4) and the brain-specific, but not redundant, temporal and spatial expression patterns of \textit{IL1RAPL} and \textit{IL1RAPL2}. Previously, we showed by \textit{in situ

![Figure 6. Exocytosis assays. Overexpression of IL1RAPL in transiently transfected PC12 cells decreases reporter growth hormone release. PC12 cells were transfected (4 \mu g/well of each plasmids) with controls pcDNA, pIRES hgy and IL1RAPL plasmids along with plasmid encoding for GH. After 2 days, the cells were washed and either incubated with 300 \mu M of ATP for 15 min or with no additions (control). Cellular GH and GH present in the medium were assayed using ELISA. GH release into the medium was expressed as a percentage of total GH level for each well and data from three separate transfections was combined.](https://academic.oup.com/hmg/article-abstract/12/12/1415/602036)
hybridization that Il1rapl, the mouse homologue of IL1RAPL, is specifically expressed in adult brain structures that are known to be involved in the hippocampal memory system (4). In contrast, Il1rapl2, the mouse homologue of IL1RAPL2, has a complementary pattern of expression and is mainly expressed in the cingulum, colliculus and lateral substantia nigra (data not shown). Although IL1RAPL2 was not implicated in cognitive impairment (unpublished data), these results suggest that both genes may have similar functions, but in different brain structures.

Data described in the literature (7) and results reported in this study suggest that IL1RAPL and its homologous protein, IL1RAPL2, function in signalling pathways different, from those described for IL-1 and IL-18 (i.e. they do not result in NF-κB activation). In order to shed light on the function of this novel class of receptors, we searched for IL1RAPL binding proteins using the yeast two-hybrid screen. One IL1RAPL binding protein corresponds to the human NCS-1. Compelling data support the existence of an interaction in vivo between IL1RAPL and NCS-1. This interaction occurs via the specific 150 amino acid C-terminal domain of IL1RAPL and the C-terminal part of NCS-1. Further definition of the IL1RAPL-interacting region suggested that the conserved amino acids DL (in position 605–606 indicated in Fig. 1) are important for the interaction. Point mutations D605A and L606R were introduced by direct mutagenesis of the C-terminal domain of IL1RAPL and tested for an interaction with NCS-1 using the two-hybrid system. The mutation L606R completely abolished IL1RAPL–NCS-1 interaction in the two-hybrid assay (data not shown). Moreover, this interaction was observed only with NCS-1, but not with other members of the NCS family, such as Calmodulin and Hippocalcin.

The non-mammalian homologue of NCS-1, Frequenin, was identified during the characterization of the molecular basis of neuronal hyperexcitability in a Drosophila mutant known as the V7 mutant (12,30,31). Subsequently, the frequenin homologue NCS-1 was identified in a variety of species, including birds and mammals (32). NCS-1–Frequenin is widely expressed in neuronal somata, dendrites and nerve terminals throughout embryonic and postnatal development (11). NCS-1–Frequenin is a pertinent example of a protein that clearly plays multiple roles in many different regulatory pathways in neurons. Compelling evidence suggests an involvement of NCS-1–Frequenin in the positive regulation of Ca2+-dependent exocytosis (13), and neurotransmission release (for a review see (11)). More recently, data reported by Tsujimoto et al. (33) and Kabbani et al. (34) suggested an involvement of NCS-1 in the activity-dependent facilitation of P/Q-type calcium currents at presynaptic nerve terminals and in the desensitization of the D2 dopamine receptor, respectively. An inhibitory role of NCS-1 via G-protein-coupled receptor regulation of calcium channels has also been reported (35). However, molecular bases for such functions are still relatively poorly understood, and little is known about the NCS-1 interaction with specific subcellular membranes at putative sites of action. Our data showing the high level of expression of IL1RAPL in specific structures of the brain, the co-localization at the membrane and the Ca2+-independent interaction between NCS-1 and IL1RAPL, and the previous characterized functions of NCS-1, suggest that IL1RAPL may be a component of a physiological pathway involved in the modulation of neurotransmitter release. Although further investigations using neuronal cells are required, this function is supported by the observation in PC12 cells of an inhibitory effect of IL1RAPL on stimulated exocytosis. Involvement of IL1RAPL in mental retardation, together with these results, provides a critical entry point to addressing the neuronal function of this novel class of proteins. The results also provide insights into physiopathological mechanisms underlying cognitive deficit resulting from a loss of function of IL1RAPL.

The potential implication of the IL1RAPL in the regulation of exocytosis and therefore in synaptic transmission is reminiscent of the physiopathological mechanism underlying mental retardation resulting from the dysfunction of zGDI (GDP dissociation inhibitor). This protein is a regulator of RAB3A and RAB3C activities, which are Rab proteins that participate in synaptic vesicle fusion (36). zGDI is encoded by the GDI1 gene that has also been shown to be implicated in a non-specific form of X-linked mental retardation (37,38). Recent data, based on the investigation of Gdi1-deficient mice, reported by Ishizaki et al. (39) have shown a function for this protein in neurotransmission release and suggested that zGDI is important in suppressing the hyperexcitability of pyramidal neurons in vivo. Further investigations, especially of Il1rapl-deficient mice will be important to allow a precise determination of the physiological role of IL1RAPL in the modulation of neurotransmitter release. These animal models should also allow us to address whether IL1RAPL and GDI1, two genes...
involved in mental retardation, act to regulate yet-to-be-defined specific aspects of synaptic activity, the dysfunction of which may result in an impairment of the development of cognitive functions.

**MATERIALS AND METHODS**

**Expression constructs**

All the cloning procedures were carried out as previously described (40). cDNA encompassing the different domains of IL1RAPL was sub-cloned in-frame with the GAL4 DNA binding domain of pGBT9 (pBD) vector and into the bacterial expression pGEX-4T1 vector. Deletion constructs of the IL1RAPL specific domain (A1, amino acids 549–644; and A2, amino acids 607–674) were also cloned into pGBT9 and pGEX 4T1. The full length cDNA of IL1RAPL was subcloned into pIREShygro using the Not1–Xho sites, and into pEGFP-N3 using the Xho–SacII sites. Constructs corresponding to full-length NCS-1, NCS-1 N-term (amino acids 1–175), NCS-1 C-Term (amino acids 174–190), Hippocalcin C-Term (amino acids 126–149) and Calmodulin C-Term (amino acids 154–193) were subcloned in-frame into the GAL4 activating domain vector pACT2 (pAD). The sequence of all these constructs was confirmed by automated sequencing (ABI 373).

**Preparation of affinity purified IL1RAPL-specific antiserum**

Two GST-fusion proteins corresponding to amino acids 569–608 and 607–644 were used to produce polyclonal antibodies (Covalab). Bleeds were taken after 8–10 weeks and tested on cell lysates. Affinity purification was performed by incubating 1 µg of the corresponding GST-fusion protein linked to Glutathione–Sepharose beads with the unpurified antiserum overnight at 4°C. The beads were then washed with PBS to remove non-specifically bound protein. Antibodies were eluted with 3 ml of glycine 0.1 M, EDTA. The antibodies were both tested for their specificity for IL1RAPL (by western blot using cell extracts) and the most specific antibody (amino acids 569–608), was chosen and named anti-IL1RAPL. Rabbit polyclonal anti-NCS-1 antibodies were characterized previously (13). Anti-GFP monoclonal antibody was purchased (Roche) and used according to the manufacturer’s instructions.

**Yeast two-hybrid system**

Screening of the cDNA library and analysis of positive clones were carried out as described by Bartel and Fields (41). Briefly, a cDNA fragment corresponding to the intracellular domain of IL1RAPL (ILRAPL-IC, amino acids 398–696), cloned in pGBT9 in frame with the GAL4 binding domain, was used as a bait for screening a human fetal brain cDNA library (Matchmaker, Clontech), cloned in pACT2, as previously described (42). The cDNA library was prepared from 19- to 22-week-old embryos. The Saccharomyces cerevisiae HF7 strain was co-transformed with the plasmids using the lithium acetate procedure. Transformants were grown at 30°C on Trp–,

Leu– and His– plates. His+ colonies were assayed for β-galactosidase activity after 2 days using a filter lift procedure (LacZ test). cDNA plasmids from positive clones were recovered using *E. coli* HB101 plated on leucine-free medium. Each positive clone was then sequenced using vector primers and a DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). In order to check the specificity of the interaction, sequenced plasmids were cotransformed into the yeast HF7 strain with SNF-1 cloned in pGBT9. SNF4 cloned in pACT2 was used as positive control. Histidine and LacZ tests were performed as described above. NCS-1 C-Term, Hippocalcin C-Term and Calmodulin C-Term were also cloned in pACT2 to check the specificity of the interaction between IL1RAPL and NCS-1.

**Cell culture, transfection and generation of stable cell lines**

CHO cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with glucose (4.5 mg/ml), 10% (v/v) fetal bovine serum, and 100 units/ml of penicillin and streptomycin. CHO and CHO-IL-1R (CHO cells stably transfected with IL-1-R) were transfected with pIREShygro-IL1RAPL, using the calcium phosphate precipitation procedure (43). Cells were then selected in DMEM containing 0.5 mg/ml of hygromycin (Sigma). Drug-resistant colonies were selected and screened for their expression of IL1RAPL by western blot. Stably transfected cell lines were maintained in supplemented DMEM with hygromycin (0.25 mg/ml).

PC12 cells were grown in DMEM supplemented with glucose (4.5 mg/ml), 5% fetal bovine serum 10% horse serum and 100 units/ml of penicillin and streptomycin. Plasmid constructs encoding IL1RAPL protein were introduced into PC12 cells (80% confluent) using GenePorter (Gene Therapy Systems) according to the manufacturer’s instructions. Stably transfected clones were selected using Hygromycin (0.5 mg/ml) and controlled for their expression of IL1RAPL by western blot analysis.

COS7 and HELA cells were maintained in DMEM containing 10% (v/v) fetal bovine serum and 100 units/ml of penicillin and streptomycin. Cells were plated at 10⁶ cells on 10 cm culture dishes and allowed 24 h to adhere before they were transfected using FUGENE 6 Transfection reagent (Roche Molecular Biochemicals). Expression was allowed for 24 h before being used for cellular and protein studies.

**Immunocytochemistry**

For immunostaining, COS7 cells and PC12 cells were grown on poly-D-lysine-coated glass coverslips and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min at room temperature. After three washes with 0.1 M PBS (5 min each), fixed cells were incubated for 1 h in TBST (10 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% Triton X100) containing 2% goat serum (DAKO) and then incubated overnight at 4°C with the primary antibody (anti IL1RAPL, 1:100, anti NCS-1, 1:100). The cells were washed three times in TBST and incubated for 1 h at room temperature with affinity purified secondary antibodies [FITC conjugated goat anti rabbit Ig or...
Texas red conjugated goat anti rabbit Ig (Jackson Immunoresearch, West Grove, PA) used at 1:200 dilution. After three washes, coverslips were mounted in Mowiol and examined with a Zeiss microscope equipped with epifluorescence illumination.

GST fusion protein production and pull down assays

GST fusion proteins were prepared in the E. coli BL21 strain and purified according to standard procedures. PC12 cells were lysed for 30 min in 500 μl of ice-cold lysis buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 5 mM EGTA, 0.1% NP40, 0.2% Triton X100, protease inhibitors mixture) in the presence of various amounts of CaCl2 to give a free Ca2+ concentration of 0, 1 or 10 μM, and cleared by centrifugation. Cellular lysates were then incubated with 30 μg of GST fusion protein immobilized on Glutathione–Sepharose 4B beads for 2 h at 4°C and washed extensively five times in the lysis buffer, and resuspended in 25 μl of SDS sample buffer. GST alone was used as control. Samples were separated by SDS–PAGE followed by western blotting and probed with NCS-1 antibody (1:1000).

Immunoprecipitation and western blot analysis

Transfected HELA cells were washed three times with ice-cold PBS and harvested in 500 μl of ice-cold lysis buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 5 mM EGTA, 5 mM trinitoacetic acid, 0.2% NP40, 0.05% CHAPS, with protease inhibitors). After a centrifugation of 30 000g for 30 min at 4°C, 500 μl aliquots of supernatant were incubated overnight at 4°C with the antibodies of interest (anti-GFP or anti-NCS-1), then protein A-agarose was added and samples were incubated for 3 h. Immunoprecipitates were extensively washed with IP buffer, then proteins were subjected to SDS–PAGE and western blot analyses. Anti-GFP (1:1000) and anti-NCS-1 (1:1000) antibodies were used for western blot detection.

125I-labelled IL1-binding assay

The CHO cells lines—CHO wild-type (CHO-WT) CHO stably transfected with IL1-R (CHO-IL1R), CHO with ILIRAPL (CHO-IL1RAPL) or with IL1-R and IL1RAPL (CHO-IL1R/IL1RAPL)—were grown in DMEM-free serum for 48 h. Cells were harvested by scraping for binding assay after a 10 min treatment with PBS containing EDTA (0.5 mM) to avoid proteolysis of receptors. Cells were then pelleted and stored at −80°C. In a second step, the frozen pellets were submitted to cryosectioning and 20 μm sections were set down in duplicate on Superfrost Plus slides. Slides were then desiccated, stored at −20°C, and transferred to 4°C before the binding experiments. 125I-labelled IL-1β and unlabelled IL-1β were resuspended in the binding medium (Tris 120 mM, 0.1% BSA, pH 7.4). For each cell line, binding experiments were carried out in duplicate using three different 125I-labelled IL-1β concentrations (5 × 10⁻⁹, 5 × 10⁻¹⁰ and 5 × 10⁻¹¹ M) with (for non-specific binding) or without (for total binding) 1000-fold molar excess of unlabelled IL-1β, as cold competitor. Binding medium (20 μL) with or without unlabelled IL-1β was incubated on the sections for 4 h at 4°C. After two steps of washing (15 min with Tris 50 mM, pH 7.4 at 4°C) both total and non-specific binding were measured in an 11 mm² window encompassing the section, using a microimager VII (Biospace Mesures, France) (44,45).

Transfection of PC12 cells and assay of human growth hormone release

PC12 cell transfection and secretion experiments were performed as previously described (13). Briefly, for transient transfections, PC12 cells were maintained in culture in 24-well trays and transiently co-transfected either with IL1RAPL or control constructs, as well as with the growth hormone (GH) plasmid used as secretion marker (4 μg of each plasmid/well were used) (46). After removal of culture medium, cells were either treated with 300 μM ATP for 15 min or non-treated before challenge with or without Ca2+ for 15 min. Buffer samples and cells were assayed for GH levels using an enzyme-linked immunosorbent assay kit (Boehringer Mannheim, UK). The amount of GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

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