Distinct effects of cAMP and mitogenic signals on CREB-binding protein recruitment impart specificity to target gene activation via CREB

Bernhard M. Mayr, Gianluca Canettieri, and Marc R. Montminy*

Peptide Biology Laboratories, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037

Edited by Tony Hunter, The Salk Institute for Biological Studies, La Jolla, CA, and approved June 28, 2001 (received for review March 28, 2001)

Ser-133 phosphorylation of the cAMP-responsive element-binding protein (CREB) is sufficient to induce cellular gene expression in response to cAMP, but additional promoter-bound factors are required for target gene activation by CREB in response to mitogen/stress signals. To compare the relative effects of different signals on recruitment of the coactivator CREB-binding protein (CBP) to CREB in living cells, we developed a fluorescence resonance energy transfer (FRET) assay. cAMP promoted the interaction of CREB with CBP in a phosphorylation-dependent manner by FRET analysis, but mitogen/stress signals were far less effective in stimulating complex formation even though they induced comparable levels of Ser-133 phosphorylation. cAMP and non-cAMP stimuli were comparably active in promoting this interaction in the cytosol; the formation of CREB·CBP complexes in response to non-cAMP signals was specifically inhibited in the nucleus. Non-cAMP signals had no effect on intrinsic CREB- or CBP-binding activities by Far Western blot assay, thereby supporting the presence of a distinct CREB·CBP antagonist. Our studies indicate that the relative effects of cAMP and mitogen/stress signals on CREB·CBP complex formation impart selectivity to gene activation through CREB phosphorylated at Ser-133.

Adenosine 3′,5′-cyclic monophosphate (cAMP) stimulates the expression of numerous genes through the protein kinase A-mediated phosphorylation of the cAMP-responsive element-binding protein (CREB) at Ser-133 (1). Ser-133 phosphorylation, in turn, promotes assembly of the transcriptional apparatus through recruitment of the coactivator paralogs CREB-binding protein (CBP) and P300 (2, 3). In addition to its role as a cAMP-responsive activator, CREB appears to function importantly in growth factor- and stress-inducible gene expression (4). Coincident with this wide profile of inducibility, CREB is a substrate for a variety of cellular kinases including pp90rsk (5), protein kinase C (PKC), Akt (6), mitogen- and stress-activated protein kinase 1 (MSK-1) (7), mitogen-activated protein kinase-activated protein 2 (MAPKAP-2) kinase (8), and calcium/calmodulin-dependent protein kinases II (CaM KII) (9) and IV (10). Overexpression of CREB kinases in transfected cells promotes target gene expression through CREB in some but not all cases. Ser-133 phosphorylation of CREB by CaM KII, for example, fails to promote target gene activation because of secondary CaM KII-dependent phosphorylation of CREB at Ser-142 (9). Whether and to what extent Ser-142 in CREB is phosphorylated in response to external stimuli has not been determined, however.

Differences in CREB activity after treatment with cAMP vs. non-cAMP signals are apparent at the promoter level. For example, a single consensus cAMP-responsive element (CRE) is sufficient for target gene activation through CREB in response to cAMP and calcium signals (10, 11). But cellular gene activation in response to nerve growth factor (NGF) requires additional promoter-bound factors that synergize with CREB in a phosphoserine-133-dependent manner (12). In this regard, activation of the c-*fos* promoter by NGF requires the CRE plus a serum-responsive element that recognizes the ternary complex factor elk-1 and the serum response factor (12).

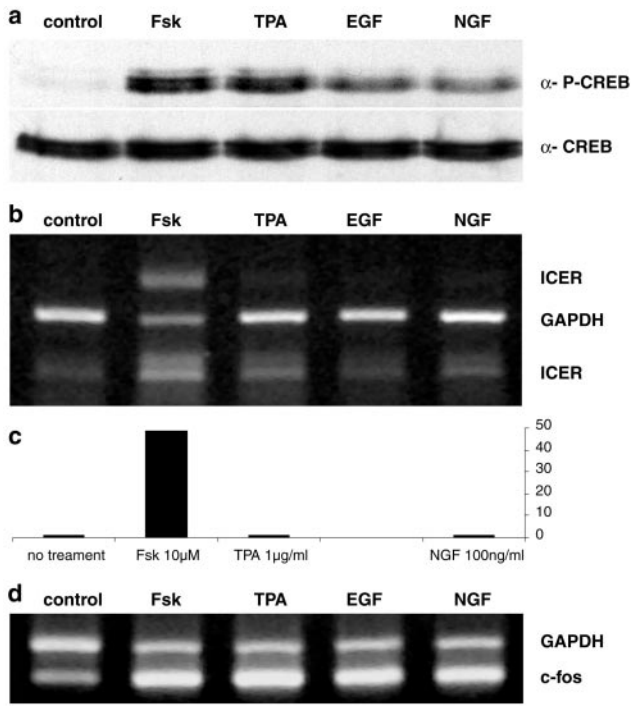

Fig. 1. Differential effects of cAMP and mitogen/stress signals on cellular gene expression. (*a*) Western blot analysis of PC12 whole-cell extracts with phospho-CREB and total CREB antisera after treatment for 15 min. (*b*) Quantitative RT-PCR of ICER mRNA with GAPDH as internal control after 1-h treatment. Alternative splicing of exons X and I of ICER results in four amplification products. (*c*) Fold-induction of ICER mRNA relative to GAPDH by TaqMan RT-PCR. (*d*) Quantitative RT-PCR of c-fos mRNA and GAPDH after 1-h treatment with various inducers.

The solution structure of the CREB·CBP complex, using relevant interaction domains referred to as KID and KIX, respectively, reveals that Ser-133 phosphorylation of CREB is both necessary and sufficient for complex formation (13). Phos-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: CREB, cAMP-responsive element-binding protein; CBP, CREB-binding protein; CaM KII, calcium/calmodulin-dependent protein kinase II; CRE, cAMP-responsive element; NGF, nerve growth factor; FRET, fluorescence resonance energy transfer; Fsk, forskolin; TPA, phorbol 12-tetradecanoate 13-acetate; EGF, epidermal growth factor; NLS, nuclear localization signal; GST, glutathione *S*-transferase; RT-PCR, reverse transcription PCR; GADPH, glyceraldehyde-3-phosphate dehydrogenase; ICER, inducible cAMP early repressor; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein.

*To whom reprint requests should be addressed. E-mail: montminy@salk.edu.

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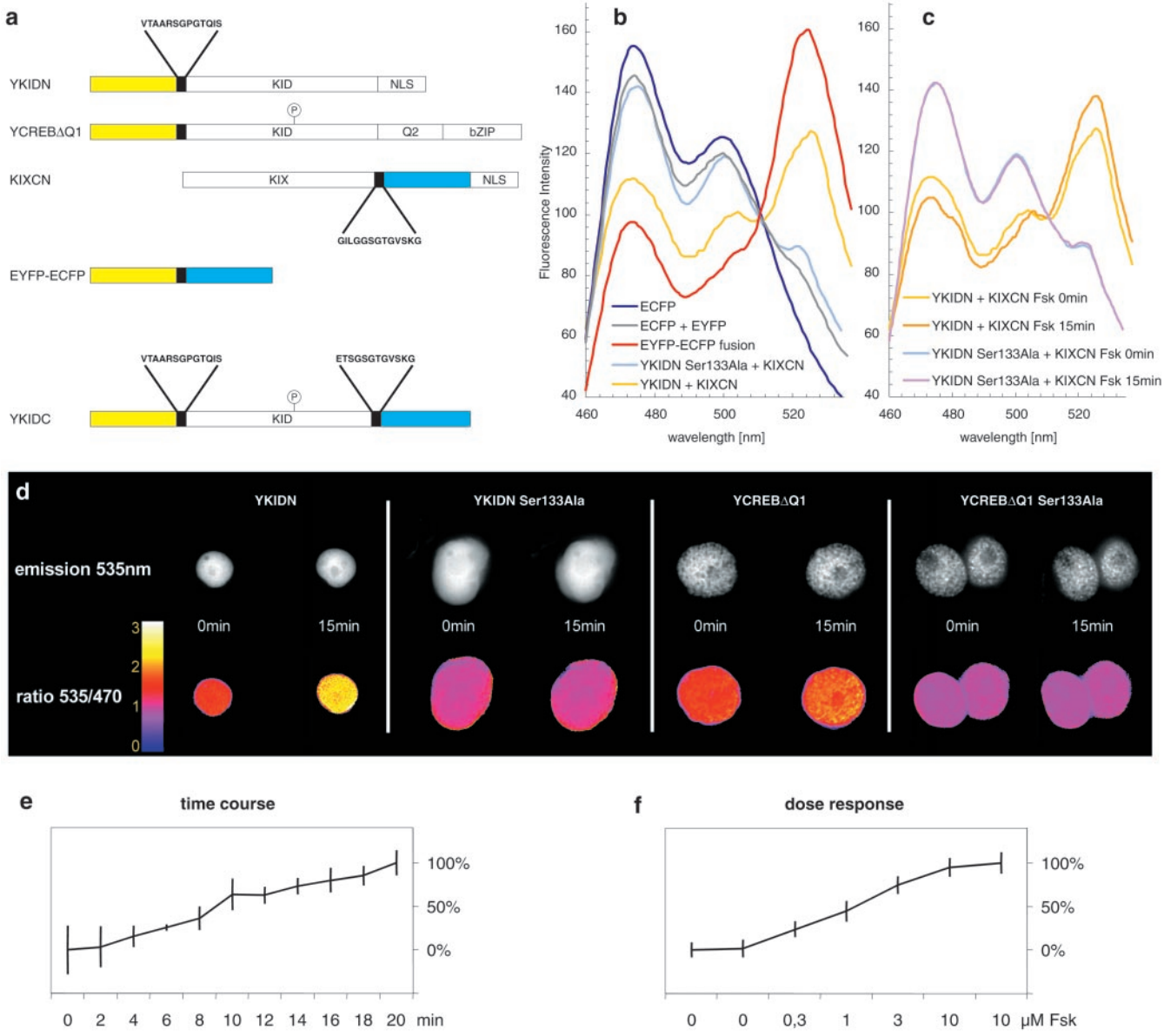

Fig. 2. Measurement of KID·KIX interaction by FRET in live cells. (*a*) Schematic representation of EYFP and ECFP fusion proteins used. YKID and KIXC are identical to YKIDN and KIXCN except for the lack of an NLS. bZIP, basic leucine zipper. (*b*) Emission spectra (excitation 430 nm) of living HEK293 cells transfected with YKIDN + KIXCN or control constructs that show either no FRET (ECFP, ECFP + EYFP, and YKIDN Ser133Ala + KIXCN) or very high FRET efficiency (EYFP-ECFP fusion). (*c*) Emission spectra of HEK293 cells transfected with YKIDN + KIXCN or YKIDN Ser133Ala + KIXCN and treated with Fsk. (*d*) Fluorescence images (*Upper*) and pseudocolor representation of the 535/470 emission ratio (*Lower*) of living HEK293 cells transfected with KIXCN and different EYFP-KID fusions before and after 15 min of Fsk treatment. An increase in the 535/470 ratio indicates formation of KID·KIX complexes. (*e* and *f*) Time course analysis (2-min intervals) in five HEK293 cells (*e*) and dose–response analysis in 14 cells (*f*) after treatment with Fsk. Average change in 535/470 ratio and the 95% confidence interval are shown.

phoserine-133 promotes this interaction by means of ion-pair and hydrogen-bond interactions with residues in KIX; the Ser-133 phosphate also stabilizes the formation of an amphipathic helix in KID that associates with a shallow hydrophobic groove in KIX (13). Notably, CREB Ser-142 projects into this hydrophobic groove in KIX, and secondary phosphorylation at Ser-142 *in vitro* blocks complex formation, suggesting a potential mechanism by which various signals may elicit different transcriptional responses (14).

Recent evidence supports the notion that cAMP and non-cAMP signals may differentially regulate formation of CREB·CBP complexes. Protein kinase C-dependent phosphorylation of CREB at Ser-133 failed to promote KID·KIX interaction either by mammalian two-hybrid assay (15) or by immunocytochemical analysis with a complex-specific antiserum directed against the KID·KIX complex (16). To monitor this important point of convergence for several major signal transduction cascades, we generated a fluorescence resonance energy transfer (FRET)-based method to visualize the interaction of KID and KIX in living cells. FRET occurs when a fluorophore is in close proximity (<100 Å) to a second fluorophore, whose excitation wavelength is similar to the emission wavelength of the first fluorophore. The result is a direct energy transfer from donor to acceptor with consecutive loss in donor emission and gain in acceptor emission (17).

By using the KID and KIX domains of CREB and CBP as interaction partners, we examine CREB activation in response to different stimuli, and we investigate the importance of Ser-142 in CREB for signal discrimination. Our studies reveal that

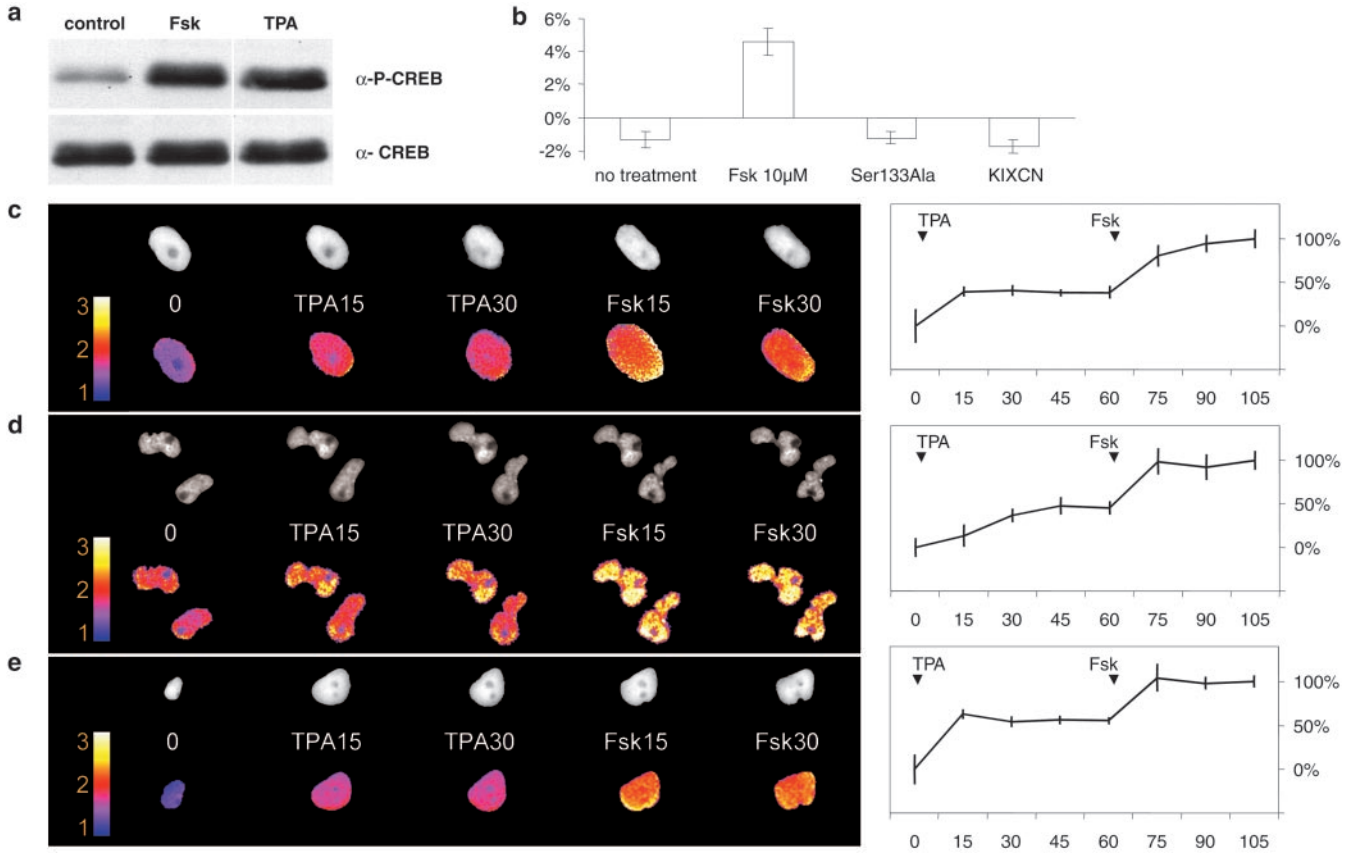

Fig. 3. Differential effects of cAMP and mitogen/stress stimuli on KID·KIX interaction in PC12 cells. (*a*) Western blot of whole cell extracts of PC12 cells transfected with YKIDN after treatment for 15 min. (*b*) FRET efficiency (±SEM) determined by photobleaching of PC12 cells transfected with KIXCN + YKIDN (±Fsk treatment), KIXCN + YKIDN Ser133Ala, and KIXCN only. (*c*–*e*) Fluorescence images and 535/470 ratio pseudocolor representation of living PC12 cells transfected with KIXCN plus YKIDN (*c*), KIXCN plus YCREBΔQ1 (*d*), or KIXCN plus YKIDN Ser142,143Ala (*e*). For *c*, *d*, and *e*, cells were treated sequentially with TPA (1 h), followed by Fsk (45 min). The numbers 15 and 30 indicate minutes after addition of TPA or Fsk. Graphs represent the average change of the 535/470 ratio and the 95% confidence interval of 10 cells as a function of time (min).

CREB·CBP complex formation is differentially regulated by cellular inputs, despite equal levels of Ser-133 phosphorylation. These differences in CBP recruitment impart selectivity to target gene activation through CREB.

Methods

Cell Cultures and Treatment. PC12 cells and HEK293 cells were maintained as previously described (18). Unless indicated, the final concentrations used for stimulation experiments were: 10 μM forskolin (Fsk), 1 μg/ml phorbol 12-tetradecanoate 13-acetate (TPA), 100 ng/ml epidermal growth factor (EGF), 100 ng/ml NGF.

Western Blotting and Far Western Blotting. Western blotting from PC12 whole cell extracts with phosphoserine-133-specific or nondiscriminating CREB antiserum was performed as previously described (18). To perform Far Western blotting, HEK293 cells were transfected with simian virus 40 nuclear localization signal (NLS)-tagged glutathione *S*-transferase (GST) KID or GST KIX, treated for 30 min, and lysed. The lysate was incubated with glutathione-Sepharose beads, washed, eluted, subjected to SDS/PAGE, blotted, and probed with either an alkaline phosphatase-KIX fusion protein or with a CREM-α protein radiolabeled with protein kinase A and [γ-^{32}P]ATP.

Reverse Transcription (RT)-PCR and TaqMan PCR. PC12 cells were treated as indicated for 1 h and processed by using the RNeasy Kit (Qiagen, Chatsworth, CA). RNA was reverse transcribed and amplified by using specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and for the inducible cAMP early repressor (ICER). For quantitative PCR, the TaqMan SYBR Green PCR reagents kit (Perkin–Elmer) was used. Samples were run in triplicate on an ABI Prism 7700 (Applied Biosystems) as above and analyzed as recommend by the manufacturer.

Construction of Fusion Proteins. Fragments of rCREB (KID: amino acids 100–160; CREBΔQ1: amino acids 100–341), rCREB mutants (KID Ser133Ala, KID Ser142,143Ala), and mCBP (KIX: amino acids 553–679) were amplified from previously published plasmids, and C- and N-terminal fusion proteins with enhanced yellow fluorescent protein (EYFP) or enhanced cyan fluorescent protein (ECFP) (amino acids 1–227) were generated by cloning into vectors derived from pECFP-N1 (CLONTECH). For nuclear targeted constructs, a simian virus 40 nuclear localization signal (NLS) was inserted at the C terminus; the CREBΔQ1 construct contains the NLS of CREB in the basic leucine zipper domain.

Spectrofluorometric FRET Measurement. HEK293 cells (10^6) were transfected for 24 h, resuspended in Hanks' balanced salt solution (GIBCO), placed in a glass cuvette, and kept in suspension with a stir bar. Emission spectra (450–550 nm) after excitation with 430-nm light were recorded on a spectrofluorometer (PTI, South Brunswick, NJ).

Cell Imaging/Deconvolution Microscopy. Cells were plated in a Delta T chamber (Bioptechs, Butler, PA) in normal growth

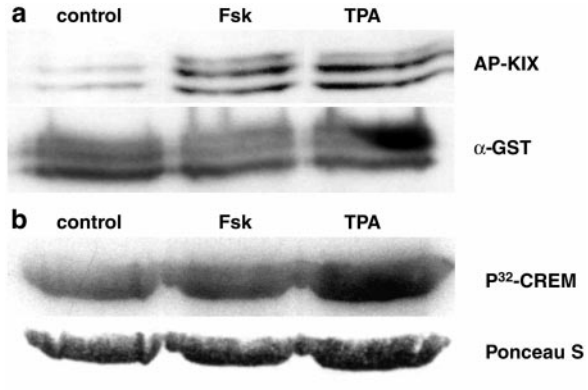

Fig. 4. Mitogen/stress signals do not alter intrinsic CBP or phosphoserine-133-CREB-binding activities. Far Western analysis of GST-KID and GST-KIX polypeptides recovered from transfected HEK293 cells treated for 30 min. (*a*) (*Upper*) CBP-binding activity of KID protein determined by Far Western blot with alkaline phosphatase-KIX (AP-KIX). (*Lower*) Western blots with anti-GST antisera (α-GST) to visualize total levels of GST KID protein. (*b*) (*Upper*) CREB-binding activity of KIX protein probed with *in vitro* ^{32}P-labeled α-CREM. (*Lower*) Ponceau S staining of the membrane to document comparable levels of GST-KIX expression.

medium and transfected by using Lipofectamine 2000 (GIBCO). After 8 h, medium was replaced with CO_2-independent (GIBCO) and cells were kept in a humidified chamber at 37°C and at ambient CO_2 levels. Images were collected by using a DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA) at 37°C. For every time point 3 images were taken with specific filter sets for ECFP (436 ± 5 nm excitation, 470 ± 20 nm emission), EYFP (500 ± 10 nm, 535 ± 15 nm) and FRET emission (436 ± 5 nm, 535 ± 15 nm).

Pixelwise calculation of the 535/470 ratio and pseudocolor representation was performed by using Corel PHOTOPAINT. Ratios of nuclear and cytoplasmic regions (535/470) were measured from the original images. FRET-efficiency was calculated as $1 - (CFP_{unbleached}/CFP_{bleached})$; i.e., the fraction of CFP emission transferred to YFP prior to 2 min photobleaching of YFP with a 525 ± 22.5 nm filter.

Results

To evaluate the correspondence between Ser-133 phosphorylation and target gene activation through CREB, we compared the effects of cAMP and non-cAMP stimuli (phorbol ester, NGF, EGF) in PC12 cells. All stimuli induced CREB Ser-133 phosphorylation after 15 min by Western blot assay (Fig. 1*a*). Induction of the cAMP-responsive *ICER* gene proceeds by way of four CREs in the promoter (19), whereas the c-*fos* promoter contains a serum-inducible element that synergizes with the CRE in response to various signals (12). Fsk induced the endogenous *ICER* gene 50-fold by quantitative TaqMan RT-PCR after 1 h; but non-cAMP signals had no effect on *ICER* expression, demonstrating that CREB Ser-133 phosphorylation per se is not sufficient for target gene activation (Fig. 1 *b* and *c*). By contrast, all treatments induced *c-fos* gene expression comparably in PC12 cells, demonstrating the importance of additional promoter-bound factors for CREB-dependent induction in response to non-cAMP stimuli (12) (Fig. 1*d*).

To determine whether cAMP and mitogen/stress stimuli have different effects on CREB·CBP complex formation, we developed a FRET-based detection system that uses the KID and KIX domains as interaction partners. The highest levels of FRET were observed when EYFP was fused to the N terminus of KID (YKIDN) and ECFP to the C terminus of KIX (KIXCN) (Fig. 2*a*), as revealed by a prominent emission peak at 527 nm in addition to normal ECFP emission peaks (Fig. 2*b*, yellow).

Consistent with enhanced formation of the KID·KIX complex, treatment with a cAMP agonist stimulated energy transfer between YKIDN and KIXCN constructs (Fig. 2*c*). No FRET was detected when a mutant Ser133Ala KID expression construct was used, however, demonstrating the importance of Ser-133 phosphorylation for this interaction (Fig. 2*c*). A significant difference between wild-type and Ser133Ala mutant KID constructs was noted even in the absence of cAMP agonist (Fig. 2*c*), reflecting high basal phosphorylation in HEK293 cells (not shown).

To visualize changes in KID·KIX complex formation with spatial and temporal resolution, we generated digital microscopic images of transfected cells and used pseudocoloring of the 535/470 ratio to document FRET changes over time (Fig. 2*d*). Interaction between nuclear YKIDN and KIXCN polypeptides was first detected after 4 min of treatment with Fsk and became maximal within 15–20 min (Fig. 2 *d* and *e*), corresponding to the time required for the protein kinase A catalytic subunit to enter the nucleus (18). The level of complex formation (and target gene activation) varied as a function of stimulus intensity from 0.3 μM to 10 μM Fsk, demonstrating the sensitivity of this pathway to external signals (Fig. 2*f*). Similar effects of Fsk on complex formation were also noted with an EYFP-CREBΔQ1 (YCREBΔQ1) construct containing the basic leucine zipper domain that targets CREB to chromatin (Fig. 2*d*).

To compare the relative potencies of different stimuli on KID·KIX complex formation, we performed FRET experiments on PC12 cells. Fsk and TPA induced equal levels of Ser-133 phosphorylation of the nuclear YKIDN polypeptide in transfected cells after 15 min (Fig. 3*a*). To calibrate FRET in absolute terms, we measured the gain in cyan fluorescent protein emission after selective yellow fluorescent protein photobleaching (Fig. 3*b*). Consistent with absence of KID·KIX complex formation under basal conditions, no energy transfer was detected in unstimulated PC12 cells (Fig. 3*b*). By contrast, treatment with a cAMP agonist promoted the KID·KIX interaction, yielding a FRET efficiency of 5% (Fig. 3*b*). TPA was much less effective in promoting KID·KIX complex formation than Fsk, however, whether TPA and Fsk were administered separately (not shown) or sequentially (Fig. 3*c*). Similar differences between cAMP and TPA on KID·KIX complex formation were noted with either free (YKIDN) or chromatin-bound (YCREBΔQ1) polypeptides, arguing against a DNA-dependent mechanism for discrimination (Fig. 3*d*). Other non-cAMP stimuli such as NGF and EGF were also unable to stimulate complex formation compared with cAMP (not shown), explaining at least in part the different promoter requirements for induction by these signals.

CaM KII-mediated phosphorylation of CREB at Ser-142 has been shown to block CREB·CBP complex formation *in vitro* and target gene activation *in vivo* (9), prompting us to examine the importance of this site in signal discrimination by mutagenesis of Ser-142 and the neighboring Ser-143 to Ala. The mutant Ser142,143Ala YKIDN polypeptide responded comparably to wild-type YKIDN after treatment with Fsk; but mutation of these residues did not abolish the difference in KID·KIX complex formation after treatment with TPA (Fig. 3*e*). These results indicate that modification of CREB at Ser-142 and Ser-143 does not account for signal discrimination via CREB.

To determine whether mitogen/stress signals prevent KID·KIX complex formation by introducing other inhibitory modifications on either KID or KIX, we performed Far Western blot analysis on GST-KID and GST-KIX fusion proteins after their over-expression and purification from HEK293 cells with glutathione-Sepharose beads. GST-KID was comparably phosphorylated in cells treated with either Fsk or TPA compared with control cells (not shown); the purified GST-KID polypeptides showed comparable levels of KIX binding activity in

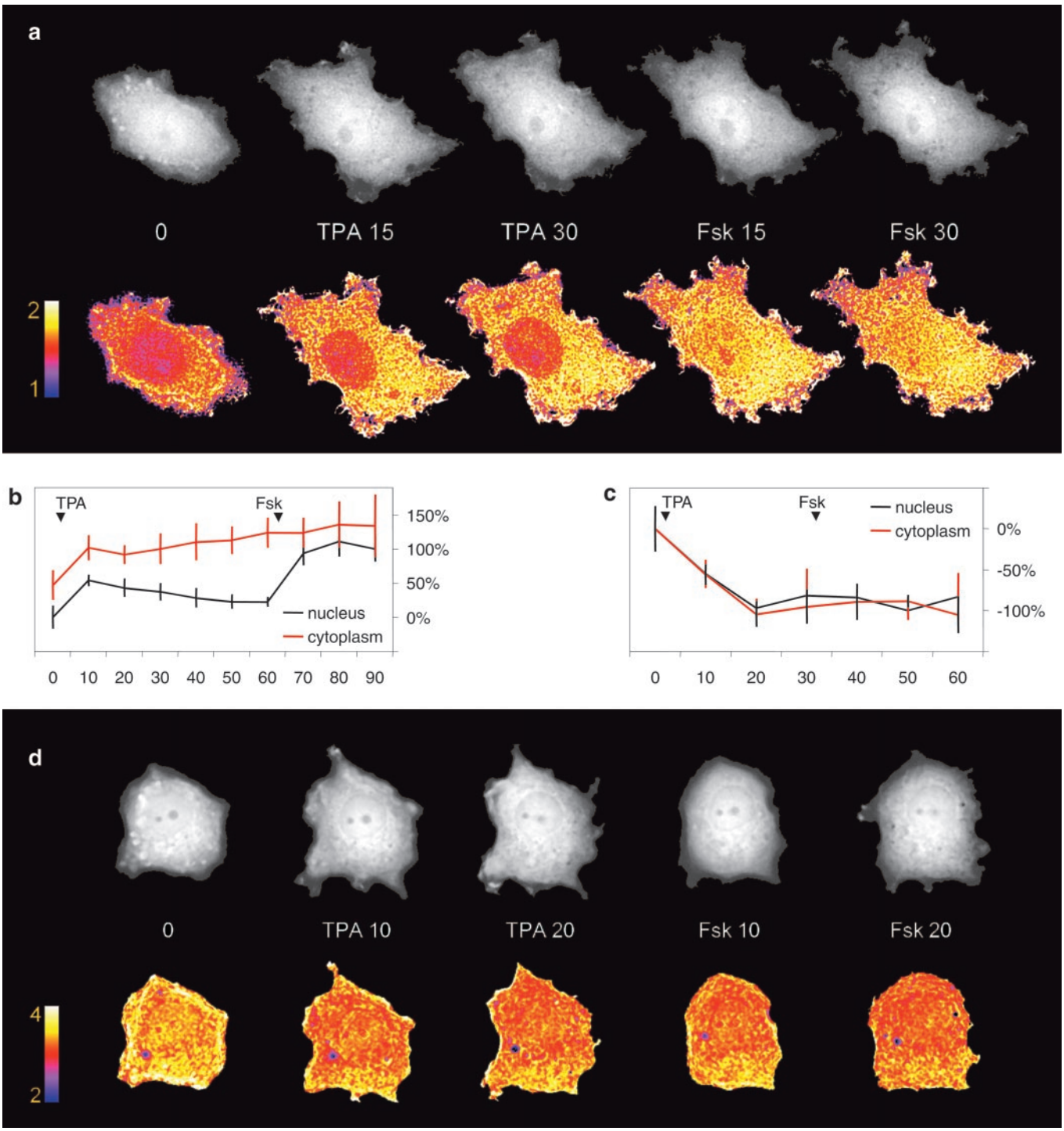

Fig. 5. Mitogen/stress stimuli induce formation of CREB·CBP complexes in cytoplasmic but not in nuclear compartments. (*a* and *b*) Comparison of nuclear and cytoplasmic KID·KIX interaction in PC12 cells in response to TPA and cAMP. (*a*) Fluorescence images and 535/470 ratio pseudocolor representation of a living PC12 cell transfected with KIXC and YKID expression vectors. Numbers indicate minutes after addition of TPA or Fsk. (*b*) Average nuclear and cytoplasmic change in 535/470 ratio and the 95% confidence interval of 10 cells as a function of time (min). (*c* and *d*) Comparison of nuclear and cytoplasmic KID phosphorylation in PC12 cells after treatment with TPA and cAMP. (*c*) Average nuclear and cytoplasmic change in 535/470 ratio and the 95% confidence interval of 7 cells. (*d*) Fluorescence images and 535/470 ratio pseudocolor representation of a living PC12 cell transfected with YKIDC.

response to either cAMP or TPA (Fig. 4*a*). Conversely, GST-KIX protein extracted from control-, cAMP-, or TPA-treated cells showed no difference in phospho-KID binding activity by Far Western blot assay with ^{32}P-labeled CREM-α protein (Fig. 4*b*). Similar results were obtained in solution-binding assays with overexpressed GST-KID and GST-KIX proteins (not shown), arguing against potential effects of denaturation in masking inhibitory modifications in either protein during Far Western blot analysis. Taken together, these results suggest that secondary covalent modifications of either CREB or CBP do not account for signal discrimination at the level of CREB·CBP complex formation.

Based on the exclusive localization of CREB and CBP in the nucleus, we examined whether the differential effects of cAMP and TPA on complex formation are also restricted to this compartment. To monitor KID·KIX complex formation in both the nucleus and the cytosol, we transfected cells with YKID and KIXC expression constructs lacking nuclear localization signals. Under these conditions, both KID and KIX constructs were diffusely distributed throughout the cell (Fig. 5*a*). After treatment with TPA, a nearly maximal response in FRET was noted throughout the cytoplasm, whereas only a low-level response was detected in the nucleus, suggesting that formation of the KID·KIX complex in response to TPA is blocked in the nucleus but not in the cytoplasm (Fig. 5 *a* and *b*). Indeed, subsequent treatment with a cAMP agonist promoted the KID·KIX interaction in the nucleus (Fig. 5 *a* and *b*). Levels of KIXC and YKID polypeptides in cytoplasm and nucleus remained constant in response to all treatments by Western blot analysis of fractionated cells (not shown).

To rule out Ser-133 phosphorylation differences between cAMP and non-cAMP signals, we studied FRET of a KID protein sandwiched between an EYFP and an ECFP (YKIDC, Fig. 2*a*). In previous studies, a similar construct has been shown to exhibit a decrease in FRET emission upon Ser-133 phosphorylation (20). After treatment with TPA, FRET emission from YKIDC decreased in the nucleus and the cytosol, but subsequent treatment with Fsk had no further effect (Fig. 5 *c* and *d*). These results indicate that KID is maximally phosphorylated in both compartments by TPA, and that cAMP does not promote KID·KIX complex formation in the nucleus because of higher levels of Ser-133 phosphorylation.

Taken together, these results support the notion that cellular discrimination between cAMP and mitogen/stress signals does not reflect differential phosphorylation of CREB. Rather, our results are consistent with a nuclear inhibitory activity that blocks CREB·CBP complex formation in cells exposed to mitogen/stress signals.

Discussion

This study was prompted by recent observations that signaling pathways differ in their ability to activate CRE-dependent transcription despite similar effects on Ser-133 phosphorylation (15, 21). The generation of a FRET-based technique to monitor the physical interaction between CREB and CBP allowed us to identify important characteristics of the underlying discriminatory signal. The discriminatory signal involves the KID·KIX interaction but does not involve direct modification of either KID or KIX. Moreover, the nature of this signal is inhibitory, but not dominant over cAMP. Finally, the inhibitory signal is found only in the nucleus and is eliminated by cAMP.

KID·KIX complex formation was not entirely blocked by TPA as might be anticipated from the almost complete block on CRE-dependent transcription. However, a total inability of CREB to bind CBP after protein kinase C or growth factor stimulation would be at variance with the well-documented requirement of phosphoserine-133-CREB for target gene activation via these pathways. Nevertheless, overexpression of the FRET constructs in our study might have outcompeted the putative inhibitor, leading us to underestimate the degree of CREB·CBP complex inhibition. Consistent with the latter notion, greater differences in complex formation between TPA- and cAMP-treated cells are apparent when lower levels of YKIDN and KIXCN are expressed (B.M. and M.M., unpublished observations).

Phosphorylation of CREB at Ser-142 has been proposed to act as a discriminatory signal for target gene activation (9). Indeed, the structure of the KID·KIX complex supports an inhibitory role for this phosphorylated residue in regulating complex formation (14). But recent studies with antisera specific for phosphorylated Ser-142 and Ser-143 indicate that these sites are not phosphorylated to a significant extent in the cell (M.M. and G.C., unpublished observations). More importantly, mutation of Ser-142 and Ser-143 to Ala did not abolish discrimination between cAMP and mitogen/stress signals at the level of the CREB·CBP complex, arguing against an important role for Ser-142 and Ser-143 phosphorylation in this process.

Our studies provide a mechanism to explain the differential activation of cellular genes by phosphoserine-133-CREB in response to various signals. Ser-133 phosphorylation of CREB in response to cAMP stimulation is sufficient to induce target gene expression through promoters containing only a CRE site. The reduced apparent affinity of phospho-CREB for CBP in cells exposed to mitogens/stressors prevents induction of cAMP-responsive genes containing only CRE promoter elements, and cellular genes containing additional promoter-bound factors may circumvent this block by cooperative interactions with phospho-CREB that permit efficient recruitment of CBP. These studies reveal an additional level of regulation subsequent to Ser-133 phosphorylation, which discriminates between cAMP- and mitogen-responsive genes and thus provides for activation of distinct genetic programs. Identification of the CREB·CBP inhibitor should provide new insights into how specificity in CREB signaling is achieved.

We thank David Chambers, Kelly Hardwicke, and Dave McDonald for helpful advice on live cell imaging and John Frangioni for sharing AP-KIX reagent. This work was supported by grants to B.M.M. from the Deutsche Forschungsgemeinschaft (MA 2205/1–1), to G.C. from Istituto Pasteur-Fondazione Cenci Bolognetti, and to M.R.M. from the National Institutes of Health (RO1-GM37828).

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