c-Raf kinase binds to N-terminal domain of c-Myc

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Abstract We have demonstrated that the 50 N-terminal amino acids of c-Myc bind a kinase activity, which phosphorylates Myc in vitro predominantly on Thr[8]. We also have shown that c-Raf, a widely known Ser/Thr kinase, involved in the Ras signaling pathway, binds to the same portion of c-Myc in vitro. In addition we were able to precipitate native c-Myc/Raf complex from various cell lysates. Physical interaction of Myc and Raf may potentially be a part of their well-known functional cooperation.

Key words: c-Raf; c-Myc; Protein phosphorylation; Protein–protein interaction

1. Introduction

This work is a part of an ongoing effort to identify the proteins interacting with an oncoprotein c-Myc, which is a nuclear phosphoprotein acting as a transcription factor that was shown to play an important functional role in cell proliferation, differentiation, neoplasia and apoptosis [1–3]. c-Myc contains two major functional domains each of which interacts with several binding factors that can modulate Myc functional activity. The C-terminal domain interacts with Max, through its leucin–zipper and helix–loop–helix domains. Max/Myc heterodimers are required for modulating transcriptional activity of Myc [3]. The N-terminal domain of c-Myc both a transactivation and transformation domain and interacts with several proteins such as the retinoblastoma-related protein p107 [4], the transcriptional regulators Yin-Yang (YY-1) [5] and AP-2 [6], the TATA-binding protein (TBP) [7], TFII-I [8]. In addition, MAP kinase has been shown to bind and phosphorylates c-Myc in this region [9]. In our previous work, we showed, that c-Myc N-terminal region is able to bind to tubulin and to microtubules [10].

c-Raf is a Ser/Thr kinase which participates in an evolutionarily conserved cascade transducing signals from plasma membrane to the nucleus (reviewed in [11]). It plays a critical role in cell growth and differentiation and can cooperate in a cotransformation assay with c- and v-Myc. Raf induces the expression of immediate early genes (*fos* and *myc* among them). Its activity is regulated by multiple protein–protein interactions and phosphorylation events. Ras activates Raf, binding to it directly and recruiting it to the plasma membrane. Activated Raf is often associated with cytoskeletal elements [12]. c-Raf can phosphorylate and activate MAP kinase kinase that in turn activates MAP kinase, which is known to bind and phosphorylate c-Myc [13,14]. In addition, Raf associates with a large number of proteins, which may modulate its activity (e.g. 14-3-3 protein) or contribute to target recognition (e.g. MEK and c-jun).

Our data show that Raf binds to amino terminal end of c-Myc protein suggesting that physical interaction of the products of these two oncogenes may underlie their biological cooperation.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP was purchased from Amersham, USA. Thrombin was purchased from Sigma. Raf monoclonal antibodies were purchased from Transduction Laboratories Inc. Monoclonal c-Myc antibodies (Ab-1) were purchased from Oncogene Science. Recombinant GST fusion proteins were prepared by bacterial expression and glutathione agarose beads (Pharmacia) affinity chromatography as described [15]. Ultra pure myelin basic protein (MBP) was purchased from UBI.

2.2. Tissue cultures

CEM (T-lymphoid) and HL60 (myeloid leukemia) human cell lines were maintained in RPMI-1640 medium with 10% fetal calf serum (Gibco-BRL). All main experiments were performed using the lysates of each of these three cell lines and identical results were obtained.

2.3. Expression of GST–Myc fusion proteins

Construction of pGEX-based plasmids (Pharmacia), containing GST–Myc fusion proteins, bearing a unique thrombin cleavage site between Myc and GST moieties, have been described [10].

2.4. Binding assays

Cells were lysed in EBC binding buffer (1 mM EDTA, 0.5% Nonidet P-40, 120 mM NaCl, 10 μg/ml leupeptin, 1 mM PMSF, 50 mM Tris-HCl, pH 8.0, 1 mM NaF, 0.2 mM orthovanadate and 1 mM DTT), kept 20 min at 0°C and then centrifuged in a microfuge for 30 min at 4°C. Cell extract (200 μl, containing 100 μg of total protein) was incubated with GST fusion proteins prebound to 20 μl of glutathione-agarose beads as described [10]. After 60 min of incubation at 4°C the agarose beads were washed 5 times. The bound proteins were separated on SDS-PAGE.

2.5. In vitro protein kinase assays with proteins bound to GST beads

GST beads with immobilized fusion proteins were incubated with cell lysates and washed as above, then equilibrated with kinase buffer, containing 10 mM $MgCl_2$, 10 mM HEPES, pH 7.0. Kinase assays were performed in 50 μl of kinase buffer with 1 μCi [γ-^{32}P]ATP. The phosphorylation reactions were performed for 20 min at room temperature and terminated by washing the beads 3 times with EBC, and subsequent addition of Laemmli sample buffer. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography.

2.6. Phosphoamino acid analysis

Phosphoamino acid analysis was performed as described earlier [16] with slight modifications. Fusion protein on beads was phosphorylated in vitro with CEM cell lysate as a source of kinase activity as described above, and cleaved with thrombin according to the pro-

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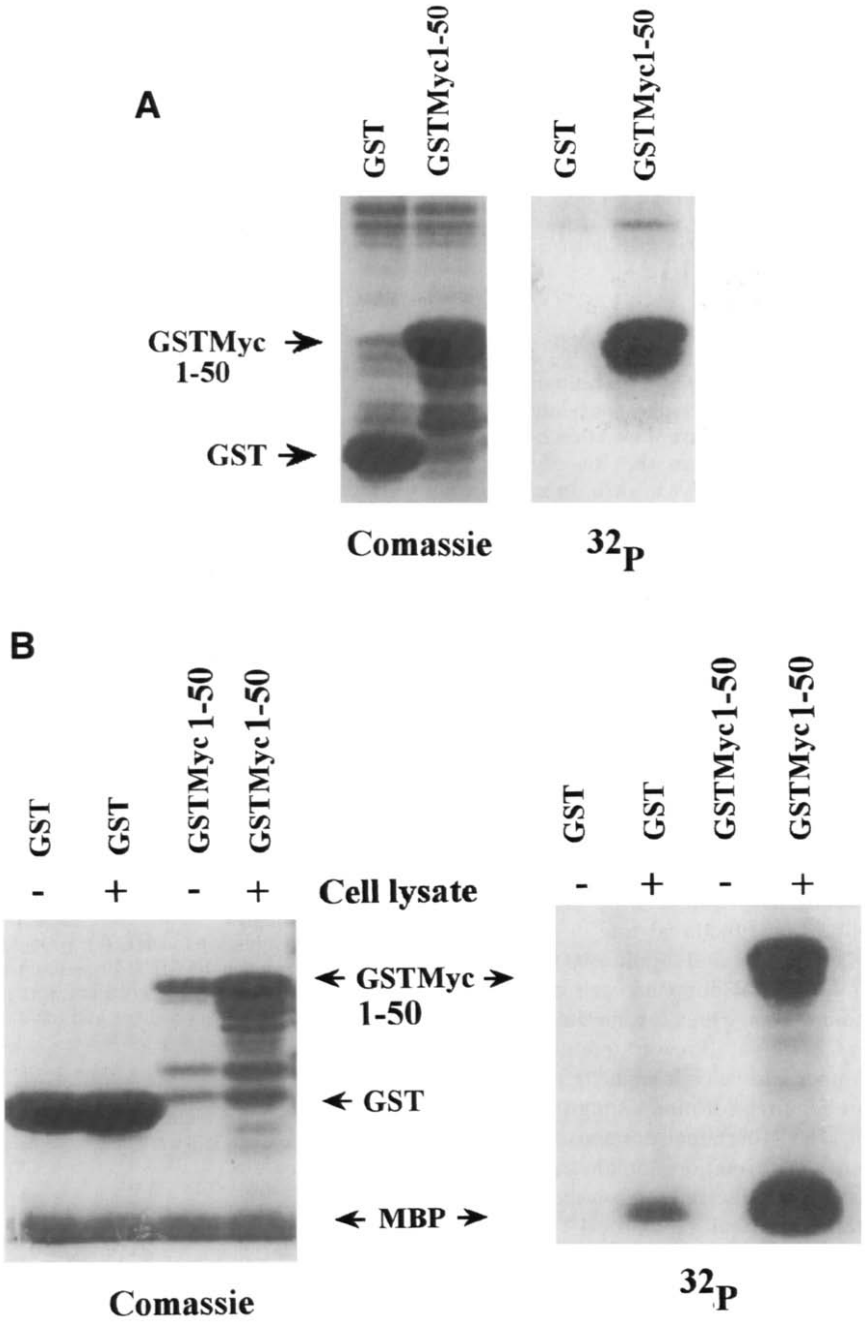

Fig. 1. Kinase activity binds to N-terminal part of c-Myc. A: Myc1-50 fusion protein or GST alone as a control were immobilized on the glutathione beads and incubated with CEM cell lysate. Kinase assay was performed in the presence of $MgCl_2$ and [γ-^{32}P]ATP. The phosphorylated proteins were examined by SDS-PAGE and subsequent autoradiography. B: Kinase assay was performed as above with an addition of 4 μg of purified MBP as an exogenous phosphorylation substrate.

cedure described in [17]. The cleaved product was purified on SDS-PAGE, eluted from the gel by incubation in 1% SDS at 67°C and acetone precipitated. Then it was resuspended in 6 N HCl (Pierce), hydrolysed for 4 h at 105°C, the acid was evaporated, hydrolysates were mixed with markers and electrophoresed on thin-layer cellulose plate (J.T. Baker) at 1100 V in 5% acetic acid, 0.5% pyridine. Markers were visualized by staining with ninhydrin solution (Sigma) and labeled phosphoamino acids by autoradiography.

2.7. Western blot analysis

After SDS-PAGE the separated proteins were transferred to Nitrocellulose membrane (Schleicher & Schull) and analyzed by Western blotting. The membrane were probed with monoclonal Ab-1 Myc antibodies or monoclonal Raf antibodies. Immune complexes were visualized using ECL procedure (Amersham) according to manufacturer's recommendations.

2.8. Immunoprecipitation

CEM cell lysate (1 mg/ml of total protein) prepared in EBC buffer with either NP-40 or Brij-96 was precipitated with c-Myc, c-Raf or unrelated antibodies, using agarose G beads (Oncogene Science) as recommended by manufacturer. Precipitated proteins were resolved by SDS-PAGE, transferred to the nitrocellulose filters and immunoblotted with c-Myc antibodies. Identical results were obtained with NP-40 and Brij lysates.

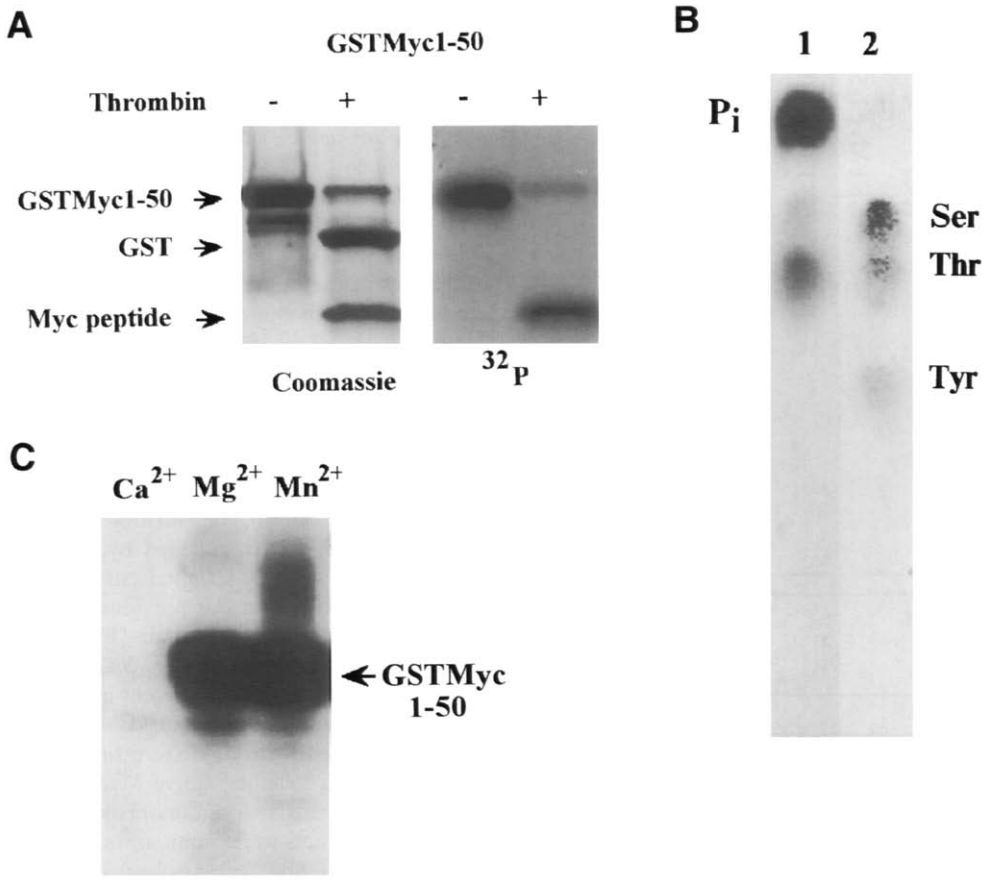

Fig. 2. Myc1-50 is phosphorylated by Mg^{2+}/Mn^{2+}-dependent Shr/Thr kinase. A: In vitro phosphorylated GSTMyc1-50 fusion protein was subjected to partial thrombin digestion, which cleaves off 1–50 peptide. Thrombin cleavage was done as in [11]. Electrophoresis was performed in SDS 8–20% gradient gel (BioRad). Myc1-50 peptide was excised and eluted from the gel and subjected to phosphoamino acid analysis. B: The purified peptide was hydrolyzed at 110°C in 6 N HCl. Hydrolysate was mixed with markers and phosphoamino acids were separated by electrophoresis on thin layer cellulose plates. Markers were visualized by ninhydrin staining and ^{32}P-labeled phosphoamino acids by autoradiography. C: In vitro kinase assay was performed in the buffer containing 10 mM HEPES, pH 7.0, and either of the following salts: 10 mM $CaCl_2$, $MgCl_2$ or $MnCl_2$.

3. Results

3.1. Protein kinase activity associates with N-terminal part of c-Myc protein

Different parts of c-Myc N-terminal exon II (amino acids 1–252) were expressed separately as glutathione-*S*-transferase (GST) fusion proteins and were designated as GSTMyc1-50, GSTMyc1-134, GSTMyc49-252, GSTMyc102-252 and GSTMycII, respectively ([10], see also Fig. 3B). Binding of protein kinases was investigated by incubation of c-Myc/GST proteins immobilized on the glutathione agarose beads with cell extracts prepared from cultured cells. After extensive washing the bound protein kinases were detected by addition of [γ-^{32}P]ATP in kinase buffer. As it is shown on Fig. 1A GSTMyc1-50, containing only the first 50 amino acids of c-Myc, was significantly phosphorylated in in vitro kinase assay, while GST was not phosphorylated. We surveyed the known sites of phosphorylation in the c-Myc molecule in order to find a candidate kinase, but none in this region were previously reported.

c-Myc protein is phosphorylated by casein kinase II at two different regions between residues 240–262 and 342–357 [18]. MAP kinase is able to bind to N-terminal part of c-Myc and to phosphorylate it on Thr^{58} and Ser^{62} [19]. Few other kinases were shown to phosphorylate the same two amino acids. GSTMyc1-50 contains neither of the above-mentioned sites nor any consensus sites for proline-dependent kinases. Thus, phosphorylation of this peptide suggested that another kinase may bind to the 1–50 amino acid region.

Control experiments were performed to show that an unidentified kinase indeed binds to the c-Myc peptide, but not to the GST sequence. (i) We added an excess of myelin basic protein (MBP) as an exogenous substrate in kinase assay. Proteins bound to GST produced very little phosphorylation, but proteins bound to Myc peptide strongly phosphorylated both MBP and the peptide itself (Fig. 1B). This experiment has shown that Myc peptide is not merely a substrate to some kinase, which binds to GST, but does not phosphorylate it. (ii) In vitro phosphorylated fusion protein was cleaved with thrombin right on the border between GST and Myc sequences. Myc peptide, but not the GST was phosphorylated (Fig. 2A).

Thus, we concluded that the N-terminal amino acids 1–50 of c-Myc are able to bind some unidentified kinase and contain in vitro phosphorylation site(s) for this kinase.

3.2. The Mg^{2+}/Mn^{2+}-dependent kinase phosphorylates c-Myc protein on Thr^{58}

We used the thrombin cleavage product obtained from in vitro labeled GSTMyc1-50 and purified by SDS-PAGE to

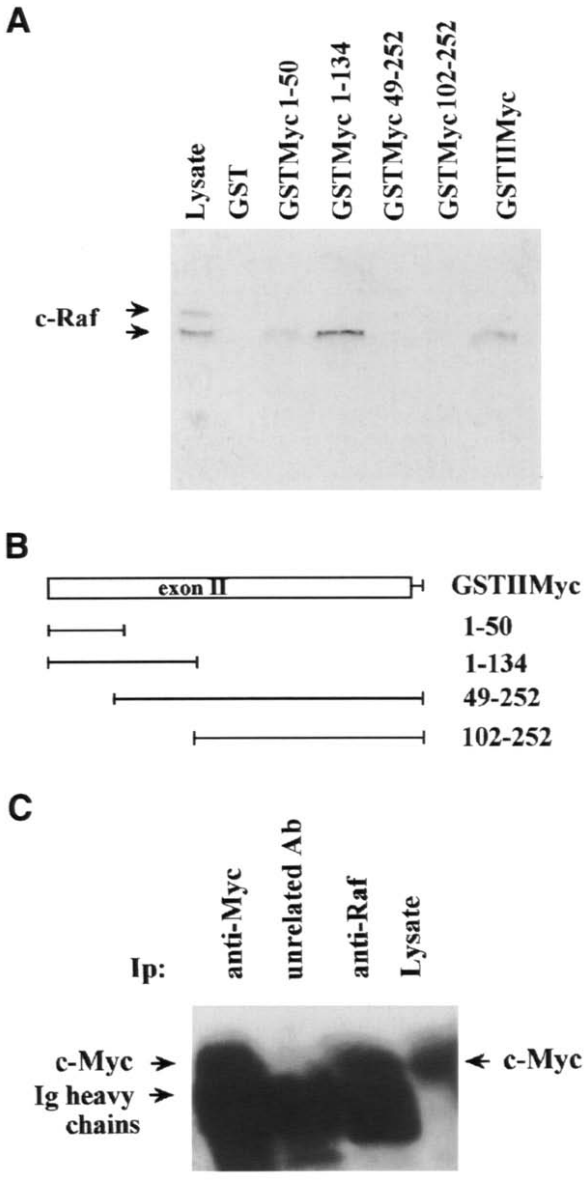

Fig. 3. Demonstration of complex formation between c-Myc and c-Raf. A,B: We used previously prepared series of MycGST fusion proteins with deletions to precipitate proteins from CEM cell lysates. Bound proteins were separated on SDS-PAGE gel and immunoblotted with c-Raf antibodies. First 50 amino acids of c-Myc are sufficient for binding to c-Raf. A: Schematic depiction of fusion proteins used. B: Immunoblot with c-Raf antibodies. C: Myc–Raf complexes were precipitated from CEM cell lysate with either Myc or Raf antibodies or unrelated antibodies as a control. Proteins were separated on SDS-PAGE and immunoblotted with c-Myc antibodies. It can be seen that c-Myc is precipitated by Raf and Myc antibodies, but not by unrelated antibodies.

perform phosphoamino acid analysis (Fig. 2B) as previously described [16]. Mostly Thr and much smaller amount of Ser appeared to be phosphorylated. No phosphotyrosine was observed. Thr^8 is the only threonine in the Myc1-50 region and therefore appeared to be the predominant site of phosphorylation. Consequently, the kinase, bound to N-terminal part of c-Myc protein belongs to a Ser/Thr kinase family.

To further characterize this enzyme, we performed kinase assays with different salts: $MgCl_2$, $MnCl_2$ and $CaCl_2$ (Fig. 2C). It appeared that the phosphorylation occurs in the presence of either Mg^{2+} or Mn^{2+}, but not in the presence of Ca^{2+}. This experiment showed, that the kinase is a Mg^{2+}/Mn^{2+} dependent one.

3.3. c-Raf kinase is able to bind to N-terminal part of c-Myc protein

In search for potential candidate kinases, we found that c-Raf, a Mg^{2+}/Mn^{2+} dependent Ser/Thr kinase is able to bind to GSTMyc II fusion protein. We used GSTMyc deletion mutants in the binding assay (Fig. 3A) to evaluate a number and location of binding sites. All fusion proteins were incubated with HL60 cell lysates and the bound proteins were separated by SDS-PAGE and immunoblotted with c-Raf antibodies. We observed that GSTMyc1-50 and GSTMyc1-134 bound to c-Raf, whereas GSTMyc49-252 and GSTMyc102-252 failed to bind. These results show that the N-terminal amino acids 1–50 of c-Myc are necessary and sufficient for Myc/Raf association, and no other binding sites are present in the part of c-Myc encoded by exon II. Therefore, c-Raf binds to the same N-terminal part of c-Myc protein, as the unidentified kinase in our previous experiments.

Notably, in HL60 cell lysates Raf antibodies reacted with two bands, p70 and p74 (Fig. 3A). It was shown previously [20] that the two bands represent hypo- (p70) and hyperphosphorylated (p74) forms of Raf. It can be seen that only hypophosphorylated form binds to c-Myc.

To find out whether native Myc and Raf proteins form a complex, we used co-immunoprecipitation assay: CEM and JD38 cell extracts were immunoprecipitated with Raf antibodies, the precipitate was resolved on SDS-PAGE gel, transferred to nitrocellulose filter and immunoblotted with Myc antibodies. Isotype matched unrelated antibodies were used as a control (Fig. 3C). It appeared that Raf immunoprecipitate contains c-Myc protein which showed that native Raf and Myc proteins may form a complex in vivo.

4. Discussion

We describe here the binding of c-Raf to c-Myc. It occurs within the N-terminal 50 amino acid region of Myc, which is necessary and sufficient for the interaction. Additionally we have shown that kinase activity with characteristics identical to that of Raf (Ser/Thr kinase, phosphorylating MBP, which can use either Mn^{2+} or Mg^{2+} as the only source of divalent cations) binds and phosphorylates this part of c-Myc predominantly on Thr^8. Whether this activity is indeed c-Raf remains to be investigated.

Although c-Myc is predominantly located in nucleus, while Raf is mainly cytoplasmic protein they may be co-localized under certain conditions. Activation of Raf kinase in response to transmembrane signals may lead to translocation of Raf to the nucleus or perinuclear space [21–24]. In HL60 cells a portion of Myc is localized in the cytoplasm [10]. In quiescent cells Myc is mainly cytoplasmic, and only after mitogenic stimulation it translocates to the nucleus [25]. Thus, the extent of Myc/Raf complex formation may depend on activation status of the cells.

Interestingly, we observed that c-Myc binds preferentially to hypophosphorylated form of c-Raf. It is not entirely clear how phosphorylation affects c-Raf activity. Although both activating and inhibiting phosphorylations may take place under different conditions, a general correlation between

phosphorylation of Raf and its activation has been observed [26]. For instance in quiescent cells Raf is usually hypophosphorylated and has very little, if any, kinase activity [27]. Mitogenic stimulation triggers hyperphosphorylation and induces Raf activity. Thus, our data suggest that the form of Raf which binds c-Myc is predominantly inactive or at least less active one. This observation does not favor a possibility of direct phosphorylation of c-Myc by Raf.

The Ser/Thr kinase, which binds to Myc1-50 peptide, detected in our experiments may represent some background kinase activity characteristic of hypophosphorylated form of Raf. Alternatively, despite preferential binding of hypophosphorylated form, a much lesser amount of hyperphosphorylated, more active Raf may also bind to fusion protein and contribute to the kinase activity. On the other hand, it may be yet another Ser/Thr kinase binding to c-Myc.

Importantly, both proteins Raf and Myc are regulated by phosphorylation and protein–protein interactions, which define their activity and compartmentalization. We have shown [10] that c-Myc is able to bind to tubulin and to microtubules. c-Raf is also known to be associated with cytoskeletal elements after recruitment by Ras to the cell membrane [12].

A number of individual proteins binding c-Myc and c-Raf have been discovered. MAP kinase binds and phosphorylates Myc in N-terminal region on Thr^{58} and Ser^{62}. Phosphorylation of these amino acids affects properties of Myc as a transcription factor [18,19]. Also the functionally important interactions of c-Myc with several proteins, including Max [28,29], the p107–Rb-related protein [4], TFII-I [8] and the TATA-binding protein [11] have been described. Raf also binds to several important proteins. Ras–Raf binding is the principal way of Raf activation [30,31]. Other proteins, interacting with Raf are: 14-3-3 protein [32], heat shock protein Hsp90 [33,34], PDGF receptor [35] and Bcl-2 [36]. Raf in its phosphorylated form binds to γ- and δ-chains of CD-3 (the component of T-cell receptor) [37]. It was also shown that Raf is able to phosphorylate nuclear oncoproteins such as p53 [38] and jun [39]. Phosphorylation of p53 leads to p53-dependent transcriptional transactivation. If, as our data suggest, Myc and Raf may form complexes in vivo, such binding could be an important link in the network of regulatory protein–protein interactions.

The results of our experiments suggest that the region containing first 50 amino acids of c-Myc was sufficient to allow binding to c-Raf. The region of Myc to which we have mapped Raf phosphorylation is crucial for Myc functioning. Amino acids 1–143 in c-Myc are required for cotransformation with ras in the rat embryo fibroblasts [40]. Amino acids 1–103 are required for the transcription regulatory activity of c-Myc [41]. The same region is crucial for Myc-dependent apoptosis [42].

It has been known for years that Raf and Myc are functionally linked: Raf can complement deregulated Myc in tumor induction in vivo [43], Raf is able to suppress Myc-mediated apoptosis and thus to cooperate with Myc in abrogation of growth factor dependence of tumor cells [44], also Myc and Raf cooperate in fibroblast cotransformation assay [45]. Traditionally the effect of Raf on Myc function was explained by activation of MAP kinase, which phosphorylates Myc. Our results allow to suppose that there may be a short-cut in this pathway and Rad can influence Myc in a more straightforward way through direct or indirect physical interaction. Whether this binding has any functional meaning will be a subject for further investigation.

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