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Molecular Characterisation of two Structurally Distinct Groups of Human Homers, Generated by Extensive Alternative Splicing

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Homer proteins bind specifically to the C termini of the metabotropic glutamate receptor mGluR1α/a and mGluR5, play a role in their targeting and modulate their synaptic properties. We have discovered that extensive alternative splicing generates a family of 17 Homer proteins. These fall into two distinct groups of 12 ''long'' Homers, which all have a coiled-coil domain at their C termini, and five ''short'' Homers, which lack such a domain. All Homers contain the N-terminal sequence responsible for their binding to mGluR1α/a receptors and can be co-localised with the recombinantly expressed mGluR1α/a protein in HEK-293 cells. The existence of the long and the short variants of each of the Homer-1, Homer-2 and Homer-3 proteins reflects the fundamental principles of Homer functions.

Keywords: Homer; VESL; splicing; coiled-coil; oligomerisation

Introduction

Homer/VESL proteins (later in this paper referred to as Homer proteins for simplicity) belong to a wider family of PDZ (post-synaptic density-95, discs large, zona occludens-1) domain-containing proteins (Craven & Bredt, 1998 and references therein; Fanning & Anderson, 1998; Hata *et al*., 1998) and contain a characteristic Gly-Leu-Gly-Phe motif within their N termini (Brakeman *et al*., 1997; Kato *et al*., 1997, 1998; Sun *et al*., 1998; Xiao *et al*., 1998). Homers form physical links with the group I metabotropic glutamate receptors (Brakeman *et al*., 1997; Kato *et al*., 1998; Xiao *et al*., 1998), and play a role in their targeting (Ciruela *et al*., 1999b,c,d), thus contributing to neuronal development and plasticity, memory acquisition and learning. The N-terminal ~115 amino acids of Homer proteins share sequence and secondary structure similarity with the WASP homology domain 1 (WH1) of the Wiskott-Aldrich syndrome family of proteins (Ramesh *et al*., 1997; Symons *et al*., 1996), with the Ena/VASP homology domain 1 (EVH1) of the Enabled protein of *Drosophila* (Ena), murine Mena, Evl and the vasodilatator-stimulated phosphoprotein (VASP) (Gertler *et al*., 1996; Haffner *et al*., 1995; Reinhard *et al*., 1992) and with the Ran-binding proteins (RanBP1) family (Callebaut *et al*., 1998). All of these proteins have been implicated in binding to proline-rich domains of various cytoskeleton-related proteins (Callebaut *et al*., 1998; Gertler *et al*., 1996; Kato *et al*., 1998; Niebuhr *et al*., 1997; Ramesh *et al*., 1997). So far three Homer proteins, which bind specifically to the C termini of the metabotropic glutamate receptor mGluR1α/a and mGluR5 have been characterised. These are Homer-1, Homer-2 and Homer-3 (Brakeman *et al*., 1997; Kato *et al*., 1997, 1998; Xiao *et al*., 1998). mRNA splicing generates three alternatively spliced forms of the Homer-1 (with the Homer-1A differing substantially from the Homer-1B and Homer-1C) and two forms of the Homer-2 (differing by 11 amino acids), expanding the Homer protein family to six related proteins, all of which share a homologous N-terminal sequence of ~130 amino acid residues, sufficient to bind group I mGluRs (Brakeman *et al*., 1997). Overall the level of amino acid sequence similarity in this domain reaches 78%. In contrast the amino

Abbreviations used: mGluR1α/a, metabotropic glutamate receptor type 1α/a; mGluR5, metabotropic glutamate receptor type 5; poly(A)$^+$, polyadenylated; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PVDF, polyvinylidene fluoride; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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acid sequence similarity between the C termini of these Homer proteins is low (~30%). Nevertheless, all of the C termini of the Homer proteins except for Homer-1A are predicted to adopt a similar coiled-coil secondary structure (Adamson *et al*., 1993; Lupas *et al*., 1991; Lupas, 1996a and references therein). This motif allows Homer proteins to form homo- or hetero-oligomeric complexes (Kato *et al*., 1998; Sun *et al*., 1998; Tu *et al*., 1998; Xiao *et al*., 1998). Alternative splicing of the Homer-1 mRNA results in a different long untranslated 3′ end of Homer-1A transcript which codes for a very short C terminus. Unlike all other Homer proteins, Homer-1A does not contain a coiled-coil domain. Homer-1A (VESL-1S) mRNA was identified by differential cloning strategies among the genes which are rapidly induced in neurones of the hippocampus and cortex by excitatory synaptic activity, following seizure or high-frequency stimulation and during development (Brakeman *et al*., 1997; Kato *et al*., 1997; Park *et al*., 1997). Following induction, the expression of Homer-1A is quickly downregulated by degradation of both the protein and its mRNA. The latter could reflect the presence of the 3′ untranslated AUUUA repeats which implicate the instability of the mRNA, and is a common feature of Immediate Early Gene mRNAs (Lanahan & Worley, 1998 and references therein). Unlike Homer-1A, the expression of other members of this protein family appears to be constitutive.

As yet, little is known about the functional role of Homer proteins. Kato *et al*. (1998) suggested their involvement in synaptic plasticity or cell signalling by means of regulating receptor cytoskeleton interaction. Xiao *et al*. (1998) and Tu *et al*. (1998) argued for a role of Homers in cross-linking of the group I mGluRs to cytoskeleton or Ca^{2+} signalling proteins. The fast induction of Homer-1A protein expression followed by its rapid downregulation led Brakeman *et al*. (1997) to suggest its involvement in negative regulation of synaptic transmission or efficacy, possibly by competing with the ''long'' Homers for their target proteins (Xiao *et al*., 1998). However, co-expression of Homer-1A with the mGluR1α/a has been shown to increase both the stability and cell surface expression of mGluR1α/a in HEK-293 cells, suggesting an important role for Homer-1A in receptor targeting (Ciruela *et al*., 1999c,d). The functional importance of the constitutively expressed Homer-2A/2B and of Homer-3 proteins has not yet been studied in detail and neither of the proteins has been found to have a corresponding ''short'' form (which would be equivalent to the Homer-1A).

Here we report that the Homer-2 and Homer-3 mRNAs can undergo diverse alternative splicing to produce four variants of Homer-2 and ten variants of Homer-3 proteins. Both Homer-2 and Homer-3 are shown to exist in both long and short forms, the latter without the C-terminal coiled-coil domains. Existence of the short and long forms of each of the Homer-1, 2 and 3 proteins suggests a common basic mode for their function, whilst extensive alternative splicing provides a means for regulating their functional properties.

Results

Cloning of the alternatively spliced forms of human Homer-2 and Homer-3 proteins

RT-PCR amplifications using various combinations of forward and reverse primers corresponding to Homer-2 and Homer-3 sequences (as described in Materials and Methods) yielded multiple cDNAs indicative of more than two known alternatively spliced forms of Homer-2 mRNA and more than one known form of Homer-3 mRNA. The results are summarised in Figures 1 and 2. We have found that Homer-2 mRNA can be alternatively spliced at two sites (Figure 1). The first site of alternative splicing is situated at the position 387 of the Homer-2 (numbering of the nucleic sequence here and for other Homer starts at the first ATG of the longest coding sequence of the corresponding Homer) and results in Homer-2A/B variants of the Homer-2 protein. The splicing at the second, newly identified site, which is situated downstream of the known Homer-2A/B splicing site, results in the deletion of a 164 bp fragment from the Homer-2 mRNA and a shift in reading frame. The new reading frame has a stop codon immediately after the splicing site. The resulting mRNA codes for either a 171 or a 182 amino acid residue long protein (depending on the absence or presence of a 33 bp fragment at the upstream splicing site), called Homer-2C and Homer-2D, respectively (Figure 1). We have also discovered that Homer-3 mRNA is subject to alternative splicing at five different sites (Figure 2). Splicing at position 303 results in the deletion of 108 bp from the Homer-3 mRNA sequence and the removal of 36 amino acid residues from the middle of the Homer-3 protein, producing Homer-3B (as opposed to the earlier cloned Homer-3, or Homer-3A, using the new nomenclature). Splicing at two sites, at positions 816 and 894, results in two insertions or deletions of three amino acid residues within the C termini of the Homer-3A/B proteins (Figure 2). Splicing at position 369 results in an 844 bp deletion from the Homer-3 mRNA sequence and produces a C-terminally truncated 145 residue long Homer-3C protein (Figure 2). Splicing at position 351 of the Homer-3 mRNA results in a 361 bp deletion from the middle of the Homer-3 mRNA. The deletion causes a reading frame shift, with the new reading frame having a stop codon soon after the splicing site. The resulting mRNA codes for a 121 amino acid residues long C-terminally truncated Homer-3D protein (Figure 2). Thus, alternative splicing can produce up to eight forms of the long Homer-3 protein differing by insertion or deletion of the 36 residue fragment in the middle of the Homer-3 protein and

of the two short fragments within their C termini (Homer-$3A_{00}$, Homer-$3A_{01}$, Homer-$3A_{10}$, Homer-$3A_{11}$, Homer-$3B_{00}$, Homer-$3B_{01}$, Homer-$3B_{10}$ and Homer-$3B_{11}$, the double figures indicate the presence (1) or the absence (0) of the first and second short fragment, respectively) and two forms of the short Homer-3 protein Homer-3C and Homer-3D without the C terminus of the long Homer-3 proteins.

Expression of Homers in transiently transfected HEK-293 cells

As we reported recently HEK-293 cells constitutively express some of the Homer proteins, of which Homer-1B/C proteins are by far the most abundant (Ciruela *et al*., 1999d). In agreement with those observations, cell extracts prepared from untransfected HEK-293 cells and immunoblotted with the pan-Homer antibody (VHr20) yielded an immunoreactive protein with an apparent molecular mass of 48 kDa (lane 1, Figure 3(a)). When the cells were transiently transfected with either the Homer-$3A_{01}$ or Homer-2A cDNAs there was a considerable increase in the immunoreactivity of the 48 kDa band (lanes 2 and 6, Figure 3(a)), as would be expected given the predicted molecular mass of these proteins. It should be noted that neither the recombinantly expressed Homer-$3A_{01}$ and Homer-2A nor the endogenous Homer-1B/C proteins can be resolved in the gel due to their similar molecular mass. Transfection of the HEK-293 cells with the newly identified cDNAs coding for Homer-$3B_{01}$, Homer-3C and Homer-3D gave rise to immunoreactive bands with apparent molecular mass of 45, 16 and 14 kDa, respectively (lanes 3, 4 and 5, Figure 3(a)). Transfection of the cells with the Homer-2C cDNA resulted in the appearance of an immunoreactive band with an apparent molecular mass of 29 kDa (lane 7, Figure 3(a)). In all cases the observed molecular mass was consistent with those calculated from the predicted amino acid sequences of their respective cDNAs. The faintly visible band of 45 kDa (Figure 3(a)) might represent the endogenous long Homer-3B proteins. The minor immunoreactive band at 63 kDa (all lanes in Figure 3(a)) corresponds in size to those reported earlier by Xiao *et al*. (1998) and by Ciruela *et al*. (1999d) and may represent a Homer-related protein or another protein cross-reacting with the anti-Homer antibody. Expression of the endogenous short Homer-2C/D and Homer-3C/D proteins in HEK-293 cells was not detected by Western blotting (Figure 3(a)) and neither was the expression of the short Homer-1A protein (Figure 3(a)). These short Homers could either be present in amounts such that they cannot be detected under the conditions used for this experiment or they may be susceptible to proteolysis (Ciruela *et al*., 1999d). Undetectably low levels of the short Homer proteins are also in agreement with the lower levels of their mRNAs (data not shown).

Interaction and subcellular co-localisation of mGluR1α/a and Homers in HEK-293 cells

To test whether the newly identified Homer proteins interact with the mGluR1α/a receptors we co-transfected the HEK-293 cells with the mGluR1α/a and Flag-tagged Homer-$3A_{01}$, Homer-$3B_{01}$, Homer-3C or Homer-3D. Immunoprecipitation using anti-Flag antibody and subsequent staining with the anti-mGluR1α/a antibody revealed that all the tested Homers co-immunoprecipitated the mGluR1α/a receptor (lanes 2, 3, 4 and 5 in Figure 3(b)). No mGluR1α/a could be co-immunoprecipitated from the cells co-transfected with mGluR1α/a and the LacZ reporter plasmid (lane 1, Figure 3(b)), thus confirming the specificity of the immunoprecipitation.

To investigate the consequences of such interaction further, various Homers and mGluR1α/a were expressed in HEK-293 cells and the intracellular distribution of the proteins was examined following fixation and permeabilisation of the cells with Triton X-100. The constitutively expressed Homer proteins in HEK-293 cells give a low level of background immunofluorescence in all cells. This endogenous fluorescence gives a weak grainy or reticular pattern (Figure 4(b)). Expression of mGluR1α/a in HEK-293 cells results in a diffuse cytoplasmic stain (Figure 4(a)) which shows some co-localisation with the endogenous Homer-related proteins (Figure 4(c)). When Homer-2A, Homer-2C, Homer-$3A_{01}$, Homer-$3B_{01}$, Homer-3C or Homer-3D were expressed alone in HEK 293 cells the fluorescence intensity of the VHr20 immunoreactive proteins increased considerably, but remained cytosolic (data not shown). This is in marked contrast with the result obtained when these Homers were co-expressed with mGluR1α/a (Figures 4(e) and (h) and 5(a), (d), (g) and (j)). Now the immunofluorescence appeared as large intracellular accumulations which were precisely co-localised with those immunoreactive for mGluR1α/a (Figures 4(d) and (g) and 5(b), (e), (h) and (k)). Therefore, co-expression of mGluR1α/a with Homer-2A/2C or with Homer-3A/3B/3C/3D caused a dramatic change in the intracellular distribution of the two proteins from a diffuse cytoplasmic/reticular pattern to large vesicular inclusions.

Homer proteins: analysis of the amino acid sequences and implications for function

Extensive alternative splicing generates a family of 17 Homer proteins (including three alternatively spliced forms of the Homer-1 and two forms of Homer-2, described previously). All the Homer proteins can be divided into the two structurally distinct groups of short and long Homer proteins. Short Homers include Homer-1A, Homer-2C, Homer-2D, Homer-3C and Homer-3D, long Homer proteins include Homer-1B, Homer-1C, Homer-2A, Homer-2B, Homer-$3A_{xx}$ and Homer-$3B_{xx}$

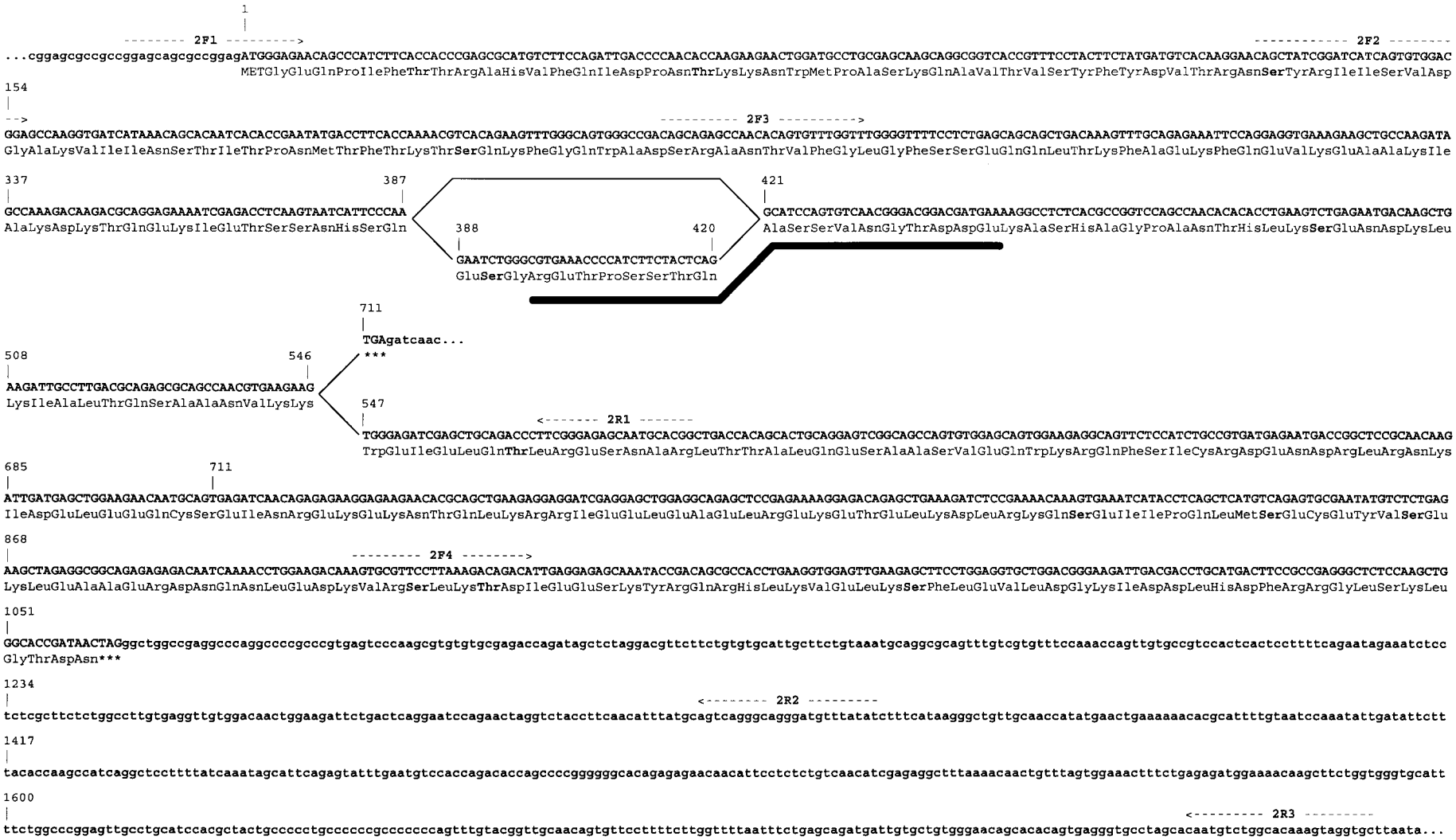

Figure 1 (*legend opposite*)

(Figure 6(a) and (b) and Figure 7(a)). The principal functional differences between the short and the long Homers are encoded in the long C-terminal tail of the long Homers. Analysis of secondary structures of the Homer-1, 2 and 3 proteins revealed that all long Homers contain ~150 amino acid residue long regions in their C termini (~175 amino acid residues in Homer-2) which adopt coiled-coil structures (Figures 6(d)-(g) and 7(b)-(e) and (f)), responsible for their intermolecular interactions (Lupas, 1996b; Lupas *et al*., 1991; Parry, 1982), and which are implicated in oligomerisation of Homer proteins cloned earlier (Kato *et al*., 1998; Tu *et al*., 1998; Xiao *et al*., 1998). Short Homer-1A and the newly identified short Homer-2C, Homer-2D, Homer-3C and Homer-3D, unlike the long Homers, do not contain that C-terminal coiled-coil domain (Figures 6(c), (h) and (i) and 7(g) and (h)). Earlier we have shown that the Homer-1A co-localises with the mGlu R1α/a receptor intracellularly and stabilises the receptor but is not associated with it at the cell surface (Ciruela *et al*., 1999b,d). The occurrence of the coiled-coil domains in both Homer-2A and Homer-3A/B resulted in the presence of widely distributed intracellular accumulations of the mGluR1α/a and the Homers within the transfected HEK-293 cells. The short forms of either Homer-3C or Homer-3D, however, gave rise to larger vesicular bodies (Figure 5). We conclude, therefore, that the alternative splicing of Homer mRNAs results in the expression of the two structurally and functionally distinct groups of short and long forms of each of the Homer proteins. The analysis of the secondary structure of Homer proteins revealed another characteristic feature of all of the Homer-1, 2 and 3 proteins. This is the existence of an additional short ~35 amino acid residue long coiled-coiled domain, most prominent in the Homer-2 proteins, which begins at around position ~100 in the Homer N-terminals (Figures 6 and 7). This region of the Homer proteins is not necessary for the homo or hetero-oligomerisation of the Homers as was shown earlier for the short Homer-1A, which does not oligomerise even though the short coiled-coil domain is fully preserved there. This region is also not necessary for the coupling to the mGluR1α/a receptor, as Homer-3 proteins, missing this domain fully or partially (Homer-3B and Homer-3D, respectively), interact with the mGluR1α/a (as shown in this study). This domain may therefore be important for the folding of Homers themselves or involved in interacting with proteins other than mGluRs. This short N-terminal coiled-coil domain is present in all Homer proteins except for the Homer-3B form in which the deletion of the 36 amino acid residues removes this domain completely (Figure 7(f)). The alternative splicing in Homer-3B might therefore provide an important means of regulating the efficiency or specificity of Homer-3 coupling.

Homer-1B/C as well as Homer-2A/B differ from each other by the insertion or deletion of a short stretch of 12 (Homer-1) or 11 amino acid residues (Homer-2) (Figure 6(a) and (b)). These alterations affect neither the first short coiled-coil region nor the long C-terminal coiled-coil region in either Homer-1B/C or Homer-2A/B (compare Figure 6(d) and (e), (f) and (g), (h) and (i)). Analysis of the secondary structure revealed that these 12 and 11 amino acid residue fragments are parts of random coils (loops) which usually allow for flexibility in the protein structure (Figure 8). In both Homer-1B/C and Homer-2A/B these fragments are located between the mGluRs binding domains (the N-terminal half of Homers) and the oligomerisation domains (the C-terminal half of Homers). Thus they link the two parts of Homer molecules together. The alternative splicing in Homer-1B/C and Homer-2A/B might therefore represent a molecular mechanism to vary either the physical distance between the mGluRs and other interacting proteins or the flexibility of such links.

In earlier studies Homer-1 was reported to contain the PEST sequence (rich in proline, glutamic acid, serine and threonine), an important feature of eukaryotic proteins with intracellular half-lives less than two hours (Rechsteiner *et al*., 1987; Rechsteiner & Rogers, 1996; Rogers *et al*., 1986), which was suggested to play a role in rapid down-regulation of the protein. Analysis of the newly identified Homer sequences (using ''PESTfind'' software, window size = 12) has indicated that some Homer-2 proteins also contain PEST sequences. Interestingly, the PEST-forming sequence in Homer-2 is subject to alternative splicing and is absent in Homer-2A (long) and Homer-2C (short) proteins (Figure 1). Thus, alternative splicing may selectively alter degradation rates of Homer-2B and Homer-2D proteins.

We have found two additional sites of alternative splicing within the amino acid sequences of

Figure 1. Nucleotide sequence and deduced amino acid sequences of the human Homer-2 proteins. Coding sequences are shown in capital letters. Asterisks denote in-frame stop codons. The numbering of the sequence starts at the first ATG of the longest open reading frame (codes for the Homer-2B). A 33 bp fragment (position 388 to 420) is present in Homer-2B/2D mRNAs and is spliced out in Homer-2A/2C mRNAs. Splicing out a 164 bp fragment (position 547 to 710) generates a stop codon by introducing a shift into the long open reading frame of Homer-2 which results in Homer-2C and Homer-2D mRNAs, coding for 171 and 182 amino acid residue long proteins accordingly. The relative position and orientation of the Homer-2 specific primers (see Materials and Methods) for PCR amplifications are indicated by broken lines above the sequence. The predicted PEST sequence (in Homer-2B/2D proteins only) scores +14.78 and is underlined.

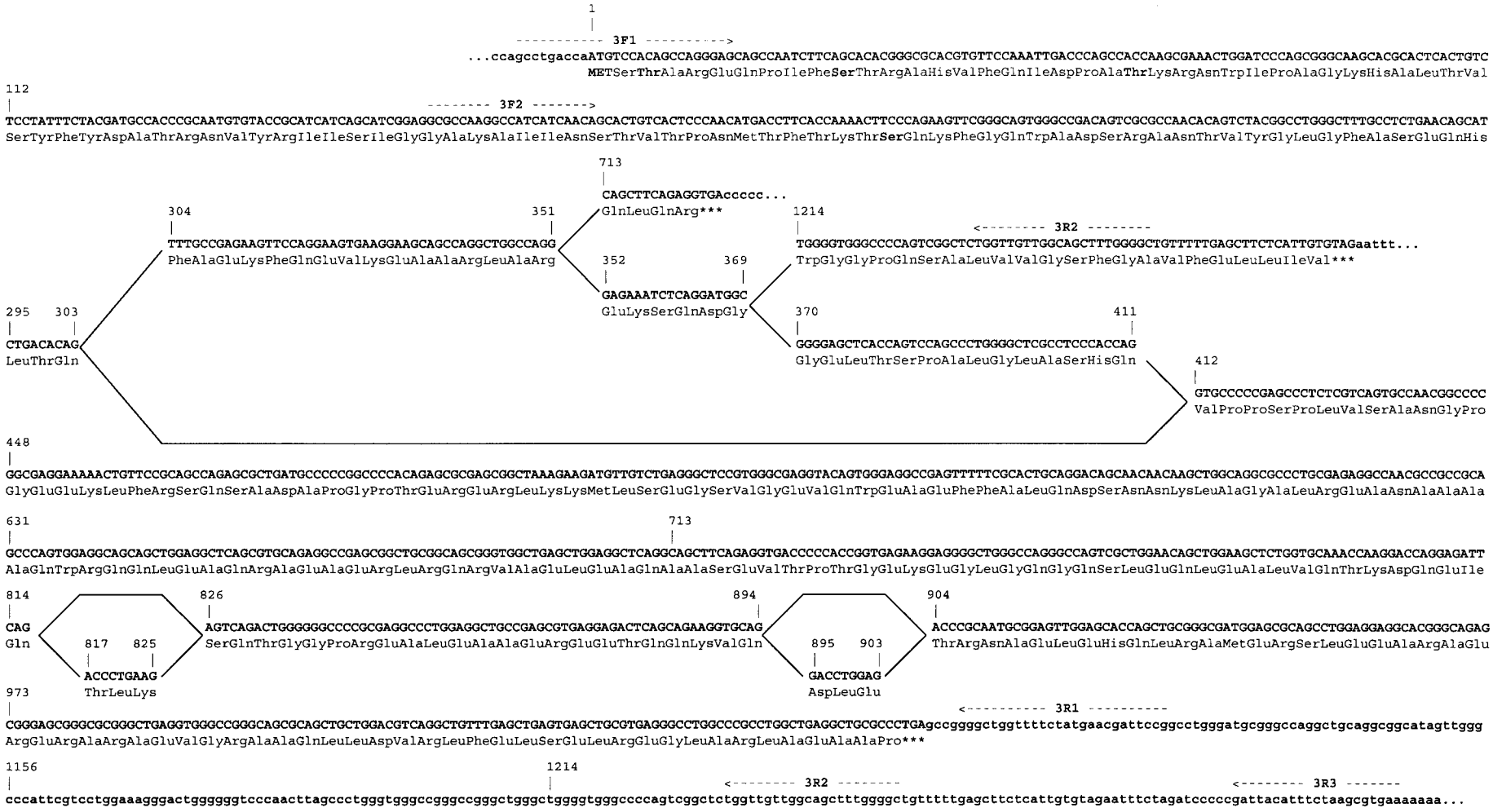

Figure 2 (*legend opposite*)

C-terminal half of Homer-3A/B (Figures 2 and 7(a)). These result in the insertion or deletion of the two, three residue long fragments (Thr-Leu-Lys and Asp-Leu-Glu) and thus produce up to four variants of each of the Homer-3A and the Homer-3B proteins. These short insertions or deletions can introduce a ''frame shift'' in the seven amino acid residue long periodical sequence forming the coiled-coils and thus may regulate their folding. The addition of the first fragment results in a marked increase in the probability of the coiled-coil formation (difference between Homer-3_{0x} and Homer-3_{1x}, amino acid residues 250-275), whilst the presence of the second fragment further extends the predicted coiled-coil structure within the C-terminal coiled-coil domains (amino acid residues 275-300) of the Homer-3_{x1} compared to Homer-3_{x0} (Figure 7(b) and (e)). The alternative splicing within the C-terminal region of Homer-3A/B might therefore be important in regulating Homer oligomerisation.

Discussion

Human cell lines such as HEK-293 or HeLa have been used for the successful functional expression of a wide range of neural receptors. Recently we showed that both HEK-293 and HeLa cells as well as another commonly used COS-7 cells constitutively express long Homer-1, 2 and 3-related proteins which appear as multiple immunoreactive bands on Western blots obtained from crude lysates from these cells (Ciruela *et al*., 1999d; Soloviev *et al*., 1999). Such cell lines can, therefore, form a convenient model for studying Homer proteins themselves. We have recently reported that the profile of Homer expression in HEK-293 and, to a lesser extent, in HeLa cells is similar to that found in mammalian brain (Soloviev *et al*., 1999) and have, therefore, used HEK and HeLa cells as a convenient source of human Homer mRNAs.

Previous reports have described a distinct set of alternatively spliced Homer-1 proteins, existing as short (186 amino acid residues) and two long (354 and 366 amino acid residues) proteins (Brakeman *et al*., 1997; Kato *et al*., 1997, 1998; Xiao *et al*., 1998). Until now Homer-2 mRNA has not been shown to code for anything but the two long proteins, differing by an 11 amino acid residue insertion (Kato *et al*., 1998; Xiao *et al*., 1998), and only one form of the Homer-3 protein (358 residues) has been described (Xiao *et al*., 1998). Our studies of Homer proteins show that Homer-2 and Homer-3 proteins also exist, like Homer-1, in different short or long forms. Our results suggest that members of the Homer family share a general organisational or regulatory principle in common, evidenced by the existence of both short and long forms of each of the proteins, with the missing C-terminal coiled-coil domains preventing homo- and/or hetero-oligomerisation of the short Homers.

All Homer-1 alternatively spliced variants share a 175 amino acid identity at their N termini, Homer-2 proteins have the same 129, and Homer-3 proteins 101 of the same N-terminal amino acids. All of the Homer proteins tested in our study, including Homer-3D and Homer-3B co-localise with the recombinantly expressed mGluR1α/a (Figures 4 and 5); moreover their interaction with mGluR1α/a was confirmed using co-immunoprecipitation (Figure 3(b)). Thus we have confined the mGluR binding domain to the N-terminal 98 amino acid residues, conserved in all Homer proteins, and shown that the absence of the short N-terminal coiled-coiled domain (as in Homer-$3B_{xx}$) does not impair Homer interaction with the mGluR1α/a.

The co-expression of the mGluR1α/a receptor with any of the Homer-2 or Homer-3 proteins tested in this work resulted in re-distribution of each of the proteins in cells in all cases. This is in agreement with our previous observations (Ciruela *et al*., 1999b,d) in which the mGluR1α/a receptor co-expressed in HEK-293 cells with either short Homer-1A or long Homer-1C proteins underwent intracellular redistribution. It is interesting to note here that in the recent report by Roche *et al*. (1999) the authors observed similarly strong intracellular re-distribution of the mGluR5 receptor in HeLa cells co-transfected with mGluR5 plus Homer-1B, but not in cells co-transfected with mGluR5 plus Homer-1A. The differences in these results might

Figure 2. Nucleotide sequence and deduced amino acid sequences of the human Homer-3 proteins. Coding sequences are shown in capital letters. Asterisks denote in-frame stop codons. The numbering of the sequence starts at the first ATG of the longest open reading frame (codes for Homer-$3A_{11}$). Splicing out a 108 bp fragment (position 304 to 411) results in Homer-$3B_{xx}$ mRNAs coding for proteins shorter by 36 amino acid residues compared to corresponding Homer-$3A_{xx}$ forms. Splicing out a 844 bp fragment (position 370 to 1213) generates a stop codon by introducing a shift into the long open reading frame of Homer-3 which results in Homer-3C mRNA, coding for a 145 amino acid residue long protein. Splicing out a 361 bp fragment (position 352 to 712) generates a stop codon by introducing a shift into the long open reading frame of Homer-3 which results in Homer-3D mRNA, coding for a 121 amino acid residue long protein. Insertion or deletion of two 9 bp fragments (positions 817 to 825 and 895 to 903) generates up to four variants of each of the Homer-3A and Homer-3B mRNAs: Homer-$3A_{00}$, Homer-$3A_{01}$, Homer-$3A_{10}$, Homer-$3A_{11}$, Homer-$3B_{00}$, Homer-$3B_{01}$, Homer-$3B_{10}$ and Homer-$3B_{11}$ (the double figures indicate the presence (1) or the absence (0) of the first and second short fragment, respectively). The relative position and orientation of the Homer-3 specific primers (see Materials and Methods) for PCR amplifications are indicated by broken lines above the sequence.

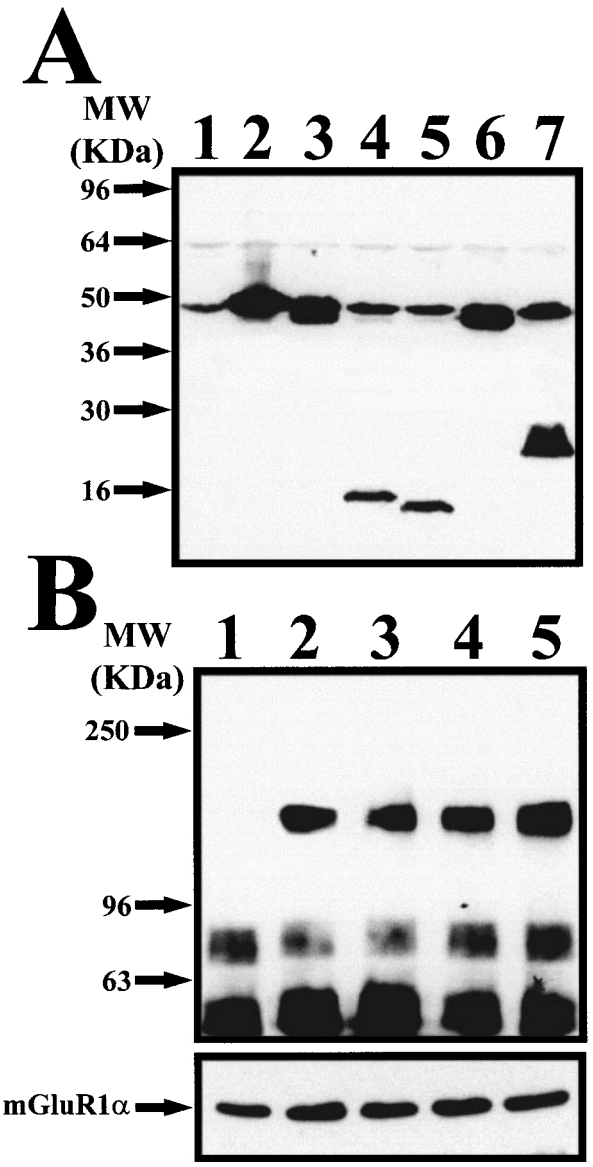

Figure 3. Expression and interaction of the Homer proteins and the mGluR1α/a receptor in HEK-293 cells. (a) Transient expression of the newly identified Homer proteins. Immunoblot of untransfected HEK-293 cells (lane 1), or cells transiently transfected with Homer-3A (lane 2), Homer-3B (lane 3), Homer-3C (lane 4), Homer-3D (lane 5), Homer-2A (lane 6) and Homer-2C (lane 7). Crude cell extracts (40 mg of protein) were analysed by SDS-PAGE and immunoblotted using anti-Homer antibody. (b) Co-immunoprecipitation of Homer proteins with the mGluR1α/a receptor. HEK-293 cells were transfected with cDNAs encoding mGluR1α/a plus either LacZ reporter (lane 1), Flag-Homer-3A (lane 2), Flag-Homer-3B (lane 3), Flag-Homer-3C (lane 4) or Flag-Homer-3D (lane 5). After immunoprecipitation with the anti-Flag antibody, immunocomplexes were separated on SDS-PAGE gel and immunobloted using F2 anti-mGluR1α/a antibody. (Insert) Immunoblotting of the crude cell extracts (samples from the same transfected cell as above) using F2 antibody shows abundant expression of the mGluR1 protein in all transfected cells. The molecular mass markers in kilodaltons are indicated on the left.

reflect the different binding affinities of different Homer proteins for the metabotropic receptors. Alternatively it could be due to HEK-293 cells and HeLa cells having different amounts and/or ratios of endogenously expressed Homer proteins and their splicing forms.

Our results extend the Homer protein family from a group of six proteins to a much wider family of 17 proteins, produced from the three genes by means of alternative splicing. The variations introduced by the alternative splicing of Homer mRNAs provide a molecular basis for the regulation of Homer proteins. Thus, splicing of each of the Homer-1, Homer-2 and Homer-3 mRNAs makes both long (coiled-coil containing) proteins which are able to oligomerise and equivalent short variants (missing the coiled-coil domain) which are unable to oligomerise. The alternative splicing may also alter the length or flexibility of the linkage regions between the N-terminal mGluR

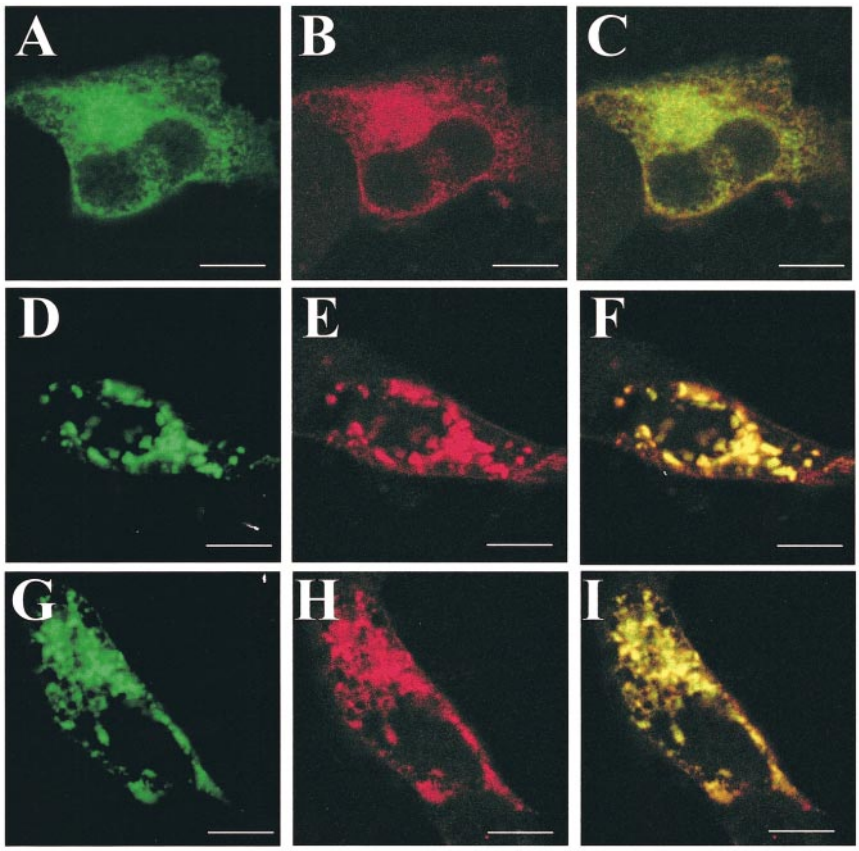

Figure 4. Co-localization of mGluR1α/a and Homer-2 in HEK-293 cells. HEK-293 cells were transiently transfected with the Flag-tagged mGluR1α/a alone ((a), (b) and (c)) or with the Flag-tagged mGluR1α/a plus Homer-2A ((d), (e) and (f)) or with the Flag-tagged mGluR1α/a plus Homer-2C ((g), (h) and (i)). Cells were stained with the anti-Flag monoclonal antibody (M2-Ab) and the anti-Homer affinity purified antibody (VHr20) as described in Materials and Methods. Cells were analysed by double immunofluorescence and confocal microscopy to detect mGluR1α/a receptor (green images in (a), (d) and (g)) and Homer proteins (red images in (b), (e) and (h)). Superimposition of images reveals Homer/mGluR1α/a co-localization in yellow ((c), (f) and (i)). The images show a single horizontal section of representative cells. The scale bar represents 10 μm.

binding domains and the C termini of Homer-1B/C and Homer-2A/B, which crosslink Homer proteins together. It may also remove the short N-terminal coiled-coil domain in Homer-3B_{xx} proteins and could alter their binding affinities or specificity. Alternative splicing may also attenuate folding of the C-terminal coiled-coil domains in the long Homer-3A/B_{xx} proteins. Finally, the degradation rates of some of the Homers could be selectively altered by splicing out of the PEST sequences, as in Homer-2A and Homer-2C proteins. In addition to these mechanisms, the fine tuning of the function of the Homer family may be achieved by varying the ratios of the Homer-1, 2 and 3 mRNAs in different brain regions or tissues. Further studies of expression levels of the individual Homers as well as their binding affinities to the metabotropic glutamate receptors and/or other interacting proteins will be needed to elucidate the functional significance of each of the individual Homer proteins. However, on the basis of our and other results published so far we can reasonably hypothesise that the stabilisation and the clustering of the mGluR1α/a receptor at the cell surface appears to be mediated by the long Homers, containing the cross-linking coiled-coil domains. The short Homers may facilitate the targeting of the receptor to the surface and may function to prevent the cross-linking of the mGluRs or other proteins before they reach their final destination(s). The existence of the short and the long forms of Homer-1, 2 and 3 proteins reveals therefore a common principle of organisation of the Homer family, whilst the existence of the numerous variants of similar Homer isoforms, which can be differentially regulated, may play an important role in maintaining the plasticity at glutamatergic synapses.

Materials and Methods

Cloning of the alternatively spliced forms of human Homer-2 and Homer-3 proteins

Human poly(A)$^+$ RNAs were isolated from 10^6 HEK-293 and HeLa cells, cDNA was synthesised using random hexanucleotide primers, as described previously (Ciruela *et al*., 1999d). PCR amplification of Homer-2 and Homer-3 cDNA fragments was carried out using synthetic oligonucleotide primers based on the sequences of human Homers (EMBL accession numbers Y17572, AF081530, AF093264 for Homer-2 and Y17573, AF093265 for Homer-3 sequences). Their positions within the Homer-2 and Homer-3 sequences are indicated in Figures 1 and 2. The primers used in this study were: 2F1 (5′-GGAGCAGCGCCGGAGATGGGAGA-3′), 2F2 (5′-CAGCTATCGGATCATCAGTGTGGA CGGA-3′), 2F3 (5′-CAGCAGAGCCAACACAGTGTTTGGTT-3′), 2F4 (5′-AGTGCGTTCC TTAAAGACAGACA-3′), 2R1 (5′-CCG TGCATTGCTCTCCCGAA-3′), 2R2 (5′-ATATAAA CATC CCTGCCCTGACT-3′), 2R3 (5′-GCACCTACTTTGTGC-CAGACATTG-3′) for amplifications of the Homer-2

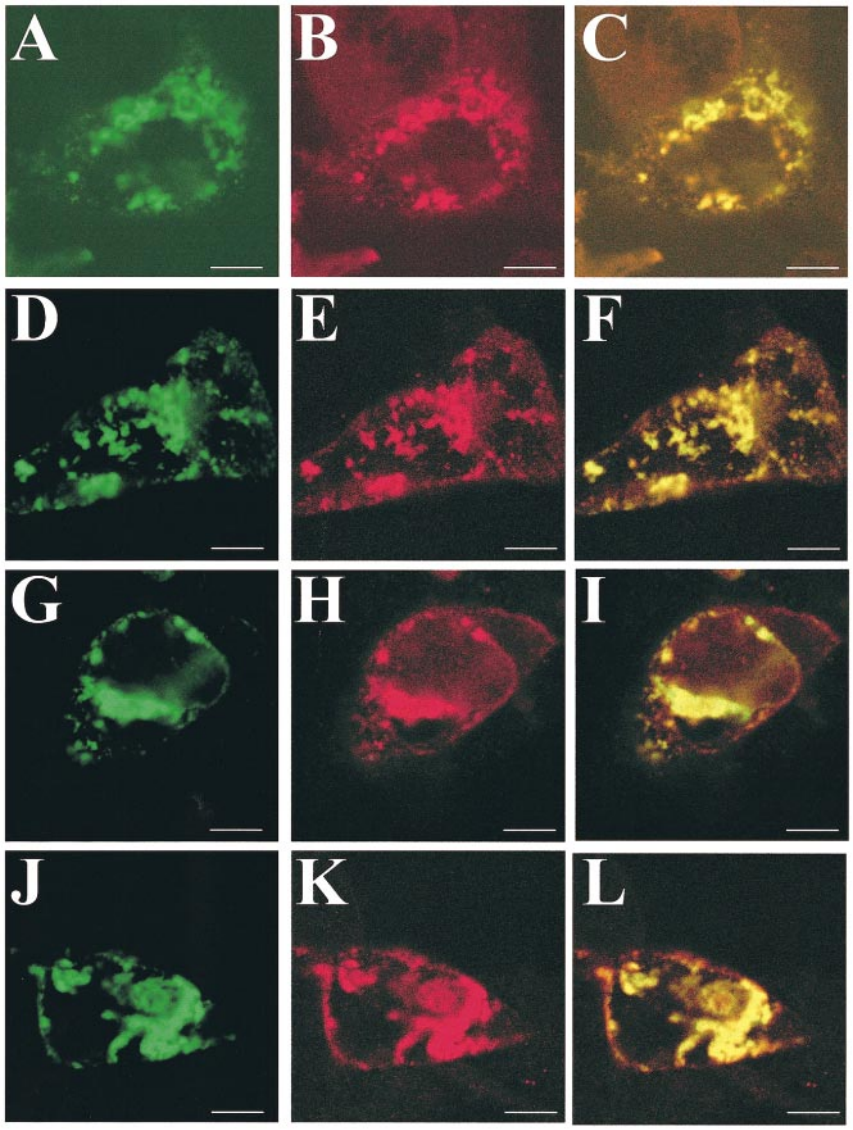

Figure 5. Co-localization of mGluR1α/a and Homer-3 in HEK-293 cells. HEK-293 cells were transiently transfected with the mGluR1α/a plus either the Flag-tagged Homer-3A ((a), (b) and (c)) or the Flag-tagged Homer-3B ((d), (e) and (f)) or the Flag-tagged Homer-3C ((g), (h) and (i)) or the Flag-tagged Homer-3D ((j), (k) and (l)). Cells were stained with the anti-mGluR1 affinity purified antibody (F1-Ab) and the anti-Flag monoclonal antibody (M2-Ab) as described in Materials and Methods. Cells were analysed by double immunofluorescence and confocal microscopy to detect the tagged Homer-3 proteins (green images in (a), (d), (g) and (j)) and the mGluR1α/a receptor (red images in (b), (e), (h) and (k). Superimposition of images reveals Homer-3/mGluR1α/a co-localization in yellow ((c), (f), (i) and (l)). The images show a single horizontal section of representative cells. The scale bar represents 10 μm.

cDNAs and 3F1 (5′-TTGGAATTC**ATG**TCCACAGC-CAGGG AG-3′), 3F2 (5′-GCGCCAAGGCCATCATCAA-CA-3′), 3R1 (5′-CGGAATCGTTCATAGAA AACC AGCCC-3′), 3R2 (5′-GCCCCAAAGCTGCCAACAAC-CA-3′) and 3R3 (5′-TTCACGC TTAGAAATGTAATC-3′) for amplifications of the Homer-3 cDNAs. All cDNAs were amplified using proof-reading *Pfu* DNA polymerase and all combinations of the forward and reverse primers. Amplification conditions were one cycle of three minutes at 96 °C, one minute at 60 °C, ten minutes at 72 °C, and 35 cycles of one minute at 96 °C, one minute at 60 °C and three minutes at 72 °C, followed by ten minutes at 72 °C. All Homer-3 amplifications were carried out in the presence of 5% (v/v) DMSO. Omitting this

Figure 6. Structure of the Homer-1 and Homer-2 proteins. (a) The three alternatively spliced forms of the Homer-1 mRNAs. Boxes indicate coding sequences. The black box indicates the coding fragment of the long 3′ untranslated Homer-1A mRNA sequence different from that of other Homer-1 mRNAs. Sequences coding for the predicted coiled-coil regions are indicated in red. A fragment coding for a 12 amino acid residues insertion in Homer-1C is shown in green. (b) The four alternatively spliced forms of the Homer-2 mRNAs. Boxes indicate coding sequences. Sequences coding for the predicted coiled-coil regions are indicated in red. Fragment coding for an 11 amino acid residues insertion in Homer-2B and Homer-2D is shown in blue. (c)-(i) The predicted coiled-coil domains in the Homer-1 and Homer-2 proteins. In all plots the vertical axis indicates the probability that the sequence will adopt a coiled-coil conformation, the horizontal axis indicates the position in the amino acid sequence of the respective Homer proteins. (d) The green bar shows the position of alternative splicing in Homer-1B, (e) the green box indicates the 12 amino acid residues insertion in the sequence of Homer-1C. Blue bars show the positions of alternative splicing in (f) Homer-2A and (h) Homer-2C, the blue box indicates the 11 amino acid insertions in the sequences of (g) Homer-2B and (i) Homer-2D. Note that the C-terminal coiled-coil conformation is not affected by these insertions.

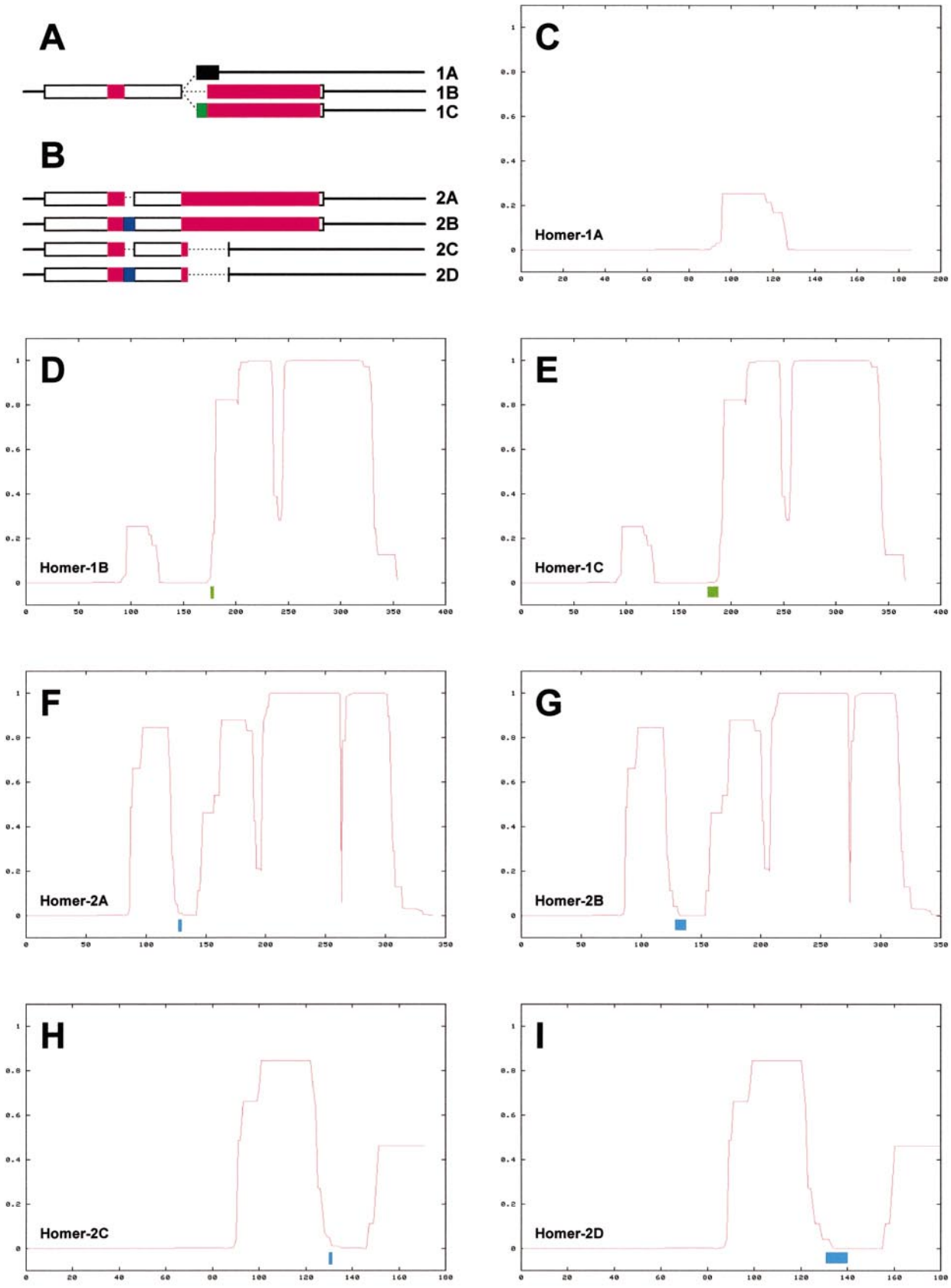

Figure 6 (*legend opposite*)

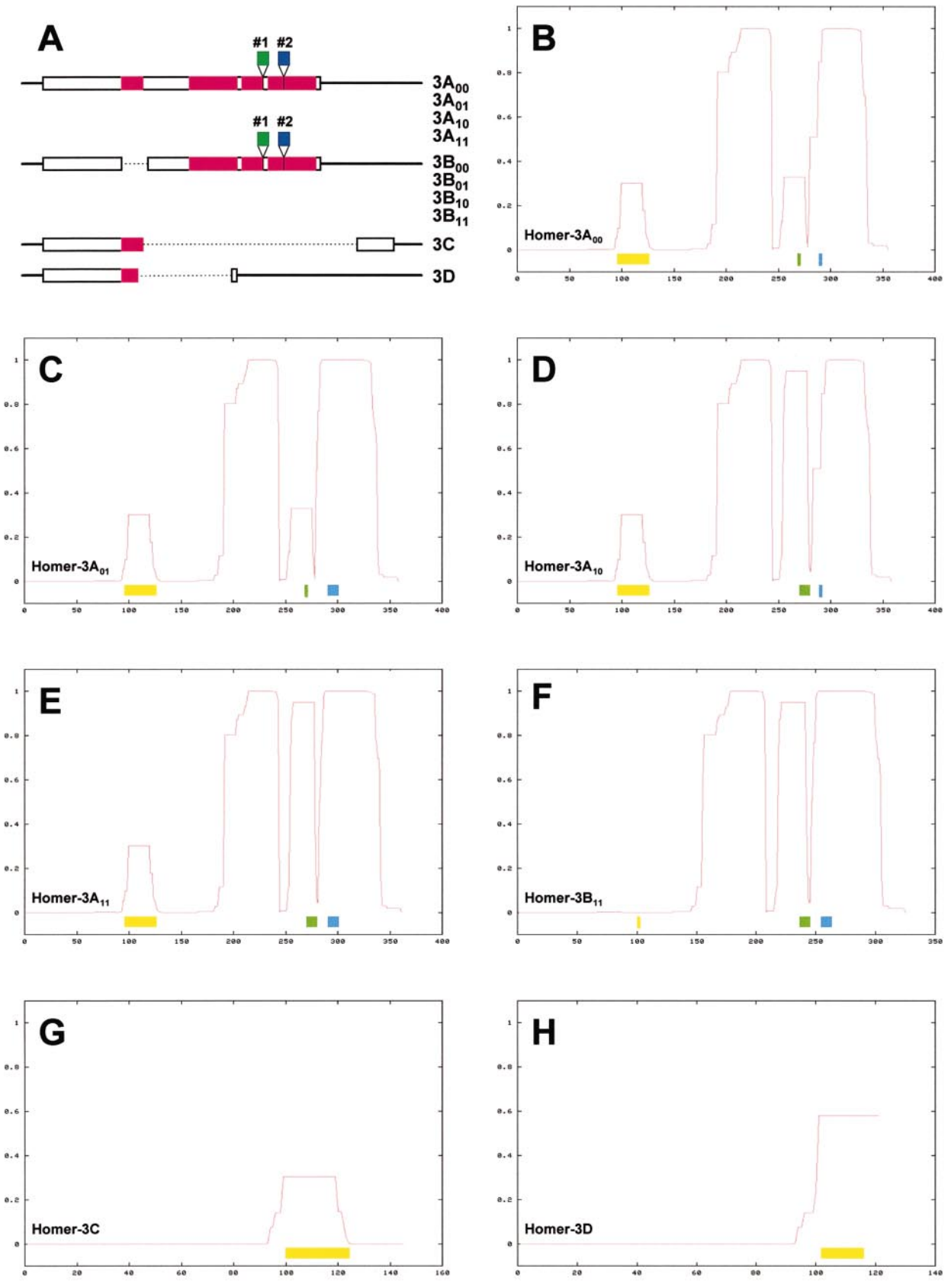

Figure 7 (*legend opposite*)

resulted in the abolition of most of the amplifications, indicative of strong RNA/DNA secondary structures within the Homer-3 nucleotide sequence. Sequencing of the amplified DNAs was carried out using ABI 373 and ABI 377 automated DNA sequencers. The EMBL accession numbers for the reported sequences are Y18894, Y18895, Y18896, Y19025, Y19026, Y19027 and Y19028. For expression studies the full-length coding sequences of the 343 amino acid residues long Homer-2A, the 171 amino acid residues long Homer-2C, the 358 amino acid residues long Homer-$3A_{01}$, the 145 amino acid residues long Homer-3C and the 121 amino acid residues long Homer-3D (all amplified from HEK-293 cells) and the 322 amino acid residues long Homer-$3B_{01}$ (amplified from HeLa cells) were cloned into the mammalian expression vector pcDNA3 (Invitrogen).

Flag-tagging of the Homer-3 proteins

Full-length Homer-3A, 3B, 3C and 3D proteins were N-terminally Flag-tagged. A cDNA coding for the Flag epitope was obtained by annealing of two synthetic oligonucleotides Flag-F (5′-AGCTATGGATTACAAAGACGATGACGATAAAC-3′) and Flag-R (5′-AATTGTTTATCGTCATCGTCTTTGTAATCCAT-3′). The obtained double-stranded DNA fragment (coding for Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was ligated between the *Bam*HI site of the pcDNA polylinker and *Eco*RI site (underlined in the sequence of the 3F1 primer, see above) of all of the Homer-3 expression constructs in frame with and immediately preceding the first methionine residue of the Homer-3 proteins (corresponding ATG codon is shown in bold in the sequence of the 3F1 primer, see above).

Mammalian cell culture and transfections

HEK-293 cells were grown in DMEM (Sigma) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin and 10% (v/v) foetal calf serum (FCS) at 37°C and in an atmosphere of 5% CO_2. Cells were passaged when 80-90% confluent. For the transient expression of proteins HEK-293 cells growing in 25 cm^2 dishes were transfected with mGluR1α/a cDNA (Ciruela *et al*., 1999d) in the absence or presence of Homer-2A, Homer-2C, Homer-$3A_{01}$, Homer-$3B_{01}$, Homer-3C and Homer-3D cDNAs by calcium phosphate precipitation (Jordan *et al*., 1996). pcDNA3 vector (Invitrogen) containing the LacZ reporter was used to maintain a constant amount of DNA in all co-transfections. The cells were harvested at 48 hours after transfection.

Antibodies

The primary antibodies were: affinity purified anti-mGluR1 antibody F1-Ab and F2-Ab (Ciruela & McIlhinney, 1997; Ciruela *et al*., 1999a), affinity purified anti-Homer polyclonal antibody VHr20 (Ciruela *et al*., 1999d) or affinity purified anti-Flag monoclonal antibody (Clone M2, Eastman Kodak Company). The secondary antibodies used were: horseradish-peroxidase(HRP)-conjugated swine anti-rabbit IgG (Dako); fluorescein(FITC)-conjugated affinity purified donkey anti-mouse IgG and Texas Red™ dye-conjugated affinity purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc).

Gel electrophoresis, immunoblotting and immunoprecipitation

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% (w/v) polyacrylamide gels (Laemmli, 1970) and proteins were immunoblotted to PVDF membranes (Immobilon-P, Millipore) using a semi-dry transfer system. After blocking with 5% (w/v) dry milk in PBS containing 0.05% Tween-20 (PBS-T), PVDF membranes were washed in PBS-T, incubated overnight at 4°C with anti-Homer polyclonal antibody VHr20 4 μg/ml in PBS-T-milk and developed with swine anti-rabbit (1:5000) secondary antibody conjugated to horseradish peroxidase using the enhanced chemiluminescence detection kit (Pierce), as described previously (Ciruela *et al*., 1999a).

For immunoprecipitation, HEK-293 cells were transiently transfected with 5 μg of the cDNA encoding mGluR1α/a plus 5 μg cDNA encoding either LacZ reporter or the Flag-tagged Homer-3A, Homer-3B, Homer-3C and Homer-3D by the calcium phosphate method and incubated during 48 hours before the experiment. Cells were solubilised in ice-cold lysis buffer (PBS, pH 7.4, containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholic acid and 0.1% (w/v) SDS) for one hour on ice. The solubilised preparations were then centrifuged at 80,000 *g* for 90 minutes and immunoprecipitated using 4 μl of the anti-Flag monoclonal antibody (Clone M2) each step of which was conducted with constant rotation at 0-4°C. After overnight incubation, 80 μl of protein G-Sepharose beads was added and then rotated for six hours as above. The beads were washed

Figure 7. Structure of the Homer-3 proteins. (a) Alternative splicing of the Homer-3 mRNAs. Boxes indicate coding sequences. Sequences coding for the predicted coiled-coil regions are indicated in red. The fragment coding for two insertions of three amino acid residues are shown in green (1) and blue (2). (b)-(h) The predicted coiled-coil domains in the Homer-3 proteins. In all plots the vertical axis indicates probability that sequence will adopt a coiled-coil conformation, the horizontal axis indicate the position in the amino acid sequence of the respective Homer proteins. Green bars show the positions of alternative splicing in (b) Homer-$3A_{00}$ and (c) Homer-$3A_{01}$, the green boxes indicate the three amino acid residues insertion in the sequences of (d) Homer-$3A_{10}$, (e) Homer-$3A_{11}$ and (f) Homer-$3B_{11}$. Blue bars show the positions of alternative splicing in (b) Homer-$3A_{00}$ and (d) Homer-$3A_{10}$, the blue boxes indicate the three amino acid residues insertion in the sequences of (c) Homer-$3A_{01}$, (e) Homer-$3A_{11}$ and (f) Homer-$3B_{11}$. Note that the C-terminal coiled-coil conformation is markedly affected by these insertions/deletions. The yellow boxes indicate the 36 amino acid residues fragment in the sequences of ((b) to (e)) Homer-$3A_{xx}$, (f) Homer-$3B_{11}$ and (g) Homer-3C, forming the N-terminal coiled-coil in these Homers, which is truncated in the sequence of the (h) Homer-3D or is absent in the sequence of the (f) Homer-$3B_{xx}$ because of alternative splicing.

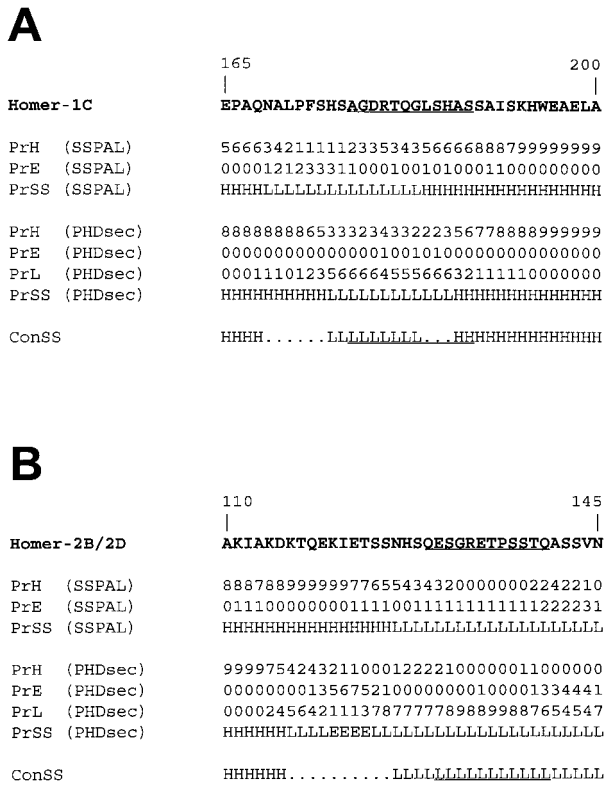

Figure 8. Secondary structure predictions for the Homer-1C and Homer-2B/2D proteins using programs SSPAL and PHDsec. (a) Fragment of the Homer-1C amino acid sequence. Position of the fragment is indicated above the sequence. The 12 amino acid residue fragment absent in the alternatively spliced Homer-1B protein is underlined as is the consensus secondary structure prediction for this fragment. (b) Fragment of the Homer-2B/2D amino acid sequence. Position of the fragment is indicated above the sequence. The 11 amino acid residue fragment absent in the alternatively spliced Homer-2A/2C proteins is underlined as is the consensus secondary structure prediction for this fragment. In (a) and (b) PrH denotes probability for assigning helical conformation; PrE, probability for assigning extended (sheet) conformation; PrL, probability for assigning loop conformation; PrSS, program output (the prediction); H denotes helix; E, sheet; L, loop; ConSS represents the consensus prediction from the two programs used.

twice with ice-cold lysis buffer, twice with ice-cold lysis buffer containing 0.1% (v/v) Nonidet P-40, 0.05% (w/v) sodium deoxycholic acid and 0.01% (w/v) SDS, once with ice-cold phosphate buffered saline (pH 7.4), and aspirated to dryness with a 28-gauge needle. Subsequently, 60 µl of SDS-PAGE sample buffer was added to each sample. Immune complexes were dissociated by incubation at 100°C for five minutes and resolved by SDS-PAGE in SDS-6% PAGE gels. The gels were run and immunoblotted as described above using F2 anti-mGluR1α/a antibody (5 µg/ml).

Immunofluorescence

For immunofluorescence staining HEK-293 cells grown on glass cover-slips were transiently transfected and incubated for 48 hours as described above. Cells were then rinsed in PBS, fixed in 4% paraformaldehyde in PBS for 15 minutes and washed in buffer A (PBS containing 20 mM glycine) to quench the aldehyde groups. Cells were permeabilised with buffer A containing 0.2% Triton X-100 for five minutes. Following 30 minutes incubation in buffer A, containing 1% BSA (buffer B), cells were incubated in buffer B (one hour at room temperature) with a mixture of anti-Flag monoclonal antibody M2-Ab (10 µg/ml) and anti-Homer polyclonal antibody VHr20 (5 µg/ml) (when staining Flag-mGluR1α/a and Homer-2 co-transfected cells) or with anti-mGluR1 antibody F1-Ab (5 µg/ml) and anti-Flag monoclonal antibody M2-Ab (10 µg/ml) (when staining mGluR1α/a and Flag-Homer-3 co-transfected cells). Following the incubation with the primary antibody, cells were washed and stained with fluorescein-conjugated donkey anti-mouse IgG antibody (1/50) and Texas red-conjugated donkey anti-rabbit IgG antibody (1/50). The coverslips were rinsed for 30 minutes in buffer B and mounted with Vectashield immunofluorescence medium (Vector Laboratories, UK). Confocal microscope observations were made with a Leica TCS NT (Leica Lasertechnik GmbH, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope.

Protein structure analysis

Protein secondary structures were analysed using two programs based on different prediction approaches: PHDsec with multiple alignment input (a neural-network based method, rating at an expected average accuracy ~72% for the three states helix, strand and loop when using multiple alignment input) (Rost & Sander, 1993a,b, 1994) and SSPAL (local alignments based method, rating at ~71% accuracy without using multiple alignment input) (Salamov & Solovyev, 1995, 1997). The analysis was performed online by using the PredictProtein server (Columbia University, USA) available at the http://dodo.cpmc.columbia.edu (PHDsec) or the protein sequence analysis tools available at the Computational Genomics Group (The Sanger Centre, UK) at the http://genomic.sanger.ac.uk (SSPAL). The analysis of protein sequences for the presence of coiled-coil domains was performed using program COILS (window size = 21, matrix = MTK) (Lupas, 1996b; Lupas *et al.*, 1991; Parry, 1982) available from the EMBnet server (Institute for Experimental Cancer Research ISREC, Switzerland) at the http://www.ch.embnet.org. The presence and location of the proteolytic signals in Homer-2 and Homer-3 proteins (PEST sequences) were predicted by using PESTfind Analysis software (Rechsteiner *et al.*, 1987; Rechsteiner & Rogers, 1996; Rogers *et al.*, 1986) available from the EMBnet Austria server (The Department for Biocomputing of the Vienna University Computer Centre VUCC, Austria) at the http://emb1.bcc.univie.ac.at/embnet/tools.

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