Phosphorylation of Adenovirus DNA-binding Protein

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SUMMARY

Evidence is presented here which indicates that the adenovirus DNA-binding protein (DBP) is phosphorylated at a tyrosine residue early in infection. This was suggested by the discovery that a proportion of the label in \(^{32}P\)-labelled DBP was resistant to alkali, and was substantiated by acid hydrolysis of DBP immunoprecipitates and by immunoblotting with a monoclonal antibody against phosphotyrosine. Treatment of \(^{35}S\)-methionine-labelled DBPs with chymotrypsin produced fragments of apparent Mr 45K and 39K whereas digestion of \(^{32}P\)-labelled DBP resulted in fragments of 45K and 26K. Consideration of the distribution of \(^{32}P\) label and its alkali stability in these fragments suggested that chymotrypsin cleaved populations of DBP at different sites depending on their phosphorylation states. The conservation, in all of the seven adenovirus serotypes sequenced, of a tyrosine residue (at amino acid 195 in adenovirus type 2) together with its surrounding residues, suggests that phosphorylation/dephosphorylation at this tyrosine residue may be important in various functions ascribed to the DBP.

INTRODUCTION

Adenoviruses encode a non-structural phosphorylated polypeptide which is first synthesized early in infection and binds to ssDNA, playing a key role in DNA replication and in other regulatory events (for review, see Hay & Russell, 1989). In adenovirus type 2 (Ad2) and 5 this polypeptide, designated the DNA-binding protein (DBP), has an apparent Mr of 72K from analysis by SDS-PAGE, a value significantly higher than the value of 59K calculated from sequence data. Isoelectric focusing has resolved purified DBP into as many as 15 subspecies, of which at least some result from differing degrees of phosphorylation (Klein et al., 1979; Linne & Philipson, 1980). However Linne & Philipson (1980) showed that even after extensive treatment with bacterial alkaline phosphatase its apparent Mr was reduced by only 2K. Therefore the mobility upon electrophoresis has been ascribed mainly to its relatively high proline content and to its highly asymmetrical configuration (van der Vliet et al., 1978).

Genetic and mutation analyses have indicated that there are at least two distinct domains in the protein (Rice & Klessig, 1984). Mutations which affect the ability of the virus to grow in monkey cells appear to be confined to the N-terminal region (Anderson et al., 1983; Brough et al., 1985) whereas the functions relating to DNA replication and to DNA- and RNA-binding are located in the C-terminal domain (Ariga et al., 1980; Cleghon & Klessig, 1986; Seiberg et al., 1989). It has been suggested (Linne & Philipson, 1980; Klein et al., 1979) that these two domains can be separated by chymotrypsin treatment of the DBP; indeed some evidence of specific breakdown can be seen in extracts of infected cells. However the exact border of the domains is unknown with reported C-terminal fragments ranging from 34K to 45K. One definition of a C-terminal domain component comes from a study by Tsernoglou et al. (1985) who carried out a controlled chymotrypsin digest of the 72K polypeptide to produce a 39K fragment via intermediates of approximately 45K. They were able to crystallize the 39K fragment and, in addition to deducing its coding sequence to be residues 174 to 525 of the parent molecule, showed it to be active in DNA replication in vitro. The other proposed chymotryptic fragment
was a 26K N-terminal polypeptide, shown to be heavily phosphorylated (Klein et al., 1979; Linne & Philipson, 1980).

Although several attempts have been made to relate the phosphorylation patterns of the DBP to functional aspects, the results have been inconclusive. Linne & Philipson (1980) suggested that a chymotryptic 45K fragment retained DNA-binding activity but had no phosphate groups, all the phosphate groups being located on the smaller 26K fragment. On the basis of these studies they concluded that the ability to bind DNA was independent of the phosphorylation events. However an earlier study by Klein et al. (1979) showed that although about 10 to 11 phosphorylated peptides were in the N-terminal fragment, one or two phosphorylated peptides could be detected also in the larger C-terminal fragment. Moreover they provided some evidence that the degree of phosphorylation of the N-terminal fragment could have an effect on the strength of DNA-binding although the latter was mediated by the C-terminal fragment. These differences, and the findings by Linne & Philipson (1980), that the degree of phosphorylation of the DBP varies with infection time, doubling when DNA replication is blocked by cytosine arabinoside, leave open the possibility that phosphorylation/dephosphorylation events play an important role in the multitude of functions already assigned to the DBP.

In this study we have re-examined the extent and nature of phosphorylation occurring throughout the course of Ad2 infection, taking care to inhibit, as far as possible, the action of phosphatases. We also re-examined the concept that chymotrypsin cleaves the DBP to give an unphosphorylated 45K C-terminal fragment and a heavily phosphorylated 26K N-terminal fragment. We have shown that the 45K and 26K fragments are both phosphorylated and more significantly that a phosphotyrosine is present in the DBP early in infection. We have interpreted our results on the basis of two subpopulations of the DBP which differ both in their phosphorylation patterns and in their chymotryptic cleavage sites.

**METHODS**

**Cells and virus.** HeLa cells were grown in suspension in Glasgow S-MEM, supplemented with 7% newborn calf serum (NCS) at a density of 3 x 10^5 to 6 x 10^5 cells per ml. Ad2 was grown in suspension cells, titrated and purified as described previously (Russell et al., 1967; Winters & Russell, 1971).

Monolayers of HeLa cells were grown in Glasgow MEM supplemented with 10% NCS. Ad2-infected and mock-infected cell extracts were prepared as follows. Subconfluent monolayers of HeLa cells in 75 cm^2 flasks were infected with 20 p.f.u./cell of Ad2. After absorption for 1 h at 37°C cells were maintained in MEM containing 2% NCS. Where required, 10 mM-hydroxyurea was added at this stage to the Ad2-infected and mock-infected cells. At 18 h post-infection (p.i.) cells were harvested and washed three times in phosphate-buffered saline containing 10 mM-ZnSO_4_ and 50 mM-NA_2_PO_4_. Cells were resuspended in 100 µl 20 mM-Tris-HCl, 30 mM-NACl, 10 µM-ZnSO_4_, 50 µM-NA_2_PO_4_, 1 mM-PMSE at pH 7.5 (Buffer A) and sonicated on ice using a MSE probe sonicator, four times for 15 s. Protein concentrations were determined by Coomassie Brilliant Blue dot blot analysis and volumes were adjusted to give protein concentrations of 10 mg/ml in infected and mock-infected extracts.

**35S- and 32P-labelling.** At various times after infection Ad2-infected and mock-infected cells growing as monolayers in 25 cm^2 flasks were labelled with [35S]methionine and [32P]orthophosphate. Medium was removed and replaced with 1 ml of phosphate-free or methionine-free MEM containing 1% NCS together with 0.5 mCi [32P]orthophosphate or 50 µCi [35S]methionine (both from Amersham). The cell monolayers were then incubated for a further 1 h and harvested as above.

**SDS-PAGE.** Samples were analysed by SDS-PAGE as previously described (Russell & Blair, 1977). Relative mobilities were determined by running standard proteins as markers. Alkali treatment of gels was carried out by placing the dried gel in 2 M-KOH, incubating for 2 h at 55°C and then washing the gel in 25% methanol, 4% acetic acid. Gels were dried and exposed to X-ray film for various times.

**Preparation of DBP and anti-DBP sera.** DBP was purified from cells infected at 100 p.f.u./cell for 22 h in the presence of hydroxyurea by submitting nuclear extracts to chromatography on DEAE-cellulose, phosphocellulose and finally denatured DNA-cellulose (Enomoto et al., 1981). Antiserum against purified DBP was prepared by standard protocols in guinea-pigs.

**Precipitation of immune complexes.** Cell extracts were sonicated using a probe (MSE probe sonicator) four times for 15 s, on ice, and clarified by centrifugation (500 g, 15 min). To 50 µl of clarified extracts, 10 µl of the polyclonal anti-DBP serum was added. The mixture was left on ice for 3 h, then 200 µl of fixed staphylococcus (Cowan strain A) was added followed by a further 30 min incubation on ice. The immune complex thus generated was sedimented at 500 g for 5 min and the pellet washed three times in 10% sucrose in Buffer A prior to dissociation and analysis by SDS-PAGE (Russell et al., 1981).
Phosphorylation of Ad2 DBP

Chymotrypsin digestion. Samples (50 µg) of Ad2-infected extracts labelled with 32P, or with [35S]methionine at 24 h p.i. were incubated at room temperature for 30 min in the presence of 0.1 to 2 µg/ml chymotrypsin. Reactions were stopped by the addition of 5 µl 20 mM-PMSF. DBP and its antigenic chymotryptic fragments were then precipitated using anti-DBP sera as described above.

In vitro phosphorylation. Thirty µl of Ad2-infected or mock-infected extracts, 10 µl of 20 mM-Tris-HCl, 40 µM-ZnSO4, 120 µM- Na3VO4 at pH 7.5, and 10 µCi [α-32P]ATP (Amersham) were mixed and incubated for 1 h at 4 °C. Reactions were stopped by boiling samples in SDS denaturing mix for 5 min prior to being analysed by SDS-PAGE as described above.

Phospho-amino acid analysis. Fifty µl of the appropriate sample was placed in a hydrolysis tube along with 90 µg of phosphotyrosine, 60 µg of phosphoserine, 40 µg of phosphothreonine and 150 µl of concentrated HCl. The tube was placed in a heating block at 108 °C for 3 h. Water (200 µl) was then added to the tube and the solution was lyophilised. High voltage paper electrophoresis of the sample was carried out according to Hunter & Sefton (1983).

Densitometry. Where quantitative information was required, autoradiograms from gels were scanned on a Vitatron TLD densitometer and peak areas were determined.

Immunoblotting. Cell extracts (150 µg of protein) and purified DBP (10 µg of protein) were separated by SDS-PAGE and electroblotting was performed essentially as described by Russell & Precious (1982). The transfer buffer was 25 mM-Tris-HCl, 133 mM-glycine, 0.5 mM-Na3VO4, 20% methanol, pH 8.5. The blotted nitrocellulose sheet was blocked in 20 mM-Tris HC1 pH 7.5, 150 mM-NaCl (TBS), 3% bovine serum albumin (BSA), for 2 h at room temperature and then incubated for 3 h with either a 1 : 250 dilution of anti-DBP polyclonal sera or a 1 : 200 dilution of an anti-phosphotyrosine monoclonal antibody (MAb) (ICN Biochemicals) in TBS-BSA. To confirm the specificity of the MAb, control blots were carried out with 40 mM-phosphotyrosine, or 40 mM-phosphoserine included in the reaction buffers (TBS contained 50 mM-Tris HC1 and adjusted to control pH).

RESULTS

Phosphorylation patterns in Ad-infected cells

Previous experiments (Linne et al., 1977; Russell & Blair, 1977) had established that during the course of Ad infection a sequence of phosphorylation events occurred in the infected cells, as observed by SDS-PAGE followed by autoradiography. A prominent early event was the phosphorylation of DBP, followed at later times of infection by other phosphorylations such as that of the virus-encoded 100K polypeptide. Our initial studies analysing in vivo 32P-labelled cells at various times after infection confirmed these observations. Further characterization of labelled polypeptides was undertaken by treating the gels with 2 M-KOH at 55 °C. This procedure has been shown to remove phosphate groups from the alkali-labile phosphoserine and threonine residues, leaving alkali-resistant phosphorylations which are attributed to phosphotyrosine residues (Hunter & Sefton, 1980).

Fig. 1 (a) shows the phosphorylation patterns from such an experiment together with the corresponding patterns after alkali treatment. An immunoprecipitate obtained by treatment of a 32P-labelled, infected cell extract with polyclonal antiserum against purified DBP was also analysed in this way. The results indicated that most of the labelled polypeptides were sensitive to alkali treatment but that an alkali-resistant 32P-labelled component was present in the immunoprecipated DBP and also in the corresponding 72K polypeptide in the infected cells. Moreover, by analysing the amount of 32P label on the DBP at different times after infection using scanning densitometry, it was clear that although the degree of labelling of the DBP increased up to 24 h the proportion of alkali-resistant phosphorylation was the greatest at the early infection times (Fig. 1 (b)).

Chymotrypsin digestion

To examine the gross distribution of phosphorylated amino acid residues within DBP, cells were labelled with 32P, for 1 h at 24 h p.i. (highest total incorporation of 32P into the DBP). A parallel labelling with [35S]methionine was also carried out. Cell extracts were prepared and treated with chymotrypsin (0-1 to 2 µg/ml) after which the DBP and its proteolytic fragments were precipitated by the polyclonal anti-DBP serum and analysed by SDS-PAGE and autoradiography. Fig. 2 (a) shows that at a chymotrypsin concentration of 1 µg/ml there are two 32P-labelled fragments with apparent Mr values of 45K and 26K (Fig. 2, lane 2). However after digestion with higher concentrations of chymotrypsin (2 µg/ml), a 32P-labelled product of 45K
could not be detected. Alkali treatment of these gels failed to give any indication of alkali-resistant $^{32}$P label in either fragment (data not shown). A parallel digestion of $^{35}$S-labelled DBP indicated that the 45K $^{35}$S-labelled fragment was digested at the higher chymotrypsin concentrations to give a fragment with an apparent $M_r$ of 39K (Fig. 2, lane 6). A similar experiment utilizing extracts of cells labelled 15 h p.i. (with a greater proportion of alkali-resistant phosphorylation, Fig. 1b) showed a similar pattern of fragmentation using 0·1 to 2 μg/ml chymotrypsin, but revealed more significantly an alkali-resistant component in the 26K fragment (Fig. 2b, lanes 2 and 3).

The chymotrypsin digests of $^{[35S]}$methionine-labelled DBP indicated that the 45K fragment was a precursor of a 39K fragment, presumably identical to the one shown by Tsernoglou et al. (1985) to be derived from the C-terminal part of the molecule. In parallel digests the 45K fragment was shown to contain the $^{32}$P label, suggesting, in contrast to the results of Linne & Philipson (1980), that this C-terminal fragment was phosphorylated.

**In vitro labelling of cell extracts with [γ-$^{32}$P]ATP**

A similar series of experiments was carried out to ascertain whether alkali-resistant phosphate groups could be incorporated into viral proteins *in vitro*. Polypeptides phosphorylated in extracts of uninfected cells and in cells infected with Ad2 for 18 h were examined by incubation with [γ-$^{32}$P]ATP in the presence of zinc and vanadate ions at 4 °C, and analysed by SDS-PAGE and autoradiography. Fig. 3 shows that many labelled species were detected. However on treating gels with 2 M-KOH at 55 °C, most of the label (as determined by
Fig. 2. Chymotryptic degradation of \textit{in vivo} \textsuperscript{32}P- and \textsuperscript{35}S-labelled DBP. (a) Extracts of cells infected for 24 h and labelled \textit{in vivo} with either \textsuperscript{32}P (lanes 1 to 3) or \textsuperscript{35}S (lanes 4 to 6), were treated with chymotrypsin (0 to 2 \textmu g/ml) prior to Ad2 DBP and its proteolytic fragments being immunoprecipitated, were analysed by SDS-PAGE followed by autoradiography (16 h exposure). Amount of chymotrypsin: lanes 1 and 4, none; lanes 2 and 5, 1 \textmu g/ml; lanes 3 and 6, 2 \textmu g/ml. (b) Extracts of cells \textsuperscript{32}P-labelled \textit{in vivo} 15 h p.i. were treated similarly with chymotrypsin (0 to 2 \textmu g/ml) prior to immunoprecipitation of DBP. Following SDS-PAGE analysis and autoradiography (16 h; left) the gel was treated with alkali and alkali-resistant proteins represented by bands were detected by further autoradiography (48 h; right). Amount of chymotrypsin: lane 1, 0 \textmu g/ml; lane 2, 2 \textmu g/ml; lane 3, 1 \textmu g/ml; lane 4, 0.2 \textmu g/ml; lane 5, 0.1 \textmu g/ml. (Note that the gel expands on treatment with aqueous alkali.) Figures show \textit{M}_r values (K) derived from protein standards.
Fig. 3. $^{32}$P in vitro phosphorylation. Extracts from cells infected for 18 h and from mock-infected cells were labelled in vitro using [$\gamma^{32}$P]ATP as described in Methods. Phosphorylated polypeptides were analysed by SDS-PAGE followed by autoradiography (16 h exposure), lanes 1 and 3. Alkali-resistant phosphorylations were detected by alkali treatment and further autoradiography of the gel (48 h), lanes 2 and 4. Lanes 1 and 2 represent uninfected cells; lanes 3 and 4, infected cells. (Note that the gel expands on treatment with aqueous alkali.) Figures show Mr values (K) derived from protein standards.

Fig. 4. Phospho-amino acid analysis. Immunoprecipitated in vivo $^{32}$P-labelled DBP was acid-hydrolysed, lyophilized and the labelled phospho-amino acids were separated by high voltage paper electrophoresis along with standards (phosphoserine, P-S; phosphothreonine, P-T; phosphotyrosine, P-Y). The figure shows the resultant autoradiogram with a densitometric scan below.

autoradiography) was removed, the exception being two components in the infected cell track, of which one, with an apparent Mr, of about 100K, was also present in the uninfected cell extract. The band specific to the infected cell pattern corresponded in mobility to the DBP, which suggests that a phosphotyrosine residue or other alkali-resistant phosphorylated residue was present in the in vitro phosphorylated product.

Phospho-amino acid analysis

The alkali resistance of a fraction of the phosphate groups on DBP suggested that a phosphotyrosine residue might be present and a more direct indication was sought by phospho-amino acid analysis. As shown in Fig. 1(b), the proportion of alkali-resistant $^{32}$P label was highest at earlier infection times. Consequently, $^{32}$P-labelled DBP was isolated by immunoprecipitation from cell extracts 15 h p.i., and subjected to acid hydrolysis. Separation of the resultant phospho-amino acids by high voltage electrophoresis revealed the presence of four components (Fig. 4). One corresponded to free phosphate whilst the other species migrated to the positions corresponding to phosphoserine, phosphothreonine and phosphotyrosine. Although the major components were clearly coincident with phosphoserine and phosphothreonine, a densitometric scanning of the autoradiogram indicated that up to 2% of the total radioactivity was associated with phosphotyrosine.
Fig. 5. Immunoblots of extracts of Ad2-infected and uninfected cells and partially purified DBP. Cell extracts were subjected to SDS-PAGE and blotted onto nitrocellulose. A parallel incubation with an anti-DBP polyclonal serum (left) and with an anti-phosphotyrosine MAb (right) resulted in the autoradiograms shown. Lanes 1, purified adenovirus; lanes 2, partially purified DBP (10 μg); lanes 3, infected and hydroxyurea-inhibited cell extracts; lanes 4, infected cell extracts; lanes 5, uninfected and hydroxyurea-inhibited cell extracts; lanes 6, uninfected cell extracts. Figures show Mr values (K) derived from protein standards.

Immunoblotting

To confirm the presence of phosphotyrosine in DBP a MAb against phosphotyrosine was used to probe infected cell extracts. In these experiments cells were grown and infected in the presence of sodium vanadate, a phosphatase inhibitor (Swarup et al., 1982). Hydroxyurea was added to some cultures to inhibit DNA replication thereby allowing only early events to occur. Extracts were prepared from mock-infected cells and cells infected for 18 h in the presence and absence of hydroxyurea. These extracts were then analysed by SDS-PAGE and the electrophoretogram was probed after blotting with the anti-phosphotyrosine MAb and with a control polyclonal anti-DBP serum. Fig. 5 shows that the anti-phosphotyrosine MAb reacted with a band corresponding to the 72K DBP in the hydroxyurea-inhibited, infected cell extracts (Fig. 5, lane 3). There was no corresponding reaction in lanes containing the mock-infected and the 18 h Ad2-infected extracts. The MAb also failed to recognize purified DBP prepared by DNA-cellulose chromatography, but did react with a protein of apparent Mr, 25K, present in both infected and uninfected cell extracts and also with a protein of apparent Mr, 100K, present only in the hydroxyurea-inhibited, infected cell extracts. In another experiment it was found that the inclusion of phosphotyrosine in the reaction buffers completely removed all bands whilst the addition of phosphoserine had no effect (data not shown).

DISCUSSION

The results presented here confirm earlier observations that the DBP is phosphorylated (Russell & Blair, 1977; Linne & Philipson, 1980). However these observations have been extended, in particular, by showing the presence of a phosphotyrosine residue within the DBP. This modification was first suggested by the presence of alkali-resistant ³²P-labelled bands on
SDS-PAGE from in vivo and in vitro labelling experiments (Fig. 1a, 2b and 3) and was confirmed by phospho-amino acid analysis (Fig. 4) and by immunoblotting with a specific anti-phosphotyrosine antibody (Fig. 5). The phosphotyrosine residue was shown to be present in DBP immunoprecipitated from cells labelled with $^{32}$P and harvested 15 h p.i. and by immunoblotting following the addition of hydroxyurea, but not in cells from later infection times nor, interestingly, in DBP purified on DNA-cellulose. The inability to detect phosphotyrosine residues at the later times by immunoblotting may to some extent, be a reflection of the relative insensitivity of the immunoblotting procedure compared to detection by alkali-stable $^{32}$P-labelling. In addition our evidence suggests that the phosphorylation event at tyrosine in the DBP occurs earlier in infection than the bulk of the phosphorylations at serine and threonine residues and therefore may not be readily detected at later times of infection.

The results of the studies using chymotrypsin (Fig. 2) suggest that a variety of chymotryptic fragments can be obtained as a function of the different digestion conditions. When $^{32}$P- and $^{35}$S-labelled cell extracts were digested in parallel, 45K, 42K and 26K phosphorylated species could readily be detected. However the $^{35}$S-labelled fragments included only a 45K species that was progressively digested to a 39K fragment at increasing concentrations of chymotrypsin. By examining the sequence of the DBP and applying the criteria for detecting putative phosphorylated residues (Leader & Katan, 1988) it can be seen (Fig. 6a) that most of these are concentrated in the N-terminal portion of the molecule. All but one of these putative phosphorylation sites are serines and threonines, there being a tyrosine with the appropriate environment at position 195. It can be seen that about 10 of the sites are conserved when the sequences of the different adenovirus serotypes (Kitchingman, 1985; Vos et al., 1988) are analysed; thus phosphorylation of these could be of functional importance. It is interesting that a recent study by Cleghon et al. (1989) showed that although a mutant DBP with a deletion from residues 23 to 105 was defective in nuclear transport it was still viable. Such a mutant would be deficient in a number of the putative phosphorylation sites but nevertheless still appeared to be phosphorylated.

Previously it has been assumed (Klein et al., 1979; Schechter et al., 1980; Brough et al., 1985) that a digestion of DBP by chymotrypsin produces two primary fragments: a 45K C-terminal polypeptide and a 26K N-terminal fragment. Whereas Schechter et al. (1980) found a 26K fragment by Coomassie Brilliant Blue staining, none of the studies of chymotrypsin digestions of $^{35}$S-labelled DBP (Klein et al., 1979; Linne & Philipson, 1980; and the present study), nor those of $[^{3}H]$leucine-labelled DBP (Klein et al., 1979) showed a 26K fragment. Originally it was presumed that the 26K fragment was grossly deficient in methionine and leucine residues, but more recent sequence studies (Kitchingman, 1985) have shown that methionine and leucine residues do occur in the proposed N-terminal fragment, although the latter is relatively depleted in leucine. The major evidence for the 26K fragment comes from phosphorylation studies and as in the experiments described here, this fragment is significantly labelled. However our studies also show that in milder digestion conditions, $^{32}$P-labelling could be detected in the 45K fragment. Further processing to the 39K fragment, consistent with the removal of residues from the N-terminal side of residue 174, appeared to be accompanied by the loss of phosphate label. If this deleted section of the molecule is assumed to be about 50 residues long it will contain three putative phosphorylation sites (Fig. 6b). The finding of alkali-resistant $^{32}$P label (presumably due to the presence of a phosphotyrosine residue) in the 26K fragment derived from DBP labelled at 15 h (Fig. 2b) suggests that this fragment must extend as far as residue 195, since this is the tyrosine residue nearest to the N terminus (Fig. 6a,b). The inability to detect an alkali-resistant 26K fragment at 24 h p.i. is presumably a reflection of the relatively low proportion of alkali-resistant label at later times of infection (Fig. 1b). Thus our results suggest that the 26K and 45K fragments are overlapping fragmentation products derived from separate subpopulations of DBP. It should also be noted that the 26K fragment was detected in chymotrypsin digests of $^{32}$P-labelled DBP but not in digests of $^{35}$S-methionine-labelled DBP. The distribution of methionine in DBP (Fig. 6a) is such that if the 26K and 45K fragments were present in equimolar amounts, both the fragments should have been detected following chymotrypsin digestion of $^{35}$S-methionine-labelled DBP. The discovery that the 26K fragment was detectable
Fig. 6. (a) Amino acid sequence of Ad2 DBP. Putative phosphorylated residues are marked with an asterisk (*). The chymotrypsin cleavage site resulting in the 39K fragment is also marked with an arrow (↓). (b) Diagrammatic representation of the Ad2 DBP polypeptide. The DBP contains 527 amino acids with the putative phosphorylation sites marked (↓). The chymotrypsin cleavage site resulting in the 39K fragment is also marked with an arrow (↓) at residue 174. Our studies suggest that the 39K fragment is a proteolytic product of the larger 45K fragment, the N terminus of which is estimated from the apparent Mr. Possible positions for the 26K fragment which also contains a phosphorylated tyrosine residue (i.e. overlapping the 45K fragment) are shown as broken lines. This fragment could arise from either one or two new sites being available to the chymotrypsin. (c) Sequence data showing the tyrosine residue at position 195 in the DBP is conserved together with its surrounding residues in all seven Ad types analysed so far (sequence data from Kitchingman, 1985; Vos et al., 1988; this latter reference highlights the conserved sequences in all seven types). The conserved acidic residues are boxed.
when derived from $^{32}$P-labelled DBP (Fig. 2) suggests that it originates from a heavily phosphorylated minor population of DBP. Furthermore, since both the 39K fragment and the 26K fragment appear to contain the critical tyrosine residue at position 195 (in a phosphorylated state in the latter fragment), they must therefore overlap. It seems likely that chymotrypsin must cleave at different sites in the DBP when this tyrosine (and possibly other putative residues on its C-terminal side) is phosphorylated, perhaps as a result of conformational changes (see Fig. 6b).

It is significant that the tyrosine residue in the DBP at position 195, with the appropriate environment of acidic residues (Leader & Katan, 1988; Hunter & Cooper, 1985), is conserved together with its surrounding residues in all seven adenovirus types so far sequenced (Fig. 6c) suggesting that this tyrosine fulfils an important role in the function of the DBP.

Also of interest in this regard is the suggestion by Brough et al. (1985), based on studies on $hr$ phenotypes, that residues 130 and 148 of the DBP in this region of the molecule could be responsible for subtle structural changes enabling the DBP to interact with cell factors required for the expression of viral late genes. These authors point out that a region of the DBP molecule centred on residues around 160 is in an extremely hydrophilic region and could function as a hinge region separating two functional domains. Could a phosphorylation/dephosphorylation event at tyrosine 195 induce modifications in this region? It is important to note that in the work reported here, vanadate and zinc, which are effective phosphatase inhibitors (Antoniw & Cohen, 1976; Swarup et al., 1982), were included at all stages, and this may explain our consistent finding of phosphorylation in the 45K fragment and of phosphotyrosine in DBP from early infected extracts. Additionally, cells were grown in the presence of vanadate in preparing the extracts to be probed with the anti-phosphotyrosine MAb. In their investigations Brough et al. (1985) failed to detect phosphotyrosine in $hr$ or $wt$ DBP and it seems likely that this was because they analysed cells infected later in infection and without taking the precautions noted above in respect of inhibiting phosphatases.

It is interesting that the putative phosphorylations of tyrosines were noted by alkali resistance (Fig. 1a, 2b and 3) and by immunoblotting (Fig. 5) in infected and uninfected cells. A number of reports (Hamaguchi et al., 1988; Kamps & Sefton, 1988) have shown that there are a variety of substrates for tyrosine protein kinases in cells. In these experiments we have not analysed further the possibility that the 100K component noted in the blocked infected cells (Fig. 5) is a virus encoded gene product of similar $M_r$ or is a transiently modified cellular protein. These investigations therefore provide further data to illustrate the complex nature of the phosphorylations occurring during adenovirus infection. A serine/threonine protein kinase has been shown to be tightly associated with purified virions (Russell & Blair, 1977) although it has never been demonstrated that this enzyme (or any other cellular protein kinase) performs a specific function during infection. A tyrosine kinase (and presumably phosphatases) may also be involved and it remains to be seen whether further studies will be successful in revealing the interplay of these enzymes and their relationship to various functional properties which have been assigned to DBP.

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