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YB-1 Acts as a Ligand for Notch-3 Receptors and Modulates Receptor Activation*

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Y-*box* (YB) protein-1 is secreted by mesangial and immune cells after cytokine challenge, but extracellular functions are unknown. Here, we demonstrate that extracellular YB-1 associates with outer cell membrane components and interacts with extracellular Notch-3 receptor domains. The interaction appears to be specific for Notch-3, as YB-1-green fluorescent protein binds to the extracellular domains and full-length forms of Notch-3 but not to Notch-1. YB-1-green fluorescent protein and Notch-3 proteins co-localize at cell membranes, and extracellular YB-1 activates Notch-3 signaling, resulting in nuclear translocation of the Notch-3 intracellular domain and up-regulation of Notch target genes. The YB-1/Notch-3 interaction may be of particular relevance for inflammatory mesangioproliferative disease, as both proteins co-localize in an experimental nephritis model and receptor activation temporally and spatially correlates with YB-1 expression.

The Y-*box* (YB)[3] protein-1 belongs to the cold shock family, which is notable for its conservation throughout evolution (1). Cold shock proteins play pleiotropic roles in gene regulation, pre-mRNA splicing (2), mRNA translocation, mRNA masking, and mRNA translation (3). The prototypic member YB-1 exhibits an exceptional high degree of phylogenetic conservation not only in the cold shock domain but also throughout the rest of the molecule.

The protein may be divided into three distinct domains, the alanine/glycine/proline-rich N-terminal part, the centrally located cold shock domain, and a C-terminal region characterized by four alternating clusters of basic and acidic amino acids. An interaction with actin has been described for the N-terminal domain (4). The cold shock domain contains basic and aromatic amino acids to attract nucleic acid backbones and to associate with DNA or RNA nucleotide bases. It forms an antiparallel β-barrel, enfolding nucleic acids in a chaperone-like manner (1). The C-terminal region of YB-1 forms a "charged zipper" with motifs that recognize specific RNA hairpins, contribute to DNA/RNA binding, and function as docking site for other proteins.

Recent findings link YB-1 with inflammatory diseases. These include allergic asthma (5) and mesangioproliferative nephritis, in which YB-1 is a downstream target of cytokine platelet-derived growth factor (PDGF)-BB (6), interferon -γ (7), and granulocyte monocyte-colony stimulating factor (5). We demonstrated that YB-1 serves as a transcriptional regulator of RANTES (CCL5) expression in atherosclerosis and renal transplant rejection (8, 9). There is emerging evidence that YB-1 is also secreted from mesangial and immune cells via a non-classical secretion pathway (10). To clarify potential extracellular protein functions, we established a two-hybrid screen with YB-1 as bait and searched for interacting proteins. We identified splicing factor SRp30c (2) and a positive clone encoding for the extracellular EGF domains 13–33 of the receptor Notch-3 as potential partner proteins.

Notch-3 belongs to a receptor superfamily encompassing Notch-1 through -4 in vertebrates. Notch signaling imparts cell fate decisions in many tissues, including the immune system (11) and vasculo- and organogenesis in multicellular organisms (12). Notch receptors constitute single-pass transmembrane proteins that contain repetitive epidermal growth factor-like domain repeats (EGF) and three cysteine-rich Notch/Lin-12 repeats within their extracellular domain. The intracellular domain encompasses seven ankyrin repeats, a nuclear localization signal, transcriptional activator domain, and a PEST sequence (13). Notch receptors are activated by membrane-anchored ligands, like Delta (or Delta-like) and Jagged/Serrate family members, on juxtaposed cells. Upon interaction, two consecutive proteolytic cleavages (S2 and S3) liberate the intracellular Notch receptor domain (ICD), that translocates to the nucleus and acts as a *trans*-activator of target gene transcrip-

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[3] The abbreviations used are: YB, Y-*box*; EGF, epidermal growth factor; HEK, human embryonic kidney; TRITC, tetramethylrhodamine isothiocyanate; HK, human kidney; CREB, cAMP-response element-binding protein; HA, hemagglutinin; IP, immunoprecipitate; ICN3, Notch-3 intracellular domain; PDGF, platelet-derived growth factor; CADASIL syndrome, cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukencephalopathy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; RIPA buffer, radioimmune precipitation assay buffer; MC, mesangial cells; CSL, CBP, Suppressor of Hairless, Lag-1; CBP, CREB-binding protein.

tion by binding to the DNA-binding protein RBP-Jκ (14), which in the absence of Notch ICD acts as a repressor of gene transcription (15). The basic helix-loop-helix transcription factors HES (Hairy Enhancer of Split) and HERP (HES-related proteins) have been depicted as immediate transcriptional targets of Notch, but an increasing number of further target genes is found.

Notch signaling pathways play prominent roles in podocyte development and likely the pathogenesis of diverse glomerular diseases, like diabetic nephropathy and lupus nephritis (16, 17). Deletion of the Notch-2 gene or treatment of developing kidneys with γ-secretase inhibitors leads to severe defects in podocyte and proximal tubule differentiation (18). Furthermore, *de novo* expression of activated Notch-1 was significantly enhanced in glomerular cells in humans with diabetic nephropathy and focal segmental glomerulosclerosis and rodent models thereof (16). Whereas insights into the role of Notch receptors in the pathogenesis of human kidney diseases are emerging, there are strong genetic links between mutations within the human Notch-3 gene and a subtype of inherited early-onset dementia (called cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukencephalopathy, CADASIL syndrome) (19). Two case reports provide evidence for renal involvement in CADASIL syndrome, namely the coexistence of mesangioproliferative IgA nephritis in kidney biopsies (20, 21). Given the primary result yielded with the two-hybrid screen, we performed an in-depth analysis of YB-1 association with receptor Notch-3 and potential effects on Notch signaling.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—A yeast two-hybrid screen (Proquest, Invitrogen) was performed as described (2).

Plasmids, Cell Lines, and Transfections—The following plasmids were kindly donated: full-length YB-1 expression plasmid (pSG5-YB-1) by J. Ting (22), YB-1-GFP by H. D. Royer (CAESAR, Germany), murine Notch-3 receptor (pcDNA3-Notch-3) by H. Karlström (23), N1(EGF)-FLAG encoding for Notch-1 EGF domains 10–36 by K. Sakamoto (24), HES1- and HES2-luc by R. Kageyama (25), 4×CSL-luc and mutated 4×CSL-luc (mut) by D. Hayward (26), and MIF-eGFP by J. Bernhagen (27). Notch-3 deletion construct N3(EGF)-HA spans EGF domains 13–33 (amino acids 494–1342) in expression plasmid pDONR201 with a relative molecular mass of 89 kDa. Human embryonic kidney (HEK293) cells stably expressing either wild-type mNotch-3 (HEK293-N3) or a mNotch-3 construct linked to a Gal4VP16 (GVP) domain (HEK293-N3-GVP) have been described (23). Transient transfections were performed using calcium phosphate precipitates, except for HK-2 and Saos-2 cells (FuGENE 6, Roche Applied Science). PDGF-BB (Sigma) and γ-secretase inhibitor compound X (Calbiochem) were purchased.

Immunoreagents—Immunoprecipitations were performed as reported (2). Antibodies used were: irrelevant mouse IgG1 (AF3), monoclonal GFP (Clontech), M2/FLAG (Sigma), Notch-3 (5E1: extracellular epitope (28)), GAPDH (Novus Biologicals); polyclonal hemagglutinin (HA) (Y11, Santa Cruz), Notch-3 (M134, Santa Cruz: intracellular epitope and BC4, intracellular epitope (28)), YB-1 (polyclonal antibody against protein N terminus, monoclonal anti-YB-1 antibody (antibodies-online), and polyclonal antibody raised against the full-length protein (5)).

Biotin Labeling of Cell-surface Proteins—HEK293T cells were transfected with YB-1-GFP or GFP expression plasmids for 24 h, incubated with PDGF-BB (20 ng/ml) for another 4 h, and thereafter scraped off the plates, washed 3 times with ice-cold PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM), and incubated for 15 min at 4 °C with non-membrane permeable NHS-SS-biotin (Pierce) at 1 mg/ml in PBS-CM. The reaction was quenched by 50 mM glycine, PBS-CM for 10 min. Cells were washed 5 times with ice-cold PBS-CM and lysed by incubation with 1 ml of RIPA buffer. Extracellularly covalently biotinylated YB-1-GFP was detected by Western blot analysis using either streptavidin (streptavidin-POD, Dianova, Germany, 1:10.000) after immunoprecipitation with anti-GFP antibody or, as control reaction, using nonspecific mouse IgG1 (Dako, Denmark). Alternatively, detection was performed after binding of biotinylated proteins to streptavidin-agarose beads (Sigma) by means of anti-GFP antibody.

Microvesicle Preparation and Trichloroacetic Acid Precipitation—To analyze the secretion of YB-1, HA-YB-1 was expressed in rat mesangial cells (MC) under control of the *Tet-Off* system (7). PDGF-BB (20 ng/ml) or PBS was added, and after 1 and 4 h supernatants were removed and sequentially centrifuged (supernatant S1, 10 min, 1000 × *g*; supernatant 2 and pellet, 30 min, 30.000 × *g*).

Alternatively, proteins in the cell supernatant were precipitated by addition of ice-cold trichloroacetic acid (20% v/v, Sigma). Precipitated proteins were pelleted by centrifugation at 20,000 × *g* for 45 min at 4 °C, washed twice with ice-cold 70% ethanol, air-dried, and resuspended in 25 μl of distilled water.

Cell Viability and Lactate Dehydrogenase Activity—Cell viability was assessed by trypan blue exclusion assay, and lactate dehydrogenase concentration was quantified in supernatants to control for cell death/lysis.

Reverse Transcription-PCR and Quantitative Real Time-PCR—Total RNA extraction and reverse transcription-PCR were performed as previously described (6) using the following Notch-3-specific primer pair: forward 5′-GGAGGGAGAAGCCAAGTC-3′ and reverse 3′-GGGAAAAAGGCAATAGGC-5′.

For quantitative real time-PCR, HEK293T or HEK293-N3 cells were seeded at 2×10^5 cells/well in 6-well plates. After 48 h medium was exchanged with conditioned culture supernatant from HEK293T cells with forced GFP or YB-1-GFP expression. Total RNA was purified, and first-strand cDNA was synthesized. Quantitative real-time-PCR was carried out using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). TaqMan primer/probe sets were obtained for human HES1 (Hs00172878_m1), human HES2 (Hs00219505_m1), and eukaryotic 18 S ribosomal RNA as the internal control (Hs99999901_s1) from Applied Biosystems.

Immunohistochemical Staining—Immunohistochemistry was performed with 5-μm paraffin sections of formalin-fixed specimen using the Vectastain avidin-biotin system (Vector Laboratories), polyclonal Notch-3 antibody (M134, Santa Cruz;

1:50), and polyclonal peptide-derived anti-YB-1 antibody (antibodies-online; 1:50).

Mesangioproliferative Anti-Thy1.1 Nephritis—Mesangioproliferative anti-Thy1.1 nephritis was induced in male Wistar rats as described (29). Renal tissue for light microscopy was fixed in Methacarn at the indicated time points and embedded in paraffin. Glomeruli were isolated using a differential sieving technique as described (30). Proteins were electrophoresed under reducing conditions and immunoblotted using a polyclonal antibody generated with a full-length YB-1 protein (kindly donated by E. E. Capowski (5)) and antibodies specific for Notch-3 (5E1, BC4). The relative band intensities were calculated after densitometry of digitalized autoradiographic images using Scion Image software (Scion Corp.).

Laser-scanning and Fluorescence Microscopy—HK-2 cells were transfected with Notch-3 (pcDNA3-Notch-3) or pcDNA3 control plasmid. After 3 washes with PBS-CM, cells were incubated for 1 h at 37 °C with lysates from HEK293T cells expressing GFP or YB-1-GFP protein, prepared by repeated freeze-thaw cycles and dilution in fetal calf serum-free RPMI medium. Cells were washed three times with PBS-CM, permeabilized and blocked as described (2), and incubated with anti-Notch-3 (BC4, 1:200) and secondary TRITC-conjugated anti-rabbit antibody (1:50; Dinova, Germany). Confocal laser scanning microscopy (Zeiss LSM 510 Meta) was performed with excitations at 488 and 543 nm.

Fluorescence-activated Cell Sorter Analysis—To test for extracellular YB-1 binding to Notch-3 carrying cells, lysates or culture medium were obtained from HEK293T cells overexpressing either YB-1-GFP, GFP, or MIF-GFP (negative control). Conditioned medium was collected from cells that had been stimulated with PDGF-BB (20 ng/ml, 4 h). Cell lysates and conditioned medium were tested for the presence of aforementioned proteins by immunoblotting and added to HEK293-N3 cells (1 h at 4 °C) that had been pretreated with γ-secretase inhibitor compound X (1 μM) to prevent receptor cleavage. Cells were washed with ice cold PBS, 5% fetal calf serum, and GFP fluorescence was quantified on a FACSCalibur flow cytometer (BD Biosciences). A total of 10,000 events was computed and analyzed with CellQuest software.

Luciferase Reporter Assays—Reporter constructs were introduced into HK-2, HEK293T, or HEK293-N3 cells. PDGF-BB (20 ng/ml) and/or compound X (at 0.02 or 1 μM) were added to the medium as indicated. Neutralization of secreted YB-1 protein (fragments) was achieved by inclusion of monoclonal anti-YB-1 antibody (3 μg/ml medium; antibodies-online). 24 h post-transfection, luciferase activity was quantified in triplicate using the Promega luciferase assay system (Mannheim, Germany) in a Sirius luminometer (Berthold Detection Systems). Results were confirmed in five independent experiments and were calculated as -fold changes relative to luciferase activity measured with control plasmid. Transfection efficiency was quantified by co-introducing pSV-β-galactosidase plasmid (Promega) and quantifying substrate turnover as described before (31).

To test extracellular YB-1 effects, HEK293T cells with ectopic YB-1-GFP, MIF-eGFP, or GFP expression were stimulated with PDGF-BB (20 ng/ml, 4 h) to induce secretion. Conditioned medium was added to HEK293-N3 cells harboring the HES1- or HES2-luc reporter construct. To assess the presence of secreted proteins, conditioned medium was collected, and proteins were precipitated with trichloroacetic acid and analyzed by Western blot analysis.

In co-culture experiments HEK293-N3-GVP cells were seeded in 6-well plates. After 24 h ligand-carrying cells (HEK293T cells transiently expressing human Jagged1) were co-cultivated for another 24 h. MH100-luc reporter constructs were present in HEK293-N3-GVP cells.

Statistical Analyses—All values are expressed as the means ± S.D. Statistical significance (defined as $p < 0.05$) was evaluated using analysis of variance and Student's t test.

RESULTS

YB-1 Accumulates in Membrane Vesicles and Associates with Extracellular Membrane Proteins—Human kidney (HK-2) cells expressing GFP-tagged YB-1 protein were cytokine-stimulated. Compared with non-stimulated cells with diffuse cytoplasmic distribution of YB-1-GFP, stimulation with PDGF-BB (20 ng/ml, 4 h) led to a change of YB-1-GFP localization, predominantly to vesicular structures in the vicinity to cell membranes (Fig. 1*A*). In keeping with this, an HA-YB-1 fusion protein was detected in the supernatant of microvesicle preparations from rat MC designed to express HA-YB-1 protein (Fig. 1*B*). In addition to full-length HA-YB-1, two smaller HA-YB-1 fragments, ~28-kDa (HA-YB-1#1) and 14-kDa (HA-YB-1#2), were also detected (Fig. 1*B*) after sequential centrifugation steps.

A thorough analysis of the mechanism resulting in YB-1 secretion has been performed and summarized in a separate publication (10). Specificity of YB-1 secretion as an active process, which is clearly distinguishable from passive cell lysis, was ascertained. MC release endogenous YB-1 protein within 1 and 4 h of PDGF-BB incubation (Fig. 1*C*), in contrast to other cytoplasmic (GAPDH) and nuclear (CREB) proteins. Notably, there were protein fragments of YB-1 in the conditioned supernatant besides full-length protein.

Next we tested whether secreted YB-1-GFP protein associates with the outer aspect of cell membranes. Biotinylation of extracellular proteins from HEK293T cells overexpressing GFP or YB-1-GFP challenged with PDGF-BB was performed. After incubation with membrane-nonpermeable biotin, cells were washed and lysed, and proteins were immunoprecipitated with anti-GFP or isotype-control IgG. Streptavidin complex addition allowed for the detection of biotinylated YB-1-GFP when YB-1-GFP-overexpressing cells were assayed (Fig. 1*D*, *lane 4*), whereas GFP-expressing cells exhibited no biotin signal (Fig. 1*D*, *lane 2*). As a control for cell lysis resulting in release of YB-1-GFP protein, we also assayed for release of GAPDH and biotinylation thereof. As shown in Fig. 1*E* (*lane 4*) GAPDH was not detected as extracellular protein. Taken together, these results indicate that YB-1-GFP is secreted in microvesicular structures upon PDGF-BB stimulation and that extracellular YB-1 associates with cell membrane components.

YB-1 Interacts with the Notch-3 Extracellular Domain—Given the detection of extracellular YB-1 and its interaction with membrane components, the results obtained by a yeast two-hybrid screen attracted our attention. The latter was per-

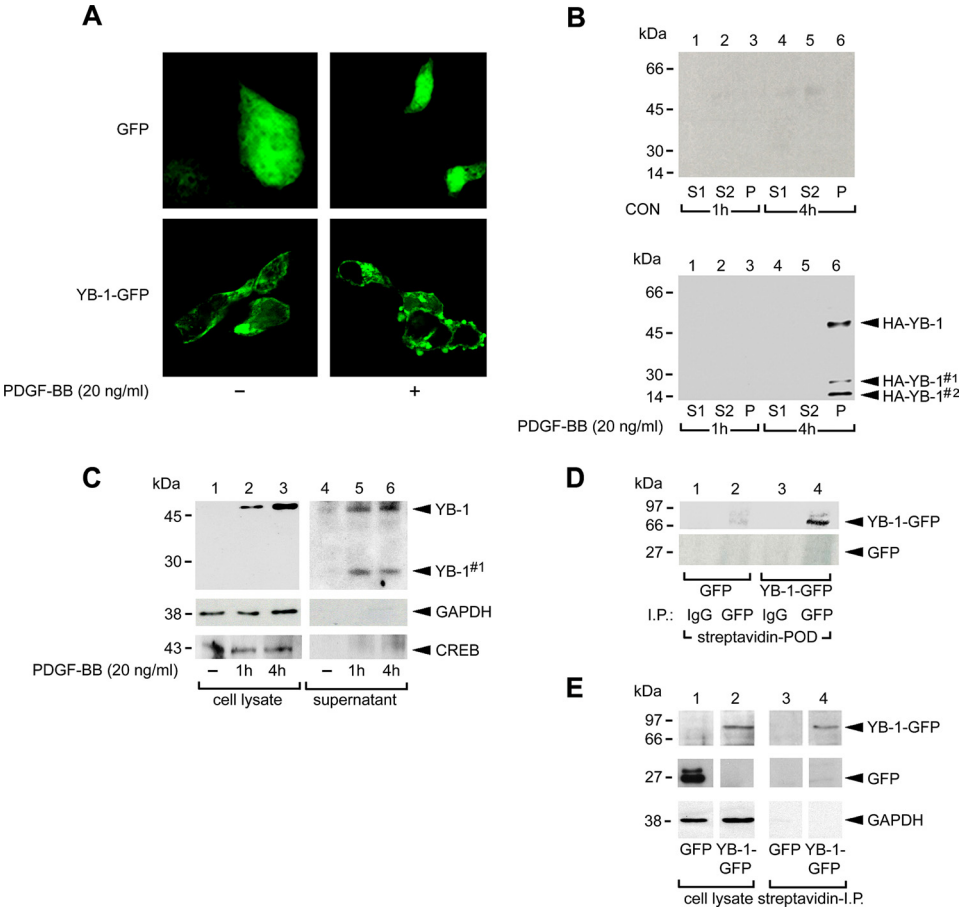


FIGURE 1. **PDGF-BB induces YB-1 secretion in microvesicular structures and binding of extracellular YB-1 to cell membrane components.** *A*, ectopically expressed YB-1-GFP was visualized by laser scanning microscopy in HK-2 cells without and subsequent to PDGF-BB stimulation for 4 h. *B*, sequential centrifugation steps allowed for the isolation of microvesicles in conditioned medium of rat MC expressing HA-YB-1 after PDGF-BB stimulation. Supernatants *S1* and *S2* as well as the pellet of the high speed centrifugation containing microvesicles (*P*) were included for Western blot analysis with an anti-HA tag antibody. Besides full-length HA-YB-1 protein, two fragments of relative molecular masses 30 and 14 kDa (denoted *HA-YB-1#1* and *HA-YB-1#2*) were detected. *CON* denotes a control experiment without cytokine challenge. *C*, rat MC were treated with PDGF-BB for 1 and 4 h. YB-1 protein was detected in cell lysates and corresponding supernatants after trichloroacetic acid precipitation. To exclude cell death/lysis after incubation with PDGF-BB, cell lysates and supernatants were tested for the presence of cytoplasmic and nuclear proteins using anti-GAPDH and anti-CREB antibodies. *D*, to test for extracellular membrane association of YB-1-GFP, HEK293T cells were transfected with either GFP or YB-1-GFP and stimulated with PDGF-BB (20 ng/ml) for 4 h. After biotinylation of extracellular membrane-associated proteins, cells were lysed, and either nonspecific immunoglobulin (IgG) or anti-GFP antibody was added for IP. Detection was performed by SDS-PAGE using streptavidin-POD. *E*, conversely, enrichment of biotinylated proteins using streptavidin beads allowed for the detection of YB-1-GFP but not GFP. The amount of YB-1-GFP protein input is provided in *lane 2*. To exclude cell death/lysis as contributing factor for biotinylation, immunoblotting for GAPDH was also performed with total cell lysates and IP.

formed with YB-1 as bait and a cDNA library generated from human mesangial cells as prey to identify a possible interaction(s) with proteins. Of 2×10^6 screened colonies, one clone encoding for the extracellular domain of the Notch-3 receptor was identified, which fulfilled all criteria for a strong protein interaction with YB-1 (phenotype: His^+, $3AT^R$, β-galactosidase$^+$, Ura^+, and 5-fluoroorotic acid$^+$). The clone corresponded to amino acids 494–1342 in the Notch-3 extracellular domain (Fig. 2*A*). The region corresponding to EGF-like repeats 13–33 was cloned into the vector pDONR201, which adds a HA tag to the C terminus of the protein (N3(EGF)-HA) (Fig. 2*A*). Protein-protein interaction between YB-1-GFP and N3(EGF)-HA was confirmed by co-immunoprecipitation experiments. To visualize the protein input whole-cell extracts were prepared and immunoblotted for the same antigens that were assayed by immunoprecipitation (Fig. 2*B*, *upper panel* (*input*) and *lower panel* (*IP*), *second* and *third lanes*, and *C*, *first*, *third*, *and fifth lanes*). Immunoprecipitation yielded a positive result, as is depicted in the *sixth lane* of Fig. 2*C*.

As all four mammalian Notch receptors are highly structurally related, we next addressed whether the YB-1 interaction was specific to Notch-3. Co-immunoprecipitation experiments were performed with corresponding domains, EGF-like repeats 10–36, from Notch-1. Co-expression of Notch-1(EGF)-FLAG and YB-1-GFP in HEK293T cells did not result in co-immunoprecipitation (Fig. 2*E*, *lane 6*). This indicates that the interaction of YB-1-GFP with an extracellular Notch domain is specific for Notch-3.

YB-1-GFP also co-immunoprecipitated with endogenous Notch-3 receptor protein in HEK293T cells (Fig. 2, *F* (*input*) and *G*, (*IP*) *second* and *third lanes* of the *upper panel*). To confirm the specificity of YB-1-GFP binding to Notch-3, a further negative control was included, namely Notch-3-negative osteoblastic osteosarcoma cells (Saos-2 (32)), in which no interaction was observed (Fig. 2*G*, *lower panel*). In these experiments care was taken that similar amounts of YB-1-GFP were expressed in HEK293T and Saos-2 cells.

Notch-3 Is Expressed by Kidney Cells and Coordinately Up-regulated with YB-1 in Mesangioproliferative Nephritis—It is known that Notch-3 is expressed in vascular smooth muscle cells (19), but little is known about other sites of expression in adult humans. The data on interaction between YB-1 and Notch-3 led us to address whether Notch-3 mRNA was expressed in mesangial cells with a myofibroblast-like phenotype (33). Notch-3 mRNA transcripts were detected in HEK293T, human tubular (HK-2) and human MC (Fig. 3*A*). Furthermore the Notch-3 intracellular domain (ICN3) was detected in non-transfected HEK293T cells after immunoprecipitation with anti-Notch-3 antibody (Fig. 3*B*, *third lane*) but not nonspecific IgG (Fig. 3*B*, *first lane*). Ectopic expression of full-length Notch-3 enhanced the ICN3 band intensity (Fig. 3*B*, *fourth lane*). By immunohistochemistry, we detected Notch-3 protein in some tubulointerstitial cells at the basal aspect but not in glomerular cells from healthy rats (Fig. 3*C*). Negative

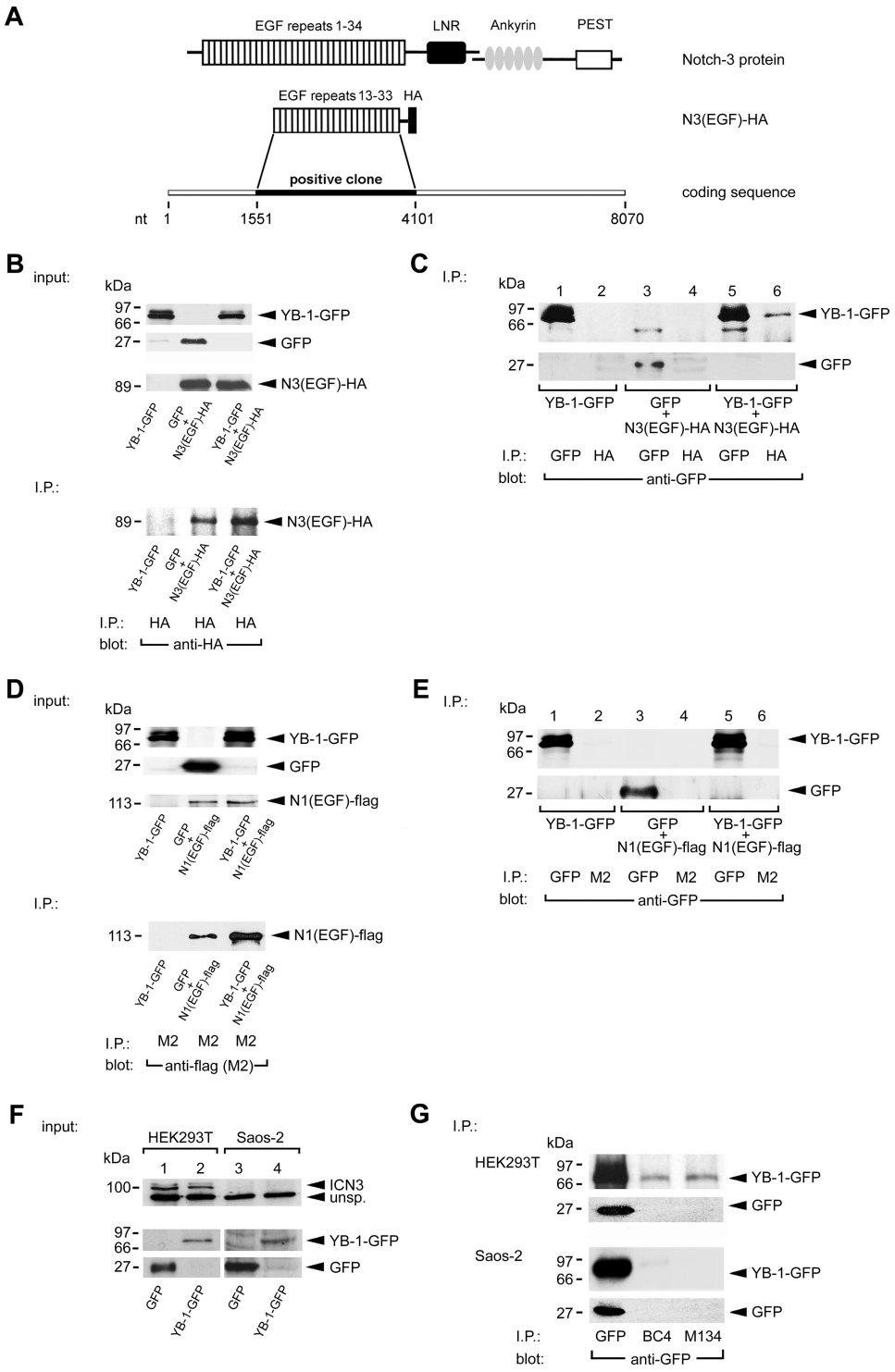


FIGURE 2. **YB-1 interacts with extracellular domains of Notch-3 receptor.** *A*, schematic diagram of Notch-3 receptor domains and the retrieved clone by two-hybrid screen. *LNR*, Notch/Lin-12 repeats; *PEST*, region rich in proline, glutamine, serine, and threonine. *B* and *C*, co-immunoprecipitation experiments were performed with lysates from HEK293T cells expressing either YB-1-GFP alone, GFP, and HA-tagged N3(EGF) or YB-1-GFP and N3(EGF)-HA. Input of expressed and immunoprecipitated N3(EGF)-HA protein is visualized in *B* by an anti-HA antibody (*second* and *third lanes*). Input of expressed and immunoprecipitated YB-1-GFP is visualized in *C* (*lanes 1* and *5*). Co-immunoprecipitated YB-1-GFP is detected in *lane 6*, whereas corresponding control reactions were negative (*lanes 2* and *4* of *C*). *D*, and *E*, co-immunoprecipitation experiments were performed with lysates from HEK293T cells expressing YB-1-GFP alone, GFP and Notch-1 EGF-domains 10–36 fused to an HA tag (N1(EGF)-FLAG), or YB1-GFP and N1(EGF)-FLAG proteins. Input of expressed and immunoprecipitated N1(EGF)-FLAG protein is visualized in *D* (*second* and *third lanes*). Input of expressed and immunoprecipitated YB-1-GFP is visualized in *E* (*lanes 1* and *5*). Co-immunoprecipitation of YB-1-GFP did not occur (*lane 6* of *E*). These results indicate specificity of the Notch-3:YB-1-GFP interaction (compare with the results in *C*). *F*, to ascertain that endogenous Notch-3 protein also specifically co-immunoprecipitates YB-1, two model systems were evaluated, HEK293T cells expressing Notch-3 receptor as well as Notch-3 receptor negative Saos-2 cells were manipulated to express YB-1-GFP. Input of Notch-3 intracellular domain, ICN3, and YB-1-GFP proteins are provided in *F*. Although anti-Notch-3 antibodies BC4 and M134 co-immunoprecipitated YB-1-GFP in HEK293T cells, they failed to do so in Saos-2 cells (*G*).

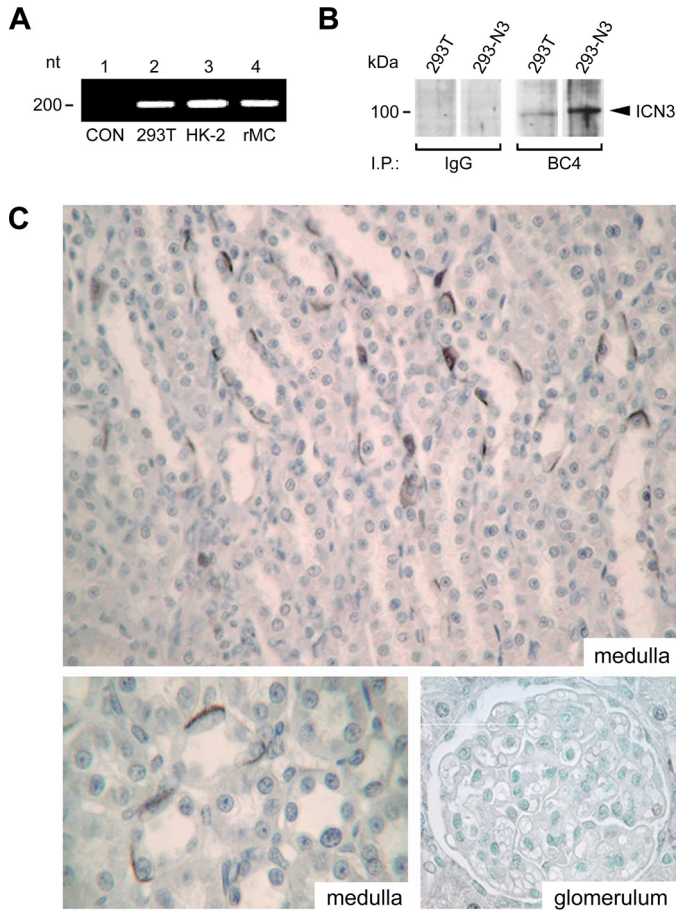


FIGURE 3. **Notch-3 expression in kidney tissue.** *A*, Notch-3 transcripts were detected in HEK293T, tubular (HK-2), and human MC (*rMC*) cells by RT-PCR with specific primers. *nt*, nucleotides. *B*, expression of endogenous Notch-3 intracellular domain (*ICN3*) protein was detected in HEK293T and HEK293 cells with constitutive overexpression of Notch-3 (HEK293-N3) after immunoprecipitation using an anti-Notch-3 antibody directed against ICN3 (*BC4*). Control with nonspecific IgG. *C*, sections of healthy rat kidneys were examined by immunohistochemistry using an antibody directed against an extracellular Notch-3 epitope. No staining was observed in glomerular cells; however, some tubular and collecting duct cells of the medulla were immunopositive at their basal membrane.

control staining reactions included nonspecific IgG antibody instead of the primary antibody (data not shown).

Recent studies indicate that YB-1 expression and subcellular localization are highly regulated in the course of mesangioproliferative disease (6). In healthy tissue YB-1 protein localizes strictly to the nuclear compartment of glomerular and tubular as well as vascular cells. Up-regulated YB-1 expression is observed in the mesangial compartment between days 4 and 9 in anti-Thy1.1 nephritis, a self-limited rodent model exhibiting similarities to human mesangioproliferative diseases. It is noteworthy that in the course of the disease the expression pattern of YB-1 is not confined to cell boundaries (Fig. 4*A*, days 7 and 9, *left panels*). We next compared the YB-1 expression pattern with that of Notch-3 in tissue collected on days 4, 7, and 9 after induction of anti-Thy1.1 nephritis. A similar expression pattern was observed for Notch-3 (ICN3) in the course of anti-Thy1.1 nephritis, and strongest staining for Notch-3 coincided with the peak of mesangial cell proliferation in this model between days 7 and 9 (Fig. 4*A*, *right panels*). Specificity of Notch-3 immunohistochemistry was confirmed for specimens from day 7 after disease induction by substituting the anti-Notch-3 antibody with an isotype-matched irrelevant antibody (Fig. 4*B*). To quantify the expression of both proteins at the different time points, sieved glomeruli were collected and immunoblotted. Over the time course of the disease, from days 2 to 9 post-disease induction, a YB-1 protein fragment was detected with an approximate molecular size of ~30 kDa (denoted YB-1#1) using a polyclonal anti-YB-1 antibody (8), whereas the initially up-regulated full-length YB-1 (50 kDa) disappeared (Fig. 4*C*, *upper panel*). Notch-3 protein was induced that peaked on day 7 of the disease and disappeared subsequent to day 14 (Fig. 4*C*, *ECN3*). Analysis with antibodies directed against the intracellular Notch-3 receptor domains yielded a similar temporal expression pattern (Fig. 4*C*, *ICN3*). These observations indicate a spatially and temporally coordinated up-regulation of YB-1 and Notch-3 protein expression in the course of inflammatory mesangioproliferative disease. Furthermore, spot urine samples from control animals and diseased animals on day 7 were analyzed for the presence of YB-1 protein and the extracellular Notch-3 receptor domains. As can be seen in Fig. 4*D*, distinct bands were detected in diseased animals with a polyclonal antibody raised against full-length YB-1 protein (5). In the same urine samples ECN3 receptor with a molecular size corresponding to cleaved extracellular Notch-3 receptor domains was only detected in diseased animals (Fig. 4*D*, *lower panel*). These data support the notion of YB-1 being secreted and present in the urine of diseased animals. At least a temporal correlation exists with the appearance of cleaved extracellular Notch-3 receptor domains in the urine, which is suggestive of receptor activation.

Extracellular YB-1 Co-localizes with Notch-3 at Cellular Membranes—To test for binding of YB-1 to extracellular membrane components, especially Notch-3, HK-2 cells overexpressing Notch-3 were incubated with cell lysates of HEK293T cells transiently expressing GFP or YB-1-GFP protein. The localization of Notch-3 receptor protein was visualized by laser-scanning microscopy using a specific antibody directed against Notch-3. In the Notch-3-overexpressing HK-2 cells, about 15% of the cells exhibited fluorescence signal that was detected predominantly at the plasma membrane (Fig. 5*A*, *lower row*). Endogenous Notch-3 receptor expression in control cells showed the same distribution but was considerably weaker (Fig. 5*A*, *upper row*). Upon extracellular addition of YB-1-GFP to HK-2 cells, GFP fluorescence was only detected in HK-2 cells overexpressing Notch-3 receptor, and the GFP signal co-localized with the Notch-3 receptor staining (Fig. 5*A*, *lower row*, to the *right*). Control experiments with GFP protein addition demonstrated an absence of binding under both conditions (Fig. 5*A*, *upper row*, to the *right*).

These results were confirmed by fluorescence-activated cell sorter analysis using stably Notch-3 overexpressing cells (HEK293-N3). Before incubation cells were pretreated with γ-secretase inhibitor to prevent ligand-induced cleavage of Notch-3 receptors. Cells were incubated with cell lysates from GFP or YB-1-GFP-overexpressing cells. GFP fluorescence was only observed after incubation with cell lysates containing

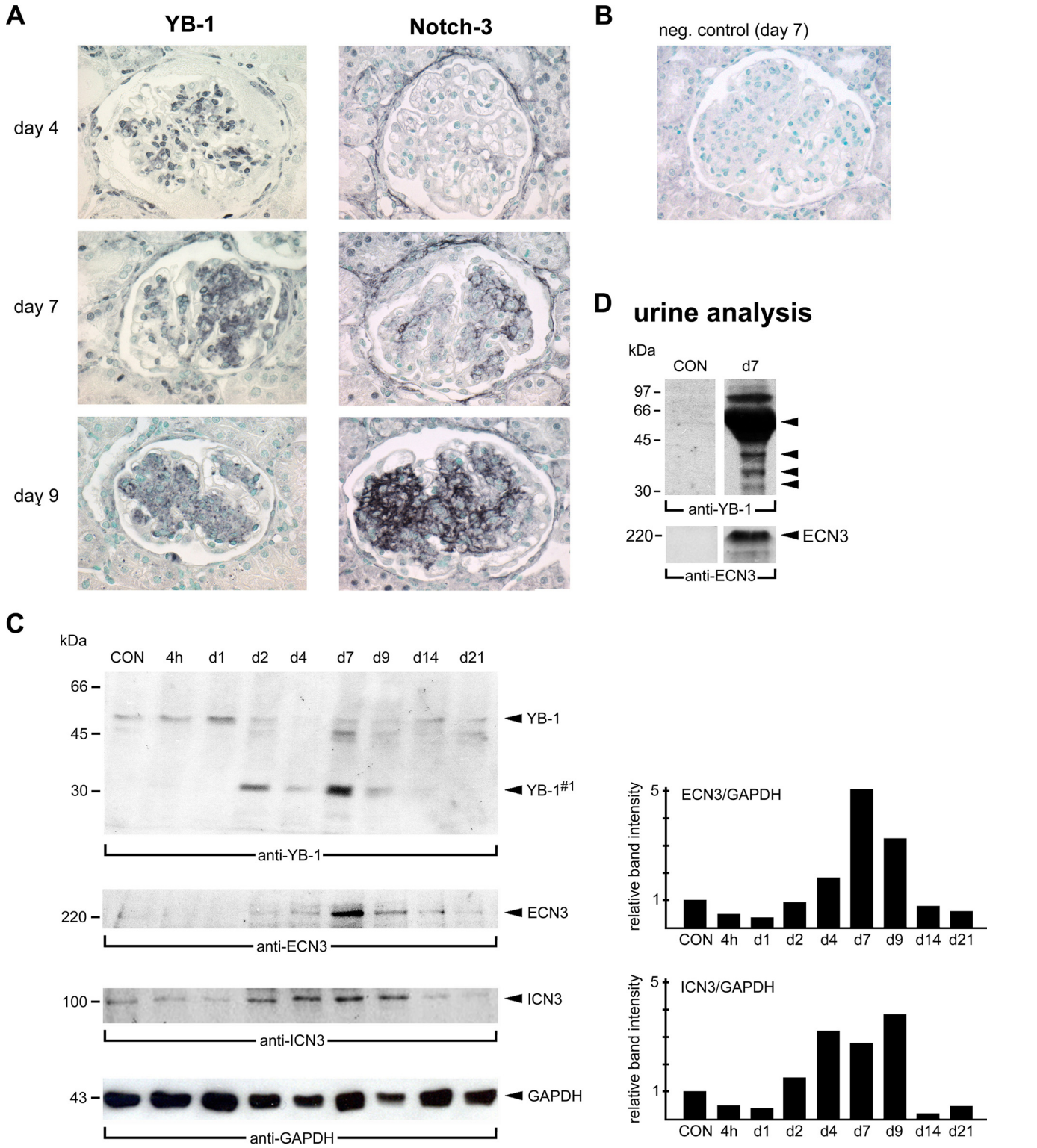


FIGURE 4. **Notch-3 is expressed by kidney cells and coordinately up-regulated with YB-1 in mesangioproliferative nephritis.** *A*, immunohistochemistry was performed for YB-1 and Notch-3 receptor in renal tissue on days 4, 7, and 9 after induction of anti-Thy1.1 nephritis in rats, and representative images for the glomerular staining patterns are provided. *B*, kidneys of rats sacrificed on day 7 after disease induction were stained with an irrelevant rabbit-IgG as primary antibody to confirm staining specificity. *C*, the expression of YB-1 and Notch-3 (intracellular, ICN3, and extracellular receptor domains, ECN3) was assessed by Western blotting in tissue lysates from sieved glomeruli collected at different time points subsequent to disease induction. Anti-GAPDH antibody was used to visualize protein load. Densitometric analyses were performed on ECN3 and ICN3 bands with normalization against values determined for GAPDH. Relative band intensities are depicted in *bar diagrams*. *D*, urine samples were obtained from healthy control animals and on day 7 after disease induction. Western blot analyses were performed to test for the presence of YB-1 and ECN3 in respective urine samples.

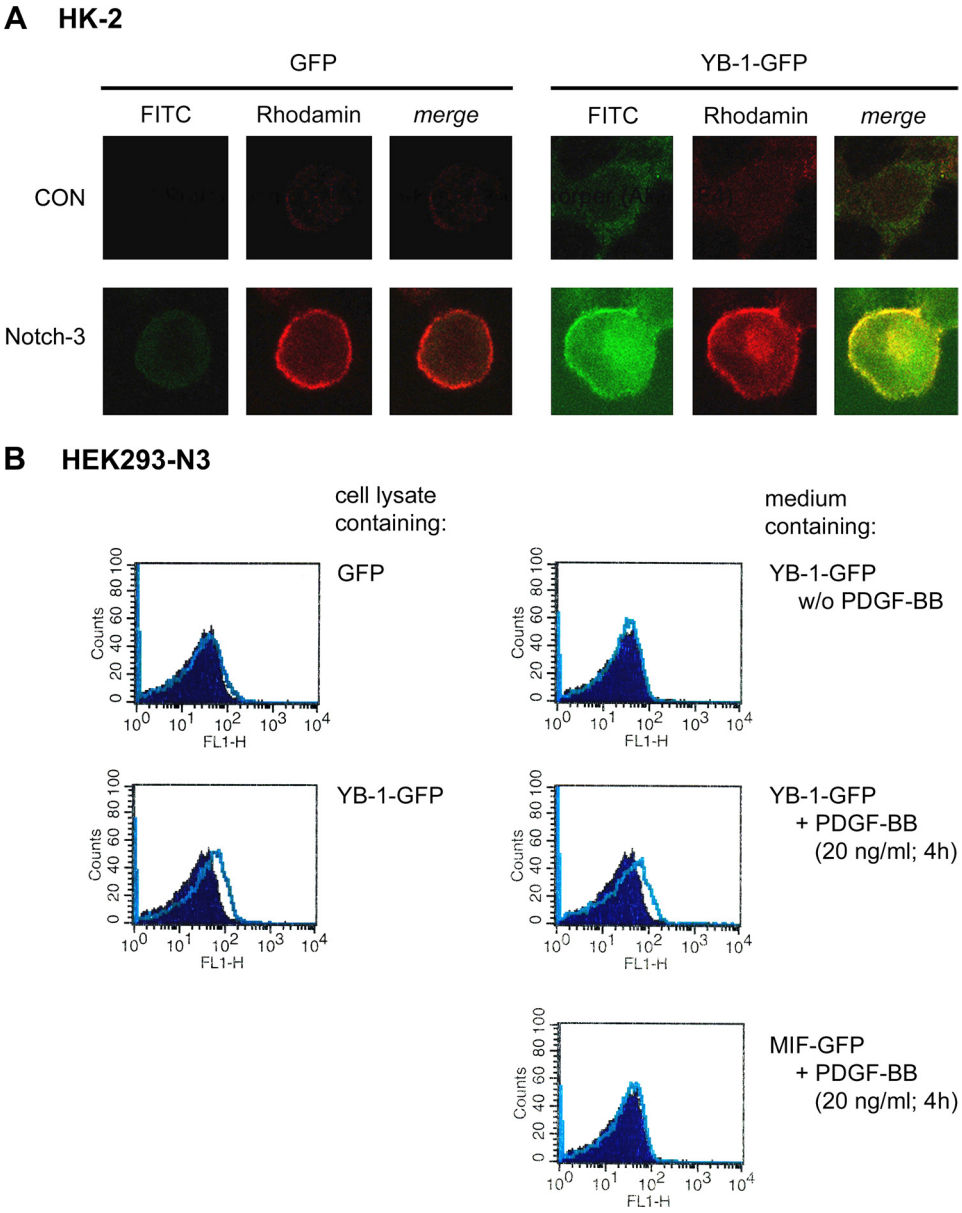


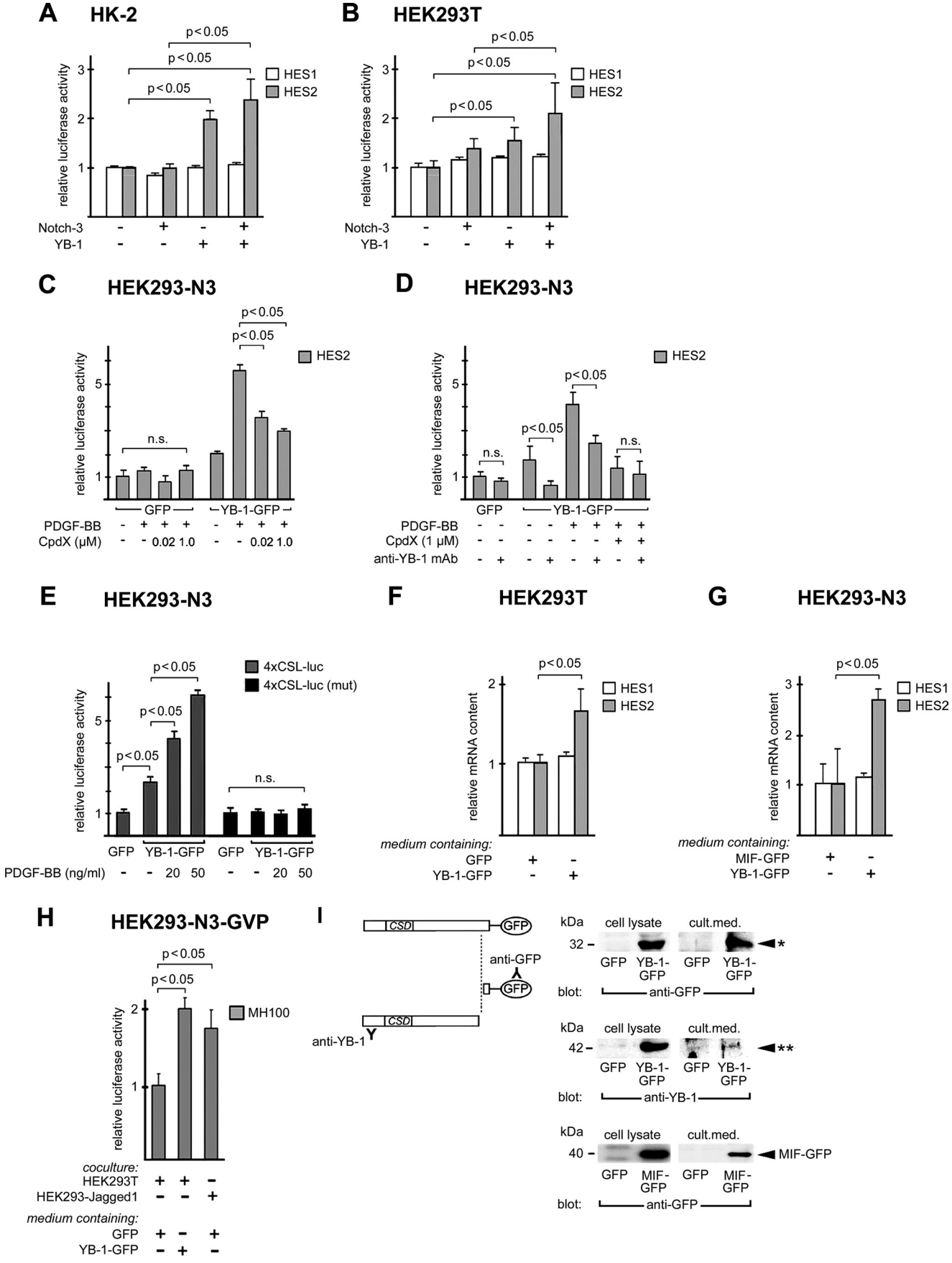
FIGURE 5. **YB-1 and Notch-3 co-localize at the outer aspect of cellular membranes.** *A*, extracellular YB-1 co-localizes with Notch-3 at cellular membranes. HK-2 cells without or with ectopic Notch-3 receptor expression were incubated with GFP or YB-1-GFP protein. Laser scanning microscopy for Notch-3 (*red*) and GFP-labeled proteins (*green*) was performed. *FITC*, fluorescein isothiocyanate. *B*, fluorescence-activated cell sorter analyses of HEK293-N3 cells pretreated with γ-secretase inhibitor compound X (1 μM) for GFP fluorescence. Cells were incubated with cell lysates containing GFP or YB-1-GFP (*left panels*) or conditioned medium harvested from cells with YB-1-GFP (with or without PDGF-BB stimulation) or MIF-GFP expression (with PDGF-BB stimulation, *right panels*).

YB-1-GFP protein, whereas GFP protein alone did not associate with Notch-3 overexpressing cells (Fig. 5*B*, *left panel*).

In a second approach conditioned medium was used that was generated with cells ectopically expressing YB-1-GFP (without or with PDGF-BB stimulation) or MIF-GFP (with PDGF-BB). Induction of YB-1 secretion upon PDGF-BB challenge led to enhanced GFP fluorescence, whereas secreted MIF-GFP as control protein had no effect (Fig. 5*B*, *right panel*).

Notch-3-dependent Target Genes Are Activated by Extracellular YB-1—Notch-3 receptor activation/signaling and subsequent release of ICN3 domains reverses the repressive effect of RBP-Jκ on transcription of specific Notch target genes (34). As HES and HERP genes are the best characterized immediate Notch downstream genes, we tested whether response elements from HES1 and HES2 genes linked to a reporter gene (HES1-luc and HES2-luc) respond to YB-1 activation of Notch-3. In the first set of experiments HK-2 and HEK293T cells were utilized that had been modified for the expression of Notch-3, YB-1, or both by introducing the respective expression plasmids (Fig. 6, *A* and *B*). Combined overexpression of YB-1 and Notch-3 with reporter constructs yielded an induction of HES2 promoter activity (*gray columns*) that reached the level of significance when compared with cells without YB-1/Notch-3 or Notch-3 expression only. In contrast, the HES1 promoter remained unstimulated throughout these experiments (Fig. 6, *A* and *B*, *white columns*). Notably, overexpressed YB-1 alone already increased HES2-dependent promoter activity in both cell lines, which may be a result of the presence of endogenous Notch-3 receptor (compare the *first* and *third pair of bars* in Fig. 6, *A* and *B*).

Given our finding that PDGF-BB is a stimulator of YB-1 secretion (compare Fig. 1), we hypothesized that cytokine-dependent release of YB-1 protein might result in increased Notch-3 receptor activation and elevated expression of Notch target genes. Therefore, HEK293-N3 cells with overexpressed GFP or YB-1-GFP protein were challenged with PDGF-BB at 20 ng/ml for 4 h, and the HES2 promoter activity was determined. PDGF-BB stimulation of YB-1-GFP-expressing cells led to a 6-fold induction of HES2 promotor activity compared with cells transfected with GFP vector (Fig. 6*C*). To assure that the observed Notch-3 signaling event is conferred by extracellular YB-1, resulting in S3 cleavage of the Notch-3 receptor by the γ-secretase complex, HEK293-N3 cells with forced YB-1-GFP expression were incubated with γ-secretase inhibitor compound X and PDGF-BB. Compound X suppressed HES2 promoter activation in a concentration-dependent manner (Fig. 6*C*). In control experiments with HEK293-N3 cells overexpressing GFP protein, such an effect was not detected (Fig. 6*C*). These observations confirm that the increase of HES2 promoter activity is dependent on


A
HK-2
relative luciferase activity
HES1
HES2
p<0.05
Notch-3
YB-1
B
HEK293T
C
HEK293-N3
n.s.
GFP
YB-1-GFP
PDGF-BB
CpdX (μM)
D
HEK293-N3
CpdX (1 μM)
anti-YB-1 mAb
E
HEK293-N3
4xCSL-luc
4xCSL-luc (mut)
PDGF-BB (ng/ml)
F
HEK293T
relative mRNA content
medium containing:
G
HEK293-N3
MIF-GFP
H
HEK293-N3-GVP
MH100
coculture:
HEK293T
HEK293-Jagged1
I
CSD
GFP
anti-GFP
anti-YB-1
kDa
cell lysate
cult.med.
blot:
MIF-GFP

Notch receptor activation followed by release of the intracellular Notch domain.

Further experiments were performed to ascertain the extracellular site of Notch receptor activation by YB-1. Secreted YB-1 protein was targeted by the addition of a monoclonal anti-YB-1 antibody to the culture medium of HEK293-N3 cells. The latter overexpressed an HES2 promoter construct and YB-1-GFP or GFP protein. An irrelevant mouse IgG1 was used as negative control. Inclusion of YB-1-specific antibody significantly abrogated YB-1-dependent activation of Notch-3/HES2 in this setup both without or with addition of secretogue PDGF-BB. Inclusion of γ-secretase inhibitor compound X again reduced HES2 promoter activity (Fig. 6*D*).

To ultimately assure specificity of the reporter gene assays for Notch signaling, we compared the responsiveness of multiple CSL-binding sites concatenated in a promoter linked to luciferase (that otherwise lacks Notch-responsive elements) with the one obtained after introduction of mutations within the CSL-binding sites. Although the transcriptional activity of the wild-type construct was readily activated in the experimental model after PDGF-BB stimulation (20 and 50 ng/ml) and YB-1-GFP secretion, the mutated promoter was unresponsive under the same conditions (Fig. 6*E*).

We next ensured that the influence of YB-1 on receptor activation is because of extracellular binding to Notch receptor domains and is independent from intracellular effects. Conditioned medium from HEK293T cells expressing YB-1-GFP was obtained after PDGF-BB stimulation. The secreted YB-1-GFP (instead of the intracellular overexpressed form) was added to HEK293T cells. An induction of HES2 mRNA was measured by quantitative real time-PCR when HEK293T cells were incubated with secreted YB-1-GFP and compared with results with GFP-containing conditioned medium (Fig. 6*F*, *gray columns*). In contrast, HES1 transcript numbers were not significantly altered by YB-1-GFP incubation (Fig. 6*F*, *white columns*).

Furthermore, we excluded the possibility that the GFP tag itself acts on receptor signaling. This was achieved by another GFP fusion protein, MIF-eGFP, that was ectopically expressed in HEK293T cells. This fusion protein is also secreted upon PDGF-BB stimulation, and the conditioned medium containing MIF-eGFP was added to the aforementioned Notch-3 readout cell system. Under these conditions no up-regulation of the HES2 promoter occurred, excluding the possibility of the GFP tag alone contributing to the observed stimulation of Notch signaling (Fig. 6*G*).

To ensure the exclusivity of YB-1 binding to Notch-3 and not to other Notch receptor subtypes, we used cells stably transfected with an artificial hybrid Notch-3 receptor construct carrying a GAL4 binding domain (HEK293-N3-GVP) that is activated after γ-secretase-dependent cleavage of Notch-3 receptor. Consecutive *trans*-regulation of a β-galactosidase reporter gene (MH100) is accomplished by the mutated intracellular N3 domain and not by other members of the Notch superfamily. The incubation of these cells with medium containing secreted YB-1-GFP led to up-regulated Notch-3 receptor activation, resulting in a 2-fold induction of β-galactosidase transcription rates compared with cells incubated with GFP-containing medium (Fig. 6*H*). This assures that extracellular YB-1 specifically activates the Notch-3 receptor.

To compare the effect of YB-1 on receptor activation directly with a well established Notch-3 ligand, Jagged1, co-culture experiments were designed with HEK293-N3-GVP and HEK293T cells. The latter were forced to ectopically express Jagged1 protein. In co-culture approaches Jagged1-overexpressing cells were able to stimulate Notch-3 signaling to a similar extent as extracellular YB-1, which is 2-fold (Fig. 6*H*).

Western blot analyses of cell culture medium utilized in those experiments were performed to assess the amount and size of secreted YB-1-GFP and MIF-eGFP. Two different antibodies were used for immunoblotting, a monoclonal antibody directed against the GFP tag and a peptide-derived polyclonal antibody directed against N-terminal domains of YB-1 protein. These immunoblots revealed the presence of a GFP-tagged proteolytic fragment of YB-1-GFP (*) with an approximate molecular mass of 32 kDa (Fig. 6*I*, *upper panel*), whereas MIF-

FIGURE 6. **Notch-3-dependent target genes are differentially regulated by extracellular YB-1.** *A* and *B*, HK-2 and HEK293T cells were tested for *trans*-regulation of HES1- and HES2-dependent reporter gene activities without and with forced expression of Notch-3, YB-1, or the combination of both. Data are the means ± S.D. of three independent experiments; $p < 0.05$ *versus* cells without overexpression of Notch-3 and YB-1. *C*, in HEK293-N3 cells GFP or YB-1-GFP and HES2 reporter constructs were overexpressed. Some of the cells were incubated with PDGF-BB (20 ng/ml) and/or γ-secretase inhibitor compound X (*CpdX*) at the indicated concentrations. Data were calculated as the means ± S.D. of five independent experiments; $p < 0.05$ *versus* cells without application of compound X. *n.s.*, not significant. *D*, HEK293-N3 cells were manipulated to express YB-1-GFP (or control GFP plasmid) and HES2 reporter construct. Incubation with PDGF-BB (20 ng/ml) and/or γ-secretase inhibitor compound X (1 μM) and/or monoclonal anti-YB-1 antibody (*mAb*) and/or nonspecific control IgG1 was performed as indicated. Data were calculated as the means ± S.D. of three independent experiments; $p < 0.05$ *versus* cells without anti-YB-1 monoclonal antibody. *E*, HEK293-N3 cells were transfected with GFP or YB-1-GFP expression plasmids and a luciferase reporter construct either containing four copies of the Notch responsive CSL-binding site (4xCSL-luc) or four copies of a mutated CSL-binding site (4×CSL-luc (mut)). Cells were incubated with PDGF-BB at the indicated concentrations for 4h before luciferase quantification. Data are the means ± S.D. of three independent experiments. $p < 0.05$ *versus* cells with overexpression of GFP protein only. *n.s.*, not significant. *F*, to ascertain that YB-1-GFP activates Notch-3 in the extracellular space, conditioned medium of GFP- or YB-1-GFP-expressing HEK293T cells was added to HEK293T cells. HES1 and HES2 transcript numbers were quantified by quantitative real-time-PCR. Data are the means ± S.D. of two independent experiments; $p < 0.05$ *versus* cells with the addition of GFP-conditioned medium. *G*, conditioned medium was obtained from HEK293T cells expressing either YB-1-GFP or GFP-tagged MIF protein after stimulation with PDGF-BB (20 ng/ml; 4 h). Medium was collected and added to HEK293-N3 cells. After 24 h HES1 and HES2 transcript numbers were quantified by quantitative real-time-PCR. Data are the means ± S.D. of two independent experiments; $p < 0.05$ *versus* cells with the addition of conditioned medium containing MIF-GFP. *H*, co-culture experiments were performed using HEK293-N3-GVP cells and the luciferase reporter construct MH100 to exquisitely detect Notch-3 signaling. Cells were co-cultured with HEK293T cells that express human Jagged1 or a control plasmid. In a similar setup, conditioned medium containing GFP or YB-1-GFP protein was added, and luciferase activities were quantified after 24 h. Data are the means ± S.D. of three independent experiments; $p < 0.05$ *versus* cells with the addition of GFP containing conditioned medium. *I*, cellular expression and secretion of proteolytic protein fragments of YB-1-GFP were assessed by Western blot analyses with trichloroacetic acid-precipitated protein from conditioned cell culture medium by a monoclonal anti-GFP antibody (fragment denoted *; ~32 kDa) and a polyclonal anti-YB-1 antibody raised against domains within its N terminus (fragment denoted *; ~42 kDa), as depicted in a schematic diagram to the *left*. Cellular expression levels and secretion of MIF-GFP protein was quantified and is shown to the right.

eGFP is secreted without proteolytic cleavage (Fig. 6*I*, *lower panel*). The corresponding N-terminal YB-1 fragment (denoted **, ~42 kDa) was visualized by means of polyclonal antibody (Fig. 6*I*, *middle panel*). By means of the anti-GFP antibody, a fragment of 32 kDa was detected. Because the GFP tag has a size of 27 kDa, we conclude that full-length YB-1-GFP is processed near the YB-1 C terminus, adding ~5 kDa to the GFP tag (depicted schematically in Fig. 6*I*). Analyses of cell lysates (HEK293T cells expressing GFP or YB-1-GFP proteins) suggest that cleavage of YB-1-GFP occurs intracellularly, as the same fragment sizes were determined with protein extracts from cell lysates (Fig. 6*I*). Comparison of the respective protein amounts in the supernatant with the cell lysates suggests that the C-terminal cleavage product is secreted more efficiently than the N-terminal portion or is degraded less rapidly. A firm statement on the relative activities of these fragments in regard to Notch-3 receptor signaling will likely be possible after purification of these protein fragments and testing in similar experiments.

DISCUSSION

Secretion of a transcription factor is unexpected at first sight but not unheard of. Similarly to high mobility group box-1 protein (HMGB-1) we determined an active secretion process for YB-1 protein via a non-classical pathway that bypasses the endoplasmic reticulum/Golgi apparatus (10). In mesangial and inflammatory cells, several cytokines like PDGF-BB, interferon-γ, oxidative stress, and lipopolysaccharide were able to act as secretogogues for YB-1. These findings combined with an observed high affinity of YB-1 for the outer aspect of the cell membrane led us to a search for possible interacting proteins at the cell surface and a two-hybrid screen identified Notch-3 as an interacting protein.

This initial finding was corroborated by co-immunoprecipitation and localization studies, which showed that YB-1 interacts with the Notch-3 receptor and that both proteins are found at the cell membrane. Interestingly, co-immunoprecipitation studies demonstrated that the interaction with YB-1 is specific for the Notch-3 receptor, as it does not occur with Notch-1. This was unexpected given the high degree of structural conservation between the two receptors, *i.e.* the extracellular Notch-3 domains of Notch-1 and Notch-3 are 45% identical, and both are composed of a series of EGF repeats and Notch/Lin-12 repeat regions. Furthermore, Notch-1 and Notch-3 appear to behave very similarly in terms of interaction with the conventional Notch DSL ligands, *i.e.* Jagged1 and Delta-like-1 (35). Our data show that the EGF-like repeats 13–33 in Notch-3 are sufficient for interaction with YB-1. This region lies outside the domain previously shown to be required for interaction with DSL ligands, which is confined to EGF-like repeat 11 and 12 (36). The YB-1-interacting domain (EGF-like repeats 13–33) may also be of interest in the context of the Notch 3-associated disease CADASIL. Notch-3 mutations in CADASIL are found in the EGF-like repeats, and several of the observed mutations fall outside the region of DSL ligand interaction but within repeats 13–33, for example, the C542Y and R1006C mutations. Besides the EGF repeats necessary for direct interaction between Notch with DSL ligands, a second functional domain in EGF-repeats 24–29 has been identified in *Drosophila melanogaster* by a class of Notch missense mutations called *Abruptex* (N^{ax}). This domain negatively regulates the interaction between Notch and DSL ligands (37), and it has been speculated that it may be necessary to invoke an altered response to a yet unidentified ligand or cofactor. Amino acid substitutions in this domain result in increased Notch activity, presumably by destroying the binding site of the postulated cofactor that functions as a negative regulator (37). Further investigations will be required to verify YB-1 binding to the *Abruptex* domain and to unravel potential modulating function(s) of YB-1 on DSL ligands.

It is of note that the observed receptor activation by YB-1 occurred in the absence of DSL ligand stimulation. The finding of YB-1 as a new ligand for Notch-3 provides further evidence for the existence of "non-conventional" Notch ligands, *i.e.* ligands that are not part of the DSL family. It has previously been reported that F3/contactin can act as a Notch ligand to control oligodendrocyte differentiation (38), and the microfibrillar proteins MAGP1 and MAGP2 were shown to induce Notch-1 signaling (39).

The Notch receptor signaling invoked by extracellular YB-1 is γ-secretase-dependent and includes HES2 promoter induction. Knowledge on HES2 promoter regulation as part of Notch signaling is rather scarce. Notch receptor-mediated induction of HES2 by membrane-bound ligand Dll4 has been reported (40), and several reports indicate an active role of HES2 in proliferative processes, such as spontaneous malignant transformation of stem cells (41) and various tumors, *e.g.* of the gastrointestinal tract (42) and Ras-transformed astrocytes (43). YB-1 knock-out mice exhibit neural tube defects with abnormal patterns of cell proliferation within the neuroepithelium (44). Given the increased HES2 transcript numbers in embryonic neural progenitor cells, one might speculate that YB-1 signaling via Notch-3 constitutes a direct link that is operative in the development of the nervous system.

In the present study care was taken to unequivocally demonstrate a direct effect of YB-1 on target gene regulation via the Notch signaling cascade. To this end, two different artificial promoter elements, a concatenated CSL-binding site and a specific promoter for Notch-3 signaling linked to the luciferase gene (MH100), as well as constructs with mutated CSL-binding sites were introduced into HEK293 cells and reproducibly demonstrate activated Notch-3 signaling by extracellular YB-1.

It was previously reported that Notch-3 is expressed in adult vascular smooth muscle cells (19), which is in keeping with the phenotype in Notch-3-deficient mice. These mice are viable, fertile, and develop largely normally but exhibit problems of vascular smooth muscle cell maturation in distal arteries (45). Our data show that Notch-3 receptor was detected at the basal membrane of some tubular and collecting duct cells within the inner renal medulla in the adult kidney, whereas no glomerular cells were immunopositive. Subsequent to induction of mesangioproliferative Anti-Thy1.1 nephritis, Notch-3 receptor was detected within the mesangial compartment of glomeruli, and its expression peaked at a time with maximal mesangial cell proliferation rates (29) and, notably, coincided temporally and spatially with increased glomerular YB-1 protein expression.

The presence of cleaved extracellular domains of Notch-3 (ECN3) in the urine of diseased rats underscores its abundant expression and activation in the course of the disease. The band pattern for YB-1 in the urine was complex and included bands that correspond to full-length protein as well as protein fragments thereof. One might speculate that a high molecular mass complex of ~90 kDa corresponds to YB-1 protein bound to albumin, given the high affinity of both proteins for each other (data not shown).

20 S proteasome-mediated cleavage of YB-1 before glycine 220 has been demonstrated before (46). The calculated sizes for the resultant fragments are in line with the observed band mobility (corresponding to 30 kDa) during mesangioproliferative nephritis. It will be furthermore of interest to test whether YB-1 protein fragments act differently on receptor activation.

The observation of Notch-3 up-regulation as part of the inflammatory response is especially notable in light of a study providing evidence that Notch-3 receptor signaling profoundly alters intracellular signaling cascades involved in inflammatory responses, namely Jak2 activation and the subsequent STAT3 phosphorylation (47). It may, therefore, be speculated that YB-1 secretion and Notch-3 receptor expression/activation may amplify the inflammatory response of MC conferred by the Jak/STAT (signal transducers and activators of transcription) pathway.

In sum, the data presented in this report provide evidence for a role of extracellular YB-1 as a ligand for Notch-3. This contributes to our understanding both of the repertoire of conventional and non-conventional ligands for Notch receptors and for the specificity of the individual receptor isoforms. It also highlights possible new roles of Notch and YB-1 in the inflammatory response. Ramifications of Notch-3 activation by YB-1 could be important in other processes such as stem cell maintenance, leukemogenesis, and lymphangiogenesis.

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