The novel coactivator C1 (HCF) coordinates multiprotein enhancer formation and mediates transcription activation by GABP

Jodi L.Vogel and Thomas M.Kristie¹

Laboratory of Viral Diseases, National Institutes of Health, Building 4, Room 133, 4 Center Drive, Bethesda, MD 20892, USA

¹Corresponding author
e-mail: thomas_kristie@nih.gov

Transcription of the herpes simplex virus 1 (HSV-1) immediate early (IE) genes is determined by multiprotein enhancer complexes. The core enhancer assembly requires the interactions of the POU-homeodomain protein Oct-1, the viral transactivator α TIF and the cellular factor C1 (HCF). In this context, the C1 factor interacts with each protein to assemble the stable enhancer complex. In addition, the IE enhancer cores contain adjacent binding sites for other cellular transcription factors such as Sp1 and GA-binding protein (GABP). In this study, a direct interaction of the C1 factor with GABP is demonstrated, defining the C1 factor as the critical coordinator of the enhancer complex assembly. In addition, mutations that reduce the GABP transactivation potential also impair the C1–GABP interaction, indicating that the C1 factor functions as a novel coactivator of GABP-mediated transcription. The interaction and coordinated assembly of the enhancer proteins by the C1 factor may be critical for the regulation of the HSV lytic–latent cycle.

Keywords: coactivator/herpes simplex virus/protein interactions/transcription

Introduction

After an initial infection with herpes simplex virus (HSV), the virus undergoes lytic replication and subsequently establishes a latent state in the neurons of the host's sensory ganglia. Various stimuli such as stress, hormonal alterations, UV exposure or tissue damage can result in the reactivation of lytic replication and recurrent disease. While the mechanisms governing the establishment of the latent state and the reactivation process remain unknown, the lytic replication cycle has been well characterized and has been a model for the biochemical analysis of gene expression studies (reviewed in Roizman and Sears, 1996).

Upon infection, the viral immediate early (IE) genes are expressed under the control of reiterated inducible enhancer elements (Mackem and Roizman, 1982; Kristie and Roizman, 1984). These elements nucleate the assembly of multiprotein complexes containing both viral (α TIF) and cellular proteins [Oct-1, GA-binding protein (GABP), Sp1 and C1] (Jones and Tjian, 1985; McKnight *et al.*, 1987; Gerster and Roeder, 1988; O'Hare and Goding, 1988; Preston *et al.*, 1988; Triezenberg *et al.*, 1988; Kristie *et al.*, 1989, 1995; Kristie and Sharp, 1990, 1993; Wilson *et al.*, 1993a,b). Oct-1 binds the common octamer sequence

in the enhancer elements while the viral transactivator, α TIF, determines the HSV-1 site specificity by interaction with both Oct-1 and HSV-specific DNA sequences directly adjacent to the octamer core (Stern *et al.*, 1989; Kristie and Sharp, 1990; Stern and Herr, 1991; Pomerantz *et al.*, 1992). This interaction is of relatively low affinity, and the formation of the stable enhancer complex further requires the cellular C1 factor (HCF) (Gerster and Roeder, 1988; Kristie *et al.*, 1989; Katan *et al.*, 1990; Kristie and Sharp, 1990; Xiao and Capone, 1990). This factor does not bind the enhancer sequences but rather interacts via direct protein–protein interactions with α TIF (Hayes and O'Hare, 1993; Kristie and Sharp, 1993; Wilson *et al.*, 1993b) and Oct-1 (data not published). A unique protein, the C1 factor is actually a family of related polypeptides resulting from site-specific proteolytic processing at reiterated amino acid sequences in the central domain of the precursor (Wilson *et al.*, 1993a, 1995; Kristie *et al.*, 1995). In addition to its role in the regulation of HSV-1 IE gene expression, this factor has also been implicated in the control of cell cycle (Goto *et al.*, 1997), although the biochemical mechanisms remain unclear.

Additional cellular transcription factors (Sp1 and GABP) have also been shown to contribute significantly to the activation of the viral IE gene expression, and DNA recognition sequences for each of these can be found adjacent to the IE enhancer cores (Jones and Tjian, 1985; Triezenberg *et al.*, 1988; LaMarco and McKnight, 1989). For GABP, point mutations in the GA-binding site result in decreased α TIF-dependent expression, suggesting that the protein plays a role in the enhancer-mediated transcription of the IE genes (Triezenberg *et al.*, 1988). A member of the ETS domain transcription factor family, GABP (NRF2/E4TF1) is a heterodimeric factor consisting of a DNA-binding subunit (α) and a transcription activation subunit (β) (Thompson *et al.*, 1991; Virbasius *et al.*, 1993; Watanabe *et al.*, 1993; Gugneja *et al.*, 1995; Batchelor *et al.*, 1998). This factor has been the focus of many studies that have defined functional domains of the protein as well as implicated the factor in the regulation of a large number of viral and cellular promoters (Triezenberg *et al.*, 1988; Virbasius *et al.*, 1993; Watanabe *et al.*, 1993; Rosmarin *et al.*, 1995; Flory *et al.*, 1996; Ouyang *et al.*, 1996; Hoffmeyer *et al.*, 1998; Vassias *et al.*, 1998). In addition, the activation of the transactivation potential of GABP has been shown to be regulated by stress-activated and mitogen-activated protein kinase pathways (Flory *et al.*, 1996; Ouyang *et al.*, 1996; Hoffmeyer *et al.*, 1998). However, while synergistic activation by GABP in conjunction with other transcription factors has been demonstrated for numerous promoters (Rosmarin *et al.*, 1995, 1998; Boccia *et al.*, 1996; Vassias *et al.*, 1998; Nuchprayoon *et al.*, 1999), no direct interaction of this factor with other proteins has been shown.

Fig. 1. The C1 factor interacts with GABP β in the yeast two-hybrid system. (A) The indicated GAL4 DNA-binding and activation domain fusion pairs were assayed for β -galactosidase expression in the yeast strain HF7c. pGBT9 and pLAM5' represent the vector and lamin fusion protein controls, respectively. (B) Schematic representation of the C1 factor indicating the fragments of the C1 factor that were fused to the GAL4 DNA-binding domain. The circles represent the sites of processing in the C1 proteolytic processing domain (PPSD). The indicated fusions were transformed into HF7c containing the GAL4 activation domain–GABP β 2 fusion and assayed for β -galactosidase expression. (–), (+) and (++) indicate the relative levels of β -galactosidase expression.

In the studies presented here, a direct interaction of GABP with the C1 factor is demonstrated, defining the C1 factor as the critical coordinator of the HSV IE enhancer assembly. In addition, residues that are critical for the transactivation potential of GABP are also critical for the GABP–C1 interaction, implying that transactivation by GABP is mediated by the novel C1 coactivator. While this interaction plays a role in the regulation of the IE genes during HSV-1 lytic replication, it may also play a critical role in the expression of the IE genes during reactivation of the virus from the latent state.

Results

Interaction of GABP with the C1 factor in a yeast two-hybrid system

While numerous studies have focused upon the roles of Oct-1 and α TIF in the regulation of HSV-1 IE genes, little is known about the function of the C1 factor due to its relatively recent isolation. To determine the roles that this protein may play in various cellular processes, the protein was divided into segments, fused to the GAL4 DNA-binding domain, and used to screen for cellular interacting proteins in the yeast two-hybrid system. In a screen using the C1-53 (amino acids 422–1021) region of the C1 protein, three positive clones were isolated. As shown in Figure 1A, one cDNA was identified as coding for amino acids 60–347 of the transcription factor GABP β 2. The interaction was specific for C1-53 as no interaction was detected between GABP β 2 and either the

GAL4 DNA-binding domain alone or the control GAL4–lamin fusion protein (Figure 1A). Furthermore, several studies have previously shown that GABP binds to sequences adjacent to the enhancer core in the HSV-1 IE regulatory domain and that these elements are critical for the enhanced α TIF-dependent transcription of these genes (Triezenberg *et al.*, 1988; Wu *et al.*, 1994; Douville *et al.*, 1995). The C1–GABP interaction and the assembly of GABP into the core enhancer complex could therefore provide a biochemical mechanism for the observed GABP– α TIF synergistic enhancement of IE gene expression.

As previously noted, GABP is a heterodimeric member of the ETS transcription factor family with an α DNA-binding subunit and a β transactivation subunit. In addition, several forms of GABP β (GA β 1 and GA β 2) have been identified that have distinct C-termini (Thompson *et al.*, 1991; de la Brousse *et al.*, 1994; Gugneja *et al.*, 1995; Batchelor *et al.*, 1998). GA β 1 contains a leucine zipper and can homodimerize to form heterotetramers with GA α , while GA β 2 does not and forms only heterodimers. Therefore, GA β 1 was tested to determine if the C-terminal domain of GA β 2 was significant for the C1–GA β interaction. As shown in Figure 1A, both GA β 1 and GA β 2 were fully capable of interacting with C1-53, suggesting that this divergent domain did not contain determinants that are critical for this interaction.

The C1 factor domain that is important for the C1–GA β interaction was mapped using clones encoding segments of the C1 protein as shown in Figure 1B. The overlapping clone C1-191 (C1 amino acids 668–1431) also exhibited a strong interaction with GA β . Therefore, a series of clones encoding segments of the C1-53/C1-191 sequences was tested. C1-53.3 and C1-53.7 interacted with GA β as strongly as the original C1-53 protein. C1-53.2 and C1-53.6 exhibited a significantly weaker interaction, while C1-53.4 and C1-53.5 did not interact. These results defined the primary determinants for C1–GA β interaction as amino acids 813–847, with sequences directly N-terminal (amino acids 758–812) contributing weakly to the interaction.

Interaction of GABP with C1 *in vitro*: requirement for the GA β transactivation domain

The interaction of GABP and C1 was confirmed by GST pull-down assay using GST–53.1 (amino acids 668–1021) and *in vitro* synthesized GA β 1 or GA β 2. Equivalent molar amounts of GST or GST–53.1 were bound to glutathione beads and incubated with 3–5 fmol of the appropriate *in vitro* translated protein. After extensive washing, the proteins were eluted, resolved by SDS–PAGE and quantitated. As shown in Figure 2A, both GA β 2 and GA β 1 bound the GST–53.1 with the same relative affinity (5.7 or 6.6% of the input protein, respectively). In contrast, no significant interaction was seen between GST–53.1 and GA α (0.1% of input). However, when GA α was co-translated with either GA β 1 or GA β 2, the heteromeric GABP was bound efficiently by the C1 protein, suggesting that the interaction is determined primarily by the GA β subunit.

The general architecture of GA β is shown in Figure 2B. The protein contains a series of ankyrin repeats at the N-terminus for interaction with GA α (Thompson *et al.*, 1991; Batchelor *et al.*, 1998), a region that is divergent between β 1 and β 2 at the C-terminus (Thompson *et al.*,

1991; Watanabe *et al.*, 1993; Gugneja *et al.*, 1995) and a well characterized 69 amino acid transactivation domain (amino acids 246–315) (Gugneja *et al.*, 1995, 1996). As shown in Figures 1A and 2A, the divergent region is not a primary determinant for C1–GAβ interaction. Therefore, to define the interaction domain further, a series of deletions in GAβ were produced by *in vitro* synthesis and tested for their ability to interact with GST-53.1 (Figure 2B, top). As shown (Figure 2B, bottom), GAΔ278 deletes 37 amino acids of the transactivation domain and exhibits only 16% of the C1–GAβ binding activity as compared with the full-length protein. Deletion of additional residues within the transactivation domain (GAΔ256) or the entire domain (GAΔ242) further compromises the ability of the protein to bind the C1 factor (8 and 2% of the full-length protein, respectively), indicating that the transactivation domain contains the primary determinants for the GA–C1 interaction.

Residues required for GAβ–C1 interaction correlate directly with those required for GABP transactivation

The transactivation domain of GAβ (Figure 3, top) contains hydrophobic clusters and multiple glutamine residues (Gugneja *et al.*, 1995). While alteration of the glutamines had no effect upon the transactivation activity, mutations in four hydrophobic clusters significantly reduce the transactivation potential (Gugneja *et al.*, 1996). To determine if these residues were also significant for GA–C1 interaction, these mutant proteins were assayed for their ability to bind GST-53.1 (Figure 3, bottom). GAβ proteins containing mutations in box 1 have little effect (63% of wild-type activity) upon either GA–C1 interaction or GABP

transactivation. In contrast, mutations in boxes 2, 3 and 4 significantly affect both C1–GA binding and GAβ transactivation. In addition, the severity of the mutation for transactivation directly correlates with the impairment of C1–GA interaction and further suggests that the hydrophobic residues in these boxes are the major determinants for both activities.

Specific repression of GABP-mediated transcription by the C1–GABP interaction domain

The significance of the C1–GABP interaction for GA-mediated transcription *in vivo* was assessed by co-transfection of HeLa cells with constructs expressing a Gal4–GA activation domain fusion protein, Gal4–luciferase reporter and C1-53.1a (C1–GABP interaction domain). As illustrated in Figure 4A, expression of the C1–GABP interaction domain significantly inhibited the Gal4–GA-mediated expression of the reporter gene in a dose-dependent manner. In contrast, expression of C1-53.1a had no effect upon the control Gal4–VP16-mediated transcription of the reporter, indicating that the repression was GABP activation domain specific. As C1-53.1a contains only the C1–GABP interaction domain and not the transactivation functions, repression of Gal4–GA-dependent transcription suggests that C1-53.1a functions as a competitive inhibitor for wild-type endogenous C1 factor.

In similar experiments, co-transfection of a construct expressing the full-length C1 factor resulted in a consistent but low level stimulation (1.7- to 2.3-fold) of the Gal4–GA-mediated transcription, possibly due to the presence of high levels of endogenous C1 factor in tissue culture cells (data not shown).

The specific interaction of these proteins is illustrated by gel shift analysis of reactions containing extracts of mammalian cells transfected with control vector, the Gal4 DNA-binding domain or the Gal4–GA fusion (Figure 4B). Inclusion of increasing amounts of purified C1-53.1 in these reactions results in the formation of a novel complex only

Fig. 4. The domain of the C1 factor required for C1–GABP interaction can inhibit GABP-dependent transcriptional activation. (A) HeLa cells were co-transfected with plasmids encoding Gal4–GA activation domain fusion protein, Gal4–luciferase reporter and increasing amounts of C1-53.1a. The results of five independent experiments are plotted relative to the expression of the reporter in the absence of C1-53.1a. The control experiment contained Gal4–VP16 in place of Gal4–GA activation domain. (B) Gel mobility assays were done as described in Materials and methods. Reactions contained nuclear extracts of HeLa cells transfected with control vector (lanes 1–3), Gal4 DNA-binding domain (Gal4, lanes 4–6) or Gal4–GA (lanes 7–9). Purified GST–53.1 (0, 100 or 200 ng) was included in the reactions as indicated at the top of the gel.

Fig. 5. The C1 factor interacts with GABP derived from either mammalian cell or bacterial expression extracts. GST pull-downs were done using GST or C1 (GST–53.1). The source of GABP was nuclear extracts of HEK293 cells (A) or bacterial strains expressing the appropriate subunits (B). The reactions were resolved on 8–16% SDS–PAGE gels, transferred to Immobilon and probed with affinity-purified antibodies to either GAα (AbGAα) or GAβ (AbGAβ). Control reactions (cont) contained no added source of GABP.

in reactions containing the Gal4–GA protein (Figure 4B, lanes 8 and 9).

Interaction of C1 with GABP derived from nuclear extracts of mammalian cells and bacterial expression strains

As shown in Figure 5A, the interaction of native GABP and C1 can be detected using mammalian cell nuclear extracts. GST and GST53.1 were incubated with nuclear extracts from HEK293 cells and the bound proteins were analyzed by Western blotting using anti-GAα and anti-GAβ antisera. Interestingly, all forms of GAβ were precipitated efficiently from the extracts. In addition, GAα was also precipitated, probably due to its association with

Fig. 6. Schematic representation of an HSV IE gene enhancer domain and the protein interactions involved in the complex assembly. The location of the reiterated enhancer cores (EC) and binding sites for GABP and Sp1 are shown. The subdomains of the Oct-1 POU-homeodomain (PS, POU-specific and PH, POU-homeo) are illustrated.

GAβ in the heterodimer. No specific protein was detected using a control anti-Oct-1 serum.

In each of the experiments presented here, the source of the GABP subunit proteins has been reticulocyte lysates or nuclear extracts from mammalian cells. Therefore, it remains possible that modification of the GABP subunits may be required for the interaction or that additional cellular proteins may play a role. It has been demonstrated that GABP transactivation activity is regulated by MAPK and SAPK pathways (Flory *et al.*, 1996; Ouyang *et al.*, 1996; Hoffmeyer *et al.*, 1998). In contrast to some other transcription factors, this regulation is not at the level of subcellular localization or DNA-binding activity, suggesting that regulation of GABP protein–protein interactions may be the target. To determine if modification or additional cellular proteins are essential for C1–GABP interaction, GABP subunits were expressed and purified from extracts of bacterial expression strains. Purified GAβ, GAα or GAα/β were tested in GST pull-down assays using either GST or GST–53.1 (Figure 5B). In these assays, GAβ bound efficiently to GST–53.1. Similarly to the results presented above, GAα did not exhibit significant binding to C1 (1% of input protein) unless present in heterodimeric form with GAβ (12% of input). The data demonstrate that while GAα may contribute to the C1 interaction, the primary interaction is determined by GAβ. Furthermore, this interaction does not require either modification of GABP subunits or additional cellular proteins.

Discussion

Regulation of the HSV-1 IE genes is determined by reiterated inducible enhancer elements that nucleate core multiprotein assemblies containing both viral (αTIF) and cellular proteins (Oct-1 and C1 factor). In addition, the regulatory domain contains recognition sites for other cellular factors such as GABP and Sp1 adjacent to the minimal enhancer core (Figure 6). The analysis of these assemblies has been a model for the regulation of RNA polymerase II-directed eukaryotic gene expression. The

assembly of the enhancer core requires the binding of the POU-homeodomain protein, Oct-1, which recognizes the octamer motif in the enhancer by virtue of cooperative binding of the POU-specific and POU-homeo subdomains (Sturm *et al.*, 1988; Kristie and Sharp, 1990; Verrijzer *et al.*, 1990; Klemm *et al.*, 1994). The viral transactivator, α TIF, binds HSV-specific DNA sequences directly adjacent to the octamer motif and interacts directly with helix 2 of the Oct-1 POU-homeobox (Stern and Herr, 1991; Pomerantz *et al.*, 1992). However, the affinity of the ternary complex is low, requiring the interaction of the cellular C1 factor for stable assembly. This unusual factor is a family of related polypeptides that are derived from a 220 kDa precursor by site-specific proteolysis at a series of 20 amino acid sites within the central domain of the protein. The factor interacts with α TIF with high affinity and with Oct-1 with low affinity (T.M.Kristie, unpublished observations), resulting in the stabilization of the quaternary or minimal enhancer complex.

Several studies have contributed to the analysis of the domain structure of the C1 factor. In addition to the proteolytic processing domain, the protein contains an N-terminal 'kelch' domain that interacts with α TIF (La Boissiere *et al.*, 1997; Wilson *et al.*, 1997; Hughes *et al.*, 1999), L-ZIP (Luman) (Freiman and Herr, 1997; Lu *et al.*, 1997, 1998) and several cellular transcription factors (J.L.Vogel and T.M.Kristie, unpublished observations). This domain (amino acids 1–380) is sufficient for the assembly of the minimal enhancer complex *in vitro*; however, additional sequences may play a significant role in the assembly and activation of the full enhancer complex. Interestingly, a temperature-sensitive mutation in the kelch domain (amino acid 134) has also implicated C1 in the control of cell cycle progression (Goto *et al.*, 1997), although rescue of the cycle block requires sequences C-terminal to the kelch region (Wilson *et al.*, 1997), again suggesting that additional domains may be critical. The C-terminus of the protein contains a nuclear localization signal that also provides a mechanism for the nuclear import of associated proteins such as α TIF (La Boissiere *et al.*, 1999) as well as a putative transcription activation domain (J.L.Vogel and T.M.Kristie, unpublished observations).

As noted, the IE regulatory domain also contains sites for additional transcription factors such as GABP and Sp1. Several studies have indicated the significance of these sites for the enhancer-dependent stimulation of HSV-1 IE transcription by either site-directed mutagenesis or *in vitro* transcription assays (Jones and Tjian, 1985; Triezenberg *et al.*, 1988; Wu *et al.*, 1994). In the studies presented here, it has been demonstrated that the C1 factor interacts directly with GABP. In addition, studies in progress indicate that C1 also interacts directly with Sp1 (J.L.Vogel and T.M.Kristie, unpublished observation), suggesting that the enhancer core assembly makes protein contacts with additional transcription factors to form a more complex regulatory unit. Interestingly, the GA-binding sites do not contribute significantly to the basal level expression of the IE genes in the absence of an induced TAATGARAT site (Wu *et al.*, 1994), suggesting that GABP activation is dependent upon the formation of the C1 enhancer complex (i.e. the presence of the C1 factor). In addition, the juxtaposition of TAATGARAT

and GA sites is conserved in the five IE gene promoters, and alteration of this spacing results in loss of the GA-dependent stimulation of the IE genes (Bailey and Thompson, 1992). These studies support the hypothesis that the GABP transcriptional activity is dependent upon the assembly of the C1 factor enhancer complex and the proximity of the C1 factor. The interaction of C1 and GABP demonstrated here thereby further defines the role of the C1 protein as the critical coordinator of the enhancer-regulatory protein complex.

GABP, a member of the ETS family of transcription factors, is a heterodimeric protein consisting of a DNA-binding subunit (α) and a transactivation subunit (β). It has been well characterized and demonstrated to be an important component of the regulation of numerous viral and cellular promoters. Interestingly, GABP has been shown to stimulate transcription synergistically with other factors such as Sp1 (Rosmarin *et al.*, 1998; Vassias *et al.*, 1998; Nuchprayoon *et al.*, 1999), C/EBP α (Boccia *et al.*, 1996) and PU.1 (Rosmarin *et al.*, 1995), although no direct interaction between these proteins has been demonstrated. In contrast, attempts to demonstrate interactions between GABP and Sp1 have been unsuccessful (Rosmarin *et al.*, 1998; J.L.Vogel and T.M.Kristie, unpublished observation). The interaction of GABP and Sp1 with the C1 factor suggests that the synergism may be a result of interactions with other factors or coactivators such as C1.

Several studies have defined the GABP transactivation domain in the GAB β subunit, and mutational analysis has indicated residues that are critical for the transactivation potential of the protein (Gugneja *et al.*, 1995, 1996). Significantly, these same residues are required for direct interaction of GAB β and the C1 factor, and the severity of the mutation for GABP transactivation correlates directly with its effect upon GABP–C1 interaction. Furthermore, the domain of the C1 factor that is required for C1–GABP interaction can significantly inhibit GA-mediated transcription *in vivo*. The data support the model that transactivation by GABP is, at least in part, mediated by the C1 factor, and defines this protein as a novel coactivator.

Interestingly, the C1 factor interacts with all of the defined proteins in the enhancer complex. Proteins such as α TIF interact with C1 via residues distinct from their independent transcription activation domains (Hayes and O'Hare, 1993; Wu *et al.*, 1994; Lai and Herr, 1997) and require the C1 protein for stable assembly with other enhancer components. In contrast, the transactivation domain of GABP itself represents the site of C1 interaction and, in this case, the function of C1 may be to mediate directly the interaction of GABP and the basal transcription apparatus. It remains to be determined if other GABP-dependent promoters also require the C1 factor for transcriptional stimulation. Similarly, it is important to note that transcription factors such as Luman (LZIP) have also been shown to interact with the C1 factor (Freiman and Herr, 1997; Lu *et al.*, 1997, 1998). These interactions may represent additional cellular regulatory circuits in which the C1 factor plays an important role in mediating or facilitating transcriptional activation.

While the significance of the enhancer complex assembly in regulation of the HSV-1 IE genes during the lytic replication cycle is clear, the role of these proteins during

the reactivation of HSV-1 from the latent state remains to be addressed. It is likely that activation of the IE genes would be a critical stage in the initiation of reactivation. However, the viral transactivator, α TIF, is not present at this time in sensory neurons (Roizman and Sears, 1996). This suggests that cellular factors play the primary role in activation of the IE genes during the initial stages of the reactivation process. The coordinated assembly of other transcription factors such as Oct-1, Sp1 and GABP by the C1 factor may represent an important mechanism for activation of these genes in an α TIF-independent manner. Significantly, in sensory neurons, nuclear localization of the C1 factor is dependent upon signals that initiate the reactivation of HSV-1 (Kristie *et al.*, 1999). In addition, these signals also induce SAPK and MAPK pathways, which have been demonstrated to regulate the transcriptional activity of GABP. These observations have led to the hypothesis that modification of GABP may alter the C1–GABP interaction in these cells, resulting in activation of HSV-1 IE genes during reactivation of the virus from the latent state (Kristie *et al.*, 1999). Regardless of the role of the C1 factor in the reactivation process, the coordination of the HSV-1 IE enhancer proteins and the direct mediation of the transcriptional activity of GABP by the C1 factor clearly define this protein as a critical coactivator.

Materials and methods

Two-hybrid analyses

A DNA fragment encoding amino acids 422–1021 of the C1 factor was inserted into pGBT9 (pGAL53). The yeast strain HF7c was transformed with pGAL53 and the resulting strain (scGAL53) was transformed with a human HeLa MATCHMAKER cDNA library (Clontech). Positive clones were selected for by growth on His[−] plates and screened for expression of β -galactosidase by filter lifts according to the manufacturer's instructions. DNAs from positive colonies were isolated and their nucleotide sequences were determined. GABP β 1 and β 2 subunits (amino acids 10–383 and 10–347, respectively) were cloned into pGAD424, transformed into scGAL53, scGBT9 and scLAM5'. The resulting strains were tested for growth on His[−] plates and, where appropriate, for β -galactosidase activity as described. Fragments of pGAL53 were constructed by PCR with the appropriate oligonucleotide primers according to standard procedures.

Purification of GABP polypeptides and production of anti-GABP antisera

Bacterial vectors for the expression of GABP subunits [pET3DNRF-2 α (GABP α), pET3DNRF-2 β 2 (GABP β 1) and pET3DNRF-2 γ 2 (GABP β 2)] were a gift of R.Scarpulla (Gugneja *et al.*, 1995). Expressed proteins were purified essentially as described (Thompson *et al.*, 1991; Gugneja *et al.*, 1995). Polyclonal antisera were produced as previously described (Kristie *et al.*, 1995) using purified GA α /GA β subunits. Antisera were purified by protein affinity chromatography as described (Kristie *et al.*, 1995) using GST fusion proteins GA α , amino acids 1–320 and GA β , amino acids 121–330.

Protein interactions

A DNA fragment encoding amino acids 668–1021 of the C1 factor was cloned into pGEX2T (pGEX53.1). GST and GST–53.1 proteins were purified as described (Kristie *et al.*, 1995). Luciferase, GA α , GA β 1, GA β 2 and GA β 2 deletions were labeled *in vitro* with [³⁵S]methionine (Promega TNT) according to the manufacturer's recommendations. Aliquots of the labeled proteins were resolved in 12% SDS–PAGE gels, transferred to nitrocellulose and quantitated by scintillation counting of the full-length products. A 3 μ g aliquot of purified GST or GST–53.1 was adsorbed to 10 μ l of glutathione beads in 200 μ l of G buffer (20 mM HEPES pH 7.9, 100 mM NaCl, 0.1% NP-40, 10 mM β -mercaptoethanol, 10% glycerol) for 30 min at 4°C and washed with 0.5 ml of buffer G. Then 3–5 fmol of the appropriate labeled protein were added to the GST or GST–53.1 beads in a total of 200 μ l of buffer G containing 200 μ g

of bacterial protein extract. After incubation at 4°C for 1 h, the beads were washed three times with 0.5 ml of buffer, and the proteins were eluted in SDS–PAGE loading buffer. Aliquots of each input and elution protein sample were resolved by SDS–PAGE, transferred to nitrocellulose and quantitated by phosphorimaging (Storm, Molecular Dynamics) prior to autoradiography. For pull-downs from nuclear extracts, 1.5 μ g of GST or GST–53.1 were adsorbed to 10 μ l of glutathione beads and mixed with 200 μ g of HEK293 cell nuclear extract protein in 200 μ l of buffer G. Bound proteins were eluted with SDS–PAGE loading buffer, resolved in an 8–16% SDS–polyacrylamide gel and transferred to Immobilon. Western blots were probed with Ab2271 (AbGA α) or Ab2275 (AbGA β) and developed using ECL-Plus (Amersham). Pull-downs using bacterially expressed and purified GABP proteins were as described above using 2 μ g of GST or GST–53.1 and 100 ng of purified GA α , GA β or GA α /GA β complex.

GABP deletions and site-directed mutagenesis

GAA278, Δ 256 and Δ 242 were generated by restriction enzyme truncation with *Pfl*MI, *Eco*RI and *Pst*II, respectively. Site-directed mutagenesis of NRF2 γ was done using the Gene Editor System (Promega Biotech) and the following mutagenic primers: Mt1, GCCATTCAGCAAGCAGCTAGTTTCAGGGGGTTC; Mt2, TCCATCTGTAACTGCTGTGGCGACTTGCTGACC; Mt3, GGAATTGGTCAGCCCGCCGCTGCGACCATGCCAGATGGAC; and Mt4, GATGGACAACAAGCATTAAACAGCACAGCAACAGAC.

Cell transfections and reporter assays

The wild-type GA β activation domain encoding amino acids 246–315 was inserted into pM (Clontech) to produce the GAL4 DNA-binding–GA β activation domain fusion pMGA/Ad. A fragment encoding the C1–GABP interaction domain (amino acids 668–933 of the C1 factor) was inserted into pCMV/myc/nuc (Invitrogen) to create pCMV53.1a. Transfections of 1×10^5 HeLa cells were performed according to the manufacturer's instructions (Superfect, Qiagen). Each transfection contained 800 ng of pG5luc (Promega) reporter plasmid, 20 ng of pRL-TK (Promega) as an internal control for transfection efficiency, 25 ng of pM, pMGA/AD or pM3-VP16 (Clontech) and 100–400 ng of pCMV/myc/nuc or pCMV53.1a. Dual luciferase assays (Promega) were carried out 46–50 h post-transfection according to the manufacturer's directions, and the resulting data points were normalized to the internal control.

Gel electrophoretic mobility shift assays

Gel electrophoretic mobility shift assays were done essentially as described (Kristie and Sharp, 1993) with reactions containing buffer A (20 mM HEPES pH 7.9, 6.25 mM MgCl₂, 0.1% NP-40, 0.2 mM EDTA, 4% ficoll), 300 μ g/ml bovine serum albumin, 100 μ g/ml poly(dI–dC), 7.5 fmol Gal4 UAS DNA probe and 6.5 μ g of nuclear extract protein derived from HeLa cells transfected with the appropriate plasmid constructs.

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