Activation Function 1 of Retinoic Acid Receptor β2 Is an Acidic Activator Resembling VP16

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The mechanisms underlying transcriptional activation are not very well understood, and knowledge is based on experiments with a small number of mostly viral activators. We have investigated the mechanism underlying transactivation by the activation domain present in the N-terminal part of retinoic acid receptor (RAR) β2 (AF-1). We show that RAR/β2 phosphorylation is not crucial for its activity although it may modulate AF-1 activity. Sequential mutation of the negatively charged residues (Asp) resulted in a stepwise decrease in activity, while mutation of all aspartic acid residues resulted in complete loss of activity. Comparison of the critical region for activation with other activators revealed moderate homology with the viral activator VP16. The hydrophobic amino acids surrounding the negatively charged residues reported to be critical for activation by VP16 are all conserved in AF-1. The hydrophobic residues are required for AF-1, since mutation of these residues resulted in a decrease in activity. Furthermore, the activity of this activator, VP16 and TA2 of RelA, is squelched by overexpression of an AF-1-containing expression construct, indicating that AF-1 is an acidic activator. Squelching experiments further indicate that AF-1 and AF-2 function by different mechanisms. Comparison of activation functions present in the AB region of other members of the steroid/thyroid hormone receptor family: RARα2, RARγ2, and GR suggested that also these receptors contain an acidic activation domain. The mechanism underlying activation by AF-1 is discussed.

Transcription of RNA polymerase II promoters requires an assembly of the preinitiation complex consisting of basal transcription factors. This process begins with the binding of TFII D to the TATA box, followed by ordered binding of the other transcription factors (TFII A, -B, -E, -F, -H) and RNA polymerase forming the initiation complex (1, 2). Transcription factors bound to promoter or enhancer sequences modulate the activity of polymerase II promoters. Transcription factors contain a DNA-binding domain (DBD)1 and an activation function (AF), which of which are interchangeable units, and generally are functioning independently when coupled to a heterologous AF or DBD (3). Activation functions/activators are regions of 30–100 amino acids in length and can be classified by their sequence similarity or the presence of predominant amino acids: acidic, glutamine-, or proline-rich (4). Presently, little is known about the exact role of the predominant amino acids (Asp/Glu, Gin, or Pro) in activators, and if it is unclear whether secondary structure is required for activation. Mutational analysis of acidic activators has shown that negative change per se is not sufficient for activation as mutation of negative to neutral or even positive amino acids does not or only marginally interfere with activation capacity (5). An amphiphatic α-helix, with negatively charged residues on one surface and hydrophobic residues on the other, could be a requirement for activation (6). However, some mutations destroying the putative α-helix also remain active (5). Based on mutational analysis, the GAL4 activator was proposed to form a parallel β-sheet structure (7). Using circular dichroism, the presence of this structure (under slightly acid conditions) was confirmed (8). Structural analysis using NMR has not provided any evidence for the presence of stable secondary structure elements in any activator analyzed so far.

The mechanism by which these activators exert their effect is currently a point of discussion. The removal of repressors interacting with a component of TFII D by activators was proposed (9). Furthermore, it has been suggested that activators can facilitate steps in the formation of the preinitiation complex by interacting with a component of this complex (10 and references therein). Thereby, the assembly of the preinitiation complex could be enhanced, and/or the number of active transcription complexes could be increased (11, 12). Also, the formation of an open complex following the formation of the initiation complex may be a target for an activator. Based on these models, activators may modulate transcription in several ways, whereby generally an interaction with one or more components of the basal transcription machinery seems to be necessary. Several activators have been shown to interact with TATA-binding protein (13–15), TFII B (16–18), or TATA-binding protein-associated factors (19, 20). In some cases, point mutants with reduced activity show also reduced in vitro binding (14, 21, 22). Occasionally, however, a bridging factor/cofactor is needed for activation, possibly indirectly connecting the activator with a component of the preinitiation complex (9). The observation that the AFs of the estrogen receptor (ER) function in a cell-specific way, and the observed promoter specificity of the AFs of ER (23) has led to the hypothesis that cofactors are required for the activity of the activators. The requirement for cofactors both in vitro and in vivo has recently been confirmed (24, 25). A different requirement for transcriptional activation may be phosphorylation. The activity of several transcription factors, e.g. CREB and c-jun, have been shown to be up-regulated by phosphorylation (for review, see Ref. 26). Also, steroid hormone receptors are phosphorylated in vivo (27). RARs belong to the steroid/thyroid hormone receptor super-
family which share a common domain structure, denoted A-F (28, 29). The C region contains the DNA-binding domain, which is most conserved among the different members of this family and consists of two zinc fingers. The hormone-binding domain is located in the E region and contains, besides the binding domain, a dimerization domain and a hormone-dependent transactivation function (AF-2). The N-terminal part of the receptor (AB) also contains an autonomous region involved in transactivation (AF-1) which functions independently of ligand, when coupled to a heterologous DNA-binding domain (28, 29). We and others have previously reported the presence of two autonomous transcriptional activation functions in RAR which activate transcription both by different, cell-type and promoter-dependent mechanisms (30, 31). The activation function present in the N-terminal part of the protein (AF-1, formerly called TAF-1), is located in the first 32 amino acids of the receptor, and functions both in the presence and absence of RA. This region is negatively charged and contains putative phosphorylation sites, but no obvious homology with known activators was observed (30).

Since no activation function present in the AB region of a member of this superfamily has been analyzed in detail so far, we decided to characterize AF-1 of RARβ2 in more detail. Here we show that AF-1 is an acidic activator, three aspartic acids present in this region are required for its activity, and the hydrophobic residues contribute to activity. Sequence comparison revealed that this activation function has homology with the acidic transactivation domain of VP16.

MATERIALS AND METHODS

Plasmids—By site-directed mutagenesis (Altered Sites Kit, Promega), we introduced a Smal site in front of the ATG of RARβ2 in the same reading frame as the Smal site of pG5-RARβ, and once with GAL-GAL-DBD, respectively, sequence 5'GACATTCAGTG3' (XhoI-Xba fragment from RARβ containing the first 76 amino acids of RARβ fused to the DBD of GB4 (1–147) (30) and pSG5-RARβ using the Smal and Xhol sites. RARβ ΔA was constructed by polymerase chain reaction using a primer (sequence: 5'cccGGGATCCATTTGACACACAGGAGG3') containing the hemagglutinin-tag and a Smal site in front of the ATG of RARβ and primer Drev (GTGCATTCTTGCTTCGAAGT); this Smal-XhoI-digested polymerase chain reaction product was cloned in the corresponding sites of pSG5-RARβ. Digestion of this plasmid with XhoI and Xhol, Klenow treatment, and ligation resulted in RARβ ΔA. RARβ Δ1–27 was made by cloning the HinfI (blunt)-XhoI fragment in Smal-XhoI-digested pSG5 RARβ, starting at amino acid 27, the first ATG. HA-tag RAR βE was made by cloning the Xhol-Xba fragment from RARβ βE in the corresponding sites of the ΔAB construct resulted in HA-tag-RARβ ΔAB,E. To generate GST-RARβ 1–76, the Smal-XhoI fragment of pSG5-RARβ was ligated after Klenow treatment in the Smal site of pGEX-ZT and transformed to Escherichia coli J m101. All constructs were sequenced to check mutations and reading frames; expression was confirmed by Western blot using a polyclonal antibody against the F region of RARβ or an anti-GAL antibody.

Transfection and CAT Assay—Transfections were carried out by calcium phosphate co-precipitation as reported before (30). 8 μg of reporter 5 × GAL-CAT (5 GAL binding sites in front of an E1b TATA box-CAT (32), mCRBP1-CAT or hRARβ2-CAT (63–256; 33), 1 μg of expression vector (GAL-RAR fusion construct pSG424 or pSGA42) and pSG5-RARβ together with 1.5 μg of SV2-LacZ as reference plasmid. For preparation of whole cell extract, 10 μg of expression construct was transfected. After removal of the precipitate, 1.0 μM RA was added when indicated, and, after a subsequent 24 h, cells were harvested. CAT assay was performed as described; for quantification a PhosphorImager (Molecular Dynamics) was employed, and percentage conversion was normalized for transfection efficiency using the β-galactosidase assay (30). Transfection was performed using at least two different batches of expression vector DNA, and data are presented as the mean (relative activity or CAT activity) of at least five independent or three duplicate experiments with the S.E. between the different experiments generally less than 15%.

Western Blotting—Whole cell extract from transiently transfected COS cells was prepared by three subsequent freeze-thaw cycles (~80 °C/4 °C) in 50–100 μl of lysis buffer (20 mM Tris, pH 7.5, 20% (v/v) glycerol, and 400 mM KCl) together with 0.2 mM phenylmethylsulfonyl fluoride and protease inhibitors (aprotinin, leupeptin, pepstatin, and chymostatin, final concentration 1.0 μg/ml of each). Equivalent amounts of extract were loaded and separated on 8–12.5% (w/v) SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose using a semi-dry blot apparatus. Membranes were blocked in 4% (w/v) nonfat dry milk in PBST (350 mM NaCl, 16 mM Na2HPO4, and 1% (w/v) Tween 20) for 1 h. Blots were incubated either with a monoclonal against GAL-DDB (1:750) or a polyclonal against the F region of RARβ (RPβf(1)112 (34) (1:750) in PBST containing 2% (w/v) nonfat dry milk for 2 h. After 7 washes in PBST, blots were incubated with peroxidase-conjugated second antibodies in PBST containing 2% (w/v) nonfat dry milk. After 7 washes with PBST, blots were developed using the ECL kit (Amersham).

In Vivo Labeling and Immunoprecipitation—Cells were transfected as described, and 24 h post-transfection medium was changed for phosphate-free DM medium containing 7.5% (v/v) dialyzed serum, and cells were labeled with 1 μCi of [32P]orthophosphate/ml (ICN), for 4 h. 10 μM RA was added together with [32P]orthophosphate (4 h). Cells were washed with cold PBSO (350 mM NaCl, 16 mM Na2HPO4, and 4 mM NaiH2PO4), scraped in PBSO, and lysed in 40 μl of lysis buffer (see Western blotting). After centrifugation, the cell lysate was predegraded in 750 μl of IP buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100) containing 0.2 mM phenylmethylsulfonyl fluoride and protease inhibitors using 1 μl of normal mouse serum and 50 μl of 50% (v/v) slurry protein A-Sepharose in 1IP buffer. 50 mM NaF, 40 μg of β-glycerophosphate, and 0.2 ml of NaVO4, were added to both lysate and IP buffer. The lysates were then incubated for 2 h with 50 μl of 12CA5 hybridoma supernatant; thereafter, 50 μl of 50% (v/v) slurry protein A-Sepharose in 1IP buffer was added and incubated for another hour. Beads were washed 3 times with 1 ml of IP buffer, once with 100 mM Tris-HCl, pH 7.5, 100 mM LiCl (1 ml), and once with 10 mM Tris-HCl, pH 7.5 (1 ml). Immunocomplexes were eluted by incubation at 100 °C for 5 min in sample buffer and run on a 10–12.5% (w/v) SDS-PAGE gel.

RESULTS

The First 32 Amino Acids of RARβ2 Are Required and Sufficient for AF-1 Activity—We have previously shown that, when coupled to the DNA-binding domain (DBD) of GAL4, the first 32 amino acids are required and sufficient for transactivation (30). RARβ lacking the AB region showed decreased transactivation capacity, which is dependent on the promoter used (30, 35). Results obtained with GAL-fusion constructs are sometimes different from results with the same activation function in its normal protein context. To determine whether the first 32 amino acids of AF-1 are also sufficient in the native context, we made several RARβ deletion constructs, as shown in Fig. 1A. All mutants were translated properly and accumulated to similar levels as judged by Western blot of extracts of COS cells transfected with these mutants; only the expression of Δ1–27 is slightly lower, because it lacks a consensus Kozak sequence (Fig. 1B). These mutant receptors were transfected in COS-1 cells together with the CRBPII promoter coupled to the CAT gene. This promoter has been shown to be activated only by AF-1 and not by AF-2 of RARβ2 (35). Fig. 1B shows a quantification of these transfections; no activity was observed in the absence of RA, and activity was dependent on cotransfection of RARs. Constructs lacking the A or AB region no longer activated the CRBPII promoter as has been reported before (35). The mutant receptors Δ1–27 and Δ11–22, which were no longer active when fused to GAL-DDB, were also unable to activate transcription of the CRBPII promoter. The receptor containing only the first 32 residues (Δ33–76), however, was still able to activate this promoter albeit to a lesser extent than the full-length receptor. From these data we conclude that the first 32 amino acids of RARβ2 are required and probably sufficient for the activity of AF-1, both when present...
in the normal receptor context and when fused to a heterologous DBD.

Phosphorylation Is Not Required for Transactivation by AF-1—The observation that RARs are phosphorylated in vivo (34, 36, 37) and recent observations by us (2) and others (38) that the activity of RARs can be up-regulated by protein kinase A, indicated that phosphorylation may be important for the activity of AF-1.

To test whether phosphorylation is involved in the activity of AF-1, we changed the tyrosine, threonine, and all serine residues present in this region to alanine and tested the ability of these mutant to activate transcription, when coupled to GAL-DBD. Fig. 2 shows the quantification of CAT assays of COS cells transfected with these mutants. It is clear from these results that all mutants are still active; only the mutation of serines 22, 24, and 25 to alanine showed a 35% reduction in activity. These transfection data indicated that the putative phosphorylation sites are not absolutely required for AF-1 activity, but that they can, however, influence the activity. A decrease in the in vivo phosphorylation levels might be expected upon mutation of the putative phosphorylation sites. Therefore, in vivo phosphorylation experiments using the indicated mutants in the HA-RARbΔE constructs (containing a hemagglutinin tag in front of the AB region in the RARb expression construct lacking the hormone-binding domain) were performed. No obvious differences in phosphorylation levels for the various mutants were observed (data not shown). Since we were not able to map the phosphorylation sites within this region, it is possible that the absence of phosphorylation is not the cause of this decrease but rather the introduction of Ala instead of Ser residues. An alternative explanation could be that the kinase responsible for this phosphorylation event is induced upon RA treatment, and a 4-h RA treatment in the in vivo phosphorylation experiment is too short to see the differences in phosphorylation levels between wild type and mutants. From these data we conclude that phosphorylation is not crucial for AF-1 activity, although it may modulate the activity of this activator.

Negatively Charged Amino Acids Are Responsible for AF-1 Activity—We have observed that RARb AF-1 when fused to GAL-DBD is capable of activating transcription synergistically upon multimerization of GAL binding sites, as has been reported similarly for VP-16 (39). Therefore, it can be hypothesized that these activators function by similar mechanisms. Experiments with VP16 have indicated that negatively charged amino acids are involved in and are required for the activity of VP-16. Moreover, the hydrophobic residues surrounding the Asp/Glu residues are required for its activity (5). Analysis of the minimal autonomous activation region of AF-1 (amino acids 1-32) indicated that this region is overall negatively charged, implying that negatively charged residues could be involved in the activity of AF-1. To test this hypothesis, we first mutated all negatively charged amino acids individually to noncharged residues (Ala) and transfected these GAL-DBD

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coupled mutants together with a GAL-responsive reporter in COS cells. We observed a considerable decrease in CAT activity of the mutant activator when compared to wild type, as shown in Fig. 3. Also, the conversion of Asp-17 to threonine resulted in a similar decrease in activity, showing that not only an aspartic acid to alanine substitution is destructive (data not shown). Subsequently, multiple aspartic acid residues were changed simultaneously to alanines causing a further decrease in activity and, upon alteration of all aspartic acid residues, nearly all activity was lost (Fig. 3).

Next we asked whether negative charge per se is needed or whether the presence of these specific negatively charged amino acids is required. Mutation of Asp-3, -6 to glutamic acid, which has been shown previously to be a poor substitute for aspartic acid in case of VP16 (5), resulted in a decrease almost as strong as the corresponding Ala mutant. We then attempted to create a stronger activator by introducing extra negative charge. Changing LDF (16–18) to aspartic acid residues (DDD) did not result in a receptor with higher activity, but instead a small decrease was observed. Above we have shown that replacement of S22A, S24A, S25A resulted in a decrease in activity (Fig. 2). Upon changing the serine residues of this putative phosphorylation site to aspartic acid, a stronger activator was created (Fig. 3), showing the importance of negative charge for activation and suggesting that phosphorylation can, by introducing extra negative charge, modulate the activity of this activator.

Hydrophobic Residues Contribute to AF-1 Activity—We then tested whether hydrophobic amino acids, like in VP16, are also required for activation. Mutation of F2P and M5P resulted in a significant decrease (38 and 35%, respectively), while alteration of the hydrophobic Val to Ala caused a larger (62%) reduction in activity (Fig. 4). The decrease in activity by mutation of hydrophobic residues is not as strong as reported for the F442P mutant of VP16 (40). These data indicate that, although the mutation of aspartic acid residues caused a stronger decrease in activity, the hydrophobic residues substantially contribute to the activity of AF-1.

Transfection of these mutants in P19 EC cells gave similar results, confirming that the negatively charged residues are most important for activation (data not shown). To confirm that the previous results are not caused by differential stability or accumulation of the various proteins, we performed Western blots using both the GAL-RARβ AF-1 fusion constructs and mutant receptors, containing the same mutations in the RARβ expression construct. All proteins migrated according to their expected molecular weight, and the variations in expression levels were not more than 2-fold (data not shown).

Next we examined whether the critical amino acids of AF-1, when present in the fusion constructs, are also the most important residues for AF-1 activity in the full-length receptor. We therefore compared the activity of each mutant AF-1 by transfecting them as GAL-fusion constructs as well as within the normal receptor context on the CRBPII promoter and on the hRARβ promoter. By comparing the transactivation capacity of all mutants (Fig. 4) with the wild type RARβ on these promoters, we observed that the amino acids found to be critical in the GAL AF-1 fusion protein were also important for AF-1 activity when present in RARβ. As expected, the activity of the CRBPII promoter was most dramatically decreased by mutations that change the negatively charged amino acids to neutral residues. Mutations that did not give a phenotype as GAL-fusion construct also caused no significant or only a weak decrease in activity as compared to the wild type receptor. The only exception was the S22D, S24D, S25D mutant which was impaired in activity on the CRBPII promoter while it was a stronger activator as GAL-fusion protein. This is possibly caused by disruption of the structure of the receptor which could be permitted in the GAL-fusion protein but not in the complete receptor. The aspartic acid residues of AF-1 were also important for activation of the RARβ promoter. These results are unexpected as Nagpal et al. (35) have shown that the A region of RARβ does not contribute to RARβ promoter activation whereas we observed that every mutant in which parts of AF-1 are deleted or mutated caused a decrease in RARβ-dependent RARβ promoter activation, comparable with the mutat RARβ lacking the complete AB region (Figs. 1 and 4; data not shown). We, however, used RAC65 cells in these experiments, whereas Nagpal et al. (35) used COS cells, probably explaining the differences in the role of AF-1 in RARβ promoter activation. This was confirmed by transfection of these mutant receptors in COS cells with both the RARβ promoter and with βRARE-tk-CAT as a reporter showing that all mutants activate these promoters to a similar extent (data not shown). Furthermore, we cannot exclude that regions within the B region contribute to the activity of AF-1 although no indications for the presence of an autonomous AF within this region were found (30). From these data we conclude that for activation by AF-1 the negatively charged amino acids are required, both as an autonomous AF and also when present in the full-length receptor.

AF-1 Is an Acidic Activator—The importance of aspartic acid and the requirement of hydrophobic amino acids surrounding these negatively charged residues lead to the hypothesis that AF-1 is an acidic activator. We therefore compared AF-1 with known acidic activation domains and observed a moderate homology with conservation of hydrophobic and negative residues (see Fig. 6). We therefore investigated whether these activators are functioning similarly by performing squelching experiments, whereby, as a consequence of overexpression of one activator, common limiting targets, also required for the other activator, are titrated away (41). Increasing amounts of AF-1 containing and AF-2 lacking cymomgalovirus or SV40 driven RARβ construct (HA-RARβ ΔE) were transfected together with the GAL-DBD-coupled activators AF-1, VP16, and RelA TA, RARβ AF-2, and GR AF-2 with the GAL-responsive promoter (32). As expected, the activity of the hormone-dependent activators (in the presence of their ligands) of RARβ and GR was not repressed by RARβ ΔE, whereas the activity of the first three activators was repressed when a 25-fold excess of squelcher was present (Fig. 5A). GAL AF-1 was most dramatically repressed by overexpression of this receptor. At lower concentrations of squelcher, the repression of VP16 and TA1 was significantly less than that of GAL AF-1. This can be
explained by the fact that these activators both consist of two autonomous activation functions (40, 42, 43), and, therefore, probably a higher level of squelcher is required for maximal repression. The specificity of this squelching by AF-1 was confirmed by performing similar experiments, with a RARβ construct lacking AF-1 and AF-2 (HA-RARβ ΔAB, E) which did not cause a decrease in activity of these activators in the presence of this construct at 2.5- or 10-fold excess; only at the highest concentration (25-fold), a 30% reduction was observed possibly caused by (artificial) activation domains still present in this construct (data not shown). These data indicate that AF-1 and AF-2 function by different mechanisms. To confirm this, we performed squelching experiments with GALAF-1, GALAF-2, and GAL GR AF-2, in the presence or absence of a 20-fold excess of RARβ constructs containing both or only one of the two activation functions. The activity of all activators was
AF-1 of RARβ2 Is an Acidic Activator

**Fig. 6.** Homology comparison of RARβ with other activators showing both amino acid and structural homology with VP16 and other (acidic) activators. Sequence alignment of various activators showing that homology is observed with AF-1 of RARβ when RARβ AF-1 is aligned around the hydrophobic residues of VP16 (5) and other activators. The hydrophobic residues (I, A, F, L, M, V, and Y) are boxed, the negatively charged residues (D and E) and positively charged residues (K, R, and H) are underlined. The relatively high activity of VP16 and TA1 of RelA compared with AF-1 of RARβ is at least 10 times lower active can be explained by the presence of two or more regions involved in activation (40, 42, 43) and also by the presence of more negatively charged amino acids of VP16 and RelA (43). In the case of RARβ, there are also two regions which contribute to the activity of AF-1 including the region around Asp-3 and Asp-6 and the region around Asp-17. The first region is homologous with acidic activators, whereas in the latter region the presence of only negative and hydrophobic residues was observed. Although we do not know how the latter region is contributing to activity, point mutants (D17A; L16F18D) as well as deletion constructs (Δ111–22; Δ111–76) indicate that it does contribute to the activity of this activator.

Phosphorylation Modulates AF-1 Activity—Phosphorylation experiments in COS cells transfected with various RARβ deletion constructs showed that multiple regions of RARβ, including the AB region, were phosphorylated (data not shown). Mutational analyses suggest that phosphorylation might modulate the activity of this autonomous activation function. The functional significance of the putative phosphorylation sites in AF-1 was established by introducing alanine residues for serine 22, 24, and 25 after which a decrease in activity became apparent whereas the contraries, upon changing these residues to aspartic acid, an increase in activity was observed. Similar findings have been reported for ER (45), c-jun (46), and p53 (47). A possible explanation is that phosphorylation introduces extra negative charge, which can be mimicked by introducing negatively charged amino acids. Recently, phosphorylation sites of the estrogen receptor (45, 48) have been mapped and found to be present also within the transactivation domain of ER, and these sites have been shown to participate in the activity of the receptor. Although all experiments performed so far are in agreement with the model that phosphorylation modulates the activity of the activator, the specific sites have not been mapped since the levels of phosphorylation and/or expression of the transfected receptor were too low to perform tryptic phosphoamino acid sequencing.

Primary and Secondary Structure of AF-1—Deletion analyses have revealed previously that the first 32 amino acids of RARβ2 are required and sufficient for AF-1 activity (30). Here we show that the negatively charged and hydrophobic residues are important for the activity of this activator. Comparable findings have been reported for GAL4 (49, 50), GCN4 (51), and VP16 (5, 52). It has been postulated that this class of activators forms an amphipathic α-helix with hydrophobic residues on one side with negatively charged residues on the other side of the helix (6). Using the Chou and Fasman (53) prediction, an α-helix could be formed over the first 9 residues, containing amino acids shown to be required for activation. This lead to the question whether structure is required for activation (54). We therefore expressed the first 76 amino acids of RARβ in E. coli, purified the protein, and performed CD and NMR analyses. Using one- and two-dimensional 1H nuclear magnetic resonance, no secondary structure could be demonstrated although the CD measurements indicated the presence of low levels of secondary structure elements. Similar results have been obtained using the active domains of VP16 (55) and TA1 of RelA (43). The absence of secondary structure elements in activation domains is in agreement with the idea of the presence of so-called acid blobs in acidic activators (56). During the preparation of this manuscript, the presence (in hydrophobic activators, in which the position of critical hydrophobic and negatively charged residues is conserved.

**DISCUSSION**

In this paper, we show that the autonomous activation function (AF-1) present at the N terminus of RARβ2, located between amino acids 1 and 32, is an acidic activator. This is supported by a number of observations. First, the activity of this activator is dependent on the presence of three aspartic acid residues, and hydrophobic residues are also required for activity. The behavior of other mutant activators is in agreement with the working mechanism of acidic activators as mutation of all nonhydrophobic/negatively charged amino acids were permissive. Furthermore, squelching experiments indicate that overexpression of an AF-1-containing construct interferes with the activity of VP16 and the recently characterized acidic activator TA1 of RelA. These activators all share the ability to activate transcription synergistically upon multimerization of binding sites (39, 42, 43). Finally, we observed sequence similarity between this activator and several other acidic activators, in which the position of critical hydrophobic and negatively charged residues is conserved.

The relatively high activity of VP16 and TA1 of RelA compared with AF-1 of RARβ (at least 10 times less active) can be explained by the presence of two or more regions involved in activation (40, 42, 43) and also by the presence of more negatively charged amino acids in VP16 or RelA (43). In the case of RARβ, there are also two regions which contribute to the activity of AF-1 including the region around Asp-3 and Asp-6 and the region around Asp-17. The first region is homologous with acidic activators, whereas in the latter region the presence of only negative and hydrophobic residues was observed. Although we do not know how the latter region is contributing to activity, point mutants (D17A; L16F18D) as well as deletion constructs (Δ111–22; Δ111–76) indicate that it does contribute to the activity of this activator.

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AF-1 of RARβ2 Is an Acidic Activator

Fig. 7. Projection of AF-1 and VP16 in a helical wheel model. The regions of RARβ and VP16 shown to be involved in transactivation and predicted to form an α-helix are projected within a helical wheel model (59). Numbers represent the amino acid numbers of RARβ AF-1. The smaller letters represent the activation domain of VP16 aligned as shown in Fig. 6; – and ϕ indicate that these positions, based on the alignments of Fig. 6, are generally negatively charged or hydrophobic, respectively.

Modeling AF-1 as α-Helix—On the basis of the assumption that the class of acidic activators indeed form an α-helix, we projected the first 8 amino acids of RARβ2, the minimal activation domain of VP16, and several other activators to a helical wheel (59) (Fig. 7) whereby in almost all cases the negative amino acids face one side of the helix and the hydrophobic residues the other side of the helix. From mutational analyses of both VP16 (5, 52) and Rta2 (EBV) (44), the Phe at position 5 in this helix is the most important and the residues close to this amino acid are generally hydrophobic while the residues at the other side of the helix (positions 3, 4, and 6) are negatively charged. The importance of negative/hydrophobic residues at these positions was confirmed by mutational analysis (52).

Recently, the activation function present in the C-terminal part of the hormone-binding domain (AF-2) of members of the steroid/thyroid hormone receptor superfamily has been characterized (60–64), shown to depend also on the presence of hydrophobic and negatively charged residues, and was proposed to form an α-helix as well (62). The position of these residues is, however, different in AF-1 (ψDψXδDψφ) as compared to AF-2 (ψψXψδψδψ). Furthermore, the presence of glutamic acid cannot be altered to aspartic acid (AF-2: 61, 63) and vice versa (AF-1: D3E,D6E) without a decrease in activity. Finally, squelching experiments showed that the activity of AF-2 cannot be repressed by overexpression of AF-1 and vice versa (Fig. 5B). Together, these findings strongly suggest that these activator functions by different mechanisms, and each fulfill a different role in the retinoid response. This is confirmed by the observation that AF-1 and AF-2 contribute differently to the activation of various RA-dependent promoters (35). The characterization of the two activation functions present in these receptors will be helpful in achieving a better understanding of the mechanism of action of these receptors in vivo.

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AF-1 of RARβ2 Is an Acidic Activator

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