Identification of integrin-stimulated sites of serine phosphorylation in FRNK, the separately expressed C-terminal domain of focal adhesion kinase: a potential role for protein kinase A

Alan RICHARDSON*, John D. SHANNON‡, Reid B. ADAMS*†, Michael D. SCHALLER*§ and J. Thomas PARSONS*¶

Department of *Microbiology, †Surgery and ‡Biomolecular Research Facility, University of Virginia, Charlottesville, VA 22908, U.S.A.

Focal adhesion kinase (pp125^{FAK}) is a protein tyrosine kinase that is localized to focal adhesions in many cell types and which undergoes tyrosine phosphorylation after integrin binding to extracellular matrix. In some cells the C-terminal non-catalytic domain of pp125^{FAK} is expressed as a separate protein referred to as FRNK (FAK-related, non-kinase). We have previously shown that overexpression of FRNK inhibits tyrosine phosphorylation of pp125^{FAK} and its substrates as well as inhibiting cell spreading on fibronectin. In this report we identify Ser¹⁴⁸ and Ser¹⁵¹ as residues in FRNK that are phosphorylated after tyrosine phosphorylation of pp125^{FAK} and in response to integrin binding to

fibronectin. Tyrosine phosphorylation of pp125^{FAK} appears to be an early event after integrin occupancy, and serine phosphorylation of FRNK occurs significantly later. Treatment of fibroblasts with a series of protein kinase A inhibitors delayed serine phosphorylation of FRNK as well as cell spreading on fibronectin and tyrosine phosphorylation of pp125^{FAK}. However, these PKA inhibitors are unlikely to delay cell spreading simply by preventing serine phosphorylation of FRNK, as overexpression of FRNK containing mutations of Ser¹⁴⁸ and Ser¹⁵¹ either singly or jointly to either alanine or glutamate residues did not significantly alter the ability of FRNK to act as an inhibitor of pp125^{FAK}.

INTRODUCTION

Focal adhesion kinase (pp125^{FAK}) was originally identified as a protein that exhibited increased tyrosine phosphorylation in cells transformed by the oncogenic tyrosine kinase v-Src [1]. Although the role of pp125^{FAK} in transformation by v-src is at present unclear, recent evidence indicates that it is likely to play a role in integrin-mediated adhesion and signalling (reviewed in [2,3]). The integrins are cell surface receptors for extracellular matrix (ECM) proteins such as fibronectin, vitronectin, laminin and collagen [4]. Integrin-dependent cell adhesion and spreading on the ECM involves the formation of focal adhesions, which are specialized regions of close cell–substrate contact formed by clustering of integrins [5]. pp125^{FAK} is localized within these focal adhesions [6]. Binding of the ECM to integrins stimulates pp125^{FAK} to undergo phosphorylation on Tyr³⁹⁷ [7], which in turn serves as a binding site for the SH2 domains of Src and Src-family tyrosine kinases [7–10]. Recent evidence indicates that pp60^{src} on associating with pp125^{FAK} may further phosphorylate and activate pp125^{FAK} [11]. This bipartite signalling complex may be responsible for the activation of multiple signal-transduction systems [2,3], providing a potential mechanism for the adhesion-dependent regulation of gene expression [12]. As shown recently, the ECM acts as a survival factor, providing a signal(s) that prevents the cell from committing to programmed cell death [13,14]. Thus pp125^{FAK} may have a role in abrogating apoptosis [15].

pp125^{FAK} is a structurally distinct kinase [6,16]. The catalytic domain is centrally located between similarly sized N-terminal and C-terminal non-catalytic domains. Both the N- and C-terminal domains appear to play a role in integrin signalling. The N-terminal domain is proposed to interact with integrin cytoplasmic domains, and may provide a direct link through which pp125^{FAK} is activated on integrin clustering [17]. In addition, the

C-terminal domain contains several regions that appear to regulate pp125^{FAK} activity. The focal-adhesion-targeting sequence is required for targeting pp125^{FAK} to focal adhesions, indicating that this sequence interacts with some as yet unidentified focal adhesion protein(s) [18]. The C-terminal domain also binds the focal adhesion protein paxillin (a candidate substrate of the pp125^{FAK}–pp60^{src} kinase complex [19,20]), the SH3 domain of GRAF (a small G-protein GTPase-activating protein [21]) and the SH3 domain of the adapter molecule p130^{cas} [22]. Finally, the C-terminus contains potential binding sites for the SH2 domains of the cell signalling molecules Grb2 and phosphatidylinositol 3-kinase. Grb2 has been shown to co-immunoprecipitate with pp125^{FAK} in an adhesion-dependent manner in mouse fibroblasts [9], but phosphatidylinositol 3-kinase association with pp125^{FAK} appears to be independent of adhesion [23]. Thus the C-terminus of pp125^{FAK} is a multifunctional domain that may recruit numerous signalling proteins.

In some cells, the C-terminal domain of pp125^{FAK} is expressed independently of pp125^{FAK} [24]. Since the C-terminal domain is apparently catalytically inert, this shortened form of pp125^{FAK} is termed pp41/43^{FRNK} (FAK-related, non-kinase). We have previously shown that overexpression of pp41/43^{FRNK} inhibited tyrosine phosphorylation of pp125^{FAK} and delayed cell spreading on fibronectin [25]. Tyrosine phosphorylation of the potential pp125^{FAK} substrates tensin and paxillin was also reduced. The inhibitory activity of pp41/43^{FRNK} is reversed by co-expression of pp125^{FAK} with pp41/43^{FRNK}, suggesting that pp125^{FAK} and pp41/43^{FRNK} compete for a common binding partner and that this partner is required for signalling by pp125^{FAK}. Whereas cell spreading on fibronectin leads to tyrosine phosphorylation of pp125^{FAK}, pp41/43^{FRNK} undergoes serine phosphorylation [24]. Serine phosphorylation of pp41/43^{FRNK} results in a decrease in the electrophoretic mobility of pp41/43^{FRNK} from 41 kDa to 43 kDa [24]. In this report, we identify two major sites of serine

Abbreviations used: CE, chicken embryo; CK-1, casein kinase 1; ECM, extracellular matrix; FAK, focal adhesion kinase; FRNK, FAK-related, non-kinase; PKA, protein kinase A; GST, glutathione S-transferase; PKC, protein kinase C.

§ Present address: Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, NC 27599, U.S.A.

¶ To whom correspondence should be addressed.

phosphorylation in pp41/43^{FRNK}, Ser¹⁴⁸ and Ser¹⁵¹, as being necessary for formation of the pp43 isoform of FRNK. However, phosphorylation of these sites does not appear to regulate the inhibitory activity of pp41/43^{FRNK} and occurs after tyrosine phosphorylation of pp125^{FAK}. In contrast, cell spreading on fibronectin and tyrosine phosphorylation of pp125^{FAK} is delayed by treatment of chicken embryo (CE) cells with inhibitors of protein kinase A (PKA). These data suggest that serine kinases play a role during integrin signalling and the activation of pp125^{FAK}.

EXPERIMENTAL

Cell culture and protein analysis

CE cells were prepared from 11-day embryos as described [26]. CE cells were transfected with RCAS-A replication-competent retroviral vector [27] containing either no insert DNA or DNA encoding pp125^{FAK} or pp41/43^{FRNK}. The high level of expression of FRNK (clone 10a [24]) obtained by this method prevented sufficient resolution of pp43^{FRNK} and pp41^{FRNK} during electrophoresis to allow their separate mapping. To overcome this, we made use of the observation that inclusion of an out-of-frame epitope tag, corresponding to the 11 C-terminal residues of simian virus 40 large T antigen [18], approx. 60 nucleotides 5' of the FRNK Kozak consensus sequence, attenuated expression of FRNK, yielding levels 5–10-fold above endogenous FRNK. Sequencing of this construct confirmed that the FRNK open reading frame was in a different frame from the epitope tag, and immunoprecipitation and immunoblotting studies indicated that the FRNK protein derived from this construct did not contain the tag (results not shown). In addition, phosphopeptide maps of mixtures of pp41^{FRNK} and pp43^{FRNK} FRNK were the same, irrespective of whether the tag was present or not. Transfected cells were cultured for 7 days at which time the majority of the cells were infected [18]. To measure cell spreading, 100 mm bacterial plastic dishes (Fisher) were coated with 10 ml of 20 µg/ml fibronectin (Sigma) for 2 h at 37 °C. CE cells were pretreated with PKA inhibitors H-89, H-88 or H-85 (Seikagaku) or solvent (DMSO) alone for 1 h at 37 °C and then collected by digestion with trypsin, washed twice with 1 mg/ml soybean trypsin inhibitor (Sigma), and 4 × 10⁶ cells were added to each dish in 5 ml of L15 medium (Gibco). Cell spreading was assessed by phase-contrast microscopy. To measure [cAMP], cells collected either after treatment with trypsin or after adhering to fibronectin were washed briefly in PBS, and lysed in 0.1 M HCl. The lysate was cleared by centrifugation (15000 g; 10 min) and cAMP determined by RIA [28]. PKA activity was measured using a PKA assay system (Gibco) according to the manufacturer's instructions as the phosphorylation of a PKA substrate peptide that was sensitive to inhibition by a PKA inhibitor peptide. To measure the effects of H-89 on phorbol ester-induced mobility shift of mitogen-activated protein kinase, adherent cells were pretreated with H-89, the protein kinase C (PKC) inhibitor GF109203X (Calbiochem) or solvent alone for 1 h before treatment with the phorbol ester, phorbol 12,13-dibutyrate (Sigma), for 10 min.

To immunoprecipitate pp125^{FAK} or pp41/43^{FRNK} from either spreading cells or cells growing continually in culture, cells were lysed in an ice-cold supplemented RIPA (S-RIPA) containing 50 mM Hepes, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P40 at pH 7.2 and 100 µM leupeptin, 10 µM pepstatin, 0.05 trypsin-inhibitory units/ml aprotinin, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM benzamidine, 20 µg/ml soya-bean trypsin inhibitor, 10 mM sodium pyrophosphate, 40 mM sodium *p*-nitrophenyl phosphate and 40 mM NaF. After

centrifugation of the cell extracts at 15000 g for 10 min, FAK or FRNK proteins were immunoprecipitated from the supernatant by incubation of up to 1.5 mg of supernatant protein with 20 µl of BC2 or 10 µl of BC3, for 2 h at 4 °C followed by incubation with 100 µl of a 50% (v/v) slurry of Protein A–Sepharose (Sigma) for 2 h. Immune complexes were collected, washed and analysed by SDS/PAGE on a 32 cm-long gel to aid resolution of pp41/43^{FRNK} isoforms. The proteins were transferred to nitrocellulose and subjected to immunoblotting with a rabbit anti-pp125^{FAK} serum (BC3), a monoclonal pp125^{FAK} antibody (2A7) or anti-phosphotyrosine (RC20; Transduction Laboratories) and detection by Enhanced Chemiluminescence (Amersham).

Generation of FRNK and FAK mutants

The FRNK cDNA (clone 10a; [24]) was subcloned into pALTER, and site-directed mutagenesis of the nucleotides encoding Ser¹⁴⁸ and/or Ser¹⁵¹ was performed with the Altered Sites mutagenesis system (Promega). The mutations were confirmed by direct sequencing. The mutated cDNAs were subcloned into the RCAS A retroviral vector and introduced into CE cells by transfection. Glutathione S-transferase (GST)–FRNK fusion proteins were generated by ligation into GEX 1AT (Pharmacia) of an *Eco*RI fragment containing the coding region of FRNK and a 5' sequence of 66 nucleotides that is normally untranslated. The resulting gene fusion encoded GST fused to FRNK by a 22-amino acid linker sequence; the linker segment did not contain any Ser residues conforming to a PKA consensus phosphorylation site.

³²P-labelling and peptide mapping

CE cells (8 × 100-mm dishes) were labelled in phosphate-free Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 1% chicken serum, 10% conditioned medium and 50–100 mCi of [³²P]P_i (NEN) for 12 h at 37 °C. The culture medium was removed and the cells lysed in S-RIPA containing 50 mM 2-glycerophosphate. ³²P-labelled pp125^{FAK} or pp41/43^{FRNK} was immunoprecipitated by incubation of labelled extracts with 160 µg of anti-pp125^{FAK} monoclonal antibody 2A7 for 2 h at 4 °C and subsequently for 2 h with 100 µl of Protein A–Sepharose previously coated with 320 µg of rabbit anti-mouse IgG (Jackson ImmunoResearch). Immune complexes were collected and resolved by SDS/PAGE and transferred to nitrocellulose. GST fusion proteins (10–50 µg) were collected on GSH–Sepharose beads and were phosphorylated as previously described [29] by incubation with either 10–50 µCi of [γ-³²P]ATP (6000 Ci/mmol) or, where specified, 100 µM unlabelled ATP and 10 units of PKA (Sigma). For phosphorylation by casein kinase-1 (CK-1), GST fusion proteins (10–50 µg) were incubated with 10–50 µCi of [γ-³²P]ATP or, where specified, 100 µM unlabelled ATP and yeast CK-1 (0.1–1 µg, a gift from Jeff Kuret, Molecular Geriatrics) as previously described [30]. The reactions were terminated by addition of electrophoresis sample buffer and labelled GST–FRNK fusion proteins purified by electrophoresis and transferred to nitrocellulose. Where indicated, labelled proteins were transferred to Immobilon for phosphoamino acid analysis [31] and analysed as previously described [32].

To generate tryptic peptides, labelled proteins immobilized on nitrocellulose were treated for 4 h at 37 °C with two sequential additions of 10 µg of trypsin (Worthington) as described [33]. This resulted in release of more than 80% of the radioactivity associated with the membrane. Phosphopeptide maps were generated as described previously [31] by electrophoresis of the indicated amount of phosphopeptide for 45 min at 1000 V in

pH 8.9 buffer [31] supplemented with 1 mM EDTA, followed by chromatography in isobutyric acid buffer [31]. All tryptic mapping experiments were replicated at least three times using proteins independently labelled either *in vivo* or *in vitro*. Where peptide mixing experiments are shown, each chromatogram was exposed to film for the same period of time as the mixture. Selected peptides were removed from thin layer plates by scraping [31] and subjected to Edman degradation as described [34].

RESULTS

Kinetics of phosphorylation of pp125^{FAK} and pp41/43^{FRNK}

pp125^{FAK} is phosphorylated on tyrosine in an adhesion-dependent time-dependent fashion [24]. To compare the kinetics of phosphorylation of pp125^{FAK} and pp41/43^{FRNK} on serine, CE cells were allowed to spread on fibronectin for various times. Tyrosine phosphorylation of pp125^{FAK} was observed within 10 min of CE cell spreading on fibronectin and little further increase was observed after 20 min (Figure 1). At the earliest time points (10–20 min), FRNK migrated on SDS/PAGE largely as the p41 isoform, and the serine-phosphorylated p43 isoform was observed only after 30–45 min. These results indicate that tyrosine phosphorylation of pp125^{FAK} is an earlier event than the phosphorylation responsible for the decrease in FRNK electrophoretic mobility.

Analysis of pp41^{FRNK}-phosphorylation sites

Metabolic labelling of CE cells overexpressing FRNK showed that both pp41^{FRNK} and pp43^{FRNK} were phosphorylated (Figure 2A). In addition, previous experiments showed that pp43^{FRNK} could be converted into pp41^{FRNK} by treatment with potato acid phosphatase [24]. Thus we concluded that pp43^{FRNK} contained additional phosphorylation sites, some of which were not present in pp41^{FRNK}. Phosphoamino acid analysis indicated that pp41/43^{FRNK} phosphorylation occurred exclusively on serine whereas pp125^{FAK} was phosphorylated on both serine and tyrosine (Figure 2B). Tryptic mapping of FRNK proteins (Figure 2C) demonstrated that pp41^{FRNK} and pp43^{FRNK} contained four major phosphopeptides, two of which, Y and Z, were common to pp41^{FRNK} and pp43^{FRNK}. In contrast, pp41^{FRNK} contained two unique peptides A and B, with peptide A being consistently more intensely labelled than peptide B. Analysis of pp43^{FRNK} also revealed two peptides, W and X, that were not observed in

Figure 2 Phosphoamino acid analysis and phosphopeptide mapping of pp41/43^{FRNK} labelled *in vivo*

(A) CE cells overexpressing pp41/43^{FRNK} were labelled *in vivo* with [³²P]P_i, and pp125^{FAK} and pp41/43^{FRNK} were isolated by immunoprecipitation and SDS/PAGE as described in the Experimental section. (B) Phosphoamino acid analysis was performed using approx. 1200 c.p.m. of pp125^{FAK} (p125), pp43^{FRNK} (p43) and pp41^{FRNK} (p41) as described in the Experimental section. S, T and Y indicate the positions of unlabelled phosphoserine, phosphothreonine and phosphotyrosine. (C) Approx. 1000 c.p.m. of ³²P-labelled tryptic phosphopeptides from pp41^{FRNK} (p41) and pp43^{FRNK} (p43) or a mixture of each (MIX) were analysed by sequential electrophoresis and chromatography. The arrows indicate the directions of electrophoresis and chromatography; peptide maps in subsequent Figures are oriented similarly.

Figure 3 Comparison of pp41^{FRNK} labelled *in vivo* and GST-FRNK labelled *in vitro*

Tryptic phosphopeptides from pp41^{FRNK} (p41; 2000 c.p.m.) labelled *in vivo* or tryptic phosphopeptides from a GST-FRNK fusion protein labelled *in vitro* with PKA (GST-FRNK/PKA; 2000 c.p.m.) were analysed either separately or after being mixed together. The directions of electrophoresis and chromatography are as described in Figure 2.

Figure 1 Kinetics of phosphorylation of pp125^{FAK} and pp41/43^{FRNK}

Untransfected CE cells were collected by trypsin treatment and allowed to spread on fibronectin-coated dishes. After the indicated time, the cells were lysed and pp125^{FAK} and pp41/43^{FRNK} were isolated by immunoprecipitation. Serine phosphorylation of p41/43^{FRNK} was analysed by immunoblotting for pp41/43^{FRNK} with antiserum BC3, and pp125^{FAK} was analysed by blotting for phosphotyrosine. Similar amounts of pp125^{FAK} were detected in each immunoprecipitate (results not shown). I.P., immunoprecipitate.

pp41^{FRNK}. Metabolic labelling of untransfected CE cells and tryptic mapping of endogenous pp41/43^{FRNK} demonstrated that the 41 kDa and 43 kDa forms of FRNK exhibited a similar characteristic distribution of peptides A, B, W, X, Y and Z (results not shown). When pp125^{FAK} was ectopically expressed in CE cells, metabolic labelling indicated that pp125^{FAK} was phosphorylated on Tyr³⁹⁷, as previously reported [7], as well as peptides Y and Z. However, phosphorylation of peptides A, B, W and X was relatively poor.

To identify the sites of phosphorylation, several protein kinases were tested for their ability to phosphorylate GST-FRNK. PKA

Table 1 Sequential Edman degradation of phosphopeptides **a** and **b**

Phosphopeptides **a** and **b** were isolated as described in Figure 3 (after different labelling reactions), subjected to Edman degradation as described in the Experimental section, and the radioactivity released was determined after each cycle of degradation.

Cycle number	Radioactivity released (c.p.m.)	
	Peptide a	Peptide b
1	60	110
2	580	5800
3	170	1040
4	60	200
5	50	240
6	50	120

was found to efficiently phosphorylate GST–FRNK on two major tryptic peptides **a** and **b** (Figure 3). These peptides were not observed using GST alone as a substrate (results not shown). In all experiments, peptide **a** was more intensely labelled than peptide **b**, reminiscent of the more intense labelling of peptide **A** in the *in vivo* peptide mapping experiments. Mixing experiments indicated that peptide **a** and **b** co-migrated with *in vivo* peptides **A** and **B** respectively (Figure 3). In addition, peptides **a** and **b** co-migrated with peptides **A** and **B** obtained from endogenous pp41^{FRNK} (results not shown).

Peptides **a** and **b** were subject to sequential Edman degradation. In each case, ³²P was released after two cycles of sequencing (Table 1). Since trypsin cleaves after arginine and lysine residues, these data indicated that the phosphorylation sites were two residues C-terminal to either a lysine or an arginine, consistent with phosphorylation *in vitro* of a PKA consensus site, K/RXS

[35]. The predicted amino acid sequence of FRNK [22] contains four serine residues conforming to this motif, Ser²⁴, Ser³⁴, Ser¹⁴⁸ and Ser¹⁵¹. A GST–FRNK fusion protein comprising residues 73 to 358 of FRNK fused to GST was also phosphorylated *in vitro* by PKA and yielded a virtually identical tryptic phosphopeptide map (results not shown). Since the shortened form of GST–FRNK lacks Ser²⁴ and Ser³⁴, we concluded that peptides **a** and **b** must include Ser¹⁴⁸ or Ser¹⁵¹.

To identify the phosphorylation sites in peptides **a** and **b**, three GST–FRNK constructs were prepared in which the codons for either Ser¹⁴⁸ or Ser¹⁵¹ or both residues (denoted FRNK/S148A, FRNK/S151A or FRNK/S148,151A respectively) were mutated to alanine. When these GST–FRNK fusion proteins were phosphorylated *in vitro* by PKA and analysed by tryptic phosphopeptide mapping, only one major phosphopeptide was detected in GST–FRNK/S148A and one in GST–FRNK/S151A (Figure 4). Neither phosphopeptide was detected in the double variant GST–FRNK/S148,151A (Figure 4). The major phosphopeptide from GST–FRNK/S148A co-migrated with peptide **b** and the major phosphopeptide recovered from GST–FRNK/S151A co-migrated with peptide **a**, indicating that the mutations did not induce PKA phosphorylation at a novel site. Since different phosphopeptides were produced by phosphorylating GST–FRNK/S148A and GST–FRNK/S151A, we concluded that **a** and **b** were not derived from partial proteolysis of a peptide containing a single phosphorylation site, and must represent separate phosphorylation sites. As mutation of Ser¹⁴⁸ leaves Ser¹⁵¹ as the only candidate phosphorylation site, Ser¹⁵¹ was predicted to be the site phosphorylated in GST–FRNK/S148A. Similarly the site phosphorylated in GST–FRNK/S151A was predicted to be Ser¹⁴⁸. Since the product of PKA phosphorylation of GST–FRNK/S151A co-migrated with peptide **a**, which in turn co-migrates with peptide **A**, we conclude that the major site of phosphorylation *in vivo*, peptide **A**, appears to contain Ser¹⁴⁸. Similarly, we conclude that peptide **B** contains Ser¹⁵¹. Thus we

Figure 4 Identification of phosphorylation sites in peptides **a** and **b**

GST fusion proteins of either FRNK (W.T.) or variants in which Ser to Ala substitutions were made at positions Ser¹⁴⁸ (S148A), Ser¹⁵¹ (S151A) or both Ser¹⁴⁸ and Ser¹⁵¹ (S148,151A) were labelled *in vitro* by PKA, purified and digested with trypsin as described in the Experimental section. Approx. 5000 c.p.m. of ³²P-labelled phosphopeptides from each variant were analysed by two-dimensional mapping (top four panels). The directions of electrophoresis and chromatography are as described in Figure 2. No predominant labelled phosphopeptides were observed in the double mutant S148,151A, although similar amounts of radioactivity were analysed, indicating that PKA probably inefficiently phosphorylated multiple sites within this variant. In the bottom three panels, approx. 5000 c.p.m. of each of S148A or S151A or FRNK were mixed before mapping.

Figure 5 Comparison of pp43^{FRNK} labelled *in vivo* and GST-FRNK labelled *in vitro*

Top three panels: the GST-FRNK fusion proteins were incubated *in vitro* with ATP and PKA followed by CK-1. In the panel designated ³²P/PKA, [γ -³²P]ATP was included during the incubation with PKA; in the panel designated ³²P/CK-1, labelled ATP was present during the incubation with CK-1. When radioactive ATP was not used, unlabelled ATP was used in its place. Tryptic phosphopeptides from the experiment designated ³²P/PKA (5000 c.p.m.) and the experiment designated ³²P/CK-1 (5000 c.p.m.) were analysed either separately or after mixing (MIX). The directions of electrophoresis and chromatography are as described in Figure 2. Bottom three panels: in the panel designated p43, FRNK was immunoprecipitated and analysed after ³²P-labelling of CE cells overexpressing FRNK. In the panel designated ³²P/CK-1, GST-FRNK was labelled *in vitro* as described above. Tryptic phosphopeptides from p43 (2000 c.p.m.) or from ³²P/CK-1 (4000 c.p.m.) were analysed either separately or after mixing together (MIX p43 and ³²P/CK-1). Note that although we identify peptide **b** as being singularly phosphorylated and peptide **w** as containing two phosphates, the larger mass of peptide **w** (see the text) can be calculated [2] to reduce its mobility to 80% of that of peptide **b**.

identified phosphopeptide **A** as LS(P)R [where S(P) represents phosphoserine] or possibly the partial cleavage peptide LS(P)RGSIER. Phosphopeptide **B** was identified as GS(P)IER. It is unlikely that **B** is LSRGS(P)IER since no release of radioactivity was observed after five cycles of Edman degradation (Table 1).

Analysis of pp43^{FRNK}-phosphorylation sites

Since peptides **A** and **B** were present in pp41^{FRNK} but missing from pp43^{FRNK}, one possibility was that pp43^{FRNK} was phosphorylated on neither Ser¹⁴⁸ nor Ser¹⁵¹. However, retrovirus-mediated expression of FRNK/S148A and FRNK/S151A variants in CE cells indicated that both phosphorylation sites were necessary to yield the pp43 form of pp41/43^{FRNK} (see below). A plausible explanation for this paradox was that the peptides unique to pp43^{FRNK}, **W** and/or **X**, were phosphorylated on both Ser¹⁴⁸ and Ser¹⁵¹ and included the sequence LS¹⁴⁸RGS¹⁵¹IER. To test this hypothesis, we took advantage of the observation that the protein kinase CK-1 phosphorylates serine/threonine residues in the consensus sequence S(P)XXS [35]. Thus phosphorylation of Ser¹⁴⁸ by PKA creates a phosphorylation site for CK-1 at Ser¹⁵¹. Two sets of experiments were performed to assess possible phosphorylation at both these residues. (1) The GST-FRNK fusion protein was incubated with PKA and unlabelled ATP followed by incubation with CK-1 and [γ -³²P]ATP (desig-

Table 2 Sequential Edman degradation of peptide **w**

Phosphopeptide **w** was isolated as described in Figure 5 from the experiments designated ³²P/CK-1 or ³²P/PKA, subjected to Edman degradation as described in the Materials and methods section, and the radioactivity released was determined after each round of Edman degradation.

Cycle number	Radioactivity released (c.p.m.)	
	Experiment ³² P/PKA	Experiment ³² P/CK-1
1	30	190
2	510	200
3	60	100
4	40	120
5	40	5860
6	40	2050
7	50	960

nated ³²P/CK-1 in Figure 5). (2) The reciprocal experiment was performed by incubation of the GST-FRNK fusion protein with PKA and [γ -³²P]ATP followed by incubation with CK-1 and unlabelled ATP (designated ³²P/PKA in Figure 5). Tryptic mapping of these labelled products revealed several phospho-

Figure 6 Phosphorylation of FRNK variants *in vivo*

(A) CE cells were transfected with individual retroviruses encoding FRNK and variant FRNK proteins, lysed and analysed by SDS/PAGE. FRNK proteins were immunoblotted with the antiserum BC3 as described in the Experimental section. MOCK designates cells transfected with empty vector. W.T., S148A, S151A and S148,151A designate cells transfected with vector containing FRNK or FRNK variants in which Ser to Ala substitutions were made at positions Ser¹⁴⁸, Ser¹⁵¹ or both Ser¹⁴⁸ and Ser¹⁵¹ respectively. (b) CE cells overexpressing the same FRNK variants as described above (but not MOCK CE cells) were labelled with [³²P]P_i *in vivo* and immunoprecipitated with mAb 2A7, transferred to nitrocellulose and detected by autoradiography. pp41^{FRNK} was isolated from cells expressing FRNK and each of its variants and 1500 c.p.m. of tryptic phosphopeptides from each of the variants was used for phosphopeptide mapping. The directions of electrophoresis and chromatography are as described in Figure 2. Although phosphopeptides B and A were observed in S148A and S151A respectively, they were usually only weakly labelled.

peptides, at least two of which, w and x, were present regardless of whether labelled ATP was added with either PKA or CK-1 (Figure 5). These peptides (w and x) co-migrated with W and X produced by *in vivo* labelling of CE cells overexpressing FRNK (Figure 5). Neither peptide w nor x was observed if incubation with PKA was omitted, nor if GST-FRNK/S148A or GST-FRNK/S151A were used as the substrate (results not shown). Sequential Edman degradation of peptide w (Table 2) released radioactivity after two cycles when peptide w was produced by ³²P/PKA (followed by unlabelled ATP/CK-1). However, when this peptide was produced by incubation with unlabelled ATP/PKA followed by [³²P]ATP/CK-1, radioactivity was released after five cycles. These data indicate that peptide w is phosphorylated on two sites and contains the sequence K/RXS(P)XXS(P). The only peptide in FRNK that contains this sequence is LS¹⁴⁸RGS¹⁵¹IER, suggesting that peptide W is phosphorylated on both Ser¹⁴⁸ and Ser¹⁵¹. We speculate that phosphorylation of both Ser¹⁴⁸ and Ser¹⁵¹ protects Arg¹⁴⁹ from cleavage by trypsin, and thus generates a longer peptide than that obtained from tryptic digestion of the protein phosphorylated at either Ser¹⁴⁸ or Ser¹⁵¹. The larger mass of peptide w is sufficient to reduce its electrophoretic mobility to less than that of peptide b, even though peptide b is singly phosphorylated and peptide w is doubly phosphorylated [31].

A similar analysis to that conducted on peptide w was carried out on peptide x. In this case, Edman degradation also released radioactivity after two and five cycles (results not shown), suggesting that peptide x is related to peptide w and may be a

partial cleavage product containing w. In addition to w and x, incubation with PKA and CK-1 also generated two peptides y and z which co-migrated with the *in vivo* labelled peptides Y and Z respectively. Thus the combination of these two enzymes appears to reproduce the major FRNK phosphorylations observed *in vivo*.

Expression of FRNK variants in CE cells

To confirm the identity of the phosphorylation sites in FRNK, S148A and S151A variants of FRNK were expressed in CE cells. In cells expressing FRNK/S148A, FRNK/S151A or FRNK/S148,151A, the 43 kDa form of FRNK was not detected by immunoblotting (Figure 6A), although it was observed in cells overexpressing unmutated FRNK. Thus the presence of Ser¹⁴⁸ and Ser¹⁵¹ appears to be necessary to generate the 43 kDa form of FRNK. Tryptic mapping of both FRNK variants revealed the presence of peptides that co-migrate with a (in FRNK/S151A) or b (in FRNK/S148A), indicating the loss of peptide A in FRNK/S148A and loss of peptide B in FRNK/S151A (Figure 6B). Both A and B were absent from maps of FRNK protein from cells expressing the double variant FRNK/S148,151A. Peptides Y and Z were present in the maps of all of the FRNK variants. Thus mutation of Ser¹⁴⁸ or Ser¹⁵¹ prevents phosphorylation of peptides A and B respectively. These data are consistent with the previous assignment of pp41^{FRNK} being phosphorylated on either Ser¹⁴⁸ or Ser¹⁵¹ and pp43^{FRNK} being phosphorylated on both Ser¹⁴⁸ and Ser¹⁵¹.

Figure 7 Inhibition of cell spreading by PKA inhibitors correlates with a delay in FRNK phosphorylation

Left, CE cells were treated with solvent alone or 10 μ M H-89, H-88 or H-85 for 1 h, collected by digestion with trypsin and allowed to spread on fibronectin-coated Petri dishes. The cells were photographed after the indicated time. Right, FRNK was immunoprecipitated from lysates prepared from the same cells and analysed by immunoblotting with serum BC3 (FRNK). The arrow denotes the position of pp43. Note that in some experiments, a third immunoreactive band was also observed at approximately 44 kDa (upper band). This species represents an additional isoform of FRNK (A. Richardson and J. T. Parsons, unpublished work). pp125^{FAK} was analysed by immunoprecipitation and immunoblotting with serum BC3 (FAK) or with RC-20, which recognizes phosphotyrosine (PTYR).

Inhibition of cell spreading and FRNK phosphorylation by PKA inhibitors

To investigate the potential role for PKA in regulating serine phosphorylation of FRNK *in vivo*, CE cells were pretreated with the PKA inhibitor H-89 [36] or with solvent alone, collected by trypsin treatment and plated on dishes coated with fibronectin. Pretreatment of the cells with H-89 resulted in a delay in spreading on fibronectin (Figure 7, left). This delay was dependent on the concentration of H-89, and was reversible, because the cells were fully spread 120 min after adhering to fibronectin, and indistinguishable from control cells (results not shown). The effects of H-89 were not mediated by inhibition of PKC, as H-89 did not block the phorbol ester-induced decrease in the mobility of mitogen-activated protein kinase, whereas a PKC inhibitor (GF109203X) did block this mobility shift (results not shown). CE cells were also pretreated with either H-88, a less potent PKA inhibitor than H-89, or H-85, which fails to inhibit PKA [36]. H-88 was a less effective inhibitor of cell spreading than H-89, whereas cells pretreated with H-85 spread at a rate indistinguishable from that of control cells (Figure 7, left). At 20 min after adherence of the cells to fibronectin, the majority of the control and H-85-treated cells were spread, whereas only a fraction of the H-88-treated cells and none of the H-89-treated cells were spread. By 45 min, the majority of the H-88-treated cells were well spread on fibronectin, whereas only a fraction of the H-89-treated cells had spread.

Pretreatment of CE cells with these PKA inhibitors also delayed the appearance of the pp43 isoform of FRNK (Figure 7, right). The most potent PKA inhibitor (H-89) delayed the appearance of pp43 more effectively than the less active PKA inhibitors. Both H-88 and H-89 blocked the appearance of pp43^{FRNK} at 20 min, but after 45 min, pp43^{FRNK} was detectable in H-88-treated cells but not in H-89-treated cells. Tyrosine phosphorylation of pp125^{FAK} was also delayed in cells pretreated with PKA inhibitors (Figure 7, right).

Measurement of intracellular cAMP showed no significant difference between cells spreading on fibronectin, or adherent to poly-L-lysine. However, detachment of cells from ECM by treatment with trypsin resulted in a significant increase (2.2 ± 0.4 -fold, $P < 0.05$, $n = 3$) in cAMP compared with cells growing in culture for 2 days. Similarly, PKA activity in cells in suspension increased 1.7 ± 0.2 -fold ($P < 0.05$, $n = 6$) above the activity measured in cells growing in culture for 2 days. Therefore release from the ECM appears to lead to an increase in cAMP, with a concomitant increase in PKA activity. Furthermore inhibition of PKA activity blocks an early step(s) in the cell-spreading process.

Role of Ser¹⁴⁸ and Ser¹⁵¹ in regulating FRNK

We have previously shown that FRNK delays cell spreading [25]. To test whether the PKA inhibitors delayed cell spreading by blocking phosphorylation of FRNK, variants of FRNK were

Figure 8 Mutation of Ser¹⁴⁸ and Ser¹⁵¹ does not alter the inhibitory activity of FRNK

(A) CE cells were transfected with empty vector (lanes 1 and 2) or retroviral vectors encoding FRNK (lanes 3 and 4), or variants of FRNK in which either Ser¹⁴⁸ (lanes 5 and 6) or Ser¹⁵¹ (lanes 7 and 8) or both Ser¹⁴⁸ and Ser¹⁵¹ (lanes 9 and 10) had been mutated to encode alanine. pp125^{FAK} was immunoprecipitated with antiserum BC2 (lanes 1, 3, 5, 7, and 9) or with preimmune serum (lanes 2, 4, 6, 8 and 10). The immunoprecipitates were divided and blotted for either pp125^{FAK} or for PTyr. (B) Tyrosine phosphorylation of FAK was analysed similarly in CE cells transfected with empty vector (lane 1) or expressing FRNK (lane 2), or variants of FRNK in which Ser¹⁴⁸ (lane 3), Ser¹⁵¹ (lane 4) or both Ser¹⁴⁸ and Ser¹⁵¹ (lanes 5) had been mutated to encode glutamate. I.P., immunoprecipitate.

expressed in which residues Ser¹⁴⁸ and Ser¹⁵¹ had been mutated either jointly or singly to either alanine (to prevent phosphorylation) or to glutamate (to mimic phosphorylation) residues. In agreement with our previous observations, pp41/43^{FRNK} inhibited tyrosine phosphorylation of pp125^{FAK}. However, mutation of residues Ser¹⁴⁸ and Ser¹⁵¹ did not substantially alter the ability of pp41/43^{FRNK} to act as an inhibitor of tyrosine phosphorylation of pp125^{FAK} (Figure 8).

DISCUSSION

Integrin binding to matrix induces serine phosphorylation of pp41/43^{FRNK} which is manifested as a decrease in the electrophoretic mobility of FRNK from 41 to 43 kDa [24]. Here, we identify Ser¹⁴⁸ and Ser¹⁵¹ as two sites of phosphorylation in pp41/43^{FRNK}. pp41^{FRNK} is phosphorylated on either Ser¹⁴⁸ or Ser¹⁵¹ and pp43^{FRNK} is phosphorylated on both these residues. We were able to phosphorylate FRNK *in vitro* with PKA, and treatment of CE cells with inhibitors of PKA delayed phosphorylation of these serine residues, tyrosine phosphorylation of pp125^{FAK} and cell spreading on fibronectin.

We have previously shown that pp41/43^{FRNK} can inhibit tyrosine phosphorylation of pp125^{FAK} and delay cell spreading on fibronectin [25] and we speculated that phosphorylation of pp41/43^{FRNK} may serve to regulate the inhibitory activity of pp41/43^{FRNK}. While identifying the integrin-stimulated sites of phosphorylation in pp41/43^{FRNK}, we observed that PKA specifically phosphorylates FRNK *in vitro* at Ser¹⁴⁸ and Ser¹⁵¹, residues

that are also phosphorylated *in vivo*. This raised the possibility that PKA may phosphorylate pp41/43^{FRNK} *in vivo*. In addition, both Ser¹⁴⁸ and Ser¹⁵¹ reside within consensus sites for phosphorylation by PKA. Treatment of CE cells with a series of PKA inhibitors delayed cell spreading and serine phosphorylation of FRNK, as indicated by the delayed formation of the pp43 isoform of FRNK. This delay correlated with potency of the compounds as inhibitors of PKA *in vitro* [36]. Although the cells were treated with relatively high concentrations of these inhibitors {10 μ M; *in vitro* IC₅₀ (H-89) = 50 nM [36]}, a similarly high concentration of this drug has previously been shown to be necessary to inhibit PKA in intact pheochromocytoma cells, probably reflecting poor membrane permeability of the drug [36]. In addition, H-89 did not act by inhibiting PKC and did not affect autophosphorylation of pp125^{FAK} or phosphorylation of GST-paxillin by pp125^{FAK} in an immune complex kinase assay (results not shown). These data suggest a role for PKA in regulating signalling through integrins.

Two lines of evidence suggest that the mechanism of action of PKA is unlikely to be solely by catalysing the phosphorylation of pp41/43^{FRNK}. First, cell spreading and tyrosine phosphorylation of pp125^{FAK} appear to precede temporally the phosphorylation of pp41/43^{FRNK} on both Ser¹⁴⁸ and Ser¹⁵¹, yet inhibition of PKA delays cells spreading and tyrosine phosphorylation of pp125^{FAK}. Secondly, we mutated Ser¹⁴⁸ and Ser¹⁵¹ either individually or jointly to either alanine (to prevent phosphorylation) or glutamate (to mimic phosphorylation). Mutation of these residues did not block the ability of pp41/43^{FRNK} to inhibit tyrosine phosphorylation of pp125^{FAK} (see Figure 8), although we cannot rule out the possibility that glutamate inadequately mimics a phosphoserine, and thereby fails to regulate pp41/43^{FRNK}.

Instead of regulating integrin signalling by catalysing phosphorylation of pp41/43^{FRNK}, PKA may play a role more proximal to integrin activation. pp125^{FAK} can be recruited to focal adhesions by integrin clustering in the absence of integrin occupancy [37] and pp125^{FAK} can bind directly to integrins [17], suggesting that recruitment of pp125^{FAK} is an early event during integrin signalling. As well as delaying cell spreading, the PKA inhibitors delayed tyrosine phosphorylation of pp125^{FAK}, suggesting a role for PKA before activation of pp125^{FAK}. In addition, an increase in cAMP and PKA activity was observed in cells in suspension compared with adherent cells, suggesting a role for PKA before integrin binding to ECM. Activation of PKA has been reported to induce the disassembly of stress fibres in a variety of cell types ([38,39] and references therein). One potential resolution of these apparently conflicting observations is that PKA may play a role in the disassembly of pre-existing focal adhesions, thereby allowing the formation of new focal adhesions. Thus pronounced elevation of intracellular cAMP might prevent assembly of new focal adhesions by stimulating their continual disassembly. Inhibitors of PKA might delay cell spreading by preventing the liberation of key focal adhesion proteins from mature focal adhesions, thereby inhibiting the formation of new focal adhesions. Such a model predicts that intracellular cAMP must be carefully regulated, consistent with the relatively small increases in cAMP and PKA catalytic activity that we observed.

Although we have not been able to ascribe a clear function to phosphorylation of Ser¹⁴⁸ and Ser¹⁵¹, there is clear evidence that these residues play an important role in signalling by pp125^{FAK} and pp41/43^{FRNK}. Most importantly, phosphorylation of these sites is triggered by receptor occupancy (integrin binding to ECM) and it is reversed by removal of the ligand (detachment of cells from ECM). There is also a striking correlation between the ability of various PKA inhibitors to delay cell spreading and to

inhibit formation of pp43^{FRNK}, emphasizing the link between integrin occupancy and FRNK phosphorylation. Furthermore analysis of cDNAs encoding human, mouse, chicken, and *Xenopus* pp125^{FAK} demonstrates the evolutionary conservation of the phosphorylation site...RLSRGS...[6,16,40,41]. Interestingly, sequence analysis of a cDNA encoding a variant of human pp125^{FAK} [40] reveals a deletion of a region encoding 21 amino acids encompassing residues corresponding to Ser¹⁴⁸ and Ser¹⁵¹.

We conclude that PKA plays a role in regulating cell spreading. It is tempting to suggest that the delay in cell spreading by PKA inhibitors reflects a role for PKA early after integrin binding to ECM. However, because cell spreading is likely to be regulated by feedback ([3] and references therein), we cannot discount a role for PKA subsequent to activation of pp125^{FAK}. The first focal adhesions formed after the initial binding of integrins to ECM probably triggers signalling events which deform the cell from its spherical morphology, thereby promoting formation of further focal adhesions (feedback). If PKA is necessary for the feedback signalling that we presume promotes formation of additional focal adhesions, inhibition of PKA would delay cell spreading and delay tyrosine phosphorylation of the large pool of pp125^{FAK} that has yet to be activated. Whatever the mechanism, PKA appears to play a complex role in both the formation and breakdown of the actin cytoskeleton, emphasizing the need to investigate further the role of serine kinases in regulating adhesion events.

We thank Jeffrey Hildebrand and Rajesh Malik for helpful discussions, C. Borgmann for technical support and T. Barbera for measuring [cAMP]. We thank Jeff Kuret (Molecular Geriatrics) for CK-1 and Mike Weber for monoclonal antibody B3B9. This work was supported by DHHS grant CA40042 and CA29243.

REFERENCES

- Kanner, S. B., Reynolds, A. B., Vines, R. R. and Parsons, J. T. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3328–3332
- Schaller, M. D. and Parsons, J. T. (1994) *Curr. Opin. Cell Biol.* **6**, 705–710
- Richardson, A. and Parsons, J. T. (1995) *Bioessays* **17**, 229–236
- Hynes, R. O. (1992) *Cell* **69**, 11–25
- Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988) *Annu. Rev. Cell Biol.* **4**, 487–525
- Schaller, M. D., Borgman, C. A., Cobb, B. C., Reynolds, A. B. and Parsons, J. T. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5192–5196
- Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. and Parsons, J. T. (1994) *Mol. Cell. Biol.* **14**, 1680–1688
- Cobb, B. S., Schaller, M. D., Horng-Leu, Z. and Parsons, J. T. (1994) *Mol. Cell. Biol.* **14**, 147–155
- Schlaepfer, D. D., Hanks, S. K., Hunter, T. and van der Geer, P. (1994) *Nature (London)* **372**, 786–791
- Xing, Z., Chen, H.-C., Nowlen, J. K., Taylor, S. J., Shalloway, D. and Guan, J. L. (1994) *Mol. Biol. Cell.* **5**, 413–421
- Calalb, M. B., Polte, T. R. and Hanks, S. K. (1995) *Mol. Cell. Biol.* **15**, 954–963
- Juliano, R. L. and Haskill, S. (1993) *J. Cell Biol.* **120**, 577–585
- Frisch, S. M. and Francis, H. (1994) *J. Cell Biol.* **124**, 619–626
- Meredith, J. E., Fazeli, B. and Schwarz, M. A. (1993) *Mol. Biol. Cell* **4**, 953–961
- Frisch, S. M., Vuori, K., Ruoslahti, E. and Chan-Hui, P.-Y. (1996) *J. Cell Biol.* **134**, 793–799
- Hanks, S. K., Calalb, M. B., Harper, M. C. and Patel, S. K. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8487–8489
- Schaller, M. D., Otey, C. A., Hildebrand, J. D. and Parsons, J. T. (1995) *J. Cell Biol.* **130**, 1181–1187
- Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1993) *J. Cell Biol.* **123**, 993–1005
- Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1994) *Mol. Biol. Cell* **6**, 637–647
- Schaller, M. D. and Parsons, J. T. (1995) *Mol. Cell. Biol.* **15**, 2635–2645
- Hildebrand, J. D., Taylor, J. M. and Parsons, J. T. (1996) *Mol. Cell. Biol.* **16**, 3169–3178
- Polte, T. R. and Hanks, S. K. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10678–10682
- Chen, H.-C. and Guan, J.-L. (1994) *J. Biol. Chem.* **269**, 31229–31233
- Schaller, M. D., Borgman, C. A. and Parsons, J. T. (1993) *Mol. Cell. Biol.* **13**, 785–791
- Richardson, A. and Parsons, J. T. (1996) *Nature (London)* **380**, 538–540
- Reynolds, A. B., Roesel, D. J., Kanner, S. B. and Parsons, J. T. (1989) *Mol. Cell. Biol.* **9**, 629–638
- Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J. and Suttrave, P. (1987) *J. Virol.* **61**, 3004–3012
- Brooker, G., Terasaki, W. and Price, M. (1976) *Science* **194**, 270–276
- Kaelin, W. G., Krek, W., Sellers, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blunar, M. A., Livingston, D. M. and Flemington, E. K. (1992) *Cell* **70**, 351–364
- Vancura, A., O'Connor, A., Patterson, S. D., Mizra, U., Chait, B. T. and Kuret, J. (1993) *Arch. Biochem. Biophys.* **305**, 47–53
- Boyle, W. J., Van Der Geer, P. and Hunter, T. (1991) *Methods Enzymol.* **201**, 110–149
- Jelinek, T. and Weber, M. J. (1993) *Biotechniques* **15**, 629–630
- Luo, K., Hurley, T. R. and Sefton, B. M. (1991) *Methods Enzymol.* **201**, 149–152
- Shannon, J. D. and Fox, J. W. (1995) *Techniques in Protein Chemistry VI* (Crabb, J. W., ed.), pp. 117–123, Academic Press, New York
- Pearson, R. B. and Kemp, B. E. (1991) *Methods Enzymol.* **200**, 62–81
- Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990) *J. Biol. Chem.* **265**, 5267–5272
- Miyamoto, S., Akiyama, S. K. and Yamada, K. M. (1995) *Science* **267**, 883–885
- Glass, II, W. F. and Kreisberg, J. I. (1993) *J. Cell. Physiol.* **157**, 296–306
- Morton, D. M. and Tchao, R. (1994) *Cell Motil. Cytoskeleton* **29**, 375–382
- Andre, E. and Becker-Andre, M. (1993) *Biochem. Biophys. Res. Commun.* **190**, 140–146
- Hens, M. D. and DeSimone, D. W. (1995) *Dev. Biol.* **170**, 274–288