

The Junction-associated Protein AF-6 Interacts and Clusters with Specific Eph Receptor Tyrosine Kinases at Specialized Sites of Cell–Cell Contact in the Brain

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Abstract. The AF-6/afadin protein, which contains a single PDZ domain, forms a peripheral component of cell membranes at specialized sites of cell–cell junctions. To identify potential receptor-binding targets of AF-6 we screened the PDZ domain of AF-6 against a range of COOH-terminal peptides selected from receptors having potential PDZ domain-binding termini. The PDZ domain of AF-6 interacts with a subset of members of the Eph subfamily of RTKs via its COOH terminus both in vitro and in vivo. Cotransfection of a green fluorescent protein-tagged AF-6 fusion protein with full-length Eph receptors into heterologous cells induces a clustering of the Eph receptors and AF-6 at sites of cell–cell contact. Immunohistochemical analysis in the adult rat brain reveals coclustering of AF-6 with

Eph receptors at postsynaptic membrane sites of excitatory synapses in the hippocampus. Furthermore, AF-6 is a substrate for a subgroup of Eph receptors and phosphorylation of AF-6 is dependent on a functional kinase domain of the receptor. The physical interaction of endogenous AF-6 with Eph receptors is demonstrated by coimmunoprecipitation from whole rat brain lysates. AF-6 is a candidate for mediating the clustering of Eph receptors at postsynaptic specializations in the adult rat brain.

Key words: postsynaptic clustering • PDZ domains • receptor tyrosine kinases • neuron physiology • Ras-binding protein

MANY cellular processes rely in large part on the correct subcellular distribution of effector proteins. At specialized sites of cell–cell contact, membrane proteins are clustered with specific adaptor proteins linked to the cytoskeleton, enabling cellular adhesion, motility, and intercellular as well as intracellular signaling events to occur (Kofron et al., 1997; Tsukita et al., 1996, 1997). Some proteins, which are localized to specific cell junctions and presumably influence their function, have been identified. In epithelial cells, the specialized site of cell contact known as the tight junction (*zonula occludens*) has been shown to be composed of transmembrane proteins such as occludin (Furuse et al., 1993) and juxtamembrane proteins such as ZO-1 (Stevenson et al.,

1986), ZO-2 (Jesaitis and Goodenough, 1994), ZO-3 (Haskins et al., 1998), cingulin (Citi et al., 1988), 7H6 (Zhong et al., 1993), and AF-6 (Mandai et al., 1997; Yamamoto et al., 1997).

In the nervous system specialized sites of cell–cell contact are vital for ensuring growth cone pathfinding during embryogenesis and neuronal signaling and information processing at developed synapses. Voltage-gated and ligand-gated ion channels are clustered at specific membrane sites at synapses in neuronal cells facilitating the coordinated transmission of electrical signals (Froehner, 1993). Shaker-type K⁺ channel and *N*-methyl-D-aspartate (NMDA)¹ receptor 2 (NR2) subunits are localized specifi-

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1. *Abbreviations used in this paper:* aa, amino acid; FLAG AF-6 PDZ, FLAG epitope-tagged AF-6 PDZ domain construct; GFP, green fluorescent protein; GST, glutathione-S-transferase; MAGUK, membrane-associated guanylate kinase; NGS, normal goat serum; NMDA, *N*-methyl-D-aspartate; NR2, NMDA receptor 2; PB, phosphate buffer; RIPA, radioimmunoprecipitation assay; RT, room temperature; RTK, receptor tyrosine kinase; SAP, synapse-associated protein.

cally at presynaptic and postsynaptic sites, respectively (Kim et al., 1995; Kornau et al., 1995). Recently, a subfamily of the membrane-associated putative guanylate kinases (MAGUKs) comprised of PSD-95/SAP90 has been shown to bind directly to these ion channels and colocalize with them at synapses in the brain (Hunt et al., 1996; Kim et al., 1995; Kornau et al., 1995; Niethammer et al., 1996). The interaction is mediated by the direct association of the COOH terminus of the ion channel receptors with the NH₂-terminal two PDZ domains present in PSD-95/SAP90. PDZ domain-containing proteins, such as that exemplified by the PSD-95 subfamily of MAGUKs, are thought to function as clustering proteins at specific synaptic membranes on the surfaces of neurons (Gomperts, 1996). In addition, these proteins may organize overall synaptic structures, since perturbations in the *Drosophila* homologue of PSD-95, discs large (DlgA), lead to aberrant synaptic structures in the fly nervous system (Lahey et al., 1994).

A number of the juxtamembrane proteins identified as components of specialized junctions in epithelial and endothelial cells such as ZO-1, ZO-2, ZO-3, and AF-6 also have PDZ domains and therefore may also function as clustering agents for as yet unidentified receptor proteins. To identify potential receptor targets of one of these proteins, AF-6, we screened the database for receptors having potential PDZ interacting termini and tested whether these termini could interact with the PDZ domain of AF-6.

We observed that AF-6 is able to interact with a specific subset of the Eph RTK proteins. Two-hybrid and in vitro binding assays confirmed that the AF-6 PDZ domain interacts specifically with the COOH terminus of the Eph receptors. Cotransfection of AF-6 with the Eph receptors in heterologous cells induces a clustering of AF-6 with the Eph receptors at sites of cell-cell contact. AF-6 is phosphorylated when cotransfected with Eph receptors but not with a kinase-deficient mutant, demonstrating that AF-6 is a substrate of Eph receptors.

Eph receptor tyrosine kinases (RTKs) are highly expressed in the nervous system (Orioli and Klein, 1997) and some can be localized to specific membrane regions of cell-cell contact on neurons (Henkemeyer et al., 1994). It is demonstrated by electron microscopy that both, AF-6 and the receptors, tightly colocalize at postsynaptic membranes of excitatory synapses in the hippocampus and at other sites of membrane specialization on neurons. This observation is further corroborated by showing that endogenous AF-6 physically interacts with Eph receptors in whole rat brain extracts.

Materials and Methods

Mammalian Two-hybrid Analysis

A partial clone of mouse AF-6 (amino acids [aa] 850–1,129) encompassing the PDZ domain was subcloned into the VP16 activation domain expression vector pSNATCH (Buchert et al., 1997). Oligonucleotide adaptors encoding the last 10 amino acids of the COOH termini of the various RTKs as listed in Fig. 1 were then cloned into the Gal4 DNA-binding domain mammalian expression vector pSNAG (Buchert et al., 1997). Human 293T cells from a 12-well tissue culture plate were transfected with 20 ng of the expression plasmids along with 50 ng of a chloramphenicol acetyltransferase (CAT) reporter gene construct and the cells were harvested 36–48 h later. A CAT enzyme-linked immunosorbent assay

(ELISA) was performed according to the manufacturer's instructions (Boehringer Mannheim).

In Vitro and Coimmunoprecipitation Assays

293T cells were transiently transfected separately with expression vectors encoding the full-length receptors, EphA7 (MDK1), EphB3 (Hek2), EphB2 (Nuk), and Ephrin-B1 (Xlerk2) along with a partial, FLAG-tagged clone of the mouse AF-6 (aa 850–1,129) encompassing the PDZ domain. 48 h after transfection cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 2.8 µg/ml aprotinin) and placed on ice for 30 min with occasional shaking. Samples were pelleted for 10 min at 4°C at 10,000 g. The supernatant was then precleared with protein A-Sepharose beads and the precleared supernatant incubated with 1 µl (2.7 µg) anti-FLAG M2 antibody (Eastman-Kodak) and incubated with rotation at 4°C for 1 h. Protein A-Sepharose beads (50 µl) were then added to the cell extracts and incubated with rotation at 4°C for a further 45 min. Cell extracts were then pelleted and washed four times with 1 ml of buffer A and resuspended in loading buffer, run on 10% SDS-PAGE gels, and then blotted as described (Harlow and Lane, 1988). Blots were probed with the anti-receptor-specific antibodies. For glutathione-S-transferase (GST)-pull down experiments the partial clone of the mouse AF-6 (aa 850–1,129) encompassing the PDZ domain was fused COOH-terminal to GST and produced and coupled to glutathionine beads as described (Ridley and Hall, 1992), except after binding, beads were washed five times in lysis buffer; PBS, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 2.8 µg/ml aprotinin. The green fluorescent protein (GFP)-peptide (EphB3pep) was visualized on the blots with a monoclonal anti-GFP antibody (Clontech) as recommended by the manufacturer.

Phosphorylation of AF-6

293T cells were transiently transfected separately with expression vectors encoding the full-length receptors EphB2 (Nuk), EphB3 (Hek2), a kinase-deficient mutant of EphB3 (EphB3K665R), and Ephrin-B1 (Xlerk2) along with a FLAG-tagged full-length AF-6 cDNA. 48 h after transfection cells were lysed in a 10-cm culture dish with 1 ml of NETN-buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40 supplemented with 1 mM PMSF, 1 mM Na₃VO₄, 2.8 µg/ml aprotinin, 50 mM NaF, and 1 mM benzamidine) and placed on ice for 15 min. Lysates were collected, transferred to precooled Eppendorf tubes, and then incubated in a shaker for another 15 min at 4°C. Samples were then pelleted for 10 min at 4°C at 10,000 g. The supernatant was then precleared with protein A-Sepharose beads and the precleared supernatant incubated with 4 µl (2.7 µg/µl) anti-FLAG M2 antibody (Eastman-Kodak) and incubated with rotation at 4°C for 1 h. Protein A-Sepharose beads (40 µl) were then added to the cell extracts and incubated with rotation at 4°C for a further 45 min. Cell extracts were then pelleted and washed four times with 1 ml of NETN-buffer, resuspended in loading buffer, run on a 7.5% SDS-PAGE gel, and then blotted as described (Harlow and Lane, 1988). Western blots were probed either with the monoclonal anti-FLAG M2 antibody, or the monoclonal anti-pTyr antibody (PY99; Santa Cruz).

Phosphorylation of AF-6 in the Cellular Lysate

293T cells were transiently transfected separately with expression vectors encoding the full-length receptors EphB2 (Nuk), EphB3 (Hek2), and a kinase-deficient mutant of EphB3 (EphB3K665R) along with a FLAG-tagged full-length AF-6 cDNA. 48 h after transfection, cells were lysed in a 10-cm culture dish with 1 ml of NETN-buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40 supplemented with 1 mM PMSF, 1 mM Na₃VO₄, 2.8 µg/ml aprotinin, 50 mM NaF, and 1 mM benzamidine) and then placed on ice for 15 min. Lysates were collected, transferred to precooled Eppendorf tubes, and then incubated in a shaker for another 15 min at 4°C. Samples were then pelleted for 10 min at 4°C at 10,000 g. Equal amounts of lysate were resuspended in loading buffer, boiled for 5 min at 95°C, run on a 7.5% SDS-PAGE gel, and then blotted as described (Harlow and Lane, 1988). Western blots were probed either with the monoclonal anti-pTyr antibody (PY99; Santa Cruz), with the monoclonal anti-FLAG M2 antibody (Eastman-Kodak), or with the anti-receptor-specific antibodies.

Coimmunoprecipitation from Whole Rat Brain Lysates

Brain coimmunoprecipitation with EphB3 was done essentially as de-

scribed (Irie et al., 1997). In brief, three fresh rat brains were homogenized in a glass teflon homogenizer in 15 ml of NL buffer: 20 mM Hepes-NaOH, pH 8.0, 1% NP-40, 0.2 M NaCl, 2 mM EGTA and inhibitors (1 mM benzamide, 1 mM PMSF, 50 mM NaF, 2.8 $\mu\text{g/ml}$ aprotinin, 1 mM Na_3VO_4). This homogenate was then vigorously shaken for 15 min at 4°C and centrifuged for 60 min at 4°C at 100,000 g to give a supernatant and a pellet fraction. The supernatant was precleared with 30 μl of protein A-Sepharose beads and then incubated for 90 min with 50 μl polyclonal rabbit anti-EphB3 antibody, 12.5 μg monoclonal anti-AF-6 antibody (Transduction Laboratories), or 12.5 μg monoclonal anti-FLAG M2 antibody (Eastman-Kodak). 60 μl of protein A-Sepharose was added for another 90 min at 4°C. Then, beads were washed five times with NL buffer and 30 μl of SDS-loading buffer were added. The pellet received after the centrifugation was extracted a second time, this time using 15 ml of the more stringent radioimmunoprecipitation assay (RIPA) buffer (which disrupts the interaction between the interacting partners): 20 mM Tris-HCl, pH 7.5, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, 137 mM NaCl, 10% glycerine, 2 mM EDTA, and inhibitors as described above. This homogenate was then processed identically as described for the homogenate and subsequently washed five times with RIPA buffer and taken up in 30 μl of SDS-loading buffer. Fractions were analyzed by SDS-polyacrylamide gel (7.5%) electrophoresis and by immunoblotting with antibodies to AF-6 and EphB3.

Brain coimmunoprecipitation with EphB2 was done as follows. Four fresh rat brains were homogenized using the FastPrep FP120 machine (BIO 101). Brains were cut in small pieces and put into FastDNA Kit tubes (BIO 101). The tubes were filled with HO buffer (50 mM Hepes-NaOH, pH 7.5, 1% Triton X-100, 0.15 M NaCl, 1 mM EGTA, 1.5 mM MgCl_2 , 10% glycerol) and inhibitors (1 mM benzamide, 1 mM PMSF, 50 mM NaF, 2.8 $\mu\text{g/ml}$ aprotinin, 1 mM Na_3VO_4) and the machine run at speed 4.5 for 15 s. To this homogenate HO buffer was added up to 20 ml and vigorously shaken for 15 min at 4°C. Subsequently, supernatant and pellet was obtained through centrifugation twice for 1 h at 13,000 g. The supernatant was precleared with 30 μl of protein A-Sepharose beads and then incubated for 90 min with 10 μg polyclonal rabbit anti-EphB2 antibody (Santa Cruz), 12.5 μg monoclonal anti-AF-6 antibody (Transduction Laboratories), or 12.5 μg monoclonal anti-FLAG M2 antibody (Eastman-Kodak). 60 μl of protein A-Sepharose was added for another 90 min at 4°C. Then beads were washed five times with HO buffer and 30 μl of SDS-loading buffer were added. The pellet received after the centrifugation was extracted a second time exactly as described for the second extraction in the EphB3 coimmunoprecipitation (see above). Fractions were analyzed by SDS-polyacrylamide gel (7.5%) electrophoresis and by immunoblotting against AF-6.

Antibodies and Immunofluorescence Microscopy

Monoclonal anti-AF-6 (Transduction Laboratories), rabbit polyclonal anti-ZO-1 (Zymed), monoclonal anti-FLAG M2 antibody (Eastman-Kodak), monoclonal anti-GFP (Clontech), and rabbit polyclonal anti-Lerk2 (Ephrin-B1; Santa Cruz) antibodies were purchased from commercial sources. Rabbit polyclonal antibodies against EphB3, EphB2, and EphA7 were kindly provided by B. Hock and K. Strebhardt (Georg-Speyer-Haus, Frankfurt, Germany [anti-EphB3]), S. Holland (Samuel Lunefeld Research Institute, Ontario, Canada [anti-EphB2]), and A. Ullrich (Max-Planck-Institute for Biochemistry, Martinsried, Germany [anti-EphA7]). All antibodies directed against the different Eph receptors and the Ephrin-B1 ligand recognize the cytoplasmic domain of their protein targets.

Immunofluorescence microscopy of 293T cells was performed as follows. Cells were cultured on a coverglass and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature (RT). The fixed sample was then treated with 0.5% Triton X-100 in PBS for 90 s at RT and washed three times with PBS. Samples were then incubated with the primary antibodies for 1 h at 4°C in 5% newborn goat serum/PBS. Samples were then washed and incubated with either rhodamine-conjugated anti-rabbit or anti-mouse IgG or FITC-conjugated anti-mouse IgG antibodies for 1 h at 4°C. Samples were then washed again and embedded in Mowiol (Hoechst) and examined with a confocal laser scanning microscope equipped with an Ar/Kr laser (model TCS4D; Leica). Bleed-through between the green and the red channel was completely blocked by adjusting the intensity of the laser beam with an acousto-optical (Leica). Images were further processed and merged with the Imaris software (Bitplane) and finally printed with a Fudjix Pictography 3000 (Fuji). For MDCK cells, samples were fixed and permeabilized in 70% methanol/30% acetone at -20°C for 20 min. Samples were washed three times with PBS and then treated as described for 293T cells.

Animals and Tissue Preparation

Oncins France strain A (OFA) line rats (180–200 g; RCC) were used in this study. Environmental conditions for housing of the rats and all procedures that were performed on them were in accordance with animal research licences granted by the Cantonal Veterinary Authority of Zürich and followed the Codes of Practice established by the University Veterinary officers. 10 rats were deeply anesthetized with halothane and then perfused transcardially with 30 ml of 0.9% NaCl, followed by 500 ml of fixative containing 3–4% paraformaldehyde and 0.2% picric in 0.1 M phosphate buffer (PB), pH 7.4, at a rate of ~18 ml/min. The brain from eight rats was removed into cold (4°C) PB. Coronal sections throughout brain were cut on a vibrating microtome at 60–70 μm . To enhance the penetration of the immunoreagents, the sections were equilibrated in 30% sucrose in PB, rapidly frozen in liquid nitrogen, and then thawed in PB. The brain from two rats was postfixed in 0.1% glutaraldehyde containing fixative for 2 h and blocked for cryo-ultramicrotomy. Tissue blocks, 0.8-mm thick, were transferred into 2 M sucrose containing 15% polyvinyl pyrrolidone solution for cryoprotection.

Immunohistochemistry

The sections were preincubated in 10% normal goat serum (NGS) in 0.05 M TBS, pH 7.4, for 45 min and then incubated in primary antibody solutions in TBS that was supplemented with 2% NGS and 2% BSA for 36–48 h at 4°C with constant gentle shaking. They were washed four times for 20 min in 1% NGS in TBS and then incubated in biotinylated goat anti-mouse or anti-rabbit IgG (1:200, Vector Labs) for 12 h at 4°C followed by 3 h of incubation in an avidin-biotin-peroxidase complex (1:100, Elite ABC; Vector Labs) at RT. Antigenic sites were visualized by incubation in 3,3'-diaminobenzidine (0.05% in TBS, pH 7.6; Sigma) in the presence of 0.0048% H_2O_2 . The reaction was stopped by several washes in TBS. Immunotreated sections were postfixed in a solution of 1% osmium tetroxide (Oxchem) in phosphate buffer, pH 7.4, for 45 min, stained with 2% uranyl acetate for 2 h, dehydrated, and flat embedded into Durcupan ACM (Fluka). The sections were examined first in Leica DMR light microscope. Areas of interest were cut from the slide and reembedded. Serial thin sections were collected on pioloform-coated copper grids, and examined in a Philips CM100 electron microscope. The specificity of the immunolabeling was proven by the absence of staining when the primary antibodies were omitted.

Immunogold Staining on Cryo-ultrathin Sections

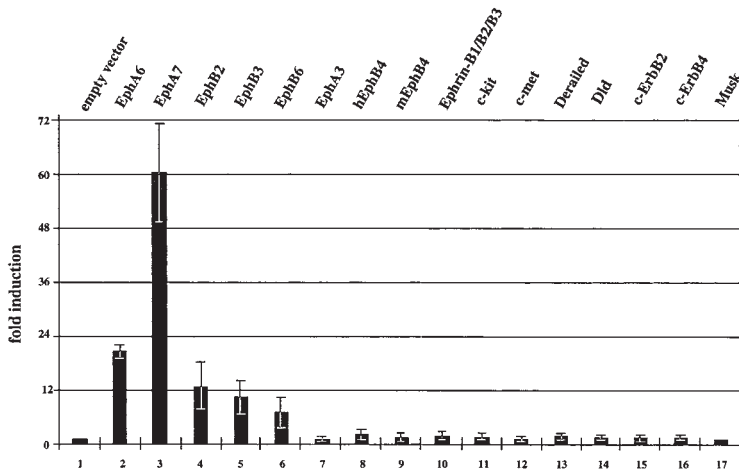
Cryo-ultrathin sections, 90-nm thick, were cut at -120°C, picked up on pioloform-coated nickel grids, and then processed according to Tokuyasu (1986) (for review see Liou et al., 1996). In brief, sections were preincubated on drops of 5% NGS in PBS for 30 min at RT, incubated either in one of the primary antibodies or in the mixture of anti-AF-6 and one of the anti-Eph receptor antibodies (1:200) in PBS containing 1% BSA overnight at 4°C. Gold-conjugated secondary antibodies (5-, 10-, and 15-nm gold-conjugated anti-mouse and anti-rabbit IgG [Nanoprobes]; 8-nm anti-rabbit IgG provided by B. Guhl [University of Zürich, Zürich, Switzerland]; 1:50) or mixture of anti-mouse and anti-rabbit IgGs, conjugated to different size gold particles, for double labeling were applied for 2 h at RT. Then sections were postfixed in 1% glutaraldehyde in PBS and embedded in 2% methylcellulose and 3% uranyl acetate mixture (9:1).

Results

AF-6 Interacts with Specific Eph Receptors

PDZ domains specifically recognize the COOH-terminal ends of target proteins, commonly XS/TXV-COOH (Saras and Heldin, 1996). The PDZ domain has been shown to link diverse membrane proteins with effector molecules inducing receptor clustering and to serve as a scaffold to assemble different components of a signaling cascade (Tsunoda et al., 1997). Different PDZ domains have been demonstrated to have distinct COOH-terminal peptide binding specificities (Saras and Heldin, 1996; Songyang et al., 1997). We surmized that the PDZ domain of AF-6 might

a



b

Transmembrane Receptor	c-terminal peptide Sequence	fold activation
	Pos: -9-8-7-6-5-4-3-2-1 0	
Gal4DBD	-H L V S Q S R L A D	1 ± 0
EphA6	-L H T L V E D I L V	20.1 ± 0.5
EphA7	-L H L H G T G I Q V	61.8 ± 10.5
EphB2	-Q M N Q I Q S V E V	12.7 ± 4.6
EphB3	-Q M N Q T L P V Q V	9.9 ± 3.2
EphB6	-H L R Q Q G S V E V	6.2 ± 2.3
EphA3	-T Q S K N G P V P V	0.8 ± 0.1
hEphB4	-G G T G G P A P Q Y	1.9 ± 0.3
mEphB4	-G G T G G P A Q Q F	1.3 ± 0.3
Ephrin-B1	-Q S P A N I Y Y K V	2 ± 0.2
Ephrin-B2	-Q S P A N I Y Y K V	2 ± 0.2
Ephrin-B3	-Q S P A N I Y Y K V	2 ± 0.2
c-kit	-S Q P L L V H D D V	1.3 ± 0.2
c-met	-T R P A S F W E T S	1.2 ± 0.1
Derailed	-E F H T Q I T R Y V	1.5 ± 0.2
Dld	-F L A E D A L N T V	1.4 ± 0.1
c-ErbB2	-P E Y L G L D V P V	1.7 ± 0.3
c-ErbB4	-P P Y R H R N T V V	1.8 ± 0.1
Musk	-C E R A E G T V S V	1.2 ± 0

Figure 1. Two-hybrid binding analysis of the AF-6 PDZ domain and COOH-terminal peptides of various receptors in 293T cells. (a) Fold induction of the receptor COOH termini and AF-6 PDZ interaction is compared with that of the empty Gal4 DBD vector. Error bars represent the standard deviation of experiments performed in triplicates. (b) Table of COOH-terminal peptide sequences used for the analysis with corresponding fold activation.

be able to recognize the COOH-terminal regions of membrane-associated proteins. Therefore, the GenBank/EMBL/DDBJ database was searched for membrane-spanning molecules having potential PDZ domain-binding COOH termini. More than 20 different receptors broadly classified among the large RTK family had COOH-terminal ends potentially able to interact with PDZ domains. Fusion proteins consisting of the DNA-binding domain (DBD) and COOH-terminal peptides, encompassing the last 10 amino acids of these various receptors, were constructed and tested in a mammalian two-hybrid interaction assay for their ability to interact with the PDZ domain of AF-6. The results are depicted in Fig. 1, a and b. Of all the different receptors analyzed, only a specific subset of members of the Eph subfamily of RTKs were able to interact with the AF-6 PDZ domain. EphA6 (Ehk2), (Ciossek et al., 1995), EphA7 (Mdk1), (Ciossek et al., 1995), EphB2 (Nuk) (Henkemeyer et al., 1994), and EphB3 (Hek2)

(Bohme et al., 1993) all activated the reporter gene between 10- and 60-fold over background levels and EphB6 (Hep) (Matsuoka et al., 1997) activated sixfold over background levels.

Interaction of the AF-6 PDZ Domain with Recombinant Eph Receptors

The binding specificities observed in the mammalian cell two-hybrid assays with the PDZ domain of AF-6 and the peptide ligands were verified with recombinant receptors in vitro. cDNAs encoding full-length EphB2, EphA7, and EphB3 were expressed in 293T cells and their ability to immunoprecipitate with a FLAG epitope-tagged AF-6 PDZ domain construct (FLAG AF-6 PDZ) was analyzed. Fig. 2 indicates that both the full-length EphB2 and EphA7 receptors, when cotransfected into 293T cells with FLAG AF-6 PDZ, specifically coimmunoprecipitate with

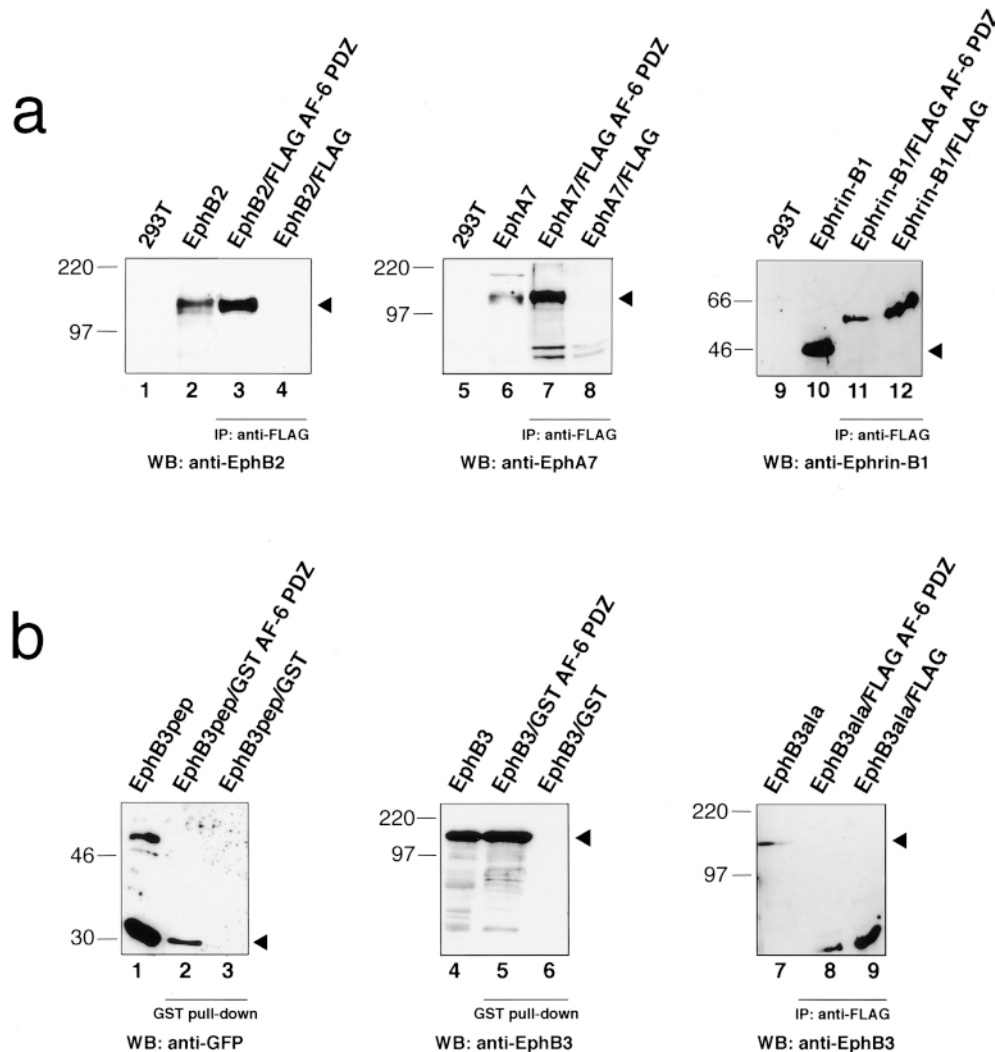


Figure 2. Binding of the AF-6 PDZ domain to recombinant Eph receptors. (a) FLAG-tagged AF-6 PDZ domain protein (FLAG AF-6 PDZ) coimmunoprecipitates with full-length EphB2 and EphA7 receptors but not with Ephrin-B1. Lanes 1, 5, and 9, direct cell lysate, untransfected cells; lanes 2, 6, and 10, direct cell lysate, receptor alone transfected; lanes 3, 7, and 11, receptors and FLAG AF-6 PDZ cotransfected; lanes 4, 8, and 12, receptors and empty vector (FLAG) transfected. (b) GST-pull down experiments of EphB3 COOH-terminal peptide (last 10 amino acids) or full-length EphB3 receptor. Lane 1, EphB3pep direct cell lysate; lane 4, EphB3 full-length receptor direct cell lysate; lanes 2 and 5, EphB3pep or EphB3 extracts mixed with GST AF-6 PDZ coupled glutathionine beads; lanes 3 and 6, GFP EphB3 peptide or EphB3 extracts mixed with GST-coupled glutathionine beads. Lanes 7–9, an EphB3ala mutant receptor (terminal valine residue changed to alanine) is unable to coimmunoprecipitate with FLAG AF-6 PDZ or empty vector (FLAG); lane 7, EphB3ala receptor direct cell lysate; lane 8, EphB3ala cotransfected with FLAG AF-6 PDZ; lane 9, EphB3ala cotransfected with empty vector (FLAG). Bands not indicated by arrows are contaminants.

the FLAG-tagged AF-6 PDZ domain fusion protein whereas no receptor immunoprecipitates with the FLAG-tagged vector alone (Fig. 2 a, FLAG, compare lanes 3 and 4 and lanes 7 and 8). As a further control the Ephrin-B1 (Xlerk2) (Jones et al., 1997) receptor ligand, which has an intracellular COOH terminus characteristic of PDZ-binding peptides but which our mammalian two-hybrid analysis had indicated did not interact with the PDZ domain of AF-6, was transfected. When the Ephrin-B1 cDNA expression vector was cotransfected with either FLAG AF-6 PDZ or the empty vector (FLAG), no Ephrin-B1 protein coimmunoprecipitated with the FLAG-tagged fusion proteins, indicating the specificity of the interaction of the AF-6 PDZ domain for only certain Eph receptors (Fig. 2 a, compare lanes 10–12).

In further experiments, a GST AF-6 PDZ domain fusion protein was coupled to glutathione beads and mixed with 293T cellular extracts expressing GFP-tagged receptor COOH termini peptides or the full-length EphB3 receptor to determine whether specific binding to the AF-6 PDZ domain was observed. Similarly a GFP-EphB3 COOH-terminal peptide containing the last ten amino acids (designated as EphB3pep) as well as the full-length EphB3 receptor were specifically retained by GST AF-6 PDZ-coupled beads but not by GST-coupled control beads (Fig. 2 b, compare lanes 1–3 and lanes 4–6). Mutation of the very COOH-terminal valine residue in the full-length EphB3 receptor to an alanine residue abolished binding of the EphB3 receptor (EphB3ala) to FLAG AF-6 PDZ (Fig. 2 b, compare lanes 7–9).

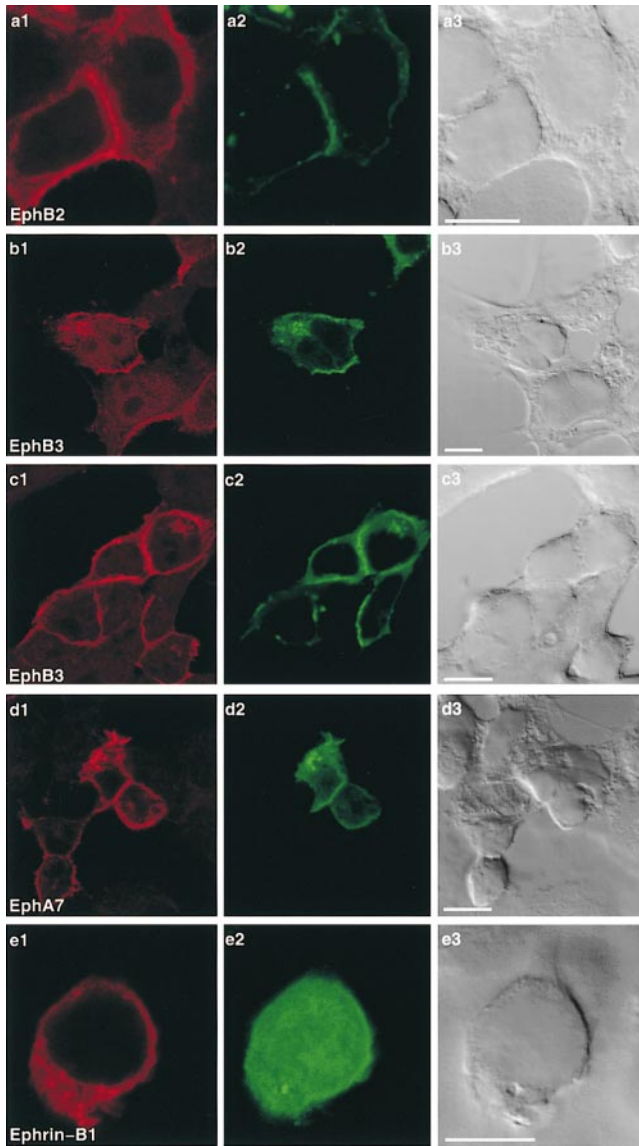


Figure 3. Transfected AF-6 and Eph receptors cocluster at sites of cell–cell contact in 293T cells. Subconfluent cells were transiently transfected with the indicated Eph receptor and a GFP-tagged AF-6 expression vector and stained with Eph receptor-specific antibodies. Red, Eph receptor localization; green, GFP AF-6 expression. GFP AF-6 is clustered with the Eph receptors at sites of cell–cell contact (a1–d3). Ephrin-B1 ligand, which does not bind to the GFP AF-6 protein at the membrane, was used as a negative control. This leads to a diffuse staining of GFP AF-6 in these cells (e1–e3). Bars, 10 μ m.

Colocalization of AF-6 and Eph Receptors at the Membrane in Transfected Cells

To observe a possible direct interaction between AF-6 and these RTK proteins, a plasmid was constructed coding for a full-length AF-6 fused to GFP at its NH₂ terminus, and cotransfected with various full-length receptors in 293T cells. The GFP-tagged AF-6 protein gave a generally diffuse fluorescent signal throughout the cytoplasm and nucleus when transfected alone (data not shown). However,

when cotransfected with full-length EphB2, EphA7, or EphB3 RTKs (Fig. 3, a1–d1), a dramatic shift in the subcellular localization of the GFP-tagged AF-6 protein occurred with a strong signal enriched at the cellular membrane, often at sites of cell–cell contact (Fig. 3, a2–d2, compare with e2). Costaining for the transfected receptors in these cells with a rhodamine-coupled antibody specific for the primary anti-receptor antibodies revealed that the GFP AF-6 signal colocalized with the membrane-associated receptor signals. As a control Ephrin-B1 and GFP AF-6 were transfected. Even though the Ephrin-B1 signal was enriched at the plasma membrane there was no colocalization of the GFP AF-6 signal and Ephrin-B1, with the GFP AF-6 signal diffusely spread throughout the cotransfected cells (Fig. 3, e1 and e2). This is consistent with the result that AF-6 is unable to associate with the COOH-terminal peptide of Ephrin-B1 in a mammalian two-hybrid experiment (Fig. 1).

Transfected Eph Receptors Colocalize with Endogenous AF-6 in MDCK Cells

It has recently been reported that AF-6 is localized at the plasma membrane at specialized sites of cell–cell contact. In the polarized epithelial cell line MDCKII, AF-6 has been shown to colocalize with the tight junction-associated protein ZO-1 at apical lateral membrane sites of cell–cell contact (Yamamoto et al., 1997). In fibroblasts, AF-6 has also been shown to colocalize with ZO-1 at cadherin-based, spot-like cell–cell adherens junction (Mandai et al., 1997). To determine the effect of exogenous Eph receptors on the subcellular localization of endogenous AF-6, EphA7 was transfected into MDCK cells. This caused an enrichment of the endogenous AF-6 protein at sites of cell–cell contact (Fig. 4, a–f), indicating that Eph receptors can be a target for the endogenous AF-6 protein. The colocalization and enrichment of endogenous AF-6 with EphA7 seems to occur only at their extreme apical positions (Fig. 4, compare e with f). This could mean that endogenous AF-6 can not freely diffuse along the lateral membrane, whereas the transfected and overexpressed receptor can do so. Alternatively, the interaction may require additional factors only present at extreme apical positions.

AF-6 and Eph Receptors Cocluster at Postsynaptic Specializations in the Rat Brain Hippocampus

Immunocytochemical techniques with light and electron microscopic analyses were combined in order to determine whether AF-6 colocalizes with Eph receptors in rat brain. In addition, the localization of ZO-1, a protein which is found together with AF-6 at tight junctions in polarized epithelial cells and at cadherin-based cell–cell junctions in fibroblasts (Mandai et al., 1997; Yamamoto et al., 1997), was examined. At low magnification, we observed overlapping spatial distributions in the immunolabeling patterns of AF-6, ZO-1, EphB2, EphA7, and EphB3. Strong immunoreactivity occurred in a variety of regions with high synaptic density, specifically the hippocampus, caudate putamen, cerebellum, superficial gray layer of superior colliculus, external cortex of inferior colliculus, medial mammillary nucleus, sensory nuclei of thalamus, and

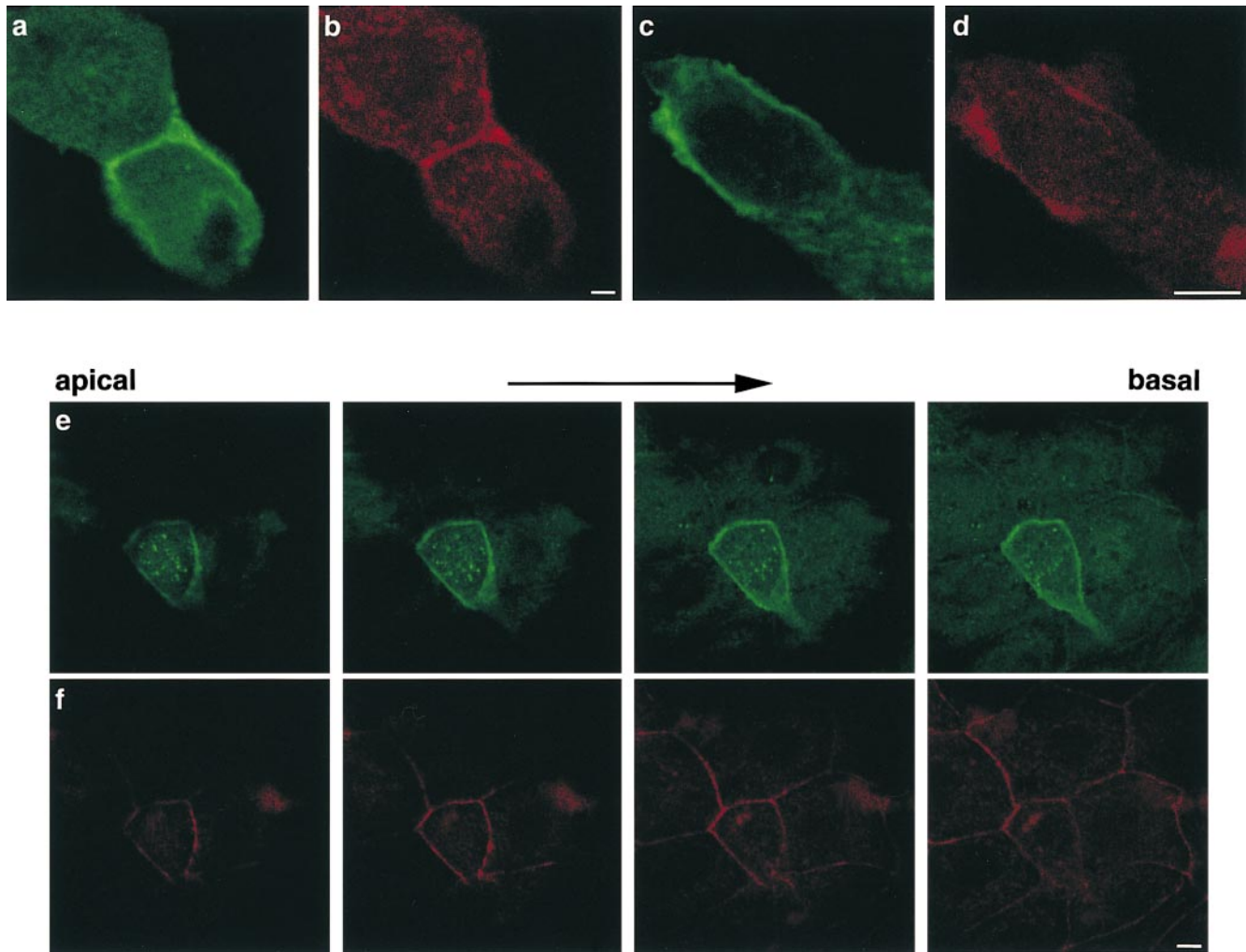


Figure 4. Transient transfection of the EphA7 receptor in MDCK cells causes a clustering of endogenous AF-6. Subconfluent MDCK cells were transfected with a full-length EphA7 receptor construct and doubly stained with anti-EphA7 (green) and anti-AF-6 (red) antibodies. AF-6 clusters with the EphA7 receptor at sites of cell–cell contact (a–d). Endogenous AF-6 is recruited to a more apical position in EphA7 transfected cells. EphA7 transient expression signal (e, green) with endogenous AF-6 expression (f, red) are shown for comparison. Bars, 10 μ m.

neocortex (data not shown). Because the architecture and function of hippocampal circuitry is well known (Johnston and Amaral, 1998), we focused our examination on the subcellular localization of AF-6, ZO-1, and Eph receptors in hippocampal regions. The strong immunolabeling of AF-6, ZO-1, and Eph receptors was found in all dendritic layers, strata oriens and radiatum of CA1 and CA3 regions, and the dentate moleculare of the hippocampus (data not shown). Electron microscopic analysis revealed the presence of immunoperoxidase reaction products at the majority of asymmetric axo-spinous and axo-dendritic synapses in stratum radiatum of the CA1 region (Fig. 5 a, c–e). Immunogold labeling of cryo-ultrathin sections of CA1 have shown that AF-6 (Fig. 6 a) and Eph receptors (Fig. 6, b and c) are localized at postsynaptic densities. Further examination of double-immunogold staining revealed colocalization of AF-6 and Eph receptors over postsynaptic specializations of excitatory synapses in CA1 (Fig. 6, d–f).

Immunoreaction products for ZO-1, in contrast, accumulated mainly in presynaptic axonal terminals that formed asymmetric synapses with either dendritic spines of pyramidal neurons (Fig. 5 b) or dendritic shafts of inhibitory interneurons. ZO-1 immunoreactivity over postsynaptic densities was rarely observed. The different localization of AF-6 and ZO-1 in the rat hippocampus contrasts with the reported interaction of these two proteins at tight junctions in polarized epithelial cells (Yamamoto et al., 1997). This implies that at least in the case of a specialized cell–cell contact, such as the synapse, AF-6 and ZO-1 do not interact with each other and might fulfill different roles.

AF-6 Is an In Vivo Substrate of Eph Receptors

To obtain some information on the biological significance of the interaction between AF-6 and Eph receptors, especially whether AF-6 is a substrate of the Eph receptor ty-

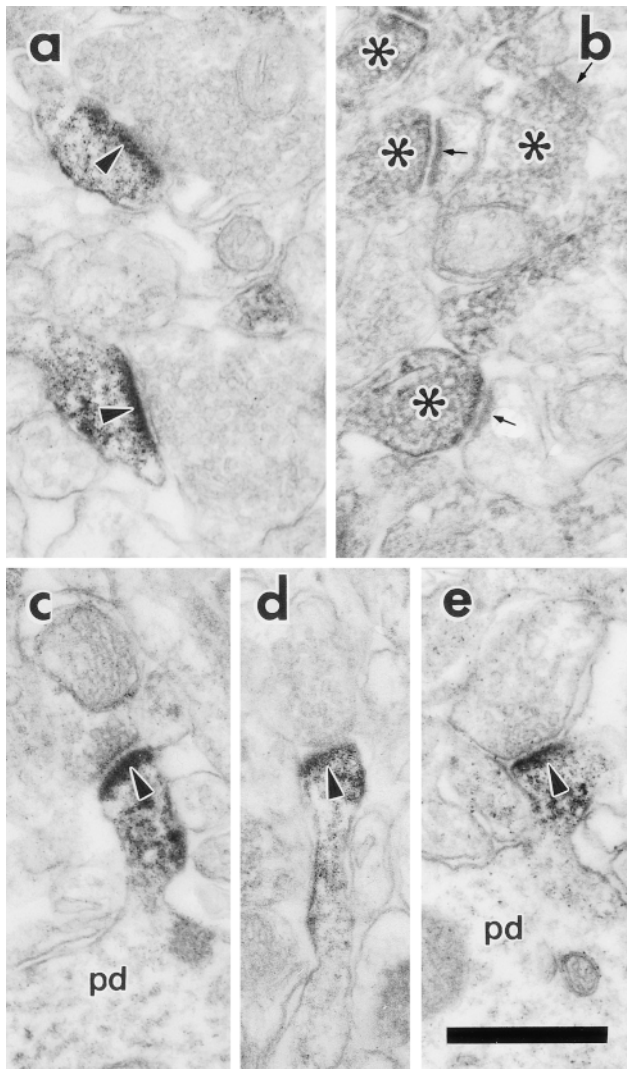


Figure 5. Electron micrographs showing localization of AF-6 (a), ZO-1 (b), EphB3 (c), EphB2 (d), and EphA7 (e) in CA1 stratum radiatum. (a, c–e) Arrowheads, peroxidase reaction products for AF-6, EphB3, EphB2, and EphA7 localized at postsynaptic membrane specialization of asymmetric axo-spinous synapses. (b) Asterisks, immunoreactivity for ZO-1 in numerous axonal terminals, which form asymmetric synapses with spines; arrows, unlabeled postsynaptic membrane specializations. In c and e, peroxidase reaction products accumulate only in spines (arrowheads) whereas parent dendrites (pd) are free of immunostaining. Bar, 0.5 μ m.

rosine kinases, we transiently transfected 293T cells with AF-6 along with either wild-type EphB3, EphB2, or a kinase-deficient mutant EphB3 receptor (EphB3K665R). The result shown in Fig. 7 a demonstrates that AF-6 is tyrosine phosphorylated only when coexpressed with wild-type EphB2 or EphB3 receptors. No phosphorylation occurred with a kinase-negative mutant receptor. Phosphorylation of AF-6 is dependent on previous activation of the receptor which is achieved by overexpression in 293T cells (Fig. 7 a and data not shown). When transfected AF-6 is immunoprecipitated from 293T cells, strong phosphorylation of the immunoprecipitated AF-6 is detected

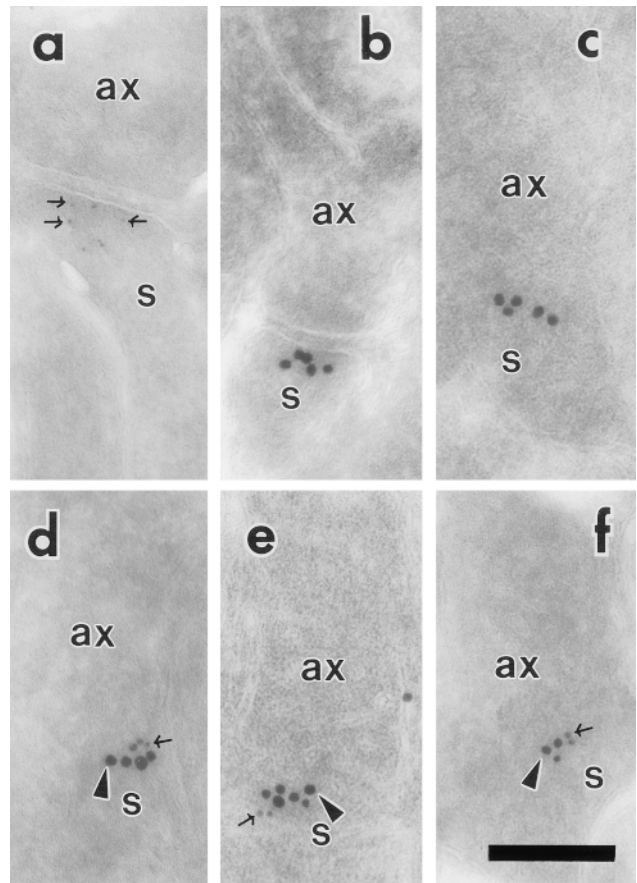


Figure 6. Immunogold labeling for AF-6 and Eph receptors in cryo-ultrathin sections of CA1 stratum radiatum. Immunogold particles for AF-6 (5-nm immunogold in a, arrows), EphB3 (15-nm immunogold in b) and EphB2 (15-nm immunogold in c) are shown at postsynaptic densities of axo-spinous synapses. (d–f) AF-6 (8-nm immunogold particles, arrows) EphB3 receptor (d and f, 15 nm immunogold particles, arrowheads), and EphB2 receptor (e, arrowhead) are shown at postsynaptic membranes. ax, axonal terminal; s, spine. Bar, 0.2 μ m.

when it is cotransfected with the wild-type EphB2 and EphB3 receptors, whereas AF-6 is not phosphorylated when transfected alone or with the kinase-negative mutant EphB3K665R (Fig. 7 e, top). Taken together, these results demonstrate that AF-6 is phosphorylated by the EphB2 and EphB3 receptors in a kinase-dependent fashion and demonstrates that AF-6 is a substrate for a subgroup of Eph receptors.

EphB2 and EphB3 Coimmunoprecipitate AF-6 from Whole Rat Brain Lysates

Finally, to confirm that these findings are also biologically relevant in the living organism, we tested whether complexes of endogenous AF-6 with Eph receptors occur in total rat brain lysates. Indeed, endogenous AF-6 coimmunoprecipitated with both EphB2 and EphB3, but not when the total rat brain extract was immunoprecipitated with an anti-FLAG M2 antibody (Fig. 8, a and b, left). The pellet, obtained after the first extraction, was reextracted with

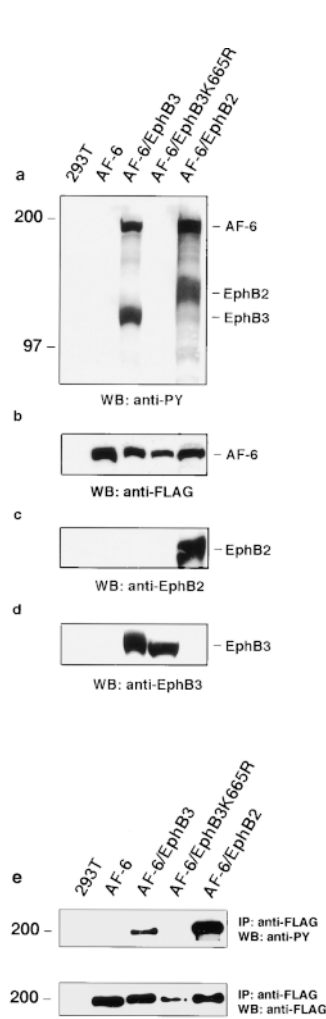


Figure 7. Phosphorylation of full-length AF-6 by the over-expressed EphB2 and EphB3 receptors but not by a kinase-deficient mutant. 293T cells were transiently transfected with either a FLAG-tagged AF-6 (AF-6) alone or together with the wild-type EphB3 receptor (EphB3), a kinase-deficient mutant (EphB3K665R) or with the EphB2 receptor (EphB2). Transfected cells were lysed and equal amounts of each lysate were probed on a Western blot (WB) either with (a) a monoclonal anti-pTyr antibody, (b) with a monoclonal anti-FLAG M2 antibody, (c) with polyclonal antisera against EphB2, or (d) against EphB3. (e) 293T cells were transiently transfected with either FLAG-tagged AF-6 (AF-6) alone or together with wild-type EphB3 (EphB3), a kinase-deficient mutant (EphB3K665R), EphB2 (EphB2). Cell lysates were immunoprecipitated (IP) with anti-FLAG M2 antibodies. Immunoprecipitates were probed on a Western blot (WB) either with a monoclonal anti-pTyr antibody (top) or with anti-FLAG M2 antibodies (bottom). Y is Tyr.

the stronger RIPA buffer, known to disrupt protein–protein interactions. This treatment destroyed the interaction even though endogenous AF-6 was still precipitable (Fig. 8, a and b, right). This further demonstrates the specificity of the interaction and suggest a physiological role for the interaction between AF-6 and Eph receptors in the brain.

Discussion

At the sites of cell–cell contact of epithelial or endothelial cells, known as the tight junction or *zonula occludens*, transmembrane proteins have been identified to form complexes with ZO-1, ZO-2, ZO-3, and AF-6. A characteristic feature of these proteins is the presence of at least one and more often several PDZ domains. The name PDZ is based on the three proteins with such domains, PSD-95, DlgA, and ZO-1. PDZ domains can mediate specific interactions to the COOH-terminal regions of target proteins and many appear to be involved in the clustering or docking of integral membrane proteins to particular sites of membrane specialization (Fanning and Anderson, 1996). The clustering function of PDZ domain-containing proteins has been most clearly demonstrated for a family of

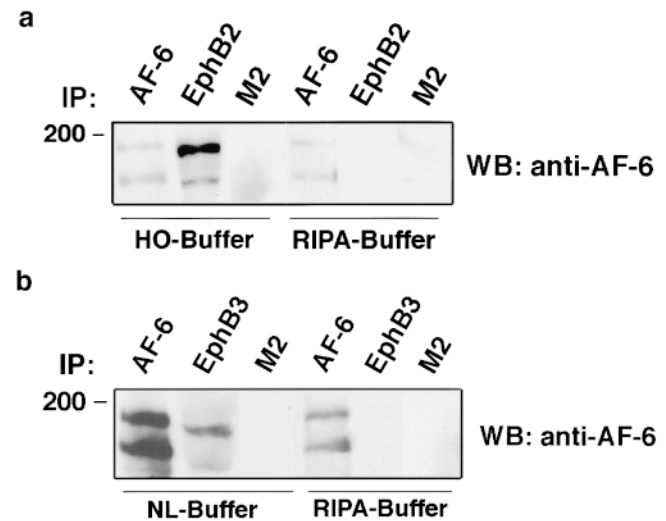


Figure 8. Endogenous AF-6 coimmunoprecipitates with EphB2 (a) and EphB3 (b) receptors from whole adult rat brain extracts prepared in HO-buffer or NL-buffer, respectively but not in RIPA-buffer. Whole adult rat brain extracts were immunoprecipitated (IP) with either monoclonal anti-AF-6 antibody, polyclonal rabbit antisera against EphB2 (EphB2), EphB3 (EphB3), or with the monoclonal M2 antibody, which recognizes the FLAG epitope. The immunoprecipitates were probed on a Western blot (WB) using the monoclonal anti-AF-6 antibody. AF-6 is recognized as a double band in the rat brain. This has also been observed by others (Yamamoto et al., 1997).

synapse-associated proteins (SAPs) which are thought to play a central role in the molecular organization of synapses (Garner and Kindler, 1996; Sheng, 1996).

Since potential PDZ-containing receptor targets of tight junction-associated transmembrane proteins have not been identified, we screened the GenBank/EMBL/DDBJ database for receptor proteins having potential PDZ domain-binding COOH-terminal ends. We noticed that a significant number of receptors in the RTK family have potential PDZ domain-binding COOH-termini (XS/TXV-COOH), suggesting that they might interact with as yet unidentified PDZ domain-containing juxtamembrane proteins. These termini were analyzed for their ability to bind to the PDZ domain of AF-6. Using a mammalian cell-based two-hybrid assay we determined that the AF-6 PDZ domain was able to interact with specific members of the Eph subfamily of RTKs but not with any of the other receptors tested. Of the 14 different members of the Eph RTK subfamily (the largest subfamily of RTKs) eight of them have COOH termini, suggesting they might interact with a PDZ domain-containing protein. Of the eight receptors tested here, five appear to interact with AF-6. Comparing the peptide sequences of receptors able to bind to the PDZ domain of AF-6 (Fig. 1 b, boldface) with each other, indicated that there seems to be a preference for a hydrophobic residue at the –2 position of the binding peptide as well as for a valine at the 0 position. Thus, the potential consensus sequence of COOH termini able to bind to AF-6 PDZ domain is XV/IXV-COOH. However, the EphA3 receptor peptide exhibited no binding to the AF-6 PDZ

domain even though it conformed to this consensus (PVPV-COOH), showing that residues at positions other than -2 and 0 are also important for binding. In a recent publication, Songyang et al. (1997) selected artificial peptides on the basis of binding to an immobilized GST-AF-6 PDZ domain fusion protein. However, their selected peptide sequences bear little relationship to the Eph receptor COOH termini able to bind to AF-6. This may indicate that the PDZ domain of AF-6 is a very flexible interaction domain, with the potential to bind to different target sequences.

Our transient transfection studies demonstrated that AF-6 is specifically recruited to sites of cell-cell contact via interaction with its cognate Eph receptor binding partners in heterologous 293T and MDCK cells. If AF-6 has a role in clustering Eph receptors at sites of membrane specialization, the endogenous proteins should colocalize in normal tissues. Additionally, the findings that Eph family receptors can be clustered at sites of cell-cell contact in the brain (Henkemeyer et al., 1994), lead us to analyze the expression patterns of the three AF-6 interacting Eph receptors and that of AF-6 in brain sections from adult rats. A very tight colocalization was observed. This was particularly the case at postsynaptic membranes of excitatory synapses in the hippocampus, indicating that the observed interaction between these receptors and AF-6 may have physiological consequences. Interestingly, ZO-1 which in polarized epithelial cells tightly colocalizes with AF-6 at the lateral apical membrane, exhibited an expression pattern with marked staining of presynaptic membrane specializations in the brain. In contrast, we found AF-6 and the Eph receptors to colocalize at the postsynaptic membrane, as demonstrated by double-immunogold labeling experiments, reinforcing the view that AF-6 may be involved in the clustering of these receptors.

The clustering of AF-6 and certain Eph receptors at postsynaptic densities may be analogous to that observed with a family of SAPs and ionotropic receptors at the same membrane location. SAPs such as SAP97, SAP90/PSD-95, SAP102, and Chapsyn110/PSD-93 contain three PDZ domains which mediate the interactions with the COOH-terminal ends of the voltage-gated K⁺ channel and the NR2 subunits of the NMDA receptor (Sheng, 1996). These interactions appear to mediate the clustering of these ion channels at synaptic membranes. AF-6 may also be involved in mediating the clustering of the Eph receptors at postsynaptic sites. In addition, like AF-6, SAP97 has also been localized to the lateral membrane between epithelial cells (Mueller et al., 1995), hence, these two proteins may also be involved in the clustering of receptors at other sites of cell-cell contact than just at synaptic membranes.

Our demonstration that AF-6 is an *in vivo* substrate for the kinase activity of a subgroup of the family of Eph receptors is in agreement with recent data (Hock et al., 1998) and supports a functional role of the Ras-binding protein AF-6 in the signal transduction mediated by Eph receptors. Moreover, endogenous AF-6 physically interacts with the two Eph receptors EphB2 and EphB3 in the adult rat brain. This is especially intriguing, considering the fact that little is known about the function of the Eph receptors in an adult organism. Most of the data on the role of Eph receptors stems from observations made dur-

ing embryonic development of the nervous system where these receptors are involved in axonal pathway selection, guidance of cell migration, and establishment of regional patterns. Therefore, our data would complement the distinct function of certain Eph receptors during embryogenesis and suggest an additional role of this class of RTKs in the developed nervous system.

It is of interest to note that the postsynaptic localization of the Eph receptors overlaps substantially with that observed for glutamate NMDA receptors. Of the three major classes of ionotropic glutamate receptors, the AMPA, kainate, and NMDA receptors, it is the latter which has been found to have a role in long-term potentiation (LTP), excitotoxicity, and synaptic development (Choi and Rothman, 1990; Bliss and Collingridge, 1993). The properties of the NMDA receptor are modulated by serine/threonine phosphorylation (Chen and Huang, 1992; Wang and Salter, 1994). Recently it has also been demonstrated that tyrosine phosphorylation of the NR2B and NR2A subunits of the NMDA receptor may regulate the properties of the NMDA receptor channel (Wang and Salter, 1994; Lau and Huganir, 1995; Wang et al., 1996; Yu et al., 1997). The tyrosine phosphorylation status of NMDA receptor subunits changes upon induction of LTP (Rosenblum et al., 1996), suggesting that regulation of the tyrosine phosphorylation of the NMDA receptor may play an important role in neuronal modulation. The identity of the kinase(s) which phosphorylate ionotropic receptor channels such as the NMDA receptor at the postsynaptic density have yet to be identified. The colocalization of some of the Eph receptors with that of NMDA receptors at the postsynaptic density suggests these Eph receptors may be candidates for kinases able to influence the phosphorylation status of other postsynaptic density associated proteins, such as the NMDA receptor. Such a role would provide a rationale for the observed clustering of these receptors and AF-6 at synaptic sites in the adult.

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