The members of the low density lipoprotein (LDL) receptor gene family bind a broad spectrum of extracellular ligands. Traditionally, they had been regarded as mere cargo receptors that promote the endocytosis and lysosomal delivery of these ligands. However, recent genetic experiments in mice have revealed critical functions for two LDL receptor family members, the very low density lipoprotein receptor and the apoE receptor-2, in the transmission of extracellular signals and the activation of intracellular tyrosine kinases. This process regulates neuronal migration and is crucial for brain development. Signaling through these receptors requires the interaction of their cytoplasmic tails with the intracellular adaptor protein Disabled-1 (DAB1). Here, we identify an extended set of cytoplasmic proteins that might also participate in signal transmission by the LDL receptor gene family. Most of these novel proteins are adaptor or scaffold proteins that contain PID or PDZ domains and function in the regulation of mitogen-activated protein kinase signaling, vesicle trafficking, or neurotransmission. We show that binding of DAB1 interferes with receptor internalization suggesting a mechanism by which signaling through this class of receptors might be regulated. Taken together, these findings imply much broader physiological functions for the LDL receptor family than had previously been appreciated. They form the basis for the elucidation of the molecular pathways by which cells respond to the diversity of ligands that bind to these multifunctional receptors on the cell surface.

The low density lipoprotein (LDL)

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1 The abbreviations used are: LDL, low density lipoprotein; apoE, apolipoprotein E; apoER2, apoE receptor-2; APP, amyloid precursor protein; GST, glutathione-S-transferase; JNK, c-Jun N-terminal kinase; LRP, LDL receptor-related protein; MAP kinase, mitogen-activated protein kinase; PBS, phosphate buffered saline; PID, protein interaction domain; PTB, phosphotyrosine binding; RGS, regulation of G-protein signaling; VLDL, very low density lipoprotein; PCRII, polymeric chain reaction; nNOS, neuronal nitric-oxide synthase; CHO, Chinese hamster ovary.
The developmental phenotypes that are caused by genetic defects of LDL receptor family members and the role the receptors may play in the development of Alzheimer’s disease are difficult to explain on the basis of impaired endocytosis of extracellular ligands alone. Rather, they suggest that these genes fulfill broad and essential roles in cellular communication such as in, but not limited to, the transmission of migratory cues in the case of Reelin, DAB1, VLDL receptor, and apoER2. It is thus likely that these genes interact with other components of the cellular signal transduction machinery besides DAB1. To allow diverse signals to be routed through a single receptor, specificity of signal transduction has to be ensured by other means. One way that nature has achieved this is through the assembly of multiprotein complexes (12). Thus, a receptor may bind multiple signaling molecules, yet activation of the appropriate intracellular signaling pathway would be dependent on the other components, e.g., coreceptors and adaptor or scaffold proteins, in the complex. Furthermore, it is unclear whether cytoplasmic adaptors that bind to the tails of LDL receptor family members interact or interfere with the endocytosis machinery. To address these questions we have studied for other cytoplasmic proteins that bind tightly to LDL receptor family tails. Our findings suggest that the role of the LDL receptor gene family in cellular signaling is not restricted to the transmission of the Reelin signal to cellular tyrosine kinases but may also involve modulation of MAP kinase activity and cell adhesion. We have investigated whether the endocytosis machinery and cellular signaling by DAB1 employ the same or overlapping sequence motifs by overexpressing DAB1 in cultured cells and determining its effect on the cellular binding and endocytosis of LDL. For the LDL receptor example, we show that adaptor binding to the tail can compete with the endocytosis machinery. This suggests a molecular mechanism by which initiation of endocytosis may serve to turn off periodically cellular signals that are routed through this class of multifunctional cell surface receptor proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and other DNA-modifying enzymes (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Roche Molecular Biochemicals and New England Bio- lysics (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Roche Molecular Biochemicals and New England Bio- lysics (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Roche Molecular Biochemicals and New England Bio- lysics (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Roche Molecular Biochemicals and New England Bio- lysics (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Roche Molecular Biochemicals and New England Bio- lysics (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Roche Molecular Biochemicals and New England Bio- lysics (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Roche Molecular Biochemicals and New England 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Identification of proteins that interact with cytoplasmic domains of the LDL receptor gene family

| Name            | Clones | Independent | Insert size | Function/pathway                  |
|-----------------|--------|-------------|-------------|-----------------------------------|
| SEMCAP-1        | 5      | 2           | 1500        | G-protein signaling; Vesicular trafficking? |
| JIP-1           | 2      | 2           | 1300        | MAP kinase scaffold               |
| PSD-95          | 3      | 1           | 800         | Synaptic organization             |
| JIP-2           | 2      | 2           | 1300        | MAP kinase scaffold               |
| Talin homologue | 2      | 1           | 1500        | Cytoskeleton                      |
| OMP25           | 2      | 2           | 1500        | Cytoskeletal attachment           |
| CAPON           | 1      | 1           | 1200        | NO signaling                      |
| DAB1            | 1      | 1           | 3000        | Neuronal migration                |
| ICP-1           | 2      | 1           | 800         | Integrin signaling                |
| MINT2           | 2      | 1           | 1500        | Synaptic vesicle docking          |
| PIP4,5-Kinase homologue | 2    | 1           | 700         | Unknown                            |
| Sodium channel brain 3 | 1  | 1           | 900         | Ion channel                        |
| APC subunit 10  | 2      | 2           | 1000        | Cell division                      |

| Lipoprotein Receptors and Signal Transduction |

Various complete and truncated cytoplasmic domains of the LDLR gene family were used to screen yeast two-hybrid libraries. Positive clones contained protein sequences that relate LRP and Megalin to signal transduction via kinases, G-proteins, ion channels, NO, the cytoskeleton, and synapses. PIP4,5-kinase is involved in phosphorylation of 4,5-phosphate of the cytoskeleton. Its PTB domain is associated with neuritic plaques in Alzheimer's disease (22). CAPON is a PTB domain-containing adaptor protein that is involved in binding neuronal nitric-oxide synthase (nNOS) and is thought to dislodge nNOS from the postsynaptic density protein PSD-95, thus inactivating the enzyme (23). PSD-95, a scaffolding protein that organizes active components of the postsynaptic neurotransmission machinery such as glutamate receptors, potassium channels, kinases, and nNOS into functional microdomains (24) was also found to interact directly with LDL receptor family tails. Although it does not contain a PTB domain, the integrin cytoplasmic domain associated protein-1 (ICAP-1) binds to an NPYX sequence motif in the integrin tail (25, 26) and thus presumably also to the NPYX motifs that are present in the cytoplasmic tails of all LDL receptor family members (2). OMP25 is an outer mitochondrial membrane protein that contains a PDZ domain and thereby interacts with the inositol phosphate synthase synaptojanin, a protein thought to be involved in the recycling of synaptic vesicles (27). Another protein that is homologous to phosphatidylinositol 4,5-kinase and therefore is presumably involved in inositol metabolism was also identified in our screen. A close homologue of the cytoskeletal protein Talin, the alpha subunit of the brain-specific sodium channel 3 and APC10, a component of the anaphase promoting complex also bound to LDL receptor family tails by yeast-two-hybrid interaction.

Next, we determined the binding specificity of each of the proteins that had been identified in the initial screens against a panel of bait constructs containing the whole or part of the cytoplasmic tails of the presently known LDL receptor family members. The tail sequences contained in these bait constructs are shown in Fig. 1A. The apoER2 tail was tested with (+) and without (−) its alternatively spliced insert. For Megalin and for LRP the complete tails and parts thereof containing a single NPYX motif were tested separately. DAB1 bound strongly to all the LDL receptor family tails but only to one of the NPYX motifs in the LRP and Megalin tail (LRP and MegA, respectively). LRP and Megalin have the longest cytoplasmic tails that contain several potential adaptor binding motifs and thus bound most of the proteins. In contrast, the LDL receptor and the VLDL receptor bound only DAB1, as noted previously (2, 3). Interestingly, the apoER2 tail containing the alternatively spliced insert bound the scaffold proteins JIP-1, JIP-2, and PSD-95, whereas the tail without this insert did not. This suggests that the alternative splicing of the apoER2 tail has important regulatory functions and may determine the ability of apoER2 to activate MAP kinase-dependent signals. A detailed account of the binding properties of JIP-1 and JIP-2 to the apoER2 tail and the importance of the alternatively spliced insert...
is given in the accompanying paper by Stockinger et al. (38).

Next, we sought to determine whether the protein interactions we had found in the yeast two-hybrid screen could be reproduced by a different approach, such as a biochemical pulldown assay (Fig. 2). The respective cDNA fragments were cut out from the yeast prey vector and cloned into a bacterial GST expression vector. GST fusion proteins were incubated with membrane extracts from liver (LRP) and kidney (Mega-

![FIG. 2. Interaction of cytoplasmic proteins with LRP and Megalin in a GST pulldown assay.](image)

membrane extracts from 129SvJ mouse liver (LRP) and kidney (Mega-

![FIG. 1. Yeast two-hybrid analysis of proteins interacting with the cytoplasmic tails of LDL receptor family members. A, amino acid sequences of cytoplasmic tails, tail fragments, and alternative splice forms used for the yeast mating assay. NPYX motifs (green), the alternatively spliced insert of apoER2 (red), PDZ recognition site (DSEV) (blue). B, prey constructs interact differentially with complete or partial cytoplasmic tails of the LDL receptor gene family. By a yeast mating assay all bait/prey interactions could be verified, with the exception of sodium channel 3a and APC subunit 10. For yeast mating analysis, clones containing bait, prey, and the reporter plasmid p8op-lacZ were selected on Trp(−), Ura(−), His(−), plates. Three individual clones were transferred to patches on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) plates deficient in Trp, Ura, His, and Leu. Growth and blue staining was graded after 3 days at 30 °C (no growth, −; weak growth and staining +, ++, or +++). Shaded areas in the table show strong interactions. The LRP tails containing the second NPYX motif and the apoER2 tail containing the insert showed some degree of self-activation but nevertheless allowed grading of the interaction. Negative controls are empty vector (LexA and pB42AD). DAB1 had previously been found to bind to LDLR and LRP and served as a positive control.

![FIG. 1. Yeast two-hybrid analysis of proteins interacting with the cytoplasmic tails of LDL receptor family members. A, amino acid sequences of cytoplasmic tails, tail fragments, and alternative splice forms used for the yeast mating assay. NPYX motifs (green), the alternatively spliced insert of apoER2 (red), PDZ recognition site (DSEV) (blue). B, prey constructs interact differentially with complete or partial cytoplasmic tails of the LDL receptor gene family. By a yeast mating assay all bait/prey interactions could be verified, with the exception of sodium channel 3a and APC subunit 10. For yeast mating analysis, clones containing bait, prey, and the reporter plasmid p8op-lacZ were selected on Trp(−), Ura(−), His(−), plates. Three individual clones were transferred to patches on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) plates deficient in Trp, Ura, His, and Leu. Growth and blue staining was graded after 3 days at 30 °C (no growth, −; weak growth and staining +, ++, or +++). Shaded areas in the table show strong interactions. The LRP tails containing the second NPYX motif and the apoER2 tail containing the insert showed some degree of self-activation but nevertheless allowed grading of the interaction. Negative controls are empty vector (LexA and pB42AD). DAB1 had previously been found to bind to LDLR and LRP and served as a positive control.](image)
vivo. In the case of DAB1, there is strong genetic and biochemical evidence that binding of this protein to the tails of the VLDL receptor and apoER2 is absolutely required for the transmission of a critical developmental signal to migrating neurons (2, 3, 10). However, as we have shown, DAB1 not only interacts with these two receptors but, in fact, bound tightly to the tails of all known members of the LDL receptor gene family. Whether these interactions occur only under in vitro conditions or also take place in vivo is not known. To address this question we chose to examine the effect of DAB1 expression on LDL receptor-mediated endocytosis in cultured cells (Fig. 3). There is currently no indication that DAB1 binding to the LDL receptor tail has a critical physiological function in vivo. However, the similarity of the NPXY motif that mediates both DAB1 binding and receptor internalization in the VLDL receptor, apoER2 and LDL receptor, as well as the availability of firmly established quantitative methods by which LDL receptor endocytosis can be studied, led us to choose this receptor as the experimental model system. Chinese hamster ovary (CHO) cells expressing the wild type human LDL receptor with (lanes 1 and 2) or without (lanes 2 and 4) its cytoplasmic tail were transfected (lanes 3 and 4) or not transfected (lanes 1 and 2) with a DAB1 expression plasmid. Protein expression was detected by immunoblotting with the indicated antibody. B, specific binding, uptake, and degradation of $^{125}$I-LDL by CHO cells expressing (open symbols) or not expressing (closed symbols) DAB1 was determined as described under “Experimental Procedures.” Cells were seeded in 6-well plates at a cell density of 60,000 cells/well in Medium A (1:1 mixture of Dulbecco’s minimal essential medium and Ham’s F-12 medium) supplemented with penicillin/streptomycin and 5% (v/v) fetal calf serum. On day 2, cells were washed twice with phosphate-buffered saline (PBS) and refed with Medium A containing 5% (v/v) newborn calf lipoprotein-deficient serum, 10% serum. On day 3 the cells were washed twice with PBS before the addition of Medium B containing 2 μg/ml $^{125}$I-LDL (specific activity 1000–1500 cpm/ng). Cells and medium were analyzed as described under “Experimental Procedures.” The experiment was performed in triplicate and repeated three times with similar results.

**Fig. 3. Effect of DAB1 on binding, uptake, and degradation of LDL by the LDL receptor.** A, immunoblot of CHO cells. CHO cells expressing human LDL receptor with (lanes 1 and 3) or without (lanes 2 and 4) its cytoplasmic tail were transfected (lanes 3 and 4) or not transfected (lanes 1 and 2) with a DAB1 expression plasmid. Protein expression was detected by immunoblotting with the indicated antibody. B, specific binding, uptake, and degradation of $^{125}$I-LDL by CHO cells expressing (open symbols) or not expressing (closed symbols) DAB1 was determined as described under “Experimental Procedures.” Cells were seeded in 6-well plates at a cell density of 60,000 cells/well in Medium A (1:1 mixture of Dulbecco’s minimal essential medium and Ham’s F-12 medium) supplemented with penicillin/streptomycin and 5% (v/v) fetal calf serum. On day 2, cells were washed twice with phosphate-buffered saline (PBS) and refed with Medium A containing 5% (v/v) newborn calf lipoprotein-deficient serum, 10 μg/ml compactin, and 100 μg/ml mevalonate. On day 3 the cells were washed twice with PBS before the addition of Medium B containing 2 μg/ml $^{125}$I-LDL (specific activity 1000–1500 cpm/ng). Cells and medium were analyzed as described under “Experimental Procedures.” The experiment was performed in triplicate and repeated three times with similar results.
functions. At E13.5 apoER2 (Fig. 4A) was expressed throughout the brain, in a pattern identical to our previously published data (3). This pattern was almost identical to that of JIP-1 (Fig. 4E) and JIP-2 (Fig. 4F). SEMCAP-1 also showed a largely overlapping expression pattern. Notably, JIP-1 and JIP-2 were predominantly expressed in the more superficial cortical plate, whereas SEMCAP-1 was mainly expressed in the subventricular zone and in the population of migrating neurons in the developing cortex. apoER2 was present throughout the cortex. In contrast, LRP expression in the developing brain was comparatively low and restricted mainly to the choroid plexus (Fig. 4B), and Megalin was almost exclusively expressed in the ventricular zone (Fig. 4C). SEMCAP-1 and JIP-1 were expressed in the same structures as LRP and Megalin.

DISCUSSION

We have used a yeast two-hybrid screening approach to identify novel components of signaling complexes that assemble on the cytoplasmic tails of the members of the LDL receptor gene family. We have found 11 novel cytoplasmic proteins that interacted with receptor tails in the yeast two-hybrid assay, and 7 of these interactions were confirmed using an in vitro GST pulldown assay. The proteins we have found can be grouped by their known or suspected functions. These include regulation of synaptic transmission, activation and modulation of MAP kinase pathways, local organization of the cytoskeleton, cell adhesion, and endocytosis. A summary of the specificity of interaction of these proteins with individual receptors is shown in Fig. 5A.

Not all protein-protein interactions that were identified in the yeast two-hybrid screen could be reproduced in the GST-pulldown assay. A reason for this may be the sensitivity of the yeast two-hybrid assay, which is much higher than that of the GST pulldown approach. It will thus detect low affinity/high off-rate interactions that cannot be shown by the other conven-
Nevertheless, such transient interactions cannot a priori be discounted as physiologically irrelevant. They may even be desirable in the dynamic context of the assembly and disassembly of signaling complexes by which cells rapidly adapt to changes in the environment. In a dimensionally restricted space, such as the inner surface of the plasma membrane, recruitment of receptor and scaffold proteins into macromolecular complexes would further drive the binding reaction due to high local protein concentrations.
Two kinds of protein domains that are well known to mediate protein-protein interactions are present in most of the genes we have identified in our screen (Fig. 5B). PTB domains are present in JIP-1, JIP-2, and CAPON. It is presumably this domain that, as in DAB1, mediates the binding of these proteins to NPXY sequence motifs in the cytoplasmic tails. Although ICAP-1 does not contain a recognizable PTB domain, it nevertheless binds to the tails of apoER2, LRP, and Megalin. This binding is likely specific as ICAP-1 was originally identified because it binds to the NPXY motif in the β1 integrin tail (26).

Binding of ICAP-1 and the interactions with a Talin-related protein that presumably also links membrane receptors to the actin cytoskeleton (Table I) suggest a function of the LDL receptor family in cell adhesion. Thrombospondin is a ligand for β1 integrins that is secreted by many cell types and has well documented roles in cell adhesion and angiogenesis (28). It also binds to LRP, integrin-associated protein (29), and another lipoprotein receptor, CD36 (28). Thrombospondin modulates focal adhesion contacts and stimulates cellular signaling pathways involving tyrosine kinases, MAP kinases and G-proteins (29, 30). Binding to β1 integrin, integrin-associated protein, and LRP involves different sites on the protein. Thrombospondin could thus ligate the extracellular domains of LDL receptor family members to integrins, thereby promoting an interaction between the distinct cytoplasmic protein complexes bound to their respective tails. Such a model is analogous to one that is currently proposed for the activation of the Reelin signaling pathway, which is thought to involve Reelin-mediated clustering of VLDL receptor, apoER2, and cadherin-like neuronal receptors (3, 31).

JIP-1 and JIP-2 are scaffolding proteins that promote the assembly and activation of several components of the JNK signaling pathway, specifically JNK, MAP kinase kinase 7, and mixed lineage kinase 3 (18). An interaction of JIP-1 with rho-GEF, an exchange factor for the small GTPase RhoA, which is involved in the rearrangement of the actin cytoskeleton (19), has also recently been reported. A functional interaction of MAP kinases and Rho proteins with LDL receptor family members is consistent with their demonstrated role in cell migration and organization of the cytoskeleton and could explain the Reelin-independent distinct phenotypes of mice lacking the VLDL receptor or the apoER2 (3).

Another type of protein interaction domain that is present in several of the tail-binding proteins uncovered in this screen is the PDZ domain. Typically, PDZ domains bind to C termini exhibiting the consensus sequence ((S/T)XV); however, in many cases PDZ domain interactions have been found that do not conform to this strict consensus motif. For instance, the PDZ domain of SEMCAP-1 (also known as GIPC) binds to the C terminus of the RGS protein GAIP, which ends on an alanine residue (21). In other cases, PDZ domains were shown to interact with sequences embedded in a polypeptide chain (32). The PDZ domain containing proteins SEMCAP-1, PSD-95, and OMP25 all specifically to the C-terminal third of the Megalin tail, which contains a perfect PDZ motif (SEV) (Fig. 1). However, they also interacted with the C-terminal half of the LRP tail, which does not conform to this consensus, and with the alternatively spliced insert of the apoER2 tail, suggesting that the interactions were mediated by an integral sequence motif.

PSD-95 is a scaffolding protein that interacts with numerous components in the postsynaptic density that are involved in neurotransmission (24). Interestingly, the PTB domain protein CAPON, which binds to the Megalin and LRP tails, binds the PDZ domain of the calmodulin-dependent nNOS at its C terminus (23). Interaction of nNOS with CAPON is thought to displace nNOS from PSD-95. This would remove the enzyme from the vicinity of glutamatergic calcium channels where calcium influx leads to nNOS activation in the wake of synaptic signal transmission (32). However, PSD-95 and CAPON may bind to the LRP and Megalin tails independently through interactions with PTB and PDZ domains, respectively. This could recruit CAPON-bound nNOS to calcium channels in the specific context of the complex that has assembled on the receptor tail. In fact, a role of LRP in synaptic signal transmission has been proposed by Zhuo et al. (33), who suggested that LRP is important for long term potentiation. Our identification of postsynaptic adaptor and scaffold proteins that interact with LDL receptor family tails and also with components of the synapse that are directly involved in neurotransmission provides a structural basis for this finding. Consistent with such a model are the observations by Stockinger et al. (34) and our own unpublished observations that localize LRP to the postsynaptic density by immunofluorescence and immunoelectron microscopy.

The physiological functions of SEMCAP-1 are currently unknown. This protein contains a single PDZ domain and an acyl carrier domain at its C terminus (21). Interactions with several proteins, including the axonal guidance molecule SemF (20) and the RGS protein GAIP (21), have been described. SEMCAP-1 and the closely related SEMCAP-2 (20) are widely expressed. In neurons and in HeLa cells, SEMCAP-1 localizes to a vesicular compartment close to the plasma membrane. This pattern resembles the subcellular localization of LRP in neurons (11) and raises the possibility that SEMCAPs may be involved in the vesicular trafficking of LDL receptor family members. The association of SEMCAP-1 with an RGS protein on the one hand and LDL receptor family members on the other may also point to a possible role of these receptors in G-protein-mediated signaling events. This hypothesis is strengthened by the findings of Goretzki and Müller (35) who described a functional association of LRP with G-protein-coupled signal transduction pathways.

Another protein that tightly binds to LDL receptor family tails is OMP25. Although the relevance of this binding is currently unclear, it may be related to the vesicular transport of the receptors. Omp25 has also been shown to interact with synaptophysin, an inositol 5'-phosphatase that functions in synaptic vesicle recycling (27). LRP and Megalin independently interact with a potential phosphatidylinositol 4,5-kinase suggesting that the receptor tails may participate in the local metabolism of inositol phosphate in vesicles or in the plasma membrane. Consistent with this are recent reports (36, 37) that suggest that phosphatidylinositol 4,5-bisphosphate regulates the adhesion energy between the cytoskeleton and the plasma membrane and that the OMP25-binding protein synaptophysin is necessary for synaptic vesicle recycling.

Finally, our finding that DAB1 binding to receptor tails competes with their clustering into coated pits and endocytosis suggests an analogous mechanism by which conversely signaling through LDL receptor family members could be turned off periodically. In such a model, assembly of the endocytosis complex on the tail would displace other scaffold and adaptor proteins that may be engaged in signaling, resulting in the recycling of a “purged” receptor to the cell surface, where it could then reenter into a new round of signal transmission.

The studies we have presented in this paper have revealed interactions of a much broader range of cytoplasmic adaptor and scaffold proteins with the LDL receptor gene family than had previously been recognized. Taken together with the avail-

A. Rohllmann, M. Gotthardt, and J. Herz, unpublished observations.
able genetic and biochemical evidence that shows central functions of this gene family in cell migration and tyrosine kinase signaling (2, 3, 9, 10, 31), the present findings strongly suggest that the LDL receptor gene family functions directly in mediating or modulating cell adhesion and the reorganization of the actin cytoskeleton. Deciphering the exact nature of the complexes that assemble on the receptor tails and that interact with various parts of the cytoskeleton and the cellular signal transduction machinery will significantly reshape our current view of the biological roles this gene family has played throughout evolution.

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