Addition and correction: the NF-κB-like DNA binding activity observed in *Dictyostelium* nuclear extracts is due to the GBF transcription factor

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SUMMARY

We have previously reported that a NF-κB transduction pathway was likely to be present in the cellular slime mold *Dictyostelium discoideum*. This conclusion was based on several observations, including the detection of developmentally regulated DNA binding proteins in *Dictyostelium* nuclear extracts that bound to bona fide κB sequences. We have now performed additional experiments which demonstrate that the protein responsible for this NF-κB-like DNA binding activity is the *Dictyostelium* GBF (G box regulatory element binding factor) transcription factor. This result, along with the fact that no sequence with significant similarity to components of the mammalian NF-κB pathway can be found in *Dictyostelium* genome, now almost entirely sequenced, led us to reconsider our previous conclusion on the occurrence of a NF-κB signal transduction pathway in *Dictyostelium*.

Key words: *Dictyostelium discoideum*, GBF transcription factor, NF-κB

INTRODUCTION

The NF-κB transduction pathway initially found in mammalian cells (Sen and Baltimore, 1986), is involved in a variety of responses to environment changes (Bauerle and Henkel, 1994). It is composed of several elements including regulatory kinases (IKK1 and IKK2), so called 'inhibitors' (IκBα, IκBβ and IκBε) and transcription factors (p65, p50, p52, cRel, RelB). Homologous systems have now been described in *Drosophila* (Steward, 1987) and *Xenopus* (Kao and Hopwood, 1991). By contrast, no NF-κB pathway was found in *C. elegans* (Ruvkun and Hobert, 1998) or yeast (Epinat et al., 1997). In a recent report, we have described results suggesting the presence of an NF-κB transduction pathway in the cellular slime mold *Dictyostelium discoideum* (Traincard et al., 1999). Our evidence was based on several approaches. Using antibodies raised against several mammalian NF-κB proteins (p65, p50, p52, IκBβ, IKK1 and IKK2) we detected homologous proteins in *Dictyostelium* extracts by western blots and we showed that the *Dictyostelium* p65 and p50-like proteins were translocated into the nucleus upon development. In addition, gel retardation experiments performed with *Dictyostelium* nuclear extracts indicated the presence of NF-κB-like DNA binding proteins. For this, we used GCR, an oligonucleotide (GC-rich) derived from the promoter of *cbpA*, a developmentally regulated *Dictyostelium* gene that carries an NF-κB-like DNA sequence (Fig. 1) (Coukell et al., 1995). The demonstration that GCR as well as mammalian bona fide NF-κB DNA sequences such as Igκ (Fig. 1) could bind specifically to developmentally regulated *Dictyostelium* nuclear proteins was considered powerful evidence for the presence of NF-κB proteins in *Dictyostelium* (Traincard et al., 1999).

However, it later occurred to us that all of the oligonucleotides used in our study contained a G-rich region with the potential to bind to a well characterized developmentally regulated *Dictyostelium* transcription factor called GBF (for G box regulatory element binding factor) (Hjorth et al., 1990; Schnitzler et al., 1994). GBF binds to DNA sequences containing two copies of a G/T interspersed sequence (Fig. 1, GBF cons.), whose spacing and orientation is flexible (Hjorth et al., 1990; Schnitzler et al., 1994). As shown in Fig. 1, there is at least one copy of the GBF-like sequences present in both GCR and in the other oligonucleotides used in the gel shift experiments described previously (Traincard et al., 1999). This observation raised the possibility that the gel retardation observed was due to GBF itself rather than to NF-κB like proteins. Here we show that this is indeed the case, leading to a re-examination of the previous conclusion on the occurrence of a NF-κB signal transduction pathway in *Dictyostelium discoideum.*

RESULTS AND DISCUSSION

In the new gel shift experiments reported here we used several new oligonucleotides (Fig. 1). Car3 is an oligonucleotide derived from the GBF activated promoter of the *Dictyostelium car3* gene with a GBF binding site that contains a GT interspersed region (Gollop and Kimmel, 1997) instead of the poly-G region found in NF-κB target sequences. Its sequence strongly differs from that of the GCR oligonucleotide used in our previous experiments.

We first established that the Car3 oligonucleotide was unable

NF-κB cons.	5'-GGGPNNYYCC-3' CCCPNNYYGG
GCR	5'-TTTTTTA GGGGGGCACC CCTCTTTTTTTTTCG GCTTTAAAAAAT CCCCCCGTGG GGAGAAAAAA
Car 3	5'-AAAAAAGA ACACACACAT CGTTTTTTTTTCT TGTGTGTGTA AGTGTGTTATTTCTT-3'
Igκ	GACAGAG GGGACTTTCC GAGAGG GTCTC CCCTGAAAGG CTCTCCCT
GBF cons.	5'-TGGGTGGTG

Fig. 1. Sequences of oligonucleotides used in gel retardation experiments. Sequences of the GCR, Car3 and Igκ oligonucleotides as well as consensus sequences of NF-κB (NF-κB cons.) and GBF (GBF cons.) DNA binding sites are shown. The GBF binding sites are underlined. N is any nucleotide; Y is any pyrimidine; P is any purine.

to bind to a NF-κB transcription factor. For this we performed a gel shift experiment with recombinant NF-κB transcription factor p50, using labelled Car3 oligonucleotide, labelled GCR or Igκ oligonucleotides as controls. As shown in Fig. 2A, row 1, the mouse recombinant p50 does not recognize the Car3 oligonucleotide, in contrast to the GCR oligonucleotide and to the bona fide Igκ NF-κB oligonucleotide. We conclude that Car3 oligonucleotide can be used as a specific GBF probe.

Next we established that the labelled Car3 oligonucleotide was retarded by nuclear extracts from *Dictyostelium* cells that had developed for 16 hours (Fig. 2A, row 2). Since Schnitzler et al. demonstrated the presence of an active GBF factor in the cytoplasm of developing cells (Schnitzler et al., 1994), a similar experiment was also performed with cytoplasmic extracts from cells that had developed for 16 hours (Fig. 2A, row 3). The extracts were used in two parallel gel retardation experiments using labelled GCR and Igκ oligonucleotides as probes. The results shown in Fig. 2A demonstrate the presence of Car3 binding factors in both the cytoplasmic and nuclear extracts from 16-hour-developed cells. As shown in Fig. 2A (row 2,3) the retardation signal obtained with the Igκ oligonucleotide is weaker than with both GCR and Car3 oligonucleotides, while all three oligonucleotides were labelled with comparable specific activities. Moreover, a larger amount (threefold) of *Dictyostelium* extract was used in the experiment performed with Igκ than in the experiments performed with GCR and Car3, suggesting that the binding capacity of *Dictyostelium* proteins to Igκ is weaker than to GCR or Car3.

As shown in Fig. 2B, the labelled GCR and Car3 oligonucleotides were competed by non-labelled GCR and Car3 oligonucleotides in gel retardation experiments performed with extracts from nuclear (row 2) and cytoplasmic fraction (row 3), indicating that the two oligonucleotides are bound to the same site of the same protein(s). Since the Car3 oligonucleotide is a specific GBF probe, our data suggest that the protein responsible for the retardation of GCR in gel shift experiments is the protein GBF and not NF-κB-like transcription factors. This conclusion is further supported by the fact that Igκ oligonucleotide binding to cytoplasmic and nuclear *Dictyostelium* proteins is competed by non-labelled Igκ and Car3 oligonucleotides (Fig. 2B), as well as by the GCR oligonucleotide (data not shown; Traincard et al., 1999).

The identification of GBF as a *Dictyostelium* protein binding to NF-κB-like *Dictyostelium* sequences leads us to reconsider our previous conclusion on the presence of an NF-κB pathway in *Dictyostelium*. The lack of such a transduction pathway in *Dictyostelium* is also indicated by searches for NF-κB-like genes in sequence databases of *Dictyostelium* genes. Although a large fraction of the genome is now covered by the *Dictyostelium* genome project, not a single sequence with significant similarity to at least one of the proteins participating in the NF-κB pathway in mammals could be identified (data not shown). Finally, attempts to isolate NF-κB-like sequences by PCR or Southern blot (S. Mesnildrey, personal communication), using *Dictyostelium* DNA were also unsuccessful.

So how can we explain the presence of homologous proteins shown by western blots previosly (Traincard et al., 1999)? The most likely hypothesis is that they correspond to the presence of common epitopes on otherwise functionally unrelated proteins. We have investigated this hypothesis in the case of the *Dictyostelium* protein reacting with the anti-p52 antibodies in a western blot. For this, we identified a unique reacting spot by bi-dimensional electrophoresis of *Dictyostelium* proteins. This spot was eluted from the gel and digested by the protease Lys-C, before Edmann sequencing of one of the peptides obtained. The sequence obtained clearly identified the protein as an ATP synthase (β-chain), unrelated to any protein of the NF-κB familly (data not shown).

In conclusion, in contrast to our previous suggestion, the present available evidence is insufficient to support the proposal that an NF-κB signal transduction pathway is present in *Dictyostelium.*

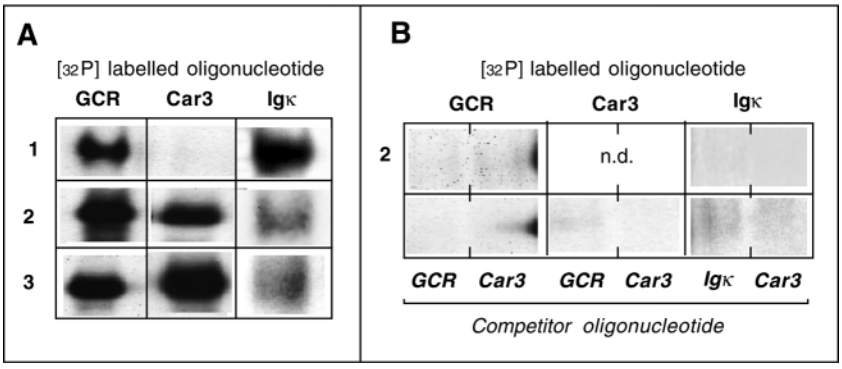

Fig. 2. Gel retardation of GCR and Car3 oligonucleotides by p50 and *Dictyostelium* cell extracts. Gel shift assays performed with [^{32}P] labelled GCR, Car3 and Igκ oligonucleotides in the presence of 100 ng of recombinant mouse p50 (row 1), 16-hour-developed *Dictyostelium* nuclear extracts (row 2) and cytoplasmic extracts (row 3). The experiments were performed in the absence (Fig. 2A) or in the presence (Fig. 2B) of a 25-fold excess of non-labelled GCR, Car3 or Igκ oligonucleotides used as competitors (italics). Gel shifts were performed as described previously (Traincard et al., 1999), except for experiments performed with Igκ as labelled probe in which threefold the amount of *Dictyostelium* extract was used. n.d., not done.

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