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3**Title: Mapping of NRF binding motifs of NF-kappaB p65 subunit**

4**Running title: NRF binding motifs within NF-kappaB p65**

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21**Abbreviations**

22aa, amino acid; CBP, calmodulin binding peptide; CREB, cAMP response element binding
23protein; HDAC1, histone deacetylase 1; IFN- β , interferon beta; IL-1, Interleukin-1; IL-8,
24Interleukin-8; iNOS, inducible nitric oxide synthase; NRE, negative regulatory element; NRF,
25NF-kappaB repressing factor; PMA, phorbol 12-myristate 13-acetate; PPI, protein-protein
26interaction; RHD, Rel homology domain; TAD, transcription activation domain; TAP, tandem
27affinity purification; TEV, tobacco etch virus; TFIID, transcription factor IID;

28**Abstract**

29NF-kappaB repressing factor (NRF) is a nuclear transcription factor that binds to a specific
30DNA sequence in NF-kappaB target promoters. Previous reports suggested that NRF
31interferes with the transcriptional activity of NF-kappaB binding sites through a direct
32interaction with NF-kappaB subunits. The aim of this study was to map specific NRF binding
33domains in the NF-kappaB proteins, p65 and p50. Our data demonstrate that NRF is able to
34interact with the p65 subunit and inhibit its transcription enhancing activity in reporter gene
35experiments. Using tandem affinity purifications (TAP), we show that NRF protein
36significantly binds to the endogenous p65, subunit but not to the p50 subunit. The selective
37binding activity of the NRF protein is consistently mediated by the N-terminal domain of NRF
38(aa 1-380). Moreover, the Rel homology domain (RHD) of p65 is sufficient for binding to the
39N-terminal domain of NRF. Using detailed peptide mapping studies, we finally identify three
40peptide motifs in p65 RHD showing distinctive binding specificities for the NRF protein.
41According to the predicted structure of p65, all three peptide motifs align within an exposed
42region of p65 and might hint at promising targets for inhibitors.

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53**Keywords**

54NF-kappaB, NRF, peptide array, protein-protein interaction, transcriptional regulation

55Introduction

56   The family of NF-kappaB transcription factors responds to diverse cell stimuli by
57activating the expression of stress response genes. Multiple signals, including cytokines and
58bacterial and viral products, induce NF-kappaB transcriptional activity . Five polypeptides,
59p50, p65, c-Rel, p52, and RelB, constitute the entire NF-kappaB family, and these proteins
60share an N-terminal Rel homology domain (RHD). The RHD consists of an N-terminal folded
61domain of 160–210 amino acids, a short flexible linker of approximately 10 amino acids and
62a C-terminal folded domain roughly spanning 100 amino acids . All NF-kappaB subunits can
63form various homo- and heterodimer combinations via their RHDs. Theoretically, the
64formation of 15 homo- and heterodimers is possible from the five NF-kappaB subunits ;
65however, only 12 could be identified *in vivo* . The predominant form is the p65-p50
66heterodimer, which assembles with a significantly greater stability than either of the
67respective p50-p50 or p65-p65 homodimers . The positions Asp-256 and Tyr-269 in p50
68symmetrically associate with positions Asn-200 and Phe-213, respectively, in p65 and
69stabilize the heterodimer through hydrogen bonds at the interface . Besides dimerization, the
70RHD is responsible for sequence-specific DNA binding and nuclear localization as well as
71interaction with the inhibitors of kappaB proteins (IkappaBs) . Only three NF-kappaB
72subunits, p65, c-Rel, and RelB, contain a transcription activation domain (TAD) bordering
73their RHD domains at the C-terminal end. The TAD is responsible for the transcriptional
74activation of NF-kappaB target genes. Consequently, NF-kappaB dimers that possess at
75least one of these subunits can act as transcriptional activators .

76NF-kappaB repressing factor (NRF) is a nuclear transcription factor that is constitutively
77expressed in variable amounts in all human tissues . Using an inducible NRF antisense
78expression approach in human epithelial cells, NRF was implicated in the basal silencing of
79NF-kappaB target genes, including IFN- β and iNOS . Additional studies on HIV-LTR and IL-8
80promoters revealed that NRF is also required for transcriptional activation upon specific
81stimulation with PMA or IL-1 . It was therefore suggested that endogenous NRF plays a dual
82role in gene transcription in concert with other accessory co-factors. Detailed characterization

83of NRF revealed that the N-terminal domain of NRF, spanning amino acids (aa) 1 to 296,
84inhibits the transcriptional activity of NF-kappaB binding sites in reporter gene experiments .
85Recently, down-regulation of NRF was associated with elevated NF-kappaB activity in cystic
86fibrosis and pancreatic cancer . Here, we report that NRF exclusively interacts with the NF-
87kappaB subunit p65. Additionally, we define three NRF-binding peptide sequences in p65
88RHD close to the p50 interaction domain.

89Results

90Reporter gene analysis demonstrates the functional interaction of p65 and NRF

91 We have previously shown that NRF represses the transcriptional activity of NF-
92kappaB binding sites , which can theoretically bind the 12 known different sets of NF-kappaB
93dimers. Here, we first attempted to characterize the functional interaction of p65 and NRF in
94HeLa cells. Therefore, we used the reporter plasmid pL6NLuc, comprising firefly luciferase
95coding sequences under the control of a DNA binding element for the *E. coli* LexA protein
96(LexA) and the NRF binding sequence from the human IL-8 promoter (NRE). This reporter
97was transfected into HeLa cells together with a constitutively active *Renilla* luciferase
98expression vector as an internal transfection control. Additionally, the pLexA-p65 vector
99encoding a fusion protein of the N-terminal LexA DNA binding domain and the C-terminal full
100length p65 protein was transfected. As shown in Figure 1, the LexA-p65 fusion protein leads
101to transcriptional activation of the reporter and significantly high expression of firefly
102luciferase. Additional expression of two different NRF proteins spanning aa 1-380 (pNRF(1-
103380)) or aa 1-690 (pNRF(1-690)) clearly decreases the firefly luciferase reporter gene
104expression. We observed slight differences in inhibition between NRF(1-380) and NRF(1-
105690). This might be based on the previously reported partial nucleolar localization of
106overexpressed NRF(1-690) , which might constrain its mobility and transcriptional activity.
107Nevertheless, full-length NRF and its N-terminal domain are both able to inhibit the
108transcriptional enhancing activity of the LexA-p65 protein.

109

110NRF does not bind p50 *in vivo*

111 To study the specificity of NRF and p65 interaction in cells, we used NRF-TAP fusion
112proteins (Fig. 2A). This TAP tag involves two affinity tags, CBP and IgG binding domains of
113protein A, separated by a specific TEV protease cleavage site. The two consecutive affinity
114purification steps provide a more stringent purification of interacting proteins compared to a
115single step procedure . Three different NRF-TAP fusion proteins as well as the TAP tag
116alone were transiently expressed in HeLa cells. NRF(1-690)TAP is composed of the NRF full

length protein and the C-terminal TAP tag. Fusion proteins NRF(1-380)TAP and NRF(1-149)TAP contain NRF aa 1-380 and aa 1-149, respectively. These mutants were initially constructed based on an isolated partial cDNA coding for aa 1-380 or by using a single restriction site (EcoRI) present in the NRF coding sequence (aa 1-149). Thus, NRF(1-149)TAP contains a nuclear localization signal (NLS) but lacks the DNA binding domain that is present in NRF(1-380)TAP. Finally, cellular extracts were first tested for equal amounts of p65 or p50 as a control (Supplemental Figure 1, total p65 or p50) and subjected to TAP analysis later. Cellular lysates were incubated with IgG-agarose for the first step of purification. Following several wash steps, the samples were tested for an equal level of bound TAP fusion protein by Western blot analysis (Supplemental Figure 1, total TAP fusion proteins). IgG-agarose was then incubated with TEV to split off the bound protein A domains. For the second purification step, the supernatants were added to calmodulin beads. Finally, the amounts of the transcription factors p65 and p50 were detected in each bound protein fraction by Western blot analysis (Fig. 2A). The data show that NRF1-690TAP and NRF1-380TAP are able to interact with the endogenous p65 protein (Fig. 2A, p65 bound). The amount of NRF1-690TAP and p65 proteins in input and bound fractions was estimated using radioactive labeled NRF1-690TAP and p65 protein standards. Based on the estimated concentrations, we calculate a dissociation constant of 10^{-8} M for NRF1-690TAP and p65 proteins (data not shown). In comparison, NRF1-149TAP and the TAP tag fail to bind to p65. Strikingly, p50 barely binds to either NRF1-690TAP or NRF1-380TAP fusion proteins. NRF and p65 were previously found to be involved in the transcriptional activation of the IL-8 promoter upon IL-1 stimulation. Therefore, we analyzed whether IL-1 treatment might affect the interaction of p65 or p50 with NRF. Importantly, upon Interleukin-1 treatment of cells (Fig. 2A, +IL-1), the endogenous p65 shows a lower binding affinity to NRF. However, p50 fails to bind to NRF either before or during IL-1 stimulation. Thus, independent of IL-1 stimulation, NRF seems to specifically interact with the p65 but not the p50 subunit *in vivo*.

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146 **NRF (1-380) interacts with p65 RHD *in vivo***

147 To assess the binding of p65 RHD to the N-terminal domain of NRF, we
148 cotransfected the NRF1-380TAP expression vector and the full-length p65 or truncated p65
149 RHD expression vectors into HeLa cells (Fig 2B). The equal expression of NRF1-380TAP
150 fusion protein was tested by Western blot analysis. The p65 antibody is a mouse monoclonal
151 antibody raised against amino acids 1-286 of human p65 that detects the endogenous and
152 overexpressed p65 as well as the p65 RHD in cellular lysates (Fig. 2B; total). Tandem affinity
153 purification experiments reveal that overexpressed p65, endogenous p65 and the truncated
154 p65 RHD bind to aa 1-380 of NRF (Fig. 2B; bound). This is in agreement with the previous
155 observation that a short central domain of NRF (aa 204-308) binds to p65 RHD . The
156 presence of p65 RHD in cellular extracts leads to apparent decrease of p65 binding to NRF
157 (Fig. 2B, bound). This might be due to a preferential formation of p65 to p65 RHD
158 heterodimers with a possible lower affinity to NRF. On the other hand, the signal intensities
159 of p65 and p65 RHD strongly depend on the antibody binding affinity and the blotting
160 characteristics of each single protein as well. Therefore, it is rather difficult to estimate and
161 compare the NRF binding affinities of p65 and p65 RHD in these experiments.

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163 **NRF-Strep binds to three short domains in p65 RHD**

164 We used the previously described SPOT technique to identify the p65 RHD amino
165 acid sequences binding to NRF. In this approach, 15-mer peptides derived from the p65
166 RHD sequence were chemically synthesized by the SPOT method and immobilized on a
167 cellulose membrane. As shown in Fig. 3A, adjacent peptides share a common sequence of
168 12 aa but differ by 3 aa at the C- or N-terminal ends. Rabbit reticulocyte lysates (RRL) were
169 used to rapidly express a relatively high concentration (120 nM) of the NRF-Strep fusion
170 protein compared to cell culture expression systems. Fusion of a C-terminal Strep tag to the
171 NRF coding sequence allows specific detection of NRF protein bound to 15-mer peptides by
172 streptavidin. Figure 3B shows that NRF-Strep fusion protein binds to three distinct peptide

173 motifs (Fig. 3B, NRF-Strep). As a control, incubation with streptavidin without (Fig. 3B,
174 control) or with rabbit reticulocyte lysates (RRL) lacking NRF-Strep protein (Fig. 3B, RRL)
175 revealed barely detectable background signals. We performed additional control experiments
176 using the strep tag or Strep-JKTBP1, and they showed no significant affinity to the p65
177 peptide spots (Supplemental Fig. 2). Together, this indicates that the NRF-Strep protein
178 binds predominantly to three amino acid motifs corresponding to p65 aa 85-114 (motif 1), aa
179 199-246 (motif 2) and aa 274-309 (motif 3). Motifs 1 and 3 represent short protein domains
180 spanning 30 and 35 amino acids, respectively. Motif 2 with 47 amino acid residues reaches
181 over a relatively large central part of p65.

182

183 **Further analysis of p65 RHD amino acid motifs**

184 We performed additional peptide array experiments to further refine the mapping of
185 the NRF binding domain in p65. The results in Fig. 3 indicate that motif 1 has a lower binding
186 affinity to NRF protein compared to motifs 2 and 3. For Motif 1, we used a sizing approach
187 that allows for the successive downsizing of binding sequences. As shown in Fig. 4A, 8- to
188 15-mer peptides corresponding to motif 1 were immobilized on a cellulose membrane. The
189 stepwise single aa extension of the peptide length from 8 to 15 aa allows the precise
190 mapping of a minimal binding motif. Adjacent peptides share an expanding common
191 sequence from 7 to 14 aa but differ by a single aa at the C- or N-terminal ends. The binding
192 procedure was performed as described above. The control shows no affinity to streptavidin
193 (Supplemental Fig. 3A). Following densitometric analysis, the signal intensities were
194 classified into four groups: background, weak, strong and very strong signals (Supplemental
195 Fig. 4). Accordingly, we searched for peptide sequences that predominantly occur in spots
196 showing strong and very strong signals. The amino acid motif YEAELCPD (aa 100-107) was
197 present in 36 spots of the peptides, mainly showing strong and very strong signals (72%).
198 Moreover, a higher number of positive spots contained the core sequence AELC. For
199 instance, motifs AELCPD lacking the first or YEAELC lacking the last two amino acids were

200found in 68 spots (Supplemental Fig. 4, table). Thus, we believe that the YEAEPCPD motif
201might be involved in the NRF-p65 interaction.

202 For Motif 2, we used another approach to isolate a possible binding motif for NRF.
203Based on the length of Motif 2, we expected two separated binding motifs. In this case, the
204length of the immobilized peptides was kept constant. As depicted in Fig. 5A, adjacent
205peptides share a common sequence of 14 aa but differ by a single aa at C- and N-terminal
206ends. The binding procedure was performed as described above. Again, no positive signals
207were obtained with streptavidin (Supplemental Fig. 3B). Strikingly, NRF-Strep binds to all
208immobilized peptides but reveals a significantly higher signal intensity with peptides
209containing the C-terminal end of motif 2 (Fig. 5B). Therefore, we suggest that amino acids
210224 to 246 might be involved in the binding of p65 to NRF-Strep protein.

211 In figure 3, motif 3 (aa 274-309) shows the strongest affinity to NRF-Strep. For further
212isolation of interacting amino acids, we used an alanine scan approach. Therefore, 15-mer
213peptides corresponding to motif 3 were synthesized and immobilized on a single array. As
214shown in Fig. 6A, adjacent peptides differ by a single aa substitution by alanine. The first 16
215peptides correspond to aa 274-288 of p65. Following a blank spot, the next 16 peptides
216correspond to aa 277-291 of p65, and each carry a single alanine substitution. Following the
217binding procedure, all peptides are able to bind to NRF-Strep (Fig. 6B). Note that blank spots
218contain no peptides as an additional control, and the control spots show negligibly low
219background binding of streptavidin (Supplemental Fig. 3C). We performed a densitometric
220analysis of the detected signals (summarized in Supplemental Figure 5). Although none of
221the amino acid exchanges to alanine cause a complete loss of NRF-Strep binding, several
222exchanges significantly decrease the signal intensity. The most prominent reduction was
223detected after substitution of glutamic acid 279 with alanine (E279A). Glutamic acid is
224negatively charged and located at an exposed position within a loop structure in p65 RHD
225(Fig. 7B). Therefore, it is conceivable that the substitution of glutamic acid with the neutral
226amino acid alanine can affect the binding of NRF-Strep to this motif.

227   Taken together, we have identified three peptide sequences in p65 RHD with
228 distinctive binding affinities for NRF protein. Thus, these peptides might represent the
229 anticipated sites of NRF-p65 interaction and can possibly be used as small interfering
230 peptides.

231 Discussion

232 Protein–protein interactions (PPIs) are the fundamental basis for many crucial
233 regulatory networks, and mapping PPIs at every scale is the key to identifying new targets for
234 therapeutic strategies. We have previously reported that NF-kappaB repressing factor (NRF)
235 directly interacts with NF-kappaB p65 protein to regulate the IL-8 gene in human epithelial
236 cells . Recently, NRF was implicated in aberrant NF-kappaB activity in cystic fibrosis and
237 pancreatic cancer, the latter being one of the most lethal and treatment-resistant forms of
238 cancer . Thus, mapping NRF and NF-kappaB interactions may lead to new strategies for
239 therapeutic intervention against these diseases.

240 In general, different sets of NF-kappaB homo- and heterodimers are able to bind to
241 the NF-kappaB DNA binding consensus sequence (GGGRNNYYCC) . X-ray structure
242 analysis of the NF-kappaB consensus sequence in complex with NF-kappaB homo- and
243 heterodimers revealed that two subunits are required to encircle the target DNA (p50/p65
244 heterodimer, Fig. 7B) . The LexA reporter assay in our study monitors LexA-p65 transcription
245 activity independent of its binding to the NF-kappaB consensus sequence. The assembly of
246 LexA-p65 proteins led to a significant increase in reporter gene expression. According to
247 previous reports, this is based on the p65 transcription activating domain (TAD) . Full-length
248 NRF or the N-terminal domain of NRF (1-380) interferes with the transcriptional activity of
249 LexA-p65 TAD. This is in agreement with our previous reports showing that NRF inhibits the
250 transcription-enhancing activity of adjacent NF-kappaB binding elements . Furthermore, the
251 TAP co-purification experiments in figure 2 confirm a selective interaction between NRF-TAP
252 and p65 in cellular extracts that contain both p65 and p50 subunits in excess (Supplemental
253 Fig. 1). A negligible amount of p50 was rarely detected in the bound fraction, which might be
254 due to a partial co-purification of p50 by binding to p65. Nevertheless, a direct interaction
255 between p50 and NRF seems very unlikely.

256 Previously, NRF was shown to differentially act on the activation of the IL-8 gene by
257 NF-kappaB upon IL-1 stimulation in human epithelial cells . Our data indicate that the binding
258 of p65 to NRF is attenuated upon IL-1 stimulation. Therefore, we suggest that the differential

259regulation of the IL-8 promoter in IL-1 stimulated cells might be at least partially due to the
260disparate mode of NRF and p65 interaction or to IL-1 mediated phosphorylation of p65 as
261reported earlier .

262 Further characterization of the NRF binding domain reveals that p65 RHD is able to
263bind to the N-terminal domain of NRF. In addition, we utilized a set of different antibodies
264including HDAC1 , CREB binding protein and TFIID to examine the binding of a variety of
265p65 binding co-factors to NRF. However, none of these cellular co-factors were able to bind
266to the NRF-TAP fusion protein (data not shown). Thus, p65 remains the only known
267transcription factor that binds to NRF so far.

268 Previous studies have shown that p65 and p50 proteins interact via their RHDs
269sharing 41% sequence homology (Fig. 7A). Together with the data presented here, we
270suggest that p65 RHD is able to bind to both NRF and p50. Notably, NRF and p50 reveal no
271significant sequence homology. Thus, it is tempting to speculate that both p50 and NRF bind
272to different sequence motifs in p65 RHD. In fact, the slight co-purification of p50 together with
273p65 bound to NRF (in figure 2A) might hint at a potential common complex of p50, p65 and
274NRF, the existence of which remains to be proven experimentally.

275 The further isolation of NRF binding domains in p65 protein reveals three distinct
276peptide motifs within the RHD. Motif 1 is exclusively found in p65 RHD and not in p50 RHD; it
277seems to be highly specific for NRF binding. The 72% of peptides containing this motif
278strongly bind NRF. Importantly, the 51 peptides that were missing the entire or part of motif 1
279in this experiment failed to bind NRF protein. In comparison, motif 2 spans 23 amino acids,
280and its C-terminal domain (aa 224-246) has an especially strong binding affinity for NRF
281protein. Although we cannot explore a core sequence in motif 2, we find an exceptional gap
282of 5 amino acid residues in this motif by comparison of p50 and p65 RHDs. Thus, these
283additional residues might be responsible for the selective binding of NRF. Motif 3 shows the
284highest binding activity for NRF and seems to interact with NRF over its entire length. Only a
285single mutation, E279A, could significantly reduce the binding of NRF, down to 30%
286(Supplemental Fig. 5).

287 X-ray structures of individual NF-kappaB dimers revealed a new mode of DNA
288 recognition wherein a dimer composed of RHDs intimately contacts the major groove of the
289 NF-kappaB target DNA sequence . We aligned the three identified NRF binding motifs in the
290 predicted structure for the p65 RHD/p50 RHD dimer . Most importantly, we find the entire
291 length of all three motifs on the surface of the p65 protein in complex with p50 RHD and DNA
292 (Figure 7B and Supplemental Fig. 6). Interestingly, the N-terminal region of motif 2 contains
293 amino acid residues 200 and 213, which approach p50 RHD to form a p50/p65 heterodimer.
294 Although NRF shows an average binding to the N-terminal tail of motif 2, we believe that the
295 binding of NRF to p65 might at least affect the formation of p50/p65 heterodimers. However,
296 extensive studies will be required to confirm this hypothesis. Given that elevated p65 activity
297 has a causative function in inflammation and transformation , the identified motifs might be
298 valuable targets for new therapeutics.

299Material and methods

300

301Plasmid constructions

302 p65 and pLexA expression plasmids were described earlier . The *NdeI/BamHI*
303fragment of the p65 expression plasmid was removed to create the p65 Rel homology
304domain (p65RHD) expression plasmid. The vector pLexA-p65 was constructed by
305transferring a *HindIII/EcoRI* fragment encoding LexA from pLexA into the p65 plasmid. The
306reporter plasmid pL6NLuc was derived from pL6 . After the insertion of the NRF binding site
307(NRE) next to the LexA site in this vector, the CAT reporter gene was replaced by firefly
308luciferase from pTA-Luc (Clontech) using the *PvuII/EcoRI* sites. NRF tandem affinity
309purification (TAP) expression plasmids were described previously . Expression plasmids
310pNRF(1-380) and pNRF(1-690) carrying NRF and linker sequences were obtained by
311inserting stop codons in the corresponding TAP vectors. The control vector pRL-Tk was
312obtained from Promega. The NRF-Strep expression plasmid (pEXPR-NRF-Strep) was
313created by the insertion of a *PvuII*/blunted *BamHI* fragment encoding full length NRF from
314NRF(1-690)TAP into pEXPR-IBA103 (IBA). For additional controls of the peptide mapping
315experiments, the vectors pEXPR-IBA105 (IBA) and pEXPR-JKTBP1 were used to express
316the strep tag and Strep-JKTBP1 *in vitro*, respectively.

317

318In vitro expression of proteins

319 The NRF-Strep expression plasmid (pEXPR-NRF-Strep) as well as the control
320vectors pEXPR-IBA105 and pEXPR-JKTBP1 were added to the *in vitro* expression kit TNT
321obtained from Promega to express NRF-Strep and control proteins. The reactions were
322performed according to manufacturer's instructions.

323 The pNRF(1-690)TAP and p65 expression plasmids were added to the *in vitro*
324expression kit TNT obtained from Promega together with [³⁵S]methionine to express
325radioactive proteins. The reactions and the determination of protein concentration were

326performed according to manufacturer's instructions. The radioactive labeled proteins served
327as standards for protein concentration in TAP experiments.

328

329**Peptide mapping**

330   The p65 RHD peptide arrays were generated by the SPOT-synthesis technique, as
331described earlier . Briefly, peptides were synthesized on an amino-functionalized cellulose
332membrane using Fmoc/*tert*-butyl chemistry. The spots consist of approximately 5 nmol of
333each peptide . For the binding assays, the peptide arrays were blocked overnight at room
334temperature with blocking buffer consisting of 2x blocking buffer concentrate (Sigma-Genosys
335Inc., # B6429) and 5% (w/v) sucrose in TBS-T (0.02 M sodium phosphate buffer with 0.15 M
336sodium chloride (pH 7), 0.05% Tween 20). Approximately 1 µg of NRF-Strep protein in
337blocking buffer was added to p65 RHD peptide arrays for 4 hours at room temperature. Next,
338the arrays were washed with a 10-fold volume of TBS-T three times and then incubated with
339AP-conjugated streptavidin (Calbiochem, #189732). Finally, a chemiluminescence reaction
340(Biorad, #170-5018) was performed to detect the bound fraction of NRF-Strep protein.

341

342**Cell lines and transfection**

343   HeLa (CCL-2, LGC Promochem) cells were maintained in DMEM with 5% FCS
344(FCS). For reporter assays, the cells were transfected by calcium phosphate coprecipitation .
345A total of 6 µg of reporter and expression plasmids together with 0.5 µg of a *Renilla*
346expression plasmid were transfected per 7.5×10^4 cells. After 48 hours, the cells were
347harvested and pooled for reporter gene assays. Firefly and *Renilla* luciferase activities were
348determined using the Dual-Luciferase reporter system (Promega Corporation) as described
349in the manufacturer's protocol. Luciferase activity was measured in a Lumat LB 9501
350luminometer (Berthold). For TAP-purification, HeLa cells were transfected with indicated
351NRFTAP expression plasmids by calcium phosphate precipitation.

352

353TAP purification

354 The TAP purification was performed as described with the following modifications.
355 HeLa cells were transfected with either TAP or NRF-TAP fusion protein- encoding plasmids.
356 Forty-eight hours later, the cells were stimulated with IL-1 (10 ng/ml) for 3 hours where
357 indicated. Approximately 10^8 cells were lysed in buffer A (10 mM HEPES/KOH (pH 7.9), 10
358 mM KCl, 1.5 mM MgCl₂, 5 mM DTT, 0.1% Nonidet P-40, 0.3 mM Na₃VO₃, 20 mM glycerol-
359 2-phosphate, 10 μ M protease inhibitor E64, 2.5 μ g/ml leupeptin, 0.3 mM PMSF, 1 μ M
360 pepstatin, 400 μ M okadaic acid (pH 7.9)). Equal aliquots of cellular extracts were used for
361 Western blot analysis using α -p65 antibody or α -p50 antibody (sc8008, sc8414, Santa Cruz
362 Biotechnology) and peroxidase antiperoxidase soluble complex antibody produced in rabbit
363 (PAP, Sigma-Aldrich) to confirm equal expression of TAP fusion proteins. Equal amounts of
364 cellular extracts were incubated with IgG-agarose (Sigma-Aldrich Inc., Munich, Germany) in
365 salt-adjusted buffer A (150 mM NaCl) at 4°C overnight to bind TAP-tagged proteins.
366 Following three washing steps with wash buffer (10 mM Tris (pH 8.0), 150 mM NaCl and
367 0.1% Nonidet P-40), the binding of TAP fusion proteins to equal aliquots of IgG-agarose
368 (10%) was examined by Western blot analysis using peroxidase anti-peroxidase soluble
369 complex antibody produced in rabbit (PAP, Sigma-Aldrich). Complexes were released from
370 IgG-agarose by tobacco etch virus (TEV)-protease (30 U; Invitrogen Life Technologies) in
371 TEV buffer (10 mM Tris-HCl (pH 8.0); 150 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT, 0.5 mM
372 EDTA, 1 μ M protease inhibitor E64). Complexes containing calmodulin binding peptide
373 (CBP)-tagged proteins were immobilized on a calmodulin affinity resin (Stratagene) for 4
374 hours at 4°C. Complexes were washed five times with CBP buffer (50 mM Tris-HCl (pH 8.0);
375 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 10 mM 2-ME, 2 mM CaCl₂). Finally, 20 μ l
376 of gel loading buffer was added to the bound protein complexes and incubated for 10 min at
377 65°C. The samples were subjected to SDS gel electrophoresis together with protein
378 concentration standards. Western blot analysis was performed using a mouse monoclonal
379 antibody against p65 (sc8008) or p50 (sc8414) and secondary goat anti-mouse antibody
380 (sc2005) from Santa Cruz Biotechnology. The dissociation constant K_d for NRF and p65 in

381TAP experiments was estimated according to the equation described for immobilized
382proteins earlier . The concentrations of NRF-TAP and p65 in input and bound fractions were
383estimated using densitometric analysis of protein standards and detected signals.

384

385**Western blot analysis**

386 Western blots were performed as described earlier . TAP fusion proteins were
387detected using peroxidase anti-peroxidase soluble complex antibody produced in rabbit
388(PAP, Sigma-Aldrich). Antibodies against p65 (sc8008) and p50 (sc8414) were obtained from
389Santa Cruz Biotechnology.

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397References

- 398(1) Hayden, M.S., and Ghosh, S. (2008) Shared principles in NF-kappaB
399 signaling. *Cell*. **132**, 344-362
- 400(2) Huxford, T., and Ghosh, G. (2009) A structural guide to proteins of the NF-
401 kappaB signaling module. *Cold Spring Harb Perspect Biol*. **1**, a000075
- 402(3) Hoffmann, A., Natoli, G., and Ghosh, G. (2006) Transcriptional regulation via
403 the NF-kappaB signaling module. *Oncogene*. **25**, 6706-6716
- 404(4) Huang, D.B., Huxford, T., Chen, Y.Q., and Ghosh, G. (1997) The role of DNA
405 in the mechanism of NFkappaB dimer formation: crystal structures of the
406 dimerization domains of the p50 and p65 subunits. *Structure*. **5**, 1427-1436
- 407(5) Ghosh, S., May, M.J., and Kopp, E.B. (1998) NF-kappa B and Rel proteins:
408 evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*.
409 **16**, 225-260
- 410(6) Hayden, M.S., and Ghosh, S. (2004) Signaling to NF-kappaB. *Genes Dev*. **18**,
411 2195-2224
- 412(7) Nourbakhsh, M., and Hauser, H. (1999) Constitutive silencing of IFN-beta
413 promoter is mediated by NRF (NF-kappaB-repressing factor), a nuclear
414 inhibitor of NF-kappaB. *EMBO J*. **18**, 6415-6425
- 415(8) Nourbakhsh, M., Kalble, S., Dorrie, A., Hauser, H., Resch, K., and Kracht, M.
416 (2001) The NF-kappa b repressing factor is involved in basal repression and
417 interleukin (IL)-1-induced activation of IL-8 transcription by binding to a
418 conserved NF-kappa b-flanking sequence element. *J Biol Chem*. **276**, 4501-
419 4508
- 420(9) Dreikhausen, U., Hiebenthal-Millow, K., Bartels, M., Resch, K., and
421 Nourbakhsh, M. (2005) NF-kappaB-repressing factor inhibits elongation of
422 human immunodeficiency virus type 1 transcription by DRB sensitivity-
423 inducing factor. *Mol Cell Biol*. **25**, 7473-7483
- 424(10) Lu, Z., Li, Y., Takwi, A., Li, B., Zhang, J., Conklin, D.J., Young, K.H., and
425 Martin, R. (2011) miR-301a as an NF-kappaB activator in pancreatic cancer
426 cells. *EMBO J*. **30**, 57-67
- 427(11) Ho, S.C., Lee, K.Y., Chan, Y.F., Kuo, L.W., Ito, K., Adcock, I.M., Chen, B.C.,
428 Sheu, J.R., Lin, C.H., and Kuo, H.P. (2009) Neutrophil elastase represses IL-
429 8/CXCL8 synthesis in human airway smooth muscle cells through induction of
430 NF-kappa B repressing factor. *J Immunol*. **183**, 411-420
- 431(12) Niedick, I., Froese, N., Oumard, A., Mueller, P.P., Nourbakhsh, M., Hauser,
432 H., and Koster, M. (2004) Nucleolar localization and mobility analysis of the
433 NF-kappaB repressing factor NRF. *J Cell Sci*. **117**, 3447-3458
- 434(13) Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B.
435 (1999) A generic protein purification method for protein complex
436 characterization and proteome exploration. *Nat Biotechnol*. **17**, 1030-1032
- 437(14) Bartels, M., Schweda, A.T., Dreikhausen, U., Frank, R., Resch, K., Beil, W.,
438 and Nourbakhsh, M. (2007) Peptide-mediated disruption of NFkappaB/NRF
439 interaction inhibits IL-8 gene activation by IL-1 or Helicobacter pylori. *J*
440 *Immunol*. **179**, 7605-7613
- 441(15) Frank, R. (2002) The SPOT-synthesis technique. Synthetic peptide arrays on
442 membrane supports--principles and applications. *J Immunol Methods*. **267**,
443 13-26
- 444(16) Frank, R. (1992) Spot-synthesis: an easy technique for the positionally
445 addressable, parallel chemical synthesis on a membrane support
446 *Tetrahedron*. **48**, 9217-9232

447(17) Omnus, D.J., Mehrtens, S., Ritter, B., Resch, K., Yamada, M., Frank, R.,
448 Nourbakhsh, M., and Reboll, M.R. (2011) JKTBP1 is involved in stabilization
449 and IRES-dependent translation of NRF mRNAs by binding to 5' and 3'
450 untranslated regions. *J Mol Biol.* **407**, 492-504

451(18) Grilli, M., Chiu, J.J., and Lenardo, M.J. (1993) NF-kappa B and Rel:
452 participants in a multiform transcriptional regulatory system. *Int Rev Cytol.*
453 **143**, 1-62

454(19) Escalante, C.R., Shen, L., Thanos, D., and Aggarwal, A.K. (2002) Structure of
455 NF-kappaB p50/p65 heterodimer bound to the PRDII DNA element from the
456 interferon-beta promoter. *Structure.* **10**, 383-391

457(20) Schmitz, M.L., and Baeuerle, P.A. (1991) The p65 subunit is responsible for
458 the strong transcription activating potential of NF-kappa B. *EMBO J.* **10**, 3805-
459 3817

460(21) Schmitz, M.L., Mattioli, I., Buss, H., and Kracht, M. (2004) NF-kappaB: a
461 multifaceted transcription factor regulated at several levels. *Chembiochem.* **5**,
462 1348-1358

463(22) Hiroi, M., and Ohmori, Y. (2003) The transcriptional coactivator CREB-binding
464 protein cooperates with STAT1 and NF-kappa B for synergistic transcriptional
465 activation of the CXC ligand 9/monokine induced by interferon-gamma gene. *J*
466 *Biol Chem.* **278**, 651-660

467(23) Schmitz, M.L., Stelzer, G., Altmann, H., Meisterernst, M., and Baeuerle, P.A.
468 (1995) Interaction of the COOH-terminal transactivation domain of p65 NF-
469 kappa B with TATA-binding protein, transcription factor IIB, and coactivators. *J*
470 *Biol Chem.* **270**, 7219-7226

471(24) Chen, Y.Q., Ghosh, S., and Ghosh, G. (1998) A novel DNA recognition mode
472 by the NF-kappa B p65 homodimer. *Nat Struct Biol.* **5**, 67-73

473(25) Ghosh, G., van Duyne, G., Ghosh, S., and Sigler, P.B. (1995) Structure of NF-
474 kappa B p50 homodimer bound to a kappa B site. *Nature.* **373**, 303-310

475(26) Karin, M., Yamamoto, Y., and Wang, Q.M. (2004) The IKK NF-kappa B
476 system: a treasure trove for drug development. *Nat Rev Drug Discov.* **3**, 17-26

477(27) Tak, P.P., and Firestein, G.S. (2001) NF-kappaB: a key role in inflammatory
478 diseases. *J Clin Invest.* **107**, 7-11

479(28) Beutling, U., Stading, K., Stradal, T., and Frank, R. (2008) Large-scale
480 analysis of protein-protein interactions using cellulose-bound peptide arrays.
481 *Adv Biochem Eng Biotechnol.* **110**, 115-152

482(29) Dikmans, A., Beutling, U., Schmeisser, E., Thiele, S. and Frank, R. (2006)
483 SC2: A Novel Process for Manufacturing Multipurpose High-Density Chemical
484 Microarrays. *QSAR Comb. Sci.* **25**, 1069-1080

485(30) Sabe, H., Kondo, S., Shimizu, A., Tagaya, Y., Yodoi, J., Kobayashi, N.,
486 Hatanaka, M., Matsunami, N., Maeda, M., Noma, T., and et al. (1984)
487 Properties of human interleukin-2 receptors expressed on non-lymphoid cells
488 by cDNA transfection. *Mol Biol Med.* **2**, 379-396

489(31) Formosa, T., Barry, J., Alberts, B.M., and Greenblatt, J. (1991) Using protein
490 affinity chromatography to probe structure of protein machines. *Methods*
491 *Enzymol.* **208**, 24-45

492(32) Schrodinger, LLC. (2010) The PyMOL Molecular Graphics System, Version
493 0.99ed^eds.
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499**Figure Legends**

500

501 **Fig. 1.** NRF inhibits the transcriptional activity of p65 protein. A schematic diagram of
 502 the transfected vectors is shown at the top of diagram. pLexA-p65 contains an MPSV
 503 promoter (MPSV) as well as the coding sequences of the LexA DNA binding domain and the
 504 p65 full length protein. pL6NLuc encodes firefly luciferase as a reporter gene. Its expression
 505 is controlled by a LexA site, containing six LexA binding motifs, and a NRF binding site
 506 (NRE). Additionally, NRF encoding plasmids pNRF(1-380) and pNRF(1-690) were
 507 transfected. pRL-Tk served as an internal control comprising a thymidine kinase promoter
 508 (Tk) and the *Renilla* luciferase gene (*Renilla* Luc). HeLa cells were transfected with the
 509 vectors pLexA-p65, pL6NLuc, internal control vector pRL-Tk and pNRF(1-380) or pNRF(1-
 510 690), where indicated. Two days after transfection, the cells were harvested and the
 511 luciferase activities were determined. Firefly luciferase activities were compared to the
 512 corresponding *Renilla* luciferase activities to calculate the relative firefly luciferase activity,
 513 shown here as the mean + SD of three transfection experiments. The relative firefly
 514 luciferase activity of cells transfected with pL6NLuc and pLexA-p65 was set to 1.

515

516 **Fig. 2.** NRF binds to endogenous p65 but not to p50. (A) HeLa cells were transfected
 517 with equal amounts of expression plasmids encoding NRF1-690TAP, NRF1-380TAP,
 518 NRF1-149TAP or TAP tag alone. Two days after transfection, the cells were stimulated with
 519 IL-1 (10 ng/ml) for 3 hours where indicated. Cellular extracts were prepared, and the amount
 520 of p65 and p50 was detected by Western blot (Supplemental Fig. 1). Next, equal aliquots of
 521 the cellular extracts were incubated with IgG-agarose. After several washing steps, 10% of
 522 the bound fraction of the TAP fusion proteins was monitored by Western blot (Supplemental
 523 Fig. 1). Following TEV digestion and the subsequent second purification step, the amounts of
 524 p65 and p50 were monitored by Western blotting and marked as bound p65 and p50. (B)
 525 HeLa cells were transfected with equal amounts of expression plasmids encoding NRF1-

526380TAP, with either p65 or p65 RHD. TAP analysis and detection were performed as
527described in section (A). Western Blot analysis of total NRFTAP, total p65 proteins and
528bound p65 proteins is shown and proteins are indicated by arrows.

529

530 **Fig. 3.** Mapping of NRF binding sequence in p65 RHD. (A) The design of the array of
53115-mer peptides corresponding to the p65 RHD protein sequences is shown. The
532superscripted numbers in the amino acid sequences correspond to p65 amino acids. (B)
533NRF-Strep fusion protein was expressed in rabbit reticulocyte lysates (RRL) and was
534incubated with the peptide array. The membrane was washed three times, and the binding of
535NRF-Strep protein was detected by streptavidin and subsequent chemiluminescence
536reaction (NRF-Strep). The first spot of each lane is indicated at the left. As controls, the
537peptide array after incubation with streptavidin alone (control) and after incubation with RRL
538lacking NRF-Strep and streptavidin (RRL) is presented. Three peptide motifs were identified
539after incubation with NRF-Strep and are indicated by the frames. The amino acid boundaries
540of each motif corresponding to the amino acids of the p65 RHD protein sequence are
541indicated at the top and bottom of the array.

542

543 **Fig. 4.** Analysis of peptide motif 1 by a sizing approach. (A) The design of the array
544composed of peptides with varying sequence length is presented. The peptides are derived
545from p65 RHD amino acids 81-114. The superscripted numbers in amino acid sequences
546correspond to p65 RHD amino acids. (B) NRF-Strep protein was incubated with the peptide
547array and detected as described in the legend of Fig. 3B. Different peptide lengths are
548indicated by arrows on the membrane and on the right. Additionally, the first spot of each
549lane is indicated on the left.

550

551 **Fig. 5.** Peptide scan of motif 2. (A) The setup of the array of 15-mer peptides
552corresponding to p65 RHD amino acids 199 – 246 is shown. The superscripted numbers in
553amino acid sequences correspond to p65 RHD amino acids. (B) NRF-Strep protein was

554incubated with the peptide array and detected as described in the legend of Fig. 3B. The first
555spot of each lane is indicated on the left.

556

557 **Fig. 6.** Alanine scan of motif 3. (A) The design of the 15-mer peptide array
558corresponding to p65 RHD amino acids 274 – 306 is shown. The superscripted numbers in
559the amino acid sequences correspond to p65 amino acids. (B) NRF-Strep protein was
560incubated with the peptide array and detected as described in the legend of Fig. 3B. The first
561spot of each lane is indicated at the left.

562

563 **Fig. 7.** NRF interacting motifs in p65 RHD sequence and structure. (A) The
564comparison of the p65 RHD (NP_068810.3) and p50 RHD (NP_003989.2) sequences is
565shown. The alignment was performed using the blastp tool at <http://www.ncbi.nlm.nih.gov/>.
566Homologous amino acids are indicated with an asterisk. NRF binding motifs are highlighted
567in grey. Amino acid E279 is indicated by an arrow at the bottom. (B) The protein structure of
568the p65 RHD-p50 RHD complex bound to DNA (PDB ID: 2I9T) is presented using PyMol
569software . The three NRF binding motifs in p65 RHD are depicted and indicated by arrows.
570Amino acid E279 is highlighted in purple and indicated by an arrow. The N- and C-terminus
571of each protein is indicated as well.