Specific inhibition of AGC protein kinases by antibodies against C-terminal epitopes

François Traincard*, Véronique Giacomoni, Michel Veron

Unité de Régulation Enzymatique des Activités Cellulaires, CNRS URA 2185, Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France

Abstract The sequences contributing to the catalytic site of protein kinases are not all comprised within the highly conserved catalytic core. Thus, in mammalian cAMP-dependent protein kinase (PKA), the C-terminal sequence participates in substrate binding. Using synthetic peptides mimicking the FxxF motif present at most C-termini of AGC kinases, we have raised highly specific antibodies which are potent and specific inhibitors of the catalytic activity of the cognate protein kinase. Taking into account the structure of PKA, these results point to the potential of the C-terminal region of protein kinases as a target for designing specific protein kinase inhibitors.
© 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Protein kinase; Inhibitor

1. Introduction

Protein phosphorylation by protein kinases serves as a universal molecular switch participating in the control of almost every cellular process [1]. Impaired function of protein kinases has been implicated in many diseases [2,3] and in the pathogenenicity of microorganisms and parasites [4,5]. Thus, intensive efforts are carried out in search for protein kinase specific inhibitors [6].

The high degree of sequence and structure conservation of the eukaryotic protein kinase catalytic core [7,8] makes it difficult to obtain inhibitory compounds able to discriminate between different kinases, in particular between isoforms within a single species or between counterparts of the same kinase in different species [9]. In contrast to the conserved 30-kDa catalytic core, the N- and C-terminal extensions of protein kinases greatly vary in size and sequence, encoding a large variety of domains implicated in activity regulation and in the binding to other proteins or nucleic acids [10].

The C-terminal extension of cAMP-dependent protein kinase catalytic subunit (PKA) participates in the structural changes involved in the transition from the open to the closed conformation of the protein [11], and is important for catalytic

activity since Tyr 330, located in the acidic cluster (D328–E334) of bovine PKA (Fig. 1A), is implicated in substrate binding [12]. In addition, the cleavage of bovine PKA between Glu 332 and Glu 333 leads to kinase inactivation [13], and a single Phe→Ala substitution in the $_{647}$FxxF-COOH motif lying at the extreme C-terminus of *Dictyostelium* PKA results in an important loss in enzyme activity and in decrease of protein stability [14].

Based on these observations, we have hypothesized that the C-terminal end of protein kinases could be a target for designing specific inhibitors. Here, we report the inhibitory properties of antibodies directed against peptides mimicking the last amino acids (AAs) of two different PKAs and of one cGMP-dependent protein kinase (PKG).

2. Materials and methods

2.1. Antibodies

Synthetic peptides corresponding to the sequences F126-Q137 and S637-F650 (peptide S) of *Dictyostelium discoideum* (*Dd*) PKA, and to A311-W325 of *Plasmodium falciparum* (*Pf*) PKA (Fig. 1) synthesized with a cysteine added at their C-terminus were coupled to BSA (FV, Boehringer Mannheim) by glutaraldehyde or 3-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) according to Schaaper et al. [15]. Rabbits were injected 3 times at one month intervals by subcutaneous injections of a mixture of 250 µg glutaraldehyde and 250 µg MBS-conjugated BSA-peptide, and 125 µg of free peptide in complete (first injection) or incomplete Freund's adjuvant. Final bleeding was performed after an additional booster injection in incomplete Freund's adjuvant 10 days before bleeding. *Dd* PKA peptides were coupled to Epoxy Activated Sepharose 6B in neutral or basic conditions according to the manufacturer's protocol (Pharmacia Biotech). Seric antibodies, eluted by glycine buffer, pH 2.3, were isolated by affinity chromatography in a column containing a 1/1 mix of neutral and basic pH peptide coupled resins. Antibodies against peptide S and against peptide F126-Q137 of *Dd* PKA are, respectively, called anti-*Dd* PKA-Ct and anti-*Dd* PKA-Nt antibodies. Crude rabbit anti-*Pf* PKA-Ct sera were used in the experiments. The antibodies against the C-terminus of human PKAα (anti-hu PKA-Ct) were from Santa-Cruz (PKAαcat (C20)), and those against the C-terminal peptide D657-F671 (peptide G) of human PKGIα (anti-PKG-Ct) or against PKCε (721–737) were from Calbiochem. Note that the C-terminal peptide sequences are identical in bovine and in human PKGIα.

2.2. Proteins and peptides

Recombinant *Dictyostelium* C309 PKA (des-(1–308)-C in [14]) and PKA from 16 h developed *Dd* cells were, respectively, obtained as described in [14,16]. Bovine PKAα was from Sigma and porcine PKAα was from ICN. Activated MAP Kinase ERK2 (rat), PKGIα (bovine) and PKCε (human) were from Calbiochem. Human PKAα C-terminal peptide was from Santa-Cruz and human PKGIα C-terminal peptide was from Calbiochem. Histone H2B was from Roche. The specific

---

* Corresponding author. Fax: +33-1-45-68-83-99.
E-mail address: traincar@pasteur.fr (F. Traincard).

A Acidic cluster

```
PKAα   mouse           309 KVEAPFIPKFKGPGDTSNFDDYEEEEIRVSINEKCGKEFTEF        I
PKGIα  bovine          634 TLTPPIIPSVASPTDTSNFDSFPEDNDEPPPDDNSGWDIDF        I
PKCε   human           680 KIKPPFKPRIKTKRDVNNFDQDFTREEPVLTLVDEAIVKQINQEEFKGFSYF*   II
RAC    D.melanogaster  535 KIPPPFKPQVTSDTDTRYFDKEFTGESVELTPPDPTGPLGSIAEEPLFPQF*   III
ßARK1  human           465 RYPPPLIPPRGEVNAADAFDIGSFDEEDTKGIKLLDSDQELYRNF*    IV
Sch9   S.cerevisiae    682 KIPPPFKPHLVSETDTSNFDPEFTTASTSYMNKHQPMMTATPLSPAMQAKFAGF*  V
S6K    human           341 KVEPPFKPLLQSEEDVSQFDSKFTRQTPVDSPDDSTLSESANQVFLGF*   VI
DBF2   S.cerevisiae    458 IADPINRLRSFEHVKRMSYFADINFSTLRSMIPPFTPQLDSETDAGYFDDF*   VII
ATPK5  A.thaliana      539 TPPEIPKPVELEKGAVSVAEAPSSQKTAAGLVLNAQKGSDNYLEFDFF     VIII
Sgk    rat             366 KITPPFNPNVSGPSDLRHFDPEFTEEPVPSSIGRSPDSILVTASVKEAAEAFLGF*  Other
```

B

```
PKAα   mouse          DDYEEEEIRVSINEKCGKEFTEF
PKAβ   mouse          ------------T------C--
PKAα   human          ------------------S--
PKAβ   human          ------D-----T---A---G--
PKAγ   human          -------L-I------A---S--
PKAα   porcine        ------------------S--
PKAβ   porcine        ------D-----T------C--
PKAα   bovine         ------------------S--
PKA    D.discoideum   EEMV--PPSSNYVDPYAHL-KD-
PKA    P.falciparum   ERVQ-DLTIADKITNENDP-YDW
PKA    P.yoelli       EKVQ-DLSIADKVINENDP-FDW
PKA    L.minor        YPDSPVDRTPALTSAQQA--NG-
PKA    C.albicans     YPE-QLDYGSQGEDPYASY-LD-
PKA    A.niger        YPE-T-QYGMAGEDPH-HL-PD-
PKA2   S.cerevisiae   YPE-DINYG-QGEDPYADL-RD-
```
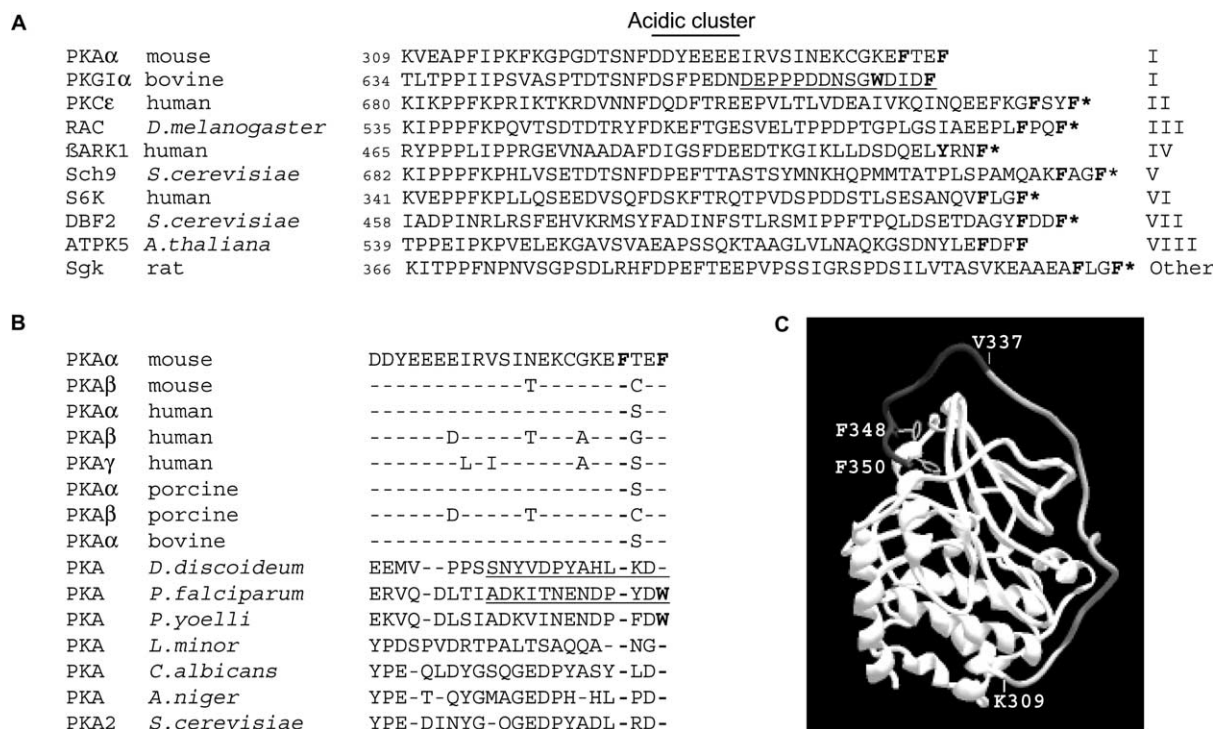
C

Fig. 1. The C-terminus of protein kinases. (A) Sequence alignment of AGC protein kinase C-terminal extensions. The sequence starts with the first residue (numbered at left) after helix αJ of the catalytic core [7]. *: Sequence continuation. Roman numbers at right of the sequences refer to the AGC sub-group of the corresponding protein. Aromatic residues of FxxF or WxxxF motifs are in bold. Sequences of the immunogen peptides are underlined. (B) Sequence alignment of the 23 last amino acids of the C-terminal extension of PKAs. Amino acids identical to mouse PKAα are indicated by (–). (C) Ribbon structure of mouse PKAα protein (1ATP). The C-terminal extension (starting at K310) is shown in light gray. The counterpart of the *Dd* PKA peptide used as immunogen (V337-F350) is shown in dark gray. Side chains of F347 and F350 are shown in light gray. Structure drawn with Swiss Pd. viewer 3.7. Abbreviations: PKC: Protein kinase C; RAC: proto-oncogenic protein kinase; βARK: β-Adrenergic Receptor Kinase; S6K: S6 kinase; DBF2: Cell cycle protein kinase; Sgk: Serum/glucocorticoid-regulated kinase.

activities of C309 PKA, porcine PKAα, and bovine PKGIα were, respectively, 40, 53, and 41 pmol ATP transferred to substrate peptide/min/μg of protein.

2.3. Protein kinase activity measurement

Dd PKA activity was measured in 10 μg *Dd* cell extracts by [^{32}P] incorporation into Kemptide for 30 min at 30 °C as described [16], using 0.05 M Tris–HCl buffer, pH 7.0, instead of MOPS, in the presence of 100 μM cAMP and 10 μg/ml BSA in the absence or presence of PKI (400 μM). Autophosphorylation of C309 (250 ng) was performed for 15 min at room temperature. PKA was then subjected to electrophoresis on a 10% polyacrylamide gel and the protein was transferred to a nitrocellulose membrane (Schleicher & Schuell, BA83) for autoradiography. Mammalian PKA activity was measured like *Dd* PKA activity except that 12 μM ATP was used. PKGIα activity was measured by [^{32}P] incorporation into Glasstide in 25 μl of 20 mM Tris–HCl buffer, pH 7.5, 10 mM Mg^{2+}-acetate, 10 mM NaF, 10 mM DTT, 0.2 mg/ml BSA, containing 200 μM [γ-^{32}P]ATP (200–500 cpm/pmol) and 100 μM Glasstide (Calbiochem), in the presence of 10 μM cGMP (30 min at 30 °C). For [^{32}P] phosphorylation of Histone H2B by PKGIα, 2 μg Histone H2B replaced Glasstide and incubation was performed at 30 °C for 1.5 h. Histone was then chromatographed on a 15% polyacrylamide gel and transferred to a nitrocellulose membrane for autoradiography.

2.4. ELISA and Western blot

Microtiter plates were coated with protein kinases or peptides at 0.5 or 1 μg/ml in phosphate-buffered saline (PBS). All washes were performed with 0.1% Tween 20 containing PBS (PBST) and antibody dilutions were done in 1% BSA containing PBST. Serial antibody dilutions were incubated in the wells (2 h at 37 °C) followed by peroxidase-labeled anti-rabbit IgG (H + L) goat antibodies diluted 1/2000 (Vector laboratories) (1 h at 37 °C), and finally by OPD/H$_2$0$_2$ substrate

solution before reading at 490 nm. Avidities of the antibodies were measured as described [17] in at least two different experiments.

Protein kinase samples (10 ng for testing with specific antibody or 1 μg for testing with heterologous antibody) were loaded onto 7.5% or 10% polyacrylamide gels and Western blotted as in [16] using anti-*Dd* PKA-Ct antibodies (500 ng/ml), anti-hu PKA-Ct antibodies (200 ng/ml) or anti-PKG-Ct antibodies (1 μg/ml).

3. Results

3.1. Specific anti-peptide antibodies against native protein kinases

Fig. 1A shows the alignment of sequences lying immediately downstream of the catalytic core of 10 kinases belonging to the 9 sub-groups of AGC protein kinases [7]. The conservation is low in this region, even between PKAs of pathogens and their human host (Fig. 1B). As shown in Fig. 1A and B, FxxF or WxxxF motifs (C-ter motif) are found in almost all sequences, although not always at their very C-terminal end.

We have used rabbit antibodies against synthetic peptides (Fig. 1) containing the C-ter motif of PKA from *Dd* and *Pf*, as well as from human PKA and PKGIα. The antibody reactivity was tested either in ELISA using synthetic peptides (sp) or in ELISA and Western blot (WB) using recombinant protein (P) (Fig. 2). Anti-*Dd* PKA-Ct antibodies did not bind peptides from human PKAα or from PKGIα in ELISA (Fig. 2A); they did not recognize porcine PKA and PKGIα proteins in WB contrary to *Dd* PKA (Fig. 2A, inset). The anti-*Dd* PKA-Ct
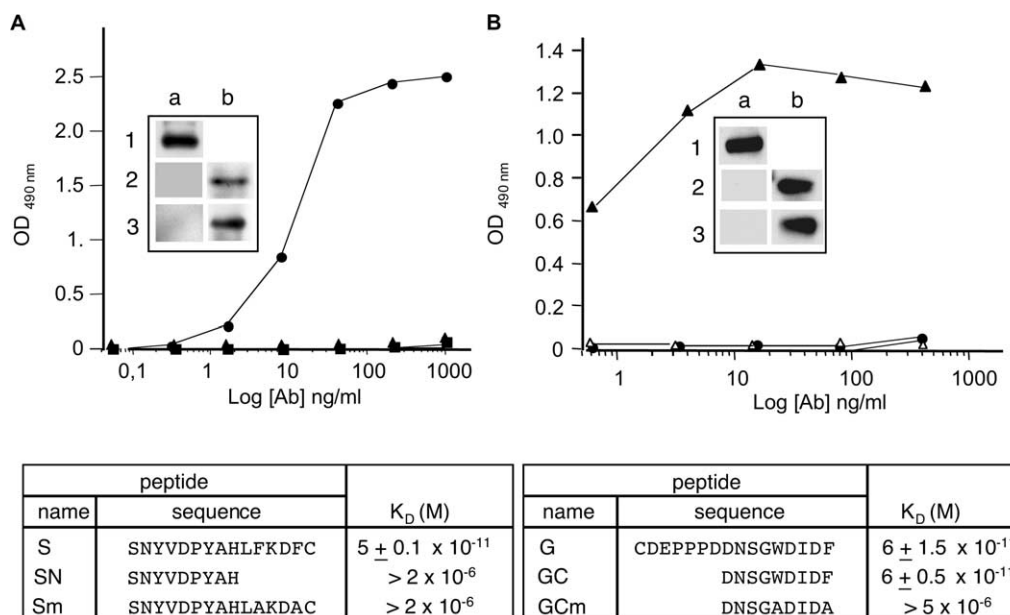
Fig. 2. Antibodies reactivity with protein kinases and peptides. (A) Anti-*Dd* PKA-Ct antibodies. ELISA of the anti-*Dd* PKA antibodies with *Dd PKA* (●), human PKAα (■), and PKGIα (▲) synthetic peptides. *Inset*: WB detection of *Dd* PKA (1), porcine PKA (2) and PKGIα (3) by anti-*Dd* PKA-Ct antibodies (a) and by antibodies directed against human PKA (2b) or PKGIα (3b). Table: Avidity of the anti-*Dd* PKA antibodies for the immunogen peptide S and for peptides SN and Sm. (B) Anti-PKG-Ct antibodies. ELISA of the anti-PKGIα antibodies with PKGIα (▲), *Dd* PKA (●) and PKCε (△) protein. *Inset*: WB detection of PKGIα (1), porcine PKA (2) and *Dd* PKA (3) by the anti-PKG-Ct antibodies (a) and by antibodies directed against human PKA (2b) or *Dd* PKA (3b). Table: Avidity of the anti-PKG antibodies for the immunogen peptide G and for peptides GC and GCm.
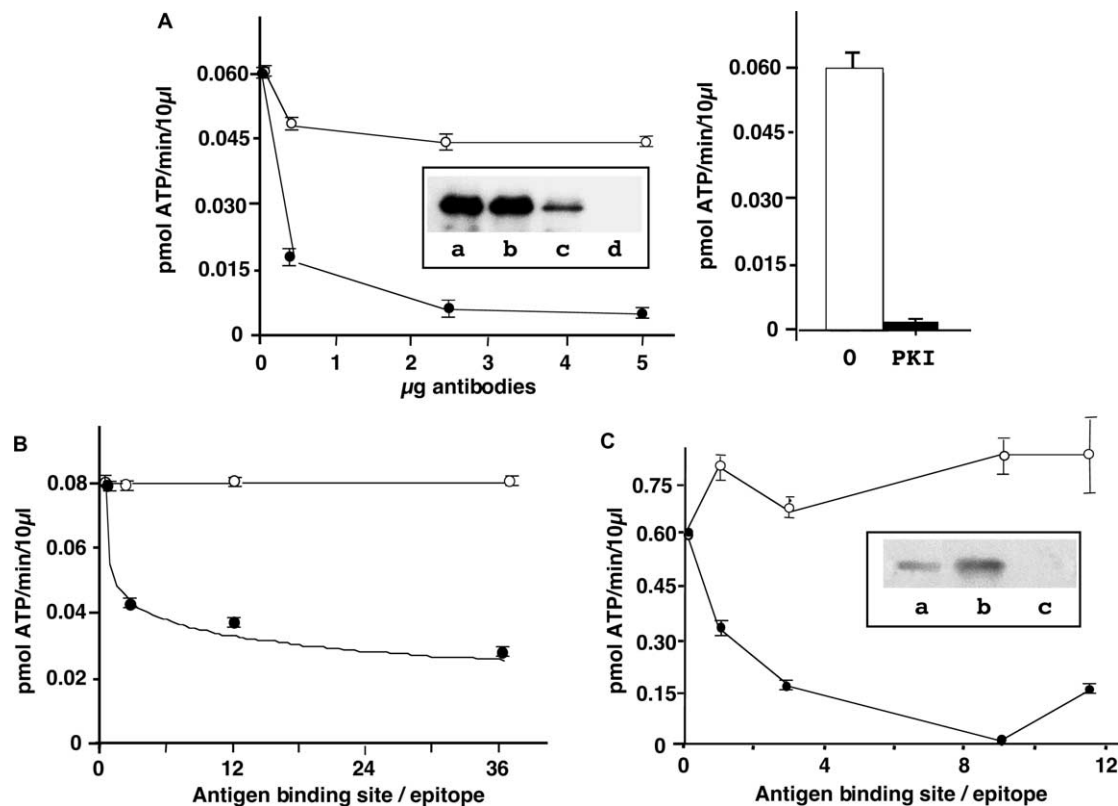

Fig. 3. Inhibition of protein kinase activity by C-ter antibodies. (A) *Dictyostelium* PKA. PKA activity of *Dd* extracts preincubated with anti-*Dd* PKA-Ct (●) or -Nt antibodies (○). *Histogram*: PKA activity in *Dd* extracts in the absence (white box) or in the presence of PKI (black box). *Inset*: Autophosphorylation of *Dd* PKA in the absence of antibody (a) or in the presence of a control antibody (b), anti-*Dd* PKA-Ct antibody (c) or PKI (d). (B) Porcine PKAα. Porcine PKAα activity after preincubation with anti-hu PKA-Ct (●) or control (○) antibodies. (C) Bovine PKGIα. PKGIα activity after preincubation with anti-PKG-Ct (●) or control (○) antibodies. *Inset*: Histone H2B phosphorylation by PKGIα alone (a) or by PKGIα preincubated with control (b) or anti-PKG-Ct (c) antibodies. The results presented were obtained in one of at least two experiments giving similar results. Kinase activities are expressed as pmol of ATP per min in 10 µl of reaction mixture.

antibodies did not recognize *Pf* PKA (sp), PKCε (P), Erk1 (sp), Erk2 (P), or yeast PKA2 (P), despite its extensive sequence similarity with *Dd* PKA (data not shown). Deletion of the C-terminal part of peptide S abolished anti-*Dd* PKA-Ct antibody binding to the corresponding SN peptide. Similarly, binding to the mutated peptide Sm was very poor ($K_D > 2 \times 10^{-6}$ M) as compared to peptide S ($K_D = 5.0 \pm 0.05 \times 10^{-11}$ M) (Fig. 2A, Table). The anti-PKG-Ct antibodies were reactive with recombinant PKGIα, but not with porcine PKA and PKCε in ELISA (Fig. 2B). In WB, they reacted with recombinant PKGIα but not with porcine PKA and *Dd* PKA (Fig. 2B, inset). These antibodies did not crossreact with *Pf* PKA (sp), Erk1 (sp), Erk2 (P) and yeast PKA2 (P) in ELISA or in WB (not shown). The anti-PKG-Ct antibodies bound the GC peptide with the same affinity as the immunogen peptide G, while the GCm peptide bound very poorly ($K_D > 2 \times 10^{-6}$ M) (Fig. 2B, Table). The anti-hu PKA-Ct and the anti-*Pf* PKA-Ct antibodies were reactive, respectively, with mammalian PKA and *Pf* PKA, but not with any of the other protein kinases or peptides tested (not shown).

In summary, antibodies against peptides from the C-terminus of PKA and PKG bind specifically the cognate kinases with high specificity, and aromatic amino acids from their conserved C-ter motif are strong epitope determinants.

3.2. Antibodies directed against the C-terminus of PKA and PKG inhibit their catalytic activity

As shown in Fig. 3A, pre-incubation of cellular *Dd* PKA with anti-*Dd* PKA-Ct antibodies, but not with anti-*Dd* PKA-Nt antibodies, resulted in a strong inhibition of PKA activity, which was dependent on antibody concentration. The phosphorylating activity in the extract was almost 100% inhibited by PKI, demonstrating that it was due to PKA (Fig. 3A, histogram). Using C309 recombinant protein, we obtained a 95% inhibition of Kemptide phosphorylation for a ratio of 2 antigen binding sites/epitope (not shown). *Dd* PKA autophosphorylation is strongly reduced in the presence of the C-ter antibodies (Fig. 3A, inset, lane c) but not by anti-*Dd* PKA-Nt antibodies (lane b). While the anti-hu PKA-Ct antibodies specifically inhibited porcine PKA activity by 65% (Fig. 3B), the anti-PKG-Ct antibodies inhibited totally and specifically the kinase activity for an antigen binding site/epitope ratio of 5 (Fig. 3C), as well as Histone 2B phosphorylation (Fig. 3C, inset). The inhibition of the kinases was completely reversed upon pre-incubation of the specific antibodies with their immunogen peptide (not shown).

4. Discussion

We have raised antibodies against synthetic peptides containing an FxxF or a WxxxF motif from protein kinases. The high specificity of these reagents is indicated by the fact that they were able to differentiate kinase counterparts belonging to different phyla like PKA from mammals and from *P. falciparum*. Despite the fact that they were obtained against synthetic peptides, the antibodies displayed good affinities for the native proteins ($2.5 \pm 0.1 \times 10^{-9}$ M for *Dd*-PKA, $3.0 \pm 1.5 \times 10^{-8}$ M for porcine PKAα, and $5.0 \pm 0.15 \times 10^{-9}$ M for PKGIα). They strongly inhibited *Dd* PKA, PKGIα, and mammalian PKAα activity as well as PKA autophosphorylation and Histone H2B phosphorylation by PKG (Fig. 3). The epitopes of the anti-*Dd*

PKA-Ct and of the anti-PKG-Ct antibodies are located in the C-terminal part of their immunogen peptides, since mutation of the aromatic residues of the C-ter motif strongly reduced antibody binding (Fig. 2, Tables).

The involvement of the Phe from the FxxF C-terminal motif of PKA in antibody binding provides a clue for their inhibitory effect. Indeed, the structure of mouse PKA shows that Phe 347 and Phe 350 are buried in the small lobe of the catalytic core with Phe 350 docked in a deep hydrophobic pocket ([12], Fig. 1C). We propose that binding of the antibodies to PKA or PKG results in movement of the terminal Phe residues out of their hydrophobic environment, leading to a destabilization of the interaction of the C-terminal domain with the catalytic domain and/or to a destabilization of the catalytic domain itself. An activating allosteric transition has been proposed for the AGC kinase PDK1 upon docking of a peptide into a similar hydrophobic pocket [18].

Several lines of evidence indicate that substrate-binding inhibition is not due to steric hindrance by the antibodies. Indeed, antibodies raised against peptide 721–737 containing the $_{728}$FSYF$_{731}$ C-ter motif from PKCε bound human PKCε with a high K_D ($6.0 \pm 1.0 \times 10^{-10}$ M), but did not inhibit its catalytic activity (not shown). Possibly, these antibodies did not inhibit the kinase because they did not bind the FSYF motif itself which is not at the C-terminus of the peptide, but surrounding regions. Regardless, they bound PKCε in a position very close to the one occupied by anti-PKA and PKGIα antibodies on their respective kinases without impeding substrate binding. In addition, the K_D of the anti-*Dd* PKA-Ct antibodies for *Dd* PKA is unchanged in the presence of mM concentrations of the substrate-like PKI peptide (not shown). Note that small peptides were used as PKA or PKG substrates (5 AA for Kemptide, 7 AA for Glasstide as compared to 16 AA for the substrate peptide of PKC and 20 AA for PKI). In conclusion, our results are in favor of an inhibition of the kinase catalytic activity by antibody-induced conformational changes.

We suggest that species-specific antibodies will be obtained against the C-ter motif sequence lying immediately downstream from the catalytic core in many other protein kinases. Such antibodies are likely to be potent phosphotransfer inhibitors in those kinases where the C-terminal extension is implicated in substrate binding, but possibly also when the C-terminal extension contributes to the specificity and activity regulation [19,20]. Finally, our results more generally underline the variable C-terminal extension of some protein kinases as a new target for designing specific inhibitors.

References

[1] Pawson, T. (1995) Nature 373, 573–580.
[2] Czech, M.P. and Corvera, S. (1999) J. Biol. Chem. 274, 1865–1868.
[3] Ni, Z., Lou, W., Leman, E.S. and Gao, A.C. (2000) Cancer Res. 60, 1225–1228.
[4] Kappes, B., Doerig, D.C. and Graeser, R. (1999) Parasitol. Today 15, 449–454.
[5] Syin, C., Parzy, D., Traincard, F., Boccaccio, I., Joshi, M.B., Lin, D.T., Yang, X.-M., Assemat, K., Doerig, C. and Langsley, G. (2001) Eur. J. Biochem. 268, 4842–4849.

[6] Cohen, P. (1999) Curr. Opin. Chem. Biol. 3, 459–465.

[7] Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science 241, 42–52.

[8] Taylor, S.S., Knighton, R., Zheng, J., Ten Eyck, L.F. and Sowadski, J.M. (1992) Annu. Rev. Cell Biol. 8, 429–462.

[9] Davies, S.P., Reddy, H., Caivano, M. and Cohen, P. (2000) Biochem. J. 351, 95–105.

[10] Shtivelman, E., Lifshitz, B., Gale, R.P., Roe, B.A. and Canaani, E. (1986) Cell 47, 277–284.

[11] Zheng, J., Knighton, D.R., Xuong, N.-H., Taylor, S.S., Sowadski, J.M. and Ten Eyck, L.F. (1993) Prot. Sci. 2, 1559–1573.

[12] Chestukhin, A., Litovchick, L., Schourov, D., Cox, S., Taylor, S.S. and Shaltiel, S. (1996) J. Biol. Chem. 271, 10175–10182.

[13] Chestukhin, A., Litovchick, L., Muradov, K., Batkin, S., Taylor, M. and Shaltiel, S. (1997) J. Biol. Chem. 272, 3153–3160.

[14] Etchebehere, L.C., Van Bemmelen, M.X.P., Anjard, C., Traincard, F., Assemat, K., Reymond, C. and Véron, M. (1997) Eur. J. Biochem. 248, 820–826.

[15] Schaaper, W.M.M., Lankhop, H., Puijk, W.C. and Meloen, R.H. (1989) Mol. Immunol. 26, 81–85.

[16] Dammann, H., Traincard, F., Anjard, C., Van Bemmelen, M.X.P., Reymond, C. and Véron, M. (1998) Mech. Dev. 72, 149–157.

[17] Friguet, B., Chafotte, A., Djavani-Ohaniance, C. and Goldberg, M. (1985) J. Immunol. Methods 77, 305–309.

[18] Biondi, R.M., Komander, D., Thomas, C.C., Lizcano, J.M., Deak, M., Alessi, D.R. and van Aalten, D.M.F. (2002) EMBO J. 21, 4219–4228.

[19] Seifert, M.H.J., Breitenlechner, C.B., Bossemeyer, D., Huber, R., Holak, T.A. and Engh, R.A. (2002) Biochemistry 41, 5968–5977.

[20] Biondi, R.M. and Nebreda, A.N. (2003) Biochem. J. 372, 1–13.