Grb4/Nckβ Acts as a Nuclear Repressor of v-Abl-induced Transcription from c-jun/c-fos Promoter Elements*

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Grb4 is an adaptor protein consisting of three src homology (SH) 3 domains and a single SH2 domain. We previously cloned Grb4 as a direct interacting partner of Bcr-Abl and v-Abl via the Grb4 SH2 domain. We now show that overexpression of Grb4 results in significant inhibition of v-Abl-induced transcriptional activation from promitogenic enhancer elements such as activator protein 1 (AP-1) and serum-responsive element (SRE). We demonstrate that the inhibitory activity of Grb4 is independent of the direct interaction of v-Abl and Grb4: a Grb4 mutant that lacks a functional SH2 domain shows an even more pronounced inhibition of AP-1/SRE. Further mutational analysis revealed that the first two SH3 domains primarily mediate the inhibitory function. The inhibitory activity of Grb4 is specific for c-jun/c-fos-regulated promoter elements and is located downstream of MEKK1 and JNK because co-expression of Grb4 resulted in down-regulation of MEKK1-induced AP-1 activity without affecting JNK activity. Thus, the nuclear pool of Grb4 is likely to mediate this inhibition. Indeed, cell fractionation and fluorescence microscopy studies revealed that the stronger inhibitory potential of the Grb4 SH2 mutant occurred in conjunction with increased nuclear localization of this mutant. Our results suggest a novel role for Grb4 in the inhibition of promitogenic enhancer elements such as 12-O-tetradecanoylphorbol-13-acetate-responsive element and SRE.

Grb4 (also referred to as Nckβ or Nck-2) belongs to an emerging class of adaptor proteins that consist of functional src homology (SH)3 domains but lack intrinsic catalytic activity (1–5). The Nck family of proteins share a common structure consisting of three SH3 domains and a C-terminal SH2 domain. SH3 domains are known to mediate interactions with proline-rich motifs; SH2 domains bind to specific phosphorylated tyrosine residues. Grb4 exhibits high homology to Nck (Nckα). For Nck, a functional role in JNK activation (6) has been proposed via Nck-interacting kinase/Ste20 kinase (7, 8). Nck-interacting kinase has been reported to bind Nck and MEKK1 and activate the JNK/stress-activated protein kinase pathway (7, 9). Nck has also been identified as an interaction partner of Sos (10), Cbl (11), WASP (12), and PAK (13, 14). Proteins reported to interact with Grb4 include PAK and Sos1 (3). Nck has been shown to transform fibroblasts (15), whereas at this time, there are somewhat contradictory reports regarding the function of Grb4 on mitogenesis. Grb4 has been shown to cooperate with v-Abl to transform NIH3T3 fibroblasts and synergistically activate the Elk-1 pathway (3). Another group demonstrated that Grb4 is a potent inhibitor of epidermal growth factor-stimulated and platelet-derived growth factor-stimulated DNA synthesis (2). We previously identified Grb4 as a direct interaction partner of Bcr-Abl (5). This interaction resulted in a significant redistribution of both proteins, most likely involving actin reorganization. Other reports also describe an involvement of Grb4 in cytoskeletal reorganization. Tu et al. (4) showed that Grb4 interacts with PINCH, a molecule that links growth factor receptors with integrin signaling. Recently, Chen et al. (16) showed that Grb4 but not Nck blocks Rac-1-mediated membrane ruffling and formation of lamellipodia. Thus, despite the high homology between Grb4 and Nck, the functional characteristics of both proteins seem to be quite different.

In this report, we show that Grb4 inhibits the transcription from promitogenic promoters such as AP-1 and SRE. AP-1 acts as a dimeric DNA sequence-specific transcriptional activator composed of homo- or heterodimers of Jun, Fos, and/or ATF. Jun homodimers and Jun-Fos heterodimers preferentially recognize the TPA-responsive element, whereas Jun-ATF and ATF-ATF dimers bind to the cyclic AMP-responsive element. Activation of AP-1 has been reported to be induced after a variety of stimuli (including TPA, growth factors, cytokines, T-cell activation, neurotransmitters, and UV radiation) and to play a role in cell proliferation and oncogenic transformation (for a review, see Refs. 17 and 18). AP-1 activity is generally regulated by the expression level of AP-1 proteins and their activity state (19). AP-1 proteins have been reported to control their own expression level. Transcription of c-Jun is usually mediated through the TPA-responsive element (TRE), which is recognized by AP-1. Transcription of c-Fos is regulated by the SRE. In addition, secondary modifications e.g. modifications (e.g. phosphorylation on serine and threonine residues) enhance the stability of these proteins (20, 21). Serine phosphorylation of c-Jun also results in increased transcriptional activity (22). A co-activator designated JAB1 has been identified, and it stabilizes AP-1-DNA complexes by interaction with c-
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Jun or JunD (23). Known inhibitors of AP-1 activity include ligands of nuclear receptors (e.g., glucocorticoid receptor), which are thought to reduce the interaction between c-Jun and the transcriptional co-activator CBP/p300 (24), and the interferon-inducible p202, which interacts directly with c-Jun and c-Fos (25). Another model proposed for the inhibition of AP-1 by ligand-activated nuclear receptors involves blocking of c-Jun phosphorylation (26).

The oncogene v-Abl has been shown to activate the serum-responsive and TPA-responsive elements (27), which is correlated with its promitogenic potential. It could be demonstrated that v-Abl-induced activation of TPA-responsive elements is inhibited by dominant negative Rac-1 but not Ras, suggesting that Rac-1 is involved in the signal transduction pathway leading to activation of AP-1 (28). In addition, dominant negative Rac also inhibited v-Abl-induced activation of SRE and other mitogenic responses such as JNK1, mitogen-activated protein kinase, and extracellular signal-regulated kinase 2 activation (28).

Searching for a functional role for Grb4 in Abl-mediated signaling, we found that Grb4 is a potent inhibitor of v-Abl-induced activation of TRE and SRE. AP-1 inhibition by various Grb4 mutants with different subcellular localization strongly suggested that the nuclear pool of Grb4 mediates this inhibition.

MATERIALS AND METHODS

Reagents and Antibodies—The anti-xpress™ antibody was purchased from Invitrogen (Groningen, The Netherlands). Anti-Abl antibodies were obtained from Pharmingen (8E9) and Calbiochem-Novabiochem (Ab) (Schwalbach, Germany), anti-c-Jun (H-79) and anti p-c-Jun (KM-1) and anti-p-JNK (G-7) antibodies were obtained from Santa Cruz (Heidelberg, Germany).

Grb4 Mutants and EY(C)FP Fusion Constructs—The Grb4 mutants were generated using polymerase chain reaction-based mutagenesis. For mutation of the SH2 domain, arginine within the FLVR motif was changed to leucine. SH3 domains were mutated by exchange of the characteristic tryptophan with lysine (29). EYFP and ECFP mutants of EGFP and the EY(C)FP-Grb4 fusion constructs were obtained as described previously (30).

Cell Culture and Transfection Methods—293, NIH3T3 and COS7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. COS7 cells were transfected using Gene Porter (GTS Inc., Biozol GmbH, Eching, Germany) according to manufacturer’s recommendations. NIH3T3 cells were transfected using Gene Porter or Superfect™ (Qiagen, Hilden, Germany). 293 cells were transfected using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethysulfate (Roche Molecular Biochemicals).

Immunoprecipitations and Immunoblotting—Immunoprecipitations and immunoblotting were done as described previously (30). Briefly, cells were harvested in cold phosphate-buffered saline containing 1 mM sodium methyl sulfate (Roche Molecular Biochemicals), and then resuspended in buffer A, and extraction was repeated. Nuclear proteins were extracted by resuspending the nuclear pellet in buffer B (20 mM HEPES, pH 8, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 300 mM sucrose, 0.1% Nonidet P-40, and a proteinase inhibitor mixture (Complete™, Roche Molecular Biochemicals). After a short centrifugation, the cytosolic supernatant was removed, the nuclear pellet was again resuspended in buffer A, and extraction was repeated. Nuclear proteins were extracted by resuspending the nuclear pellet in buffer B (20 mM HEPES, pH 8, 20% glycerol, 100 mM KCl, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and a proteinase inhibitor mixture). After 10 s of sonication and a pulse centrifugation, the supernatant containing the nuclear fraction was frozen. For shift and supershift assays, 3 µg of protein was used and incubated with the ³²P-labeled AP-1 probe at room temperature for 30 min. Antibodies (4 µg) were added as indicated. Complexes were separated by nondenaturing PAGE (8%). Gels were dried, and DNA-protein complexes were visualized by autoradiography.

Visualization of EGFP Fusion Constructs in Fixed Cells—Fixation and detection of EGFP fusion constructs in fixed cells was done as described previously (5). Briefly, COS7 cells were cultured and transfected on gelatin-coated chamber slides (Nunc GmbH, Wiesbaden, Germany). 72 h after transfection, cells were starved for at least 6 h, washed with cold phosphate-buffered saline containing 1 mM vanadate, and fixed with 3.7% paraformaldehyde for 15 min. Cells were then washed twice with phosphate-buffered saline, overlaid with mounting medium (Molecular Probes Europe BV, Leiden, The Netherlands), covered with a coverslide, and visualized using a fluorescence microscope (Olympus Optical Co. GmbH, Hamburg, Germany) connected to a digital imaging system (T.I.L.L. Photonics, Munich, Germany). For detection of EGFP and EYFP fluorochromes, the appropriate filter sets were used (Chroma; AHF AG, Tuebingen, Germany).

Grb4SH2 and v-Abl Bidistrionic Construct—The Mig-IRES-v-abl construct was cloned by ligating v-abl cDNA (a kind gift from J. Wang, University of California San Diego, San Diego, CA) cut with BamHI/ HindIII together with the ECMV-IRES sequence cut with EcoRI/BamHI into the MigIR vector (a kind gift from W. Pear, University of Pennsylvania Medical Center, Philadelphia, PA) cut with EcoRI and HindIII. The FLAG epitope-tagged Grb4-SH2 mutant cDNA was digested from the pcDNA3.1-Zeo vector (Invitrogen, Karlsruhe, Germany) with PmeI and cloned into the HpoI site of the Mig-IRES-v-abl construct, generating Mig-Grb4SH2-v-abl.

Generation of Retroviral Stocks and Infection Procedure—Viral particles were produced by transiently transfecting the ecotropic Phoenix cell line (kindly supplied by G. Nolan, Stanford University School of Medicine, Standford, CA) with the different constructs, as described previously (31). 10⁵ NIH3T3 cells were infected in 6-well plates in the presence of 4 µg/ml polybrene overnight. After 2 days, cells were analyzed for v-abl expression by intracellular staining for Abl, as described previously (32).

Soft Agar Assays—Equal numbers of infected cells were plated in duplicate in soft agar medium according to procedures described previously (31). The plates were monitored for colony growth, and photographs were taken after 20 days of culture.

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**Preparation of Nuclear and Cytosolic Extracts and AP-1 DNA Binding Shift Assay**—Nuclear and cytosolic extracts were prepared as follows: transiently transfected 293 cells were pelleted, washed once in ice-cold phosphate-buffered saline, and lysed in buffer A (10 mM HEPES, pH 8, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 300 mM sucrose, 0.1% Nonidet P-40, and a proteinase inhibitor mixture (Complete™, Roche Molecular Biochemicals). After a short centrifugation, the cytosolic supernatant was removed, the nuclear pellet was again resuspended in buffer A, and extraction was repeated. Nuclear proteins were extracted by resuspending the nuclear pellet in buffer B (20 mM HEPES, pH 8, 20% glycerol, 100 mM KCl, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and a proteinase inhibitor mixture). After 10 s of sonication and a pulse centrifugation, the supernatant containing the nuclear fraction was frozen. For shift and supershift assays, 3 µg of protein was used and incubated with the ³²P-labeled AP-1 probe at room temperature for 30 min. Antibodies (4 µg) were added as indicated. Complexes were separated by nondenaturing PAGE (8%). Gels were dried, and DNA-protein complexes were visualized by autoradiography.

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results

Complex Formation between v-Ab1 and Grb4 Depends on the SH2 Domain of Grb4—We previously identified Grb4 as a specific interaction partner of Bcr-Ab1 binding to the Ab1 portion of Bcr-Ab1 (5). Glutathione S-transferase binding assays revealed that this interaction is mediated by the SH3 as well as the SH2 domains of Grb4 and that Grb4 was able to bind to v-Ab1 in vitro (5). To investigate the role of the SH2 and SH3 domains for complex formation of Grb4 and v-Ab1 in vivo in more detail, we created a Grb4 SH2 mutant (Grb4SH2) and a Grb4 mutant with loss of function mutations in all three SH3 domains (Grb4SH3-1,2,3) of Grb4 (29). In a third mutant, SH2 and SH3 mutations were combined (Grb4SH2/SH3-1,2,3) (Table 1). Co-immunoprecipitation experiments revealed that co-expression of Grb4 and v-Ab1 resulted in complex formation between v-Ab1 and Grb4 independent of the kinase activity of v-Ab1 (Fig. 1A, top panel, lanes 1 and 5). The complex between Grb4 and kinase-defective v-Ab1 (v-Ab1KD) was dependent on the SH3 domains but independent of the SH2 domain of Grb4

Fig. 1. Complex formation between v-Ab1 and Grb4. A, kinase-defective v-Ab1 (KD) or v-Ab1 was co-expressed with either tagged (xpress™) Grb4, the triple SH3 Grb4 mutant (Grb4SH3-1,2,3), the SH2 mutant (Grb4SH2), or the combined mutant (Grb4SH2/SH3-1,2,3) in COS1 cells. The Grb4 mutants were immunoprecipitated (IP) using anti-xpress™ antibodies (first and third panels) or nonspecific rabbit anti-mouse antibody (RAM) as control (second panel). Immunoprecipitations were analyzed by αAb1 antibody (first and second panel) or anti-xpress™ antibody (third panel) to demonstrate immunoprecipitation of Grb4. Lysates analyzed by αAb1 antibody demonstrate equal expression of v-Ab1 in all samples (fourth panel). B, kinase-defective Bcr-Ab1 (KD) was co-expressed with tagged Grb4 WT (lane 1), and Bcr-Ab1 was co-expressed with either Grb4 or Grb4SH2 (lanes 2 and 3). The Grb4 proteins were immunoprecipitated using αexpress antibody (first and third panels) from lysates containing equal amounts of Bcr-Ab1 (fourth panel). Control immunoprecipitations were performed using equal amounts of rabbit anti-mouse antibody (RAM) (second panel). Immunoprecipitations were analyzed by Western blotting using the antibodies indicated.

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Fig. 2. Grb4 acts as an inhibitor of v-Ab1-induced AP-1 activation. A, 293 cells were co-transfected with the AP-1 reporter construct (1 μg), a plasmid containing the β-GAL gene (100 ng), v-Ab1 (1 μg), and tagged Grb4 constructs or vector control (0.1 μg) as indicated. Luciferase activity was normalized for transfection efficiency using β-GAL values. Data represent the relative luciferase activity of three independent experiments. B, lysates of 293 cells transfected with tagged Grb4 constructs in triplicates (A–C) as indicated were run on SDS-PAGE and analyzed for expression of Grb4 and Grb4SH2. (Fig. 1A, top panel, lanes 2–4). In contrast, complex formation between Grb4 and kinase-active v-Ab1 was significantly reduced by a mutation in the SH2 domain of Grb4 (Fig. 1A, top panel, lane 7). Mutation of the SH3 domains had little effect on complex formation with kinase-active v-Ab1 (Fig. 1A, top panel, lane 6). A similar result was obtained when we analyzed complex formation with the other oncogenic variant of Ab1, Bcr-Ab1 (Fig. 1B). Both kinase-defective Bcr-Ab1 (Bcr-Ab1KD) and kinase-active Bcr-Ab1 can co-immunoprecipitate Grb4 (Fig. 1B, top panel, lanes 1 and 2). According to the data obtained with v-Ab1, the complex between Grb4 and Bcr-Ab1 was also abrogated by a mutation in the SH2 domain of Grb4 (Fig. 1B, top panel, lane 3). Similar to v-Ab1, kinase-defective Bcr-Ab1 binds to Grb4 independent of the SH2 domain but dependent on the SH3 domains (data not shown). Thus, in vivo complex formation between Grb4 and kinase-active v-Ab1 or Bcr-Ab1 is mediated mainly by the SH2 domain of Grb4. Interestingly, the SH3 domain only contributes to the complex of kinase-defective v-Ab1 and Grb4, suggesting that the SH3-mediated binding of Grb4 to v-Ab1 might be negatively regulated by phosphorylation.

Grb4 Is an Inhibitor of v-Ab1-induced AP-1 Activation—We have previously shown that co-expression of Grb4 with v-Ab1 resulted in inhibition of v-Ab1-induced activation of an AP-1 reporter construct in a concentration-dependent manner (5). We were interested in discovering whether disruption of the direct interaction between v-Ab1 and Grb4 would have an effect on the Grb4-mediated AP-1 inhibition. Surprisingly, co-expression of v-Ab1 and the Grb4SH2 mutant that no longer binds to v-Ab1 caused an even stronger inhibition of v-Ab1-mediated activation of an AP-1 reporter construct (Fig. 2A). Co-expression of v-Ab1 and Grb4SH2 resulted in about 30% more inhibition of AP-1 activity compared with that induced by the same amount of wild-type (WT) DNA (Fig. 2A, compare bars 2 and 5). Western blot analysis of the lysates showed that the expression level of the Grb4SH2 mutant was even lower than that of WT Grb4 (Fig. 2B). In turn, compared with WT Grb4, the same extent of Grb4SH2-induced inhibition was obtained when only a third of the WT Grb4 DNA was transfected (76% versus 79% inhibition; Fig. 2A, compare bars 4 and 5). Therefore, the difference in the inhibitory activity is not due to the expression level of both proteins but is specific for the introduced muta-
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**FIG. 3. Grb4-mediated inhibition of MEKK1-induced AP-1 activity.** A, 293 cells were co-transfected with the AP-1 reporter construct (1 μg), a plasmid containing the β-GAL gene (100 ng), constitutively active MEKK1 (50 ng), and tagged Grb4 constructs (1 μg) or vector control (Ø; 1 μg). Luciferase activity was normalized for transfection efficiency using β-GAL values. B, 293 cells were co-transfected with the SRE reporter construct (1 μg), a plasmid containing the β-GAL gene (100 ng), constitutively active MEKK1 (25 ng), and tagged Grb4 constructs (1 μg) or vector control (Ø; 1 μg). Luciferase activity was normalized for transfection efficiency using β-GAL values. C, 293 cells were co-transfected with a plasmid containing the β-GAL gene (100 ng), constitutively active MEKK1 (25 ng), and tagged Grb4 constructs (1 μg) or vector control (Ø; 1 μg). Luciferase activity was normalized for transfection efficiency using β-GAL values. D, 293 cells were co-transfected with the SMAD-dependent luciferase reporter construct (1 μg), a plasmid containing the β-GAL gene (100 ng), constitutively active MEKK1 (25 ng), and tagged Grb4 constructs (1 μg) or vector control (Ø; 1 μg). Luciferase activity was normalized for transfection efficiency using β-GAL values. Data represent the relative luciferase activity of three independent experiments.

**FIG. 4. Inhibition of MEKK1-induced AP-1 activity by different Grb4 mutants.** 293 cells were co-transfected with the AP-1 reporter construct (1 μg), a plasmid containing the β-GAL gene (100 ng), constitutively active MEKK1 (50 ng), and tagged Grb4 constructs (1 μg) or vector control (Ø; 1 μg). Luciferase activity was normalized for transfection efficiency using β-GAL values. Data represent the relative luciferase activity of three independent experiments.

Grb4-mediated Inhibition of MEKK1-induced AP-1 Activity—To further define the target in the signaling cascade of Grb4-mediated AP-1 inhibition, we tested whether MEKK1-induced AP-1 activity is affected by Grb4. Similar to v-Abl, co-expression of constitutively active MEKK1 and Grb4 resulted in inhibition of MEKK1-induced AP-1 activation in a concentration-dependent manner (Fig. 3A). The SH2 mutant of Grb4 again exhibited significantly increased inhibition. The same result was obtained when we measured the effect of Grb4 on MEKK1-induced SRE activation using a different reporter construct (Fig. 3B). These results confirmed the data obtained with v-Abl. In addition, the inhibitory effect of Grb4 is also demonstrable with another promitogenic enhancer element such as SRE. Importantly, Grb4 did not show any detectable suppression of β-GAL activity (Fig. 3C) or of transforming growth factor β-inducible activation of a SMAD-responsive reporter construct (Fig. 3D). Thus, Grb4 specifically inhibits the activation of promitogenic reporter systems such as SRE and TRE downstream or at the same level of MEKK1.

Role of the Different Grb4 Domains for the Inhibition of Promitogenic Enhancer Elements—Grb4 consists of one C-terminal SH2 domain and three SH3 domains (Table I). Our data show that disruption of the SH2 domain even enhances the inhibitory effect on transcription from TRE/SRE-regulated reporter constructs. To investigate the role of the Grb4 SH3 domains for the inhibition of these promitogenic enhancer elements, we created three additional single SH3 mutants of Grb4 (SH2/SH3-1, SH2/SH3-2, and SH2/SH3-3) and a combination thereof by changing the central tryptophan to lysine within each SH3 domain (Table I) (29). These mutants were co-expressed with MEKK1 and analyzed for their ability to down-regulate AP-1-dependent luciferase activity (Fig. 4). Compared with the Grb4SH2 mutant, the triple SH3 mutant (Grb4SH2/SH3-1,2,3) failed to inhibit MEKK1-mediated AP-1 activation (Fig. 4, compare bars 2 and 6). When the single SH3 mutants in combination with the SH2 mutation were co-expressed with MEKK1, mutation of the first and the second SH3 domains resulted in significantly weaker inhibition compared with that of the mutation affecting the third SH3 domain (Fig. 4, compare bars 3, 4, and 5). Similar results were obtained for v-Abl-induced AP-1 activity (data not shown). These findings suggest that the first two SH3 domains of Grb4 mediate the inhibition of transcription from promitogenic enhancer elements such as TRE in this assay.

Grb4 Does Not Inhibit MEKK1-induced Activation of JNK—JNK is a direct effector of MEKK1 (33). JNK regulates the transcriptional activation domain of c-Jun for both the SRE and TRE enhancer elements. Therefore, we wished to determine the activity of JNK because it is located further downstream within the AP-1 signaling cascade. To test whether the inhibi-
tion of MEKK1-induced AP-1 activation is due to a decrease in JNK activity, we measured JNK activation and AP-1-dependent luciferase activity within the same cells (Fig. 5). MEKK1-induced JNK activity measured by a kinase assay using glutathione S-transferase-Jun as a substrate (Fig. 5A, top two panels) or analyzed in cell lysates by Western blot using a phosphospecific anti-JNK antibody (Fig. 5A, bottom panel) was not affected by the co-expression of Grb4 or Grb4SH2. Again, co-expression of Grb4 resulted in about 50% inhibition of MEKK1-induced AP-1 luciferase activity, whereas the SH2 mutant showed about 75% inhibition (Fig. 4B). These data suggested that Grb4-mediated AP-1 inhibition is downstream of JNK using a phosphospecific JNK antibody (bottom panel). Similar results were obtained in an independent experiment. B, the other half of the cells were used for luciferase/β-GAL measurement. Luciferase activity was normalized for transfection efficiency using β-GAL.

**Fig. 5.** Grb4 does not inhibit MEKK1-induced activation of JNK. 293 cells were co-transfected with the AP-1 reporter construct (1 μg), a plasmid containing the β-GAL gene (100 ng), constitutively active MEKK1 (50 ng), hemagglutinin-tagged JNK1 (2 μg), and express-tagged Grb4 constructs (4 μg) or vector control (Ø; 4 μg). Half of the cells were used to determine JNK activity (A). Lysates were incubated with glutathione S-transferase-c-Jun bound to glutathione-agarose beads. Subsequently, a kinase assay was performed. Bound fractions were analyzed for phosphorylation of c-Jun using a phospho-c-Jun antibody (ap-c-JunSer63; top panel). Equal amounts of glutathione S-transferase-c-Jun were verified by analyzing the blot for c-Jun (middle panel). Lysates of the same cells were analyzed for activated endogenous JNK using a phosphospecific anti-JNK antibody (bottom panel). Similar results were obtained in an independent experiment. B, the other half of the cells were used for luciferase/β-GAL measurement. Luciferase activity was normalized for transfection efficiency using β-GAL.

**Fig. 6.** Grb4 does not inhibit MEKK1-induced AP-1-DNA complex formation. AP-1 mobility shift DNA binding assays were performed as described under “Materials and Methods.” Nuclear fractions of 293 cells were incubated with a 32P-labeled oligonucleotide containing a single AP-1 binding site. Complexes were incubated with the indicated antibodies. This experiment was repeated at least six times, and similar results were obtained.

**Fig. 7.** The Grb4-mediated negative regulation of transcription from AP-1-responsive elements is independent of Abl. NIH3T3 abl−/− cells (A) or c-Abl reconstituted abl−/− cells (B) were co-transfected with the AP-1 reporter construct (1 μg), a plasmid containing the β-GAL gene (100 ng), constitutively active MEKK1 (25 ng), and tagged Grb4 constructs (1 μg) or vector control (Ø; 1 μg). Luciferase activity was normalized for transfection efficiency using β-GAL values.
Subcellular localization of Grb4 and Grb4SH2. A, nuclear and cytosolic fractions of 293 cells overexpressing MEKK1 and tagged Grb4 constructs were prepared as described under “Materials and Methods.” Equal amounts of protein were separated on SDS-PAGE and analyzed for the presence of Grb4 using the Grb4 antibody (top panel). The purity of the fractionation was controlled by Western blot analysis of retinoblastoma susceptibility protein RB (middle panel) and cyclin A (bottom panel). This experiment was repeated twice, and similar results were obtained. B, EYFP-Grb4 chimeras were obtained by N-terminal fusion of EYFP to different Grb4 mutants and expressed in COS7 cells grown on slides. Cells were imaged using fluorescence microscopy. Representative cells are shown. N-EYFP Grb4, left panel; N-EYFP Grb4SH2, middle panel; and N-EYFP Grb4SH2/SH3-1,2,3, right panel; N, nucleus; C, cytoplasm.

Co-localization of N-ECFP v-Abl and N-EYFP Grb4. N-ECFP v-Abl and different N-EYFP Grb4 mutants were expressed in COS7 cells as indicated. Distribution of N-EYFP Grb4 protein is shown in green, and distribution of N-ECFP v-Abl is shown in red. Co-localization of both proteins in the overlay picture is shown in yellow. N, nucleus; C, cytoplasm.

Grb4-mediated Negative Regulation of AP-1 Is Independent of c-Abl—Our previous data showed that Grb4 can interact with kinase-active and -inactive v-Abl. This allowed us to speculate that Grb4 is a possible interaction partner of c-Abl as well. Nuclear c-Abl has been implicated in transcriptional regulation (34, 35). We therefore wished to test whether the inhibitory effect on transcriptional activity mediated by Grb4 is dependent on the presence of c-Abl. MEKK1-induced AP-1 activity in c-Abl-null fibroblasts (abl−/−) was inhibited in a similar way by Grb4SH2, and not by the triple SH3 mutant of Grb4SH2 (Fig. 7A). A similar result was obtained in c-Abl reconstituted knockout cells (Fig. 5B). The percentage of Grb4-mediated inhibition in fibroblasts was comparable to that obtained in 293 cells. Thus, Grb4-mediated transcriptional inhibition is independent of c-Abl. This result also shows that the effect of Grb4 on AP-1 activity is demonstrable in different cell lines.

The Grb4 SH2 Mutant Accumulates in the Nucleus—The hypothesis that the negative regulation of transcriptional activity mediated by Grb4 is connected to its localization to the nucleus raised the question of whether Grb4 WT and the SH2 mutant are present in the nucleus. Western blot analysis of nuclear and cytosolic extracts from cells expressing MEKK1 together with either Grb4 or Grb4SH2 mutant showed that Grb4 was localized to both the cytosol and the nucleus, whereas Grb4SH2 was present mainly in the nucleus (Fig. 8A). This differential subcellular localization correlates with the extent of transcriptional inhibition. To demonstrate the quality of the cell fractionation, the same extracts were analyzed for other nuclear proteins such as the retinoblastoma susceptibility protein (RB) and cyclin A. This observation indicates that the different extent of transcriptional inhibition by Grb4 and the Grb4SH2 mutant might be linked to their differential subcellular localization. The differential localization of the Grb4 WT and the SH2 mutant of Grb4, on the other hand, is compatible with the hypothesis that the Grb4-mediated negative regulation of AP-1 takes place within the nucleus.

Subcellular Localization of Grb4 and Co-localization with v-Abl—The differential subcellular localization of WT Grb4 and the Grb4SH2 mutant and the crucial role for the SH3 domains in mediating the inhibitory function of Grb4 led us to directly investigate the localization pattern of the different Grb4 mutants fused to EYFP using fluorescence microscopy in intact cells. In normally growing COS7 cells, the N-EYFP-Grb4 construct exhibited both cytosolic and nuclear localization, with most cells showing strong cytosolic staining (Fig. 8B, left panel). In contrast, the SH2 mutant of Grb4, N-EYFP-Grb4SH2, was predominantly present in the nucleus in nearly every cell expressing this construct (Fig. 8B, middle panel). This finding is compatible with the subcellular fractionation experiment showing nuclear accumulation of the SH2 mutant (Fig. 8A). In addition, the nuclear pool of Grb4SH2 exhibited enhanced dot-
Like distribution within the nucleus (Fig. 8B, middle panel). The triple SH3 mutant of Grb4 SH2 also showed predominantly nuclear localization (Fig. 8B, right panel). However, in contrast to wild-type Grb4 and primarily in contrast to the Grb4SH2 mutant, it exhibited a diffuse nuclear distribution (compare Fig. 8B, middle and right panels). This suggests that dot-like distribution within the nucleus depends on the integrity of the SH3 domains of Grb4. Thus, the integrity of the Grb4 SH3 domains is not only a prerequisite for Grb4-mediated AP-1 inhibition but also determines the distribution of Grb4 within the nucleus in punctate aggregates. Indeed sites of active transcription show dot-like structures (speckles) in the nucleus, as demonstrated by staining proteins involved in transcriptional events (36, 37).

To examine the subcellular localization of Grb4 upon co-expression with v-Abl, we created an ECFP-v-Abl chimera by N-terminal fusion of ECFP to v-Abl. Co-expression of N-ECFP-v-Abl together with N-EYFP-Grb4 resulted in the redistribution of the nuclear pool of Grb4 to the cytoplasm and the co-localization of both chimeric proteins in the cytoplasm and in juxta-nuclear aggregates (Fig. 9, top row). In contrast, in cells co-expressing N-ECFP-v-Abl with N-EYFP-Grb4SH2 mutant, the nuclear pool of Grb4SH2 was not influenced by v-Abl and maintained its nuclear dot-like localization (Fig. 9, middle row). Like Grb4SH2, the nuclear pool of the triple SH3 mutant of Grb4SH2 failed to co-localize with v-Abl in the cytoplasm (Fig. 9, bottom row). Again, the nuclear distribution of the triple SH3 mutant of Grb4SH2 was diffuse, and there was no formation of nuclear punctate aggregates. These observations suggest that phosphorylserine-dependent SH2-mediated direct interaction between v-Abl and Grb4 sequesters Grb4 in the cytoplasm, thereby depleting the nuclear pool of Grb4. If, as our results suggest, Grb4-mediated inhibition of promitogenic pathways depends on its nuclear localization, then v-Abl-mediated redistribution of Grb4 to the cytoplasm negatively regulates the inhibitory capacity of Grb4 on transcriptional activity from TRE and SRE enhancer elements.

Grb4SH2 Reduces v-abl-induced Anchorage-independent Growth of NIH3T3 Cells—We examined the effect of Grb4SH2 on v-abl-induced colony formation of retrovirally transduced NIH3T3 cells in soft agar assays (Fig. 10). Although the expression level of v-Abl in the cells plated in soft agar was comparable (Fig. 10B, top panel), co-expression of Grb4SH2 resulted in a smaller size and a reduced number of colonies (Fig. 10A, left panel) compared with colony formation of cells expressing v-Abl alone (Fig. 10A, right panel). In addition, a reduction of medium acidification was noted. This effect could be due to the capacity of Grb4SH2 to significantly inhibit v-Abl-induced AP-1 activity.

**DISCUSSION**

We now show that Grb4, previously identified as an interaction partner of Bcr-Abl, is a potent inhibitor of v-Abl-induced transcriptional activity from promitogenic enhancer elements such as AP-1 and SRE. Our results indicate that the nuclear pool of Grb4 mediates the inhibitory activity. This conclusion can be drawn from several lines of evidence. First, the fact that Grb4 also inhibited MEKK1-induced AP-1 activity without affecting JNK activity or influencing the DNA binding activity of AP-1 strongly suggests that the inhibitory effect of Grb4 in the signaling cascade is located downstream of the formation of AP-1-DNA complexes. Secondly, direct complex formation be-
between v-Abl and Grb4 depends mainly on an intact SH2 domain of Grb4. However, the SH2 domain does not contribute to the Grb4-mediated inhibition of v-Abl-induced transcriptional activity. The SH2 mutant of Grb4 unable to bind v-Abl was even more effective in AP-1 inhibition. This mutant of Grb4 exhibited increased nuclear localization and also showed enhanced formation of punctate aggregates within the nucleus. It has been shown that dot-like structures (speckles) in the nucleus can indeed represent sites of active transcription (36–38). Thus, there is a correlation between AP-1 inhibition and localization to subnuclear dot-like structures. Third, a triple SH3 mutant of Grb4SH2 showed significantly reduced inhibition of MEKK1- or v-Abl-induced AP-1 activity. Mutations destroying the core region of each SH3 domain did not affect the general nuclear localization but completely abrogated dot-like subnuclear localization and induced a homogenous distribution of Grb4 within the nucleus.

We show that co-expression of Grb4SH2, which no longer binds v-Abl, together with v-Abl results in a significantly reduced colony formation of NIH3T3 cells compared with the colony formation induced by v-Abl alone. This result could be interpreted as the in vivo correlation to our observation that Grb4SH2 inhibits AP-1 activation, a promitogenic v-Abl-induced pathway.

It might be speculated that Grb4 interferes with transcriptional co-activators/repressors, e.g. JAB1 or CBP/p300. Activation of transcription by JAB1 is thought to involve the stabilization of AP-1 complexes and AP-1 binding sites (23). Inhibition of AP-1 by activated nuclear receptors is thought to involve titration of CBP/p300, which has a high affinity to phosphorylated c-Jun but is also involved in nuclear receptor binding (24). It would be interesting to determine whether Grb4 is able to interact with these proteins or pathways known to be critical in AP-1 regulation. Because we show that Grb4 was also able to inhibit MEKK1-induced SRE activity, additional studies are necessary if inhibition of AP-1 and SRE is due to Grb4-mediated targeting of a common repressor of both pathways.

By performing a yeast two-hybrid screen with Grb4, we were able to identify hnRNP as a direct interaction partner of Grb4.² hnRNP-K has been reported to possess transcriptional repressor function (39, 40) and to physically and functionally interact with C/EBPβ (39), which is known to interact with AP-1 transcription factors (41). The interaction between Grb4 and hnRNP-K is independent of tyrosine phosphorylation because no active tyrosine kinase was co-expressed in yeast. Both proteins clearly co-localized in dot-like subnuclear structures. However, at this point, it is unclear if and how the interaction between Grb4 and hnRNP-K could be involved in AP-1 inhibition.

The Grb4 SH2 domain seems to be involved in regulating the amount of Grb4 present in the nucleus because the SH2 mutant of Grb4 exhibited enhanced nuclear localization as analyzed by subcellular fractionation and fluorescence microscopy. It is possible that under physiological conditions, the SH2 domain-mediated binding of Grb4 to tyrosine-phosphorylated cytoplasmic proteins regulates the amount of Grb4 present in the nucleus. Because the nuclear pool of Grb4 upon co-expression of v-Abl or Bcr-Abl is redistributed to the cytosol, it is tempting to speculate that the antimitogenic activity of Grb4 is negatively regulated by these oncoproteins. The fact that the inhibitory effect of Grb4 WT on v-Abl-induced AP-1 activity showed a concentration-dependent increase possibly reflects the saturation of Grb4 binding to v-Abl under the conditions of overexpression. This hypothesis is consistent with the increased inhibitory activity of Grb4SH2 unable to bind to v-Abl. It is possible that this redistribution of inhibitory proteins shuttling between cytoplasm and nucleus due to recruitment to cytoplasmic oncoproteins represents a general mechanism contributed to the malignant transformation of cells. Another explanation for the differential subcellular localization may be the differential phosphorylation status of the Grb4 mutants. The SH2 mutant of Grb4 might exhibit reduced phosphorylation upon co-expression of v-Abl because co-localization of both proteins is impaired. Therefore transfer to the nucleus of the less phosphorylated Grb4 SH2 mutant could be facilitated per se or again by reduced binding of Grb4 to SH2-containing cytoplasmic proteins. Accordingly, tyrosine phosphorylation of Nck has been shown only to be specific for the cytoplasmic pool (42).

A recent publication showed that Grb4 (Nckβ), in contrast to Nck (Nckα), blocks platelet-derived growth factor- and Rac-L62-induced membrane ruffling and formation of lamellipodia (16). This effect was dependent on the SH2-mediated binding of Grb4 to platelet-derived growth factor or constitutive membrane targeting of Grb4. Because nuclear localization and mutation of the Grb4 SH2 mutation enhance AP-1 inhibition, both observations must reflect different and distinct roles of Grb4.

The finding that in vivo complex formation between Grb4 and phosphorylated v-Abl or Bcr-Abl is regulated by the SH2 domain of Grb4 whereas complex formation between kinase-defective v-Abl or Bcr-Abl and Grb4 is dependent on the SH3 domain indicates that the mode of interaction between Grb4 and Bcr-Abl with and without kinase activity is different. The hypothesis that autophosphorylation of tyrosine residues within the SH3-binding region within the Abl part of v-Abl or Bcr-Abl inhibits the binding of Grb4 SH3 domains is most appealing. It has been shown that the interaction between WASP and PSTPIP is regulated in a similar way (43). In the case of an in vivo interaction of Grb4 and Abl, the differential binding mode could then regulate SH3 domain-mediated binding of Grb4, depending on the phosphorylation status of c-Abl. Nonphosphorylated c-Abl would bind Grb4 via its SH3 domains, whereas phosphorylation of c-Abl would induce a Grb4-SH2-dependent interaction. The SH3 domains of Grb4 could then recruit other proteins to the c-Abl-Grb4 complex. This speculation appears even more interesting in the light of a recent study showing activation of Abl by Nck-SH3 domains (44). However, use of c-Abl knockout cells in this study did not show any important role for c-Abl in Grb4-mediated inhibition of transcription from promitogenic enhancer elements. Regulation by the c-Abl homologue Arg in Abl knockout cell lines could be responsible for this lack of experimental evidence.

Taken together, our results suggest a role for Grb4 in the inhibition of v-Abl-induced transcription from promitogenic enhancer elements such as TRE and SRE on the nuclear/transcriptional level. Redistribution of the nuclear inhibitory pool of Grb4 to the cytoplasm by oncoproteins such as v-Abl or Bcr-Abl may represent a mechanism contributing to the malignant transformation of cells.

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