Phosphorylation of the Transactivation Domain of Pax6 by Extracellular Signal-regulated Kinase and p38 Mitogen-activated Protein Kinase

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The transcription factor Pax6 is required for normal development of the central nervous system, the eyes, nose, and pancreas. Here we show that the transactivation domain (TAD) of zebrafish Pax6 is phosphorylated in vitro by the mitogen-activated protein kinases (MAPKs) extracellular-signal regulated kinase (ERK) and p38 kinase but not by Jun N-terminal kinase (JNK). Three of four putative proline-dependent kinase phosphosylation sites are phosphorylated in vitro. Of these sites, the serine 413 (Ser413) is evolutionary conserved from sea urchin to man. Ser413 is also phosphorylated in vivo upon activation of ERK or p38 kinase. Substitution of Ser413 with alanine strongly decreased the transactivation potential of the Pax6 TAD whereas substitution with glutamate increased the transactivation. Reporter gene assays with wild-type and mutant Pax6 revealed that transactivation by the full-length Pax6 protein from paired domain-binding sites was strongly enhanced (16-fold) following co-transfection with activated p38 kinase. This enhancement was largely dependent on the Ser413 site. ERK activation, however, produced a 3-fold increase in transactivation which was partly independent of the Ser413 site. These findings provide a starting point for further studies aimed at elucidating a post-translational regulation of Pax6 following activation of MAPK signaling pathways.

Pax6 is a member of the paired box-containing Pax gene family of transcription factors containing nine human (PAX1-PAX9) and murine (Pax1-Pax9) family members (1). The paired box was first discovered in Drosophila as an encoding a conserved 125–128 amino acid paired domain unique to this family of developmental control genes (2). Pax6 was initially cloned from human (3), mouse (4), zebrafish (5), and quail (6). Subsequently, the Drosophila eyeless gene was shown to be a Pax6 homolog and Pax6 homologs have now been described in other invertebrates such as flatworm, ribbonworm, Caenorhabditis elegans, squid, sea urchin, and ascidian (reviewed in Ref. 7) as well as in amphioxus (8). Pax6 is expressed in the developing central nervous system, the eyes, nose, and pancreas in higher vertebrates (4, 5, 9, 10) and plays a pivotal role in the development of these organs (7, 11, 12). Loss of a functional Pax6 allele results in the Mendelian syndromes aniridia, Peter’s anomaly, and congenital cataracts in man (13) and Small eye in rodents (14). Pax6 acts high up in the regulatory hierarchy controlling eye development in both vertebrates and invertebrates (reviewed in Ref. 15). Eyeless, ribbonworm-, squid-, ascidian-, zebrafish-, or mouse Pax6 are all able to induce ectopic eyes in Drosophila upon targeting their expression to different imaginal discs (16–20). We have recently found that zebrafish contain two Pax6 genes, Pax6.1 and Pax6.2, which are expressed in both overlapping and distinct regions during development of the eyes and the central nervous system. Both these genes are able to induce ectopic eyes in Drosophila (18).

The paired domain is a bipartite DNA-binding domain containing an N- and a C-terminal subdomain each with a helix-turn-helix motif (21, 22). Pax6, like Pax3, Pax4, and Pax7, also harbors a second DNA-binding domain, the paired-type homeodomain (2, 7, 23). In Pax6 this domain is separated from the N-terminal located paired domain by a flexible, acidic linker region (5, 7). The region C-terminal to the homeodomain is enriched in proline, serine, and threonine residues (PST-rich) and acts as a transactivation domain (TAD) (24–27).

It has previously been shown that quail Pax6 proteins expressed in the neuroretina are phosphoproteins and phosphoamino acid analysis revealed phosphoserine and a minor proportion of phosphothreonine (28). The activity of many transcription factors is regulated in a rapid and reversible manner by specific phosphorylation events mediated by protein kinases acting in signaling cascades initiated by extracellular stimuli (reviewed in Refs. 29 and 30). Mitogen-activated protein kinases (MAPKs) are proline-directed serine/threonine protein kinases activated by heterogenous extracellular stimuli, including growth factors, hormones, cytokines, antigens, and physical-chemical stimuli such as oxidative stress, heat shock, osmotic imbalance, and UV light. MAPK cascades play essential roles in regulating many critical cellular processes, including cell growth and division, differentiation, apoptosis, and stress-related responses (reviewed in Refs. 31 and 32). These cascades are organized into modules of three protein kinases where a MAPK kinase kinase (e.g. Raf-1) activates a MAPK kinase (e.g. MEK1) which subsequently activates a MAPK (e.g. ERK1) (31, 33). Following activation the MAPKs translocate to the nucleus to phosphorylate nuclear substrates. Currently, four distinct MAPK cascades are known in vertebrates. How-
ever, more will probably be found since the budding yeast contains five such cascades that orchestrate responses to different physiological stimuli (34). The extracellular-signal-regulated kinase (ERK) pathway mainly conveys signals from mitogenic and differentiation stimuli. The same may be true for the p38 MAPK pathway (reviewed in Ref. 31). A growing number of transcription factors have been identified as nuclear targets of the ERK pathway, JNK1–3 in the JNK pathway and p38 MAPK. Each of these three pathways contains five such cascades that orchestrate responses to different stimuli. The same may be true for the p38 MAPK pathway.

**TABLE I**

Sequences of oligonucleotides used as polymerase chain reaction primers for plasmid constructions and site-directed mutagenesis and as double-stranded binding site probes in gel mobility shift assays.

| Name          | Sequence                                      |
|---------------|-----------------------------------------------|
| G4-P6(299)    | 5'GCCGGATCCCTAATGCTCAAGT-3'                   |
| pSG424.3'     | 5'TGTTTCAATTCTAAGTCCGTAAGTTT3'               |
| 5eukGST       | 5'ATGCCGCAGCCGTGCGCTGACGACG-3'               |
| S413E         | 5'CGG-GTT-TAA-TGG-ACC-TGG-TGG-CGG-3'         |
| T358A         | 5'GATGCGCACCCGCAGCCGAGTT-3'                 |
| T375A/S376A   | 5'GATGCGCACCCGCAGCCGAGTT-3'                 |
| T322A/T323A   | 5'GATGCGCACCCGCAGCCGAGTT-3'                 |
| P6COns        | 5'TCAGGATYTYGAAAATTTTTCACGCTTGAGTTCACAGCTCGAGTA-3' |

**Materials and Methods**

**Cell Culture—**HeLa cells (ATCC CCL 2) were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (HyClone), nonessential amino acids (Life Technologies, Inc.). 2.5 mg/l glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). NIH 3T3 fibroblasts (passage 123) were purchased from the American Type Culture Collection (ATCC CRL 1658) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (HyClone, Logan, Utah), penicillin (100 units/ml), and 10 μg/ml streptomycin (Life Technologies, Inc.).

**Plasmid Constructs—**GAL4-Pax6 fusions were made by cloning parts of the zebrafish Pax6 coding region in-frame with the DNA-binding domain of yeast GAL4 (1–147) in pSG424 (50). All GAL4-Pax6 fusions are named according to the included parts of the Pax6 protein with the amino acid positions shown in parentheses. GAL-Pax6 (174–47) was made by cloning the 1296-base pair EdIII-XbaI fragment of Pax6 into the Snal-XbaI sites of pSG424. The 64-base pair MboII fragment containing the C-terminal TAD of Pax6 was inserted into the Snal site of pSG424 to give GAL-Pax6 (299–437). GAL-Pax6 (299–395) was made from the 299–437 construct by deleting the SpeI-Sacl fragment from GAL-Pax6 (299–437). To construct GAL-Pax6 (299–352) a HindIII-Sacl fragment was deleted from GAL-Pax6 (299–437). GAL-Pax6 (353–437) was made by ligation of a HindIII (made blunt)-XbaI fragment into the BamHI (made blunt)-XbaI sites of pSG424. GAL-Pax6 (374–437) was constructed from GAL-Pax6 (299–437) by deletion of the SpeI (made blunt)-HindIII fragment and ligation of the rest of the vector to a HindIII-Sacl fragment from pSG424. GAL-Pax6 (396–437) was also made from GAL-Pax6 (299–437) by deleting the NdeI (made blunt)-HindIII fragment followed by ligation of the rest of the vector to a HindIII-Sacl fragment from pSG424. GAL-Pax6 (374–395) was made from GAL-Pax6 (374–437) by cutting it with NdeI and SalI, making the ends blunt and religating them. GAL-Pax6 (353–374) was derived from GAL-Pax6 (353–437) by digestion with BsiWI, making the ends blunt, and religating them. The GAL-Pax6 (353–395) construct was made from GAL-Pax6 (353–437) by deletion of the NdeI-Sacl fragment followed by religation of the rest of the plasmid.

**Specific PCR primers (G4-P6(299) and pSG424.3′)**; see Table I were used to transfer selected Pax6 constructs from pSG424 to pFA-CMV (Stratagene), where the expression of the GAL4-Pax6 fusions is driven by a CMV promoter. All constructs were verified by sequencing and the expression and correct sizes of fusion proteins following transfection of HeLa cells were verified by Western blotting.

The GST-Pax6 (353–437) construct was made by ligating the HindIII (made blunt)-EcoRI Pax6 fragment into the Smal-EcoRI site of pGEX-3X (Pharmacia). GST-Pax6 (299–437) was constructed by inserting a 640-base pair EcoRI fragment from GAL-Pax6 (299–437) into the EcoRI site of pGEX-1 (Pharmacia). The Pax6 expression vector pCI-Pax6 and the reporter vectors pGM1TATA-CAT, pTKG-CAT, and pG5TATA-LUC have been described previously (18, 51).
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*In Vitro Mutagenesis—*In vitro mutagenesis was performed according to the instruction manual for the “Quick-Change Site-Directed Mutagenesis Kit” (Stratagene). All mutagenized constructs were checked by sequencing. The specific mutagenesis primers used are shown in Table II.

*In Vitro Phosphorylation Assays—*GST-Pax6 fusion proteins were purified from Escherichia coli LE392 extracts using glutathione-agarose beads (Pharmacia). The proteins were not eluted, but left on the beads and stored at 4 °C in phosphate-buffered saline containing 1% Triton X-100. To equalize the amounts of proteins used in the kinase assays the concentrations of GST fusion proteins were estimated by Coomassie Blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The beads were washed twice with the respective kinase buffers before use in *in vitro* kinase assays. GST-Pax6-containing agarose beads (5–20 μl) were mixed with either: 1) 2 μl of 10 × MAPK buffer (500 mM Tris-Cl, pH 7.5, 100 mM MgCl2, 10 mM dithiothreitol, 10 mM EGTA), 2) 1 μl of 1 mM ATP, 0.5 μl of [γ-32P]ATP (100 μCi/μmol), and 1 unit (1 μg) of recombinant active p38 kinase reaction buffer (250 mM Heps, pH 7.5, 100 mM magnesium acetate), 500 μM ATP, 0.5 μl of [γ-32P]ATP (100 μCi/μmol), and 1 μl (1 μg) of recombinant active p38 kinase (Stratagene) in a total volume of 40 μl. The reactions were left at 30 °C for 30 min, flicking the tubes every fifth min. The agarose beads were subsequently washed with 500 μl MAPK buffer, 500 μl PBS, and 500 μl proteinase K-containing PBS before 2 × SDS-PAGE gel loading buffer. The proteins were loaded on a 10% SDS-polyacrylamide gel, and run at 20 mA for 1.5–2 h in a Tris glycine buffer and the other half was washed twice in the TBST buffer for 30–60 min. The secondary antibodies for 1 ha troom temperature. The membrane was then washed 5–6 times in Tris-buffered saline, pH 7.5, 0.1% Tween 20 (TBST) and 5% non-fat dried milk. The following primary antibodies were used: anti-GST, anti-GAL4 DBD antibodies and protein A/G-Sepharose beads (both from Santa Cruz Biotechnology) were used for immunoprecipitation of the GAL4-Pax6 fusion proteins. The beads were washed 3 times with the lysis buffer before boiling in 30 μl of gel-loading buffer and electrophoresis on a 10% SDS-polyacrylamide gel. The proteins were blotted onto a PVDF membrane and 32P-labeled proteins were detected by autoradiography.

**Western Blot—*HeLa cells were seeded at 4 × 10^5 cells per 10-cm dish the day before transfection. The relevant expression constructs were transfected using calcium phosphate coprecipitation. Ten μg of pCI-Pax6 or the vector control pCI-neo was co-transfected with 5 μg of expression vector for p38 kinase in combination with 5 μg of expression vector for MKK6b(EE) or vector control for the latter or with 5 μg of expression vector for MEK1(EE) or its vector control (see Fig. 8). In all transfections 1 μg of pCMV-βgal (Stratagene) was included to allow measurement of β-galactosidase activities that were used to normalize for variations in transfection efficiencies. The cells were harvested after 2 days either by directly scraping them into 100 μl of 2 × SDS-PAGE gel loading buffer or into the Dual-Light lysis buffer (Tropix Inc.) to set the condition for a β-galactosidase activity measurement. The remaining was mixed with 5 × SDS gel loading buffer and boiled. For some blots nuclear extracts prepared as described (57) were used. When indicated in figure legends the amount of protein loaded on the gel had been adjusted according to measurements of β-galactosidase activity. Proteins run on a 10% SDS-PAGE gel were blotted onto a PVDF membrane and blocked overnight at 4 °C in a buffer consisting of Tris-buffered saline with 0.1% Tween 20 (TBST) and 5% non-fat dried milk. The following primary antibodies were used: anti-GST, anti-GAL4 DBD (diluted 1:1,000; Santa Cruz Biotechnologies), serum 14 (28) (diluted 1:5,000), and P6C; affinity purified anti-Pax6 C-terminal antibodies (58, 59) (diluted 1:800). The primary antibodies were applied for 1 h at room temperature. The membrane was then washed 5–6 times in the TBST buffer for 30–60 min. The secondary antibodies (anti-rabbit IgG-AP, Santa Cruz Biotechnology or anti-mouse IgG-AP, Sigma) were diluted 1:2,000 or 1:2,000, respectively, in the blocking buffer and left for 1 h at room temperature. The washing described above was repeated. CDP-Star substrate (Roche Molecular Biochemicals) was used according to the manufacturers instructions to visualize the specific protein bands.

**Transient Transfections, CAT, and Luciferase Assays—*The conditions used for transfections, extract preparation, and measurements of CAT activity were as described earlier (60). For transfections for luciferase assays, 4 × 10^4 cells were seeded per well in 6-well dishes the day before transfection. Fresh medium was added to the cells 2 h before the DNA on the day of the transfection. Whenever the Pax6 expressing construct contained a cytomegalovirus (CMV) promoter or LipofectAMINE Plus (Life Technologies, Inc.). For ERK-mediated phosphorylation, 10 μg of GAL4-Pax6 TAD fusion constructs and 5 μg of expression vector for an activated mutant of MEK1, MEK1(EE) (56), or the respective vector control was used in co-transfections. In *in vivo* labeling with [32P]orthophosphate was done essentially as described above with the exception that the cells were harvested in HA lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100) for experiments with p38 kinase and JNK lysis buffer (25 mM Heps, pH 7.7, 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.15 Triton X-100) for experiments with activated MEK1. Both lysis buffers contained the following inhibitors: 20 mM p-nitrophenyl phosphate, 50 mM sodium fluoride, 50 μM sodium vanadate, 5 mM benzamidine, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, 0.2 mM benzamidine, 0.5 mM p-chloro- methyl p-nitroaniline, 0.7 μg/ml pepstatin A. Polyvalent anti-GAL4 DBD antibodies and protein A/G-Sepharose beads (both from Santa Cruz Biotechnology) were used for immunoprecipitation of the GAL4-Pax6 fusion proteins. The beads were washed 3 times with the lysis buffer before boiling in 30 μl of gel-loading buffer and electrophoresis on a 10% SDS-polyacrylamide gel. The proteins were blotted onto a PVDF membrane and 32P-labeled proteins were detected by autoradiography.
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**RESULTS**

The Transactivation Domain of Pax6 Includes the Entire C-terminaL PST Domain—The 139-amino acid long C-terminal transactivating region of zebrafish Pax6.1 (hereafter referred to as Pax6) is enriched in proline (P), serine (S), and threonine (T) residues comprising 45.4% of the total amino acids in this so-called PST domain. The over-representation of these residues imposes a hydrophilic nature to this domain (63% hydrophilic amino acids), but it is also noteworthy that there is both a significant under-representation of charged residues and a over-representation of methionine residues. The sequence of this region is extremely conserved among vertebrate Pax6 proteins with zebrafish Pax6 being 93.6% identical (98.6% similar) to human Pax6 (see Fig. 1A). If amphioxus, squid, and sea urchin Pax6 proteins are included in the comparison, the sequence divergence increases but there is a striking conservation of two C-terminal sequence motifs (GLISPGVSVP(V/I)QVPG and YW(P/S)R(L/I)Q in Fig. 1A).

**Fig. 1. Localization of putative phosphorylation sites for proline-dependent protein kinases and predicted secondary structure elements in the Pax6 transactivation domain (TAD).** A, sequence alignment of the Pax6 TAD including sequences from zebrafish (Zpax6.1 and Zpax6.2) (15, 18), human (Hpax6) (3), quail (Qpax6) (6), amphioxus (Apax6) (8), squid (Spax6) (19), and sea urchin (Spax6) (25). Putative phosphorylation sites for proline-dependent protein kinases are indicated with asterisks above the alignment. Residues displayed on a black background are identical in all the compared species whereas other residues conserved in most of the proteins aligned are indicated by two shades of gray. Dashes indicate gaps introduced to facilitate optimal alignment. B, predicted secondary structure elements within the Pax6 TAD displayed together with the primary sequence of the zebrafish Pax6.1 TAD (positions 299 to 437). Black bars indicate β-sheets and an open bar an α-helix. These elements were predicted using both the PHD neural network method with a multialignment as input (72) and the Alexis program of the Seqsee program suite (73). Vertical arrows indicate turns predicted using the ProtScale resource at the Expasy server (http://www.prospector.expasy.org) and putative phosphorylation sites for proline-dependent protein kinases (asterisks).

In order to define the extent of the Pax6 TAD we performed a deletion study by fusing parts of the Pax6 TAD (from amino acids 299 to 437) to the yeast GAL4 DNA-binding domain (DBD) and assayed transactivation by co-transfection of HeLa cells using two different reporters. One reporter,
co-transfected into HeLa cells with reporter plasmids containing 5 GAL4-binding sites either upstream of the adenovirus E1b TATA minimal promoter (pG2,E1bTATA-CAT) or the thymidine kinase promoter (pTKG5CAT). A, schematic drawing of the different constructs. The numbers in parentheses refer to amino acid positions defining the parts of the Pax6 protein included in the fusions. The locations of the paired domain (PD), the homeodomain (HD), and the TAD are indicated. B and C, deletions from either the N- or C-terminal part of the TAD lead to a sharp decrease in transcriptional activity. The results are presented as percent activity relative to the complete TAD (aa 299–437) which was set to 100%. The data represent the means of four to seven independent experiments performed in triplicates for each construct. For all transfections 2 μg of both effector and reporter plasmids, supplemented with 2 μg of sonicated salmon sperm DNA, were used. The cells were kept in 10% serum and harvested 48 h post-transfection. GAL4-AH and GAL4-VP16 were included as controls of a weak and a very strong transactivation domain, respectively.

pG2,E1bTATA-CAT, contained 5 GAL4-binding sites upstream of the TATA box from the minimal promoter of the adenovirus E1b gene (62) allowing activation of a core promoter from a TATA-proximal position to be measured. The other, pTKG5CAT (51), contained the same GAL4-binding sites inserted upstream of the herpes simplex virus thymidine kinase promoter facilitating measurements of both activation and repression of a more complex promoter. The CAT activity obtained by the GAL4-Pax6(299–437) construct was set to 100% and the activities of the truncated constructs are given in percent relative to this value. The expression and correct sizes of all the different fusion proteins were verified by Western blotting of extracts from transfected cells (data not shown). As can be seen from Fig. 2, deletions of the TAD from either the N- or C-terminal end dramatically reduced the transactivation potential. Both reporters produced a similar picture but the reporter containing the minimal E1b promoter was more sensitive and we therefore refer to the results obtained with this reporter in the following. Deletion of 55 amino acids from the N-terminal end of the TAD (retaining positions 353–437) reduced transactivation by 65%. Further deletion of 23 amino acids (retaining positions 375–437) yielded a residual activity of only 5% while no significant activity could be measured when only the C-terminal 42 residues from position 396 to 437 were assayed following fusion to the GAL4 DBD. However, deletion of the C-terminal 43 amino acids showed that, although inactive by itself, this region is very important for full activity since the TAD from position 299 to 395 has lost 80% of the activity of the full-length TAD. Further deletion of the region between positions 395 and 375 reduced only slightly this residual activity while all activity was lost upon removal of an additional 23 amino acids from the C-terminal end (retaining position 299–352). We also measured the activity of the middle region from position 353 to 395 and the two halves of this region as well as two internal deletions in this area. The middle region showed very low activity while the two halves were devoid of activity. The internal deletion of residues 353 to 374 reduced transactivation by more than 50% while deletion of amino acids 375 to 395, removing one of the two repeat modules, had no effect. Taken together, these results clearly show that the entire C-terminal PST-rich region of Pax6 acts as an unusually long TAD with no minimal activation domain. In contrast to what has been found for the Pax2, -5, and -8 subfamily of Pax proteins (63) there does not seem to exist any inhibitory regions within the Pax6 TAD.

To assess the relative potency of the transactivation domain of Pax6 we compared the level of transactivation from the pG2,E1bTATA-CAT reporter of GAL4-Pax6(299–437) to that of the weak activator GAL4-AH and the extremely strong activator GAL4-VP16 in HeLa cells. From three independent experiments performed in triplicate we found the Pax6 TAD to be only 2.5-fold weaker than the VP16 TAD and 40-fold stronger than AH. This clearly indicates the strong transactivation potential of the isolated Pax6 TAD upon fusion to the GAL4 DBD. Interestingly, when the region from amino acids 175 to 437, comprising part of the linker region, the homeodomain, and the entire TAD, was fused to the GAL4 DBD a low activity was measured from the minimal promoter reporter compared with that of the isolated Pax6 TAD.

**The Transactivation Domain of Pax6 Is Phosphorylated in Vitro by the Mitogen-activated Protein Kinases ERK2 and p38, but Not by JNK**—It has previously been demonstrated that the quail Pax6 protein is phosphorylated in neuroretina cells mainly on serine residues but with some phosphorylation of threonine also (28). Inspection of the sequence of the Pax6 TAD revealed four potential phosphorylation sites for mitogen-activated protein kinases at Thr235, Ser376, Thr388, and Ser413 (indicated with asterisks in Fig. 1). As an initial experiment a GST fusion protein of Pax6 (amino acids 353–437), expressed and purified from *E. coli*, was used in *in vitro* kinase assays with the activated MAPKs ERK2, p38 kinase, and JNK (Jun N-terminal kinase). Activated ERK2 phosphorylated the GST-Pax6 fusion protein, whereas GST alone or GST fusions of the C-terminal TADs of Pax2, Pax3, Pax9a, or Pax9b were not.
Phosphorylated at all. The same result was obtained with activated p38 kinase. However, when JNK immunoprecipitated from UV-irradiated NIH 3T3 cells was used only GST-Jun and neither the Pax6, Pax2, Pax9a nor Pax9b GST fusions were phosphorylated (data not shown). To map the phosphorylation site(s) for ERK2 and p38 kinase in the Pax6 TAD, mutations were introduced at the three most C-terminal candidate sites described above by substituting the respective serine and threonine residues with alanine. Upon expression in E. coli the GST-Pax6(353–437) fusion protein gives a band of 35 kDa representing the full-length fusion protein and three other bands of lower molecular masses which are specific proteolytic fragments representing “deletions” from the C-terminal end of the Pax6 TAD (Fig. 3A, lower panel). This specific fragmentation pattern proved beneficially for mapping of the sites that are phosphorylated in vitro. Thus, in vitro phosphorylation of GST-Pax6(353–437) mutant proteins with ERK2 showed that the Thr388 site is not phosphorylated since the T388A mutant protein gives the same phosphorylation pattern as the wild-type (wt) protein (Fig. 3A, lane 4). Due to the specific band pattern, one can easily see that both the Ser376 and Ser413 sites are phosphorylated in vitro. In the S376A mutant protein the lower molecular weight fragments are not phosphorylated (Fig. 3A, lane 3) whereas they are in the T388A and S413A mutants. Phosphorylation at Ser413 caused a mobility shift that can no longer be seen in the S413A mutant (Fig. 3A, lane 5). Since the Thr323 site was not included in this set of experiments we prepared GST-Pax6 fusions harboring the entire C-terminal TAD from amino acid 299 to 437 representing a panel of single and double mutants as well as a triple mutant including all three phosphorylation sites (T322A/T323A, T375A/S376A, and S413A) sites. (For simplicity, we refer to the T322A/T323A and T375A/S376A mutants as single mutants, since only one putative MAPK phosphorylation site is mutated in each of them.)

Following in vitro phosphorylation both ERK2 and p38 kinase gave the same phosphorylation pattern (Fig. 3B). The GST-Pax6(299–437) fusion protein gives two bands, where the slowest migrating band have the expected size for the full-length fusion protein (about 41 kDa), while the faster migrating band is a degradation product containing GST and a short fragment of the Pax6 TAD encompassing the Thr323 phosphorylation site. As is evident when lanes 3, 8, and 10 are compared with the other lanes in Fig. 3B, the T322A/T323A mutation prevents phosphorylation of the faster migrating band showing that the Thr323 site is a phosphorylation site for both ERK2 and p38 kinase in vitro. None of the other single site mutations affected the phosphorylation pattern. The two double mutants (T322A/T323A, S413A and T375A/S376A, S413A) both caused a marked decrease in phosphorylation intensities when ERK2 was used for in vitro phosphorylation (Fig. 3B, lanes 8 and 9). For the p38 kinase, however, only the T322A/T323A, S413A double mutation lead to a decreased phosphorylation indicating that the Thr323 site is more important for phosphorylation by p38 kinase than the Ser376 site. For both ERK2 and p38 kinase the triple mutation (Fig. 3B, lane 10) prevented phosphorylation completely. This proves that Thr323, Ser376 and Ser413 are the only phosphorylation sites used in vitro by ERK2 and p38 kinases in the C-terminal TAD (amino acids 299–437) of zebrafish Pax6.

**Pax6 Is Phosphorylated In Vivo by ERK and p38 MAPKs**—As described above, three of the four potential phosphorylation sites for proline-dependent kinases in the TAD of Pax6 are phosphorylated by ERK2 and p38 kinase in vitro. To see if the Pax6 TAD could be phosphorylated in vivo, NIH 3T3 cells were transfected with a eukaryotic expression vector for GST-Pax6(353–437) and in vivo labeled with [32P]orthophosphate following serum starvation. The cells were harvested after stimulation with 10% serum, to activate ERK1 and -2, and the GST-Pax6 fusion protein was purified from the cell lysate by
the use of glutathione-agarose beads. There is some phosphorylation of the fusion protein in serum-starved cells, but addition of serum enhanced this phosphorylation 2.1-fold (Fig. 4A). The membrane was stained with an antibody raised against the Pax6 TAD to control equal loading of the lanes. We then utilized the finding that phosphorylation at the evolutionary conserved Ser413 site causes a mobility shift of the GST-Pax6(353–437) fusion protein to study the time course of phosphorylation of this site following serum stimulation. As monitored by Western blotting, Ser413 is phosphorylated after 15 min of serum stimulation with a slight increase at 60 min followed by a rapid decrease 4 h after addition of serum (Fig. 4B). Thus, the kinetics of phosphorylation of the Ser413 site of the Pax6 TAD is similar to the kinetics of ERK activation. Using in vivo labeling with [32P]orthophosphate followed by serum stimulation of NIH 3T3 cells we confirmed the in vitro data that suggested the Ser413 site as the cause of the phosphorylation-induced mobility shift. When the Ser376 site is mutated the lower migrating band disappears, while the shifted band disappears, while the shifted band is present (Fig. 4C).

To further confirm that the Ser413 site is phosphorylated in vivo by ERK1 and -2 we stimulated HeLa cells transfected with expression vectors for GST-Pax6(353–437) wt and S413A mutant proteins with the phorbol ester TPA for 15 min. TPA is a rather specific inducer of the MEK-ERK pathway in these cells. We also employed a specific inhibitor of MEK, PD 98059 (64), to determine if MEK-induced activation of ERK is required for phosphorylation of Ser413 following stimulation with TPA. As seen from the Western blot in Fig. 5A, TPA induced the mobility shift indicative of phosphorylation of Ser413 while pretreatment with PD 98059 abolished the TPA-induced phosphorylation of Ser413. To confirm that TPA leads to ERK activation under these conditions we determined the phosphorylation of a GST-ElkC fusion protein following immunoprecipitation of ERK. Thus, TPA treatment (100 ng/ml) for 15 min induced phosphorylation of the GST-ElkC fusion protein purified from serum-stimulated NIH 3T3 cells, but addition of serum enhanced this phosphorylation 2.1-fold (Fig. 4A). The membrane was stained with an antibody raised against the C-terminal TAD of Pax6 (Fig. 4B). In vivo, the mobility shift induced by phosphorylation of Ser413 is clearly evident at 15 min (Fig. 4C, upper panel). This phosphorylation experiment clearly suggest that the Ser413 site is contributing much more than the Ser376 site to the total phosphorylation of GST-Pax6(353–437). As a final proof that the mobility shift observed for the GST-Pax6(353–437) fusion protein is indeed due to phosphorylation of Ser413 we treated 35S-labeled GST-Pax6 fusion proteins purified from serum-stimulated NIH 3T3 cells with λ-phosphatase which specifically dephosphorylates phosphoserine and phosphothreonine residues in proteins. As shown in Fig. 4D, phosphatase treatment leads to loss of the shifted band.

FIG. 4. The Pax6 TAD displays serum-induced phosphorylation at Ser413 in vivo with kinetics similar to the activation of ERK1 and -2. A, in vivo phosphorylation of the Pax6 TAD is increased following addition of 10% serum to quiescent NIH 3T3 fibroblasts for 15 min. NIH 3T3 cells transfected with a GST-Pax6(353–437) fusion protein expression vector were metabolically labeled with [32P]orthophosphate, stimulated with serum for 15 min and GST-fusion protein purified as described under “Materials and Methods.” Following SDS-PAGE and transfer to a PVDF membrane quantitation of phosphorylation was performed using a PhosphorImager (top panel) and the fusion protein was detected by chemiluminescence using P6C, an affinity purified antibody raised against the C-terminal TAD of Pax6 (bottom panel). Note the specific increase of the upper shifted band due to serum-induced phosphorylation of the Ser413 site. B, phosphorylation of Ser413 mimics the kinetics of ERK activation in serum stimulated NIH 3T3 cells with an increase from 15 to 60 min and a subsequent decrease 4 h following serum addition. NIH 3T3 cells transfected with the GST-Pax6(353–437) fusion protein expression vector were deprived of serum for 16 h and stimulated with 10% serum for the indicated times before extract preparation and detection of the phosphorylation shift by Western blotting using the same antibody as in A. C and D, phosphorylation of Ser413 induces a mobility shift of the GST-Pax6(353–437) fusion protein. NIH 3T3 cells transfected as above were grown in 10% serum and labeled with [32P]orthophosphate (C) or [32S]methionine/cysteine (D). In C, the upper panel shows [32P]labeled fusion proteins following purification on glutathione-agarose beads while the lower panel is a Western blot of these proteins with the P6C anti-Pax6 antibody to verify protein loading on the gel. Note that the upper shifted band is not present for the S413A mutant protein and that the weak phosphorylation signals of the T388A mutant protein is due to less protein being loaded on the gel. D, the mobility shift induced by phosphorylation of Ser413 is removed by treatment with a serine-threonine phosphatase. Purified fusion proteins labeled in vivo with [32S]methionine/cysteine were either untreated or treated with λ phosphatase (λPhase) before SDS-PAGE, electrophoresis to a PVDF membrane, and autoradiography.

FIG. 5. TPA-induced phosphorylation of the Ser413 site is blocked upon pretreatment with the specific MEK inhibitor PD 98059. A, HeLa cells transfected with expression vectors for GST-Pax6(353–437) wt and S413A fusion proteins were either untreated or pretreated with the MEK inhibitor PD 98059 (50 μM) for 30 min before being stimulated with TPA (100 ng/ml) for 15 min. GST-Pax6 fusion proteins were subsequently purified on glutathione-agarose beads and analyzed for phosphorylation induced mobility shift by SDS-PAGE and Western blotting with the P6C antibody against the Pax6 TAD. B, TPA treatment (100 ng/ml) for 15 min induce ERK-activation in HeLa cells to a similar extent as 10% serum. Phosphorylation of a GST-ElkC fusion protein purified from E. coli by ERK immunoprecipitates (upper panel) using agarose-conjugated polyclonal anti-ERK antibody (Santa Cruz Biotechnology). Immunoprecipitated ERK was visualized by Western blot using a monoclonal antibody to ERK2 (Upstate Biotechnology) and chemiluminescent detection (lower panel).
ERK activation to the same extent as 10% serum (Fig. 5B).

These results show that the Ser413 site is phosphorylated upon activation of ERK MAPKs in serum- and TPA-stimulated cells. Furthermore, the Ser376 is also phosphorylated following serum stimulation but not as efficiently as the Ser413 site.

The above-mentioned in vivo phosphorylation experiments were conducted with GST fusion proteins that do not contain a nuclear localization signal. Immunostaining of transfected cells revealed that the fusion proteins were located in the cytoplasm (data not shown). To include the entire Pax6 TAD and to study in vivo phosphorylation of nuclear localized fusion proteins, we constructed expression vectors where wt and mutant Pax6 TAD (299–437) were fused in-frame with the GAL4 DBD. We then asked whether activation of ERK and p38 kinase could lead to phosphorylation of the Pax6 TAD in the nucleus of transfected cells. Thus, in one set of experiments the GAL4-Pax6 fusion constructs were co-transfected with expression plasmids for activated MEK1. NIH-3T3 cells were transfected, deprived of serum, and in vivo labeled with [32P]orthophosphate before harvesting and immunoprecipitation with antibodies against the GAL4 DBD. As seen from lane 1 in Fig. 6A, the GAL4 DBD is phosphorylated upon co-transfection with activated MEK1. We found that there are two putative MAP kinase sites in the N-terminal region of the GAL4 DBD. However, the wt GAL4-Pax6 TAD fusion protein is clearly phosphorylated and migrates as a doublet due to the mobility shift induced by phosphorylation of the Ser413 site (lanes 2 and 3).

Fig. 6. Activation of ERK or p38 kinase leads to phosphorylation of the Pax6 TAD in the nucleus. A, co-transfection of activated MEK1 with the Pax6 TAD fused to the GAL4 DNA-binding domain increases the phosphorylation of the Pax6 TAD. Note that the mobility shift due to phosphorylation of Ser413 (lanes 2 and 3) is absent when this site is mutated to alanine (lanes 4 and 5). The upper panel shows an autoradiography of [32P]-labeled GAL4-Pax6(299–437) fusion proteins immunoprecipitated with and antibody to the GAL4 DNA-binding domain. The lower panel displays a Western blot of the immunoprecipitates with the anti-Pax6 TAD antibody. B, co-transfection of MKK6(EE) and p38 kinase with GAL4-Pax6 TAD expression constructs reveal nuclear phosphorylation of the Pax6 TAD. All experiments were performed in NIH 3T3 cells which were labeled with [32P]orthophosphate for 6 h in 0.1% serum before preparation of extracts as described under "Materials and Methods." The Pax6 TAD fusions to the GAL4 DNA-binding domain were expressed from the FA-CMV vector.

ERK activation to the same extent as 10% serum (Fig. 5B). These results show that the Ser413 site is phosphorylated upon activation of ERK MAPKs in serum- and TPA-stimulated cells. Furthermore, the Ser376 is also phosphorylated following serum stimulation but not as efficiently as the Ser413 site.

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Fig. 6A. The background level of phosphorylation in serum-starved NIH 3T3 cells (lane 2) is increased 2.4-fold (following subtraction of the contribution by phosphorylation of the GAL4 DBD) upon co-transfection with an expression vector for activated MEK1 (lane 3). In the S413A mutant the phosphorylation shift is abolished. In another set of experiments, GAL4-Pax6 fusion constructs were co-transfected with expression plasmids for p38 kinase and the constitutively active mutant upstream kinase, MKK6b(EE). The p38 kinase is inactive by
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Mutation of the Evolutionary Conserved Ser413 Site Strongly Affects the Transactivating Ability of the Isolated Pax6 TAD—In order to test whether mutation of the MAPK phosphorylation sites would affect the transactivating activity of the isolated Pax6 TAD we transfected HeLa and NIH 3T3 cells with GAL4-Pax6(299-437) wt and mutant expression constructs using pG5E1bTATA-LUC as the reporter. Following transfection, the cells were left in 10% serum for 24 h before harvesting. As shown in Fig. 7, the T322A/T323A and T375A/S376A mutations had little or no effect on the transcriptional activity. However, when these two mutations are combined the transactivation is reduced by 50% in HeLa cells. In NIH 3T3 cells, this effect is greatly reduced. In both cell lines the S413A mutant, the two double mutants, and the triple mutant (all containing the S413A mutation) displayed markedly decreased transactivation compared with the wt TAD (from 60 to 80% reduction in activity). When the serine in position 413 was mutated to glutamate (S413E) to mimic the effect of a phosphorylation, an increase in transcriptional activation was observed. In some experiments the positive effect of the S413E mutation was even more dramatic than shown here. Such a difference between Ala and Glu mutants were not observed for the 376 and 388 sites (data not shown). Furthermore, we also mutated a putative casein kinase II phosphorylation site at position 425 both to Ala and Glu without observing any differences in transactivation. Taken together, this shows that it is not simply an increase in negative charge that increases transactivation or loss thereof that decreases it. Rather, the evolutionary conserved Ser413 residue is clearly important for full transactivation by the isolated Pax6 TAD, most likely by serving as an important phosphorylation site.

Phosphorylation of the Ser413 Site Positively Modulates the Transactivating Activity of Pax6—To see if phosphorylation of the TAD affected the transactivation potential of the full-length Pax6 protein, HeLa cells were transfected with pCI-Pax6 wt, the phosphorylation site mutants T322A/T323A, T375A/S376A, S413A, or a triple mutant containing all three mutations. As a reporter p6CON-LUC (18), containing six consensus Pax6 paired domain-binding sites (65) upstream of the adenovirus E1b minimal promoter, was used. Co-transfection with activated MEK1 (lane 3) does not affect the expression level of Pax6 protein. Measurements of β-galactosidase activity were used to compensate for differences in transfection efficiencies so that equal amounts of cell lysate (measured as units of β-galactosidase activity) were loaded on the gel. B, activation of p38 kinase strongly enhances the transactivation potential of Pax6 via phosphorylation of Ser413. HeLa cells were transfected as described in A, except that expression vector for p38 kinase together with vector control for MKK6b(EE) (p38) or expression vector for p38 kinase together with expression vector for MKK6b(EE) (p38 + MKK6b(EE)) were used instead of MEK1(EE) vector itself and is activated following co-transfection with an expression vector for MKK6b(EE) (48). As can be seen from Fig. 6B, the wt Pax6 TAD is strongly phosphorylated following co-expression of MKK6b(EE) and p38 kinase with all of the labeled protein displaying the mobility shift indicative of Ser413 phosphorylation. The S413A mutant is also phosphorylated but does not display the mobility shift seen for the wt fusion protein (compare lanes 3 and 5). In the experiments shown twice as much protein was loaded in lane 5 compared with the others. When this is taken into account and the background due to phosphorylation of the GAL4 DBD is subtracted we measured a 1.8-2.0-fold reduced phosphorylation in the S413A mutant compared with the wt. A similar experiment including also the triple mutant, where all MAPK phosphorylation sites in the TAD are mutated, showed no increase in the signal above the background due to phosphorylation of the GAL4 DBD (data not shown). Taken together, the results shown in Figs. 4–6 show that the Pax6 TAD can be phosphorylated following activation of both ERK and p38 MAP kinases in vivo.
compared with co-transfection with the vector control (Fig. 8A). This induction was not caused by increased levels of Pax6 proteins, since a Western blot of Pax6 co-transfected with activated MEK1 or the vector control displayed similar amounts of Pax6 protein. The T322A/T323A and T375A/S376A mutants showed elevated transcriptional activation potentials compared with wt Pax6 but where less inducible by activated MEK (2.0- and 1.8-fold, respectively). The S413A mutant demonstrated only half of the transactivation potential of wt Pax6, but was still induced 2.0-fold by MEK1(EE). The triple mutant was less active than the wt protein, but more active than the S413A mutant, and inducible by activated MEK by a factor of 2.2.

We have found that expression of the activated MEK1 mutant alone is sufficient to produce maximal activation of a GAL-Elk-1 fusion protein suggesting that the levels of endogenous ERK1 and -2 are not limiting. To ensure that activated MEK1 gives maximal activation of Pax6 transactivation we also performed experiments where an expression vector for ERK1 was co-transfected with activated MEK1. The results obtained were similar to those obtained with activated MEK1 alone (data not shown) confirming that the levels of endogenous ERK1 and -2 are not limiting.

When the same set of pCI-Pax6 plasmids were co-transfected with expression vectors for p38 kinase and MKK6b(EE), wt Pax6 was induced 16-fold compared with transfection with p38 kinase alone (Fig. 8B). The T322A/T323A and T375A/S376A mutants had nearly the same transcriptional activity as the wt. The S413A mutant showed about 50% of the wt transactivation activity, and was only induced about 6-fold following co-transfection with MKK6b(EE) and p38 kinase. The triple mutant was only stimulated 2.7-fold indicating some contribution from the Thr323 and Ser376 sites to the overall transcriptional activity following activation of p38 kinase. The Western blot in Fig. 8B demonstrates that equal amounts of Pax6 protein was expressed in HeLa cells transfected with Pax6 and p38 kinase compared with cells transfected with Pax6, p38 kinase, and MKK6b(EE). The striking increase in transactivation by Pax6 observed following activation of p38 kinase is therefore not caused by an increase in the expression level or stability of the Pax6 protein.

The DNA-binding Affinity of the Paired Domain Is Neither Affected by Phosphorylation Site Mutations in the TAD nor ERK or p38 Kinase Activation—It has previously been shown that mutations in the homeodomain of Pax3 affect the DNA binding of the paired domain and vice versa (74–76), and that deletion of C-terminal amino acids 332–416 of quail Pax6 aborts DNA binding (24), suggesting that structural constraints arising from other parts of the molecule may modulate the DNA binding by the paired domain. Gel mobility shift assays were performed to determine if the phosphorylation site mutations in the TAD affected DNA binding by the paired domain. Nuclear extracts from HeLa cells transfected with the wt and mutant Pax6 expression vectors were used in gel mobility shift assay with the P6CON probe containing a single consensus Pax6 paired domain-binding site (61) on ice for 20 min, and run on a 5% polyacrylamide gel for 2 h at 220 V. B, mutations of the phosphorylation sites in the TAD do not affect the expression level of Pax6 and the Pax3 protein level is not increased upon co-transfection with activated MEK1 or activation of p38 kinase. Three μg of nuclear extract was loaded in each lane of a 10% SDS-polyacrylamide gel and Western blot performed using the P6C antibody (1:800 dilution). The differences in the number of detected bands between lanes 1–6 and lanes 7–10 are due to the use of different batches of primary antibody, membrane type, and detection system. The Western blot shown as lanes 1–6 was performed as described under “Materials and Methods,” while for the Western blot shown as lanes 7–10 a Hybrid membrane (Amersham) and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Transduction Laboratories) were used with the ECL detection system (Amersham). The location of a Pax6 isoform lacking the paired domain due to expression from an internal start codon (28) is indicated (Pax6ΔPD).

Fig. 9. The DNA binding affinity of the Pax6 paired domain is not affected by mutation of the phosphorylation sites or by co-transfection with activated MEK1 or p38 kinase. A, gel mobility shift assay of nuclear extracts from HeLa cells transfected with expression vectors for Pax6 wt and mutant proteins (lanes 2–6). Lanes 8–11 contain nuclear extracts from cells transfected with expression vectors for wt Pax6 in combination with an expression vector for activated MEK1 (lane 9) or expression vectors for both p38 kinase and MKK6b(EE) (lane 11) and their respective controls (lanes 8 and 10). For experimental details, see “Materials and Methods.” Nuclear proteins (3 μg) were incubated with the P6CON probe containing a single consensus Pax6 paired domain-binding site (61) on ice for 20 min, and run on a 5% polyacrylamide gel for 2 h at 220 V. B, mutations of the phosphorylation sites in the TAD do not affect the expression level of Pax6 and the Pax3 protein level is not increased upon co-transfection with activated MEK1 or activation of p38 kinase. Three μg of nuclear extract was loaded in each lane of a 10% SDS-polyacrylamide gel and Western blot performed using the P6C antibody (1:800 dilution). The differences in the number of detected bands between lanes 1–6 and lanes 7–10 are due to the use of different batches of primary antibody, membrane type, and detection system. The Western blot shown as lanes 1–6 was performed as described under “Materials and Methods,” while for the Western blot shown as lanes 7–10 a Hybrid membrane (Amersham) and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Transduction Laboratories) were used with the ECL detection system (Amersham). The location of a Pax6 isoform lacking the paired domain due to expression from an internal start codon (28) is indicated (Pax6ΔPD).

starved before nuclear extracts were made. Phosphorylation of Pax6 caused by co-transfection of activated MEK1 or p38 kinase/MKK6b(EE) (Fig. 9A, lanes 8 and 10) does not display any marked effects on DNA binding compared with the controls (Fig. 9A, lanes 8 and 11). As shown by Western blotting (Fig. 9B), the nuclear extracts used for gel mobility shift assay contained similar amounts of Pax6 proteins.
Phosphorylation of Pax6 by ERK and p38 Kinase

In this report we show that the complete region C-terminal to the homeodomain of Pax6 is necessary for maximal transcriptional activation. The Pax6 TAD does not contain any internal sequence elements that may inhibit transactivation as found for the Pax2, -5, and -8 subfamily of Pax proteins (63). While this work was in progress Tang et al. (27) came to a similar conclusion using deletions corresponding to the exons encoding the human Pax6 TAD. Thus, taken together, these two studies using different deletion constructs of human and zebrafish Pax6 proteins confirm that Pax6 contains an unusually long TAD which has been strongly conserved both in primary sequence and function during vertebrate evolution. The large size of the TAD suggests that it may provide interaction surfaces for several cofactors and/or components of the basal transcriptional machinery. Interestingly, both we and Tang et al. (27) found that the presence of the homeodomain in GAL4-Pax6 TAD fusions inhibited the transcriptional activation potential compared with the TAD alone. This has also been observed with the Pax3 homeodomain in a similar GAL4-Pax3 TAD fusion, where inclusion of the homeodomain reduced the activation by approximately 20-fold (66). Different models may account for this behavior. The homeodomain could exert a direct negative effect either by interfering with TAD function or the reduced activity could reflect a titration of the fusion protein away from the reporter promoter due to binding of the Pax6 homeodomain to chromosomal sites. However, Chalepaki et al. (67) have shown that the Pax3 homeodomain itself causes repression of a thymidine kinase promoter when fused to GAL4 making it likely that the homeodomain may recruit a repressor.

Vertebrate Pax6 TADs contain four potential MAPK phosphorylation sites. We found that three of these sites are phosphorylated in vitro by both ERK and p38 kinase. JNK was unable to phosphorylate the Pax6 TAD in vitro and the C-terminal TADs of zebrafish Pax2, Pax9a, and Pax9b were not phosphorylated by any of the three MAP kinases, even though they too contain putative MAPK phosphorylation sites. Furthermore, the murine Pax3 TAD was not phosphorylated by ERK2 or p38 kinase. Of the three sites phosphorylated in the Pax6 TAD the Ser413 is conserved from sea urchin to man while the other two sites are conserved in the highly similar vertebrate proteins but not in amphioxus, squid, and sea urchin. In vivo phosphorylation experiments using GST-Pax6 TAD wild-type and mutant proteins demonstrated that the Pax6 TAD is an ERK substrate also in vivo. Since the two nuclear localization signals of Pax6 reside in the paired domain and directly N-terminal to the homeodomain, respectively (24), the GST-Pax6 TAD fusions contain no nuclear localization signals and were only expressed in the cytoplasm. Apart from demonstrating that the Pax6 TAD is phosphorylated in vivo following activation of ERKs by serum and phorbol ester, particularly on the evolutionary conserved Ser413 site, our results with these fusion proteins are also of interest since the paired-less isoform of Pax6 (28) is distributed between the nucleus and cytoplasm with most expression in the cytoplasm (our own observations and Ref. 24). Using Gal4-Pax6 TAD fusions in co-transfection experiments we found that both activation of ERKs and p38 kinase lead to phosphorylation of the Ser413 site in the nuclear compartment.

Specific phosphorylation events may regulate the activity of transcription factors via several different mechanisms involving changes in protein stability, DNA binding activity, subcellular localization, or protein-protein interactions (reviewed in Refs. 30 and 68). We have shown by gel-mobility shift assays and Western blots that for Pax6 neither the DNA binding activity nor the protein stability are affected by mutations of phosphorylation sites or activation of ERK or p38 kinase. Since the full-length Pax6 protein is localized in the nucleus (data not shown and (24) subcellular localization is not regulated by phosphorylation of the Pax6 TAD. Both in the contexts of fusions to the GAL4 DBD and in the full-length Pax6 protein mutation of the Ser413 site to an alanine greatly reduced the transactivation potential. This could be due to structural effects not reflecting any direct role for phosphorylation. However, mutation to a glutamate increased the activity of the GAL-Pax6 TAD fusion and activation of p38 kinase strongly enhanced the activity of the wt Pax6 protein whereas the activities of the Ser413 and triple mutant (where all three MAPK phosphorylation sites in the TAD are mutated to alanines) showed greatly reduced responses to p38 kinase activation.

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The different efficiencies of ERK and p38 kinase activation in
enhancing the transactivation potential of Pax6 is most readily explained by differences in activity toward the substrate in vivo. This is illustrated in Fig. 6 where only part of the wt GAL-Pax6 TAD fusion proteins displays the mobility shift due to phosphorylation of Ser413 following co-transfection with activated MEK1 while all of the protein is shifted following activation of p38 kinase.

This work represents the first report of induced phosphorylation of a Pax protein. Dörfler and Busslinger (63) stimulated B cells with various interleukins, serum growth factors, or phorbol esters in an effort to demonstrate a link between transcriptional activation of Pax5 and signaling pathways, but failed to identify such a link. A challenge for future studies is to determine the role of phosphorylation of the Pax6 TAD in more physiologically relevant settings. Pax6 can potentially receive signals mediated by MAP kinase cascades in all the tissues where it is expressed during development and/or in the adult organism. The nature of the external signal could differ according to the specific tissue, and the final response elicited by phosphorylation of Pax6 will most likely depend on tissue- and cell-type specific parameters such as availability of specific cofactors and other transcription factors acting together with Pax6 on specific regulatory sites.

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