The R1 Subunit of Herpes Simplex Virus Ribonucleotide Reductase Is a Good Substrate for Host Cell Protein Kinases but Is Not Itself a Protein Kinase

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The N terminus of the R1 subunit of herpes simplex virus type 2 ribonucleotide reductase is believed to be a protein kinase domain mainly because the R1 protein was phosphorylated in a protein kinase assay on blot. Using Escherichia coli and adenovirus expression vectors to produce R1, we found that, whereas the reductase activity of both recombinant proteins was similar, efficient phosphorylation of R1 and loss in the presence of MnCl2 was obtained only with the R1 purified from eukaryotic cells. Phosphorylation of this R1, in solution or on blot, results mainly from the activity of casein kinase II (CKII), a co-purifying protein kinase. Labeling on blot occurs from CKII leakage off the membrane and its subsequent high affinity binding to in vivo CKII-phosphorylated R1. CKII target sites were mapped to an acidic serine-rich segment of the R1 N terminus. Improvement in purification of the R1 expressed in eukaryotic cells nearly completely abolished its phosphorylation potential. An extremely low level of phosphorylation observed in the presence of Mg2+ with the R1 produced in E. coli was probably due to an unidentified prokaryotic protein kinase. These results provide evidence that the herpes simplex virus type 2 R1 does not possess an intrinsic protein kinase activity.

The herpes simplex virus type 1 and type 2 (HSV-1 and -2)1 ribonucleotide reductases, which convert ribonucleoside diphosphates to the corresponding deoxyribonucleotides, play a key role in the synthesis of viral DNA (1). A peculiar feature of the HSV-1 and HSV-2 ribonucleotide reductases was found in the amino acid sequence of their R1 proteins; in contrast to the R1 of other species, including those of other herpesviruses, the HSV-1 and -2 R1 subunits possess an N-terminal extension of about 350 amino acids (2, 3). It has been clearly shown that this extension, which appears to be linked to the reductase domain by a protease-sensitive region, is dispensable for ribonucleotide reduction (4–7).

From sequence comparisons with eukaryotic PKs, Chung et al. (8) were the first to propose that the unique N-terminal domain of HSV R1 could be a PK domain. Among the experimental evidence that has been accumulated thereafter in favor of this hypothesis, the more convincing are the following: (i) the N terminus of the HSV-2 R1 produced with a bacterial expression system and purified by immunoprecipitation is able to phosphorylate histones and calmodulin (9); (ii) both HSV-1 and -2 R1 are labeled by the ATP analogue [14C]FSBA, which co-valently binds to the active-site lysine of eukaryotic PKs (10, 11); (iii) both HSV-1 and -2 R1 produced in eukaryotic cells retrieve their capacity to be phosphorylated after migration on a denaturing polyacrylamide gel and renaturation on blot (11, 12); (iv) a protein exhibiting a weak homology with the N-terminal domain of HSV-2 R1 (termed FAST) was described as a PK involved in the phosphorylation of TIA-1 during Fas-induced apoptosis (13).

However, subsequent observations indicated that the R1 N-terminal domain should belong to a novel type of PK. Deletions of different parts of the protein showed that several of the classical PK consensus sequences could be removed without loss of protein phosphorylation (10, 11). [14C]FSBA, which inhibits the R1 labeling by γ-32P[ATP, does not bind to residues located in the putative PK domain but to a site in the reductase domain (10). Phosphorylation of histones observed with the HSV-1 and -2 R1 proteins purified following expression in Escherichia coli was shown to be the result of a contaminant prokaryotic kinase; surprisingly, this kinase appears to be able to phosphorylate, in the presence of MnCl2, several other eukaryotic proteins. After elimination of the prokaryotic kinase responsible for the histone phosphorylation, both types of R1 retain a weak capacity to be phosphorylated in the presence of MnCl2 (1 R1 molecule/~2,400 being labeled in 30 min). From these observations, even if the rate of 32P incorporation was very low, it was concluded that these proteins had intrinsic PK activity (10, 14). However, in the above mentioned studies, the possibility that the R1 phosphorylation was accomplished by residual contaminant PK(s) was never completely ruled out.

During the development of E. coli and adenovirus expression vectors for the production of HSV-2 R1, we observed that efficient phosphorylation of R1 protein and of casein was obtained only with preparations purified from eukaryotic cells. As improvement in the purification of the HSV-2 R1 expressed in eukaryotic cells greatly diminished the capacity of the protein

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† The abbreviations used are: HSV, herpes simplex virus; PK, protein kinase; FSBA, p-fluorosulfonylbenzoyl 5'-adenosine; CKII, casein kinase II; DTT, dithiothreitol; NDPK, nucleoside diphosphate kinase; PAGE, polyacrylamide gel electrophoresis.

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Phosphorylation of R1 was critically reanalyzed in its origin(s) of R1 phosphorylation for both recombinant proteins. We found that the PK activity attributed to R1 was due to contaminating cellular PKs.

EXPERIMENTAL PROCEDURES

Reagents—Casein, b calf thymus histones, calmodulin, heparin, polylysine, and protamine sulfate were from Sigma. \( \gamma^\text{-32P} \)ATP (4,500 Ci/\text{mmol}) and \( \alpha^\text{-32P} \)ATP (4,000 Ci/\text{mmol}) were from ICN and \( \text{[H]} \text{CDP} \) (18 Ci/\text{mmol}) from Amersham Life Science. Affi-Prep 10 support, bovine serum albumin standard, and the Bio-Rad protein assay kit were from Bio-Rad. Polyclonal antibodies directed against a peptide corresponding to residues 70–91 of the CKII beta was kindly provided by S. Pelech (15). True human recombinant CKII was from Boehringer Mannheim. Purified HSV-1 R1 (DN247) was kindly provided by Joe Conner (14).

K Purification—The recombinant adenovirus Ad5BM5-R1 was used to produce BSM5-R1 in human 293S cells as described previously (16). Crude cytoplasmic extracts of infected cells were prepared by Dounce homogenization of phosphate-buffered saline washed cells suspended in ice-cold buffer A (50 mM Hepes pH 7.6, 2 mM DTT) followed by a 12,000 \( \times \)g centrifugation. The resulting supernatant (S1) was clarified by centrifugation at 100,000 \( \times \)g for 1 h at 4 °C (S2). The production and purification of the HSV-2 R1 in E. coli with a pET vector (pET-R1) were performed as described by Furlong et al. (17) with the exception that the proteins were precipitated by 25% ammonium sulfate instead of 45%.

The peptidolluxinity method used for the R1 purification was modified from the work developed for R1 expressed in HSV-1 infected cells (18). Briefly, 100–150 mg of S100 proteins were diluted at 2 mg/ml in buffer A containing 2.5 mM bacitracin and loaded on a 40-ml column of Affi-Prep-coupled peptide. After washing with 50 column volumes of buffer A, the R1 protein was eluted with 15 ml of buffer A containing 200 \( \mu \)M peptide acetyl-YAGAIVNDL. In some cases, a high salt wash (40 ml of 2 M NaCl in buffer A) was performed before the R1 elution. Ultrafiltration with Centriprep-30 (Amicon) was used to concentrate the eluted R1 and to reduce the concentration of the eluting peptide below 0.2 \( \mu \)M. The protein purity was assessed by densitometric scanning of Coomassie Blue-stained gels as determined by laser densitometric comparison with R1 standard. To evaluate the percentage of recombinant R1 in protein extracts was done by densitometric scanning of Coomassie Blue-stained gels and by densitometric scanning of a lane containing 10 \( \mu \)g of protein on a Coomassie Blue-stained gel and by laser densitometric analysis performed with a polyclonal rabbit antiserum to the recombinant Ad5BM5-R1. Purification of R1 (23). Briefly, purified R1 protein was incubated for 15 min at 37 °C usually at a concentration of 2 \( \mu \)M with 0–10 \( \mu \)M \( \text{[\text{-32P}]} \text{ATP} \) in 150 \( \mu \)l of the phosphorylation assay mixture containing either 5 mM MgCl2 or 1 M NaCl. Ultrafiltration used to measure free ATP concentration was performed as we recently described (19). The specific binding of the nucleotide to the R1 was calculated by subtracting the nonspecific binding measured in the absence of R1.

RESULTS

HSV-2 R1 Produced in E. coli Does Not Efficiently Incorporate \( \gamma^\text{-32P} \)ATP—To study both domains of the HSV-2 R1, the R1 gene was introduced in the adenovirus Ad5\DeltaE1A3E3 to create the recombinant Ad5BM5-R1 (16), and in the bacterial expression vector pET11c, which gave pET-R1. Purification of both types of recombinant R1 by peptidolluxinity gave protein preparations, which contained <5% of host cell protein contaminants (Fig. 1). Initial phosphorylation assays were performed with MgCl2 using conditions optimized for HSV-1 R1 purified from HSV-1-infected cells (18). Surprisingly, whereas the reductase activity of BM5-R1 and pET-R1 was similar (Table I), phosphorylation of the R1 protein and casein could be observed only with preparations purified from eukaryotic cells (Fig. 2, lanes 1–4). Phosphoamino acid analysis of the \( \text{[\text{-32P}]} \text{ATP} \) detected phosphorylation on serine and threonine residues. In addition, we noticed that the rate of BM5-R1 phosphorylation among different batches of purified protein varied largely from 0.04 to 0.64 nmol/min/mg with a parallel variation in the extent of the phosphorylation of either casein (from 0.01 to 0.17 nmol/min/mg) or other substrates. Histones were \( \approx \)32-fold poorer phosphate acceptors than casein. Calmodulin was also efficiently phosphorylated but only when incubated in the presence of 10 \( \mu \)M polylysine. IgGs (either polyclonal or monoclonal) were not phosphorylated at the rate of HSV-2 ribonucleotide reductase were not phosphorylated at all. Interestingly, the formation of the ribonucleotide reductase holoenzyme by the addition of R2 did not alter the rate of R1 phosphorylation.

With pET-R1, undetectable phosphorylation (<0.5 fmol/min/mg) was further observed in assays that contained only radiactive ATP with or without adding protamine sulfate, a basic
protein that has been reported to increase 10-fold the labeling of the HSV-1 R1 expressed in E. coli also with a pET vector (10, 14). However, with the assay conditions used in the studies quoted above (MnCl₂ instead of MgCl₂ and only radioactive ATP), a very weak R1 phosphorylation (2 fmol/min/mg) could be detected, which was not enhanced by the addition of protamine sulfate. Casein, calmodulin, IgGs, and the HSV-2 R2 subunit were not phosphorylated when added to 2 μg of pET-R1, whereas histones were labeled just above background. As the maximal level of phosphorylation that we obtained for pET-R1 was 50-fold lower than the one reported by Cooper et al. (10), we first suspected that a deleterious mutation had been introduced in the N-terminal domain of the R1 gene during the vector construction. This possibility was made unlikely by the construction of two other pET-R1 expression vectors (pET-R1b and pET-R1c) using the HSV-2 R1 coding sequences contained in the pAdBM5 transfer vector, which had been sequenced prior to its use to create the BM5-R1 recombinant. The R1 protein purified from E. coli bearing either pET-R1b or -c did not incorporate higher amounts of 3²P than pET-R1 (data not shown). Second, we considered the possibility that our purification procedure had not completely eliminated phosphatases, ATP hydrolyses, and other inhibitory molecules abundantly present in crude bacterial extracts (24). As nucleotide phosphatase activity was undetectable in our pure R1 preparations, ATP degradation could not be involved in the nearly complete incapacity of pET-R1 to be labeled by γ-3²P]ATP. In addition, the low labeling of pET-R1 could not be attributed to the presence of inhibitory molecules in pET-R1 purified preparations as mixtures of pET-R1 with BM5-R1 did not impair the high 3²P incorporation observed with the latter protein (data not shown). On the contrary, when a 10-fold lower amount of BM5-R1 was introduced in the mixture, a strong increase in the R1 labeling was observed indicating that pET-R1 could be a substrate either of BM5-R1 or of a co-purifying PK (Fig. 2, lanes 9 and 10). The latter possibility became more likely when a strong R1 labeling was obtained upon the mixing of pET-R1 with a crude extract of Ad5AE1AE3-infected 293 cells (Fig. 3, lane 2). Moreover, using a purified preparation of an HSV-1 R1 protein deleted of its first 247 amino acids (DN247; see Fig. 1, lane 7), evidence was obtained that an E. coli protein could phosphorylate pET-R1. Hence, a mixture of 2 μg of DN247 (unlabeled itself) with pET-R1 produced a 16-fold increase in the phosphorylation of the later. Phosphoaminoacid analysis of 3²P-labeled pET-R1 identified serine as the only modified residue (data not shown).

Altogether, these results suggested that HSV-2 R1 does not possess an intrinsic autophosphorylating activity but instead is a good substrate for PK originating from the host cell. To further substantiate this, we next searched to identify the PK(s) co-purifying with BM5-R1 and, more importantly, to understand how such an enzyme could produce the R1 phosphorylation seen on blot.

A High Ionic Wash Decreased the BM5-R1 Phosphorylation—To demonstrate that a PK activity was co-purifying with R1, we attempted to separate the enzyme from its substrate by washing with 2 M NaCl the BM5-R1 protein bound to the affinity column. As shown in Fig. 2, this wash produced a 40-fold reduction in the R1 (compare lanes 6 and 7) and casein phosphorylation (Table I). Calmodulin and histone phosphorylations were no longer detectable (data not shown). The addition of a small fraction of the NaCl wash desalted and concentrated by ultrafiltration to separate free and bound PK(s) co-purifying with BM5-R1 and, more importantly, to demonstrate that a silver-stained gel and a ribonucleotide reductase assay (data not shown). In Table I, the capacity of the protein to incorporate 3²P, after each step of purification is compared with its reductase activity. In addition, the activity of NDPK, a highly active cellular enzyme (650 μmol/min/mg; see Refs. 25 and 26), is also presented. Whereas the specific activity of ribonucleotide reductase increased 10-fold in parallel with the concentration of the R1 subunit indicating that the purification procedure did not affect the subunit structure, the R1 phosphorylation exhibited an overall decrease of 300-fold. The observation that the decrease was much less significant after the affinity step than after the NaCl wash (8-fold compared with 40-fold) is also an indication that a co-purifying PK could be responsible of the R1 labeling. The more efficient removal for NDPK by the affinity step (30-fold compared with 5-fold) strengthens this hypothesis. It is noticeable that the residual NDPK activity is 1,000-fold greater than the rate of R1 phosphorylation. Nevertheless, it would be difficult to conclude that this activity is catalyzed by the R1 protein itself because, once again, pET-R1 did not exhibit such an activity (<1 pmol/min/mg). Thus, it is far more plausible to estimate, from the known specific activity of human NDPK, that our pure BM5-R1 preparations contain a 0.0002% level of NDPK contamination than to conclude that the HSV-2 R1 is a primitive NDPK exhibiting 8 × 10⁻⁴ less activity than the human enzyme.

As some of the motifs present in the R1 N-terminal domain are found in ATP-binding proteins which are not PKs, the ATP binding capacity of the highly purified BM5-R1 was assayed using filter ultrafiltration to separate free versus bound nucleotides and was found to be below the detection limits of our assay (Kₐ > 0.1 mM for one binding site). As a positive control of the assay, binding of CDP (one of the substrates of the reductase domain) was evaluated. These measurements gave a Kₐ value of 6 μM with 1.6 mol of CDP bound/mol of R1 subunit. A very low level of ATP binding was detected using 100 μg of the protein preparation exhibiting the highest phosphorylation potential, consistent with the presence of a trace amount of contaminating PK.
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**TABLE I**
Comparison of enzymatic activities at different stages of HSV-2 R1 purification

BM5-R1 and pET-R1 proteins were purified by peptidoaffinity with (+) or without (−) a NaCl wash during the R1 binding to the column. Enzymatic activities were measured as described under “Experimental Procedures.”

| Purification step | R1 concentration | Ribonucleotide reductase | Phosphorylation[a] | NDKP |
|-------------------|------------------|-------------------------|--------------------|------|
|                   |                  | (nmol/min/mg)           | (pmol/min/mg)      | (nmol/min/mg) |
| BM5-R1            |                  |                         |                    |      |
| S100              |                  | 2.92                    | 315                | 154.3 |
| Purified − NaCl wash | 92[b]         | 26.6                    | 40                 | 5.4  |
| Purified + NaCl wash | 95[c]         | 29.2                    | 1                  | 1.14 |
| pET-R1            |                  | 25.3                    | <0.1               | <0.001 |
| BM5-R1            |                  | 11.7                    | <0.1               | ND[c]|
| HSV-1 R1 (D247)   |                  |                         |                    |      |
| Purified − NaCl wash | 93[d]         |                        |                    |      |
| Purified + NaCl wash |              |                        |                    |      |
| BM5-R1            |                  |                         |                    |      |

[a] Measured with standard assay conditions.
[b] Measured by densitometric comparison with a standard of pure R1.
[c] ND, not determined.
[d] Evaluated by laser densitometric scanning of a lane containing 10 μg of protein on a Coomassie Blue-stained gel.

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**Fig. 2. In vitro phosphorylation of recombinant HSV-2 R1.** Analysis of 32P-labeled proteins by SDS-PAGE followed by autoradiography after incubation in the standard conditions described under “Experimental Procedures.” Left panel, comparison of BM5-R1 (2 μg, lanes 1 and 2) and pET-R1 (2 μg, lanes 3 and 4) labeling in the absence (lanes 1 and 3) or in the presence of 10 μg of casein β (lanes 2 and 4). Middle panel, BM5-R1 labeling after different steps of purification using a similar amount (2 μg) of R1 in each case: lane 5, S100; lane 6, R1 from a standard purification; lane 7, R1 from a purification with a 2 M NaCl wash; lane 8, same as in lane 7 plus 1/10 of the NaCl wash. Right panel, pET-R1 (1 μg, lane 10) labeling produced by unwashed BM5-R1 (0.1 μg); lane 9, BM5-R1 control.

**Fig. 3. Phosphorylation of recombinant HSV-2 R1 in standard and blot assays.** Left panel, analysis of 32P-labeled proteins as described in Fig. 2. Lane 1, 20 μg of an extract of Ad5ΔE1ΔE3-infected 293 cells; lanes 2–4 contain 2 μg of pET-R1 either alone (lane 2), or with 20 μg of the lane 1 extract (lane 3), or 0.5 unit of CKII (lane 4). Right panel, purified BM5-R1 (5 μg, lane 5), extract of Ad5ΔE1ΔE3-infected cells (50 μg, lane 6), same extract as in lane 6 plus 5 μg of pET-R1 (lane 7), and 5 μg of pET-R1 (lane 8) were subjected to SDS-PAGE and blotted. After denaturation and renaturation of the proteins, the membrane was incubated in the blot assay mixture, washed, and dried before autoradiography.

**R1 Phosphorylation on Blot Is Produced by a Movable PK—** The HSV2-R1 labeling seen in PK assays on blot has been taken as a nearly irrefutable proof for the autophosphorylation potential of this protein because, in such an assay, the denatured proteins are separated by SDS-PAGE before their blotting onto a membrane (11). Two hypotheses were successively considered to explain how a contaminating eukaryotic PK could be responsible for the R1 labeling on the blot. First, comigration of the contaminating PK with the R1 protein on the gel appears likely from initial observations made in PK assays on blot similar to the one presented in the right panel of Fig. 3. As can be seen, BM5-R1 from preparations unwashed with NaCl exhibited a high degree of phosphorylation (lane 5), whereas pET-R1 was not labeled (lane 8). A faint labeled band was seen at the R1 position in an adjacent lane, which contained a crude extract of Ad5ΔE1ΔE3-infected 293 cells. To test if this contaminating protein could be responsible for the R1 labeling, an aliquot of the Ad5ΔE1ΔE3 extract was mixed with pET-R1 in gel loading buffer and immediately boiled to prevent protein phosphorylation prior to gel electrophoresis. The added extract did not produce any increase in the signal seen at the R1 position (lane 7), making it unlikely that the protein involved in the R1 labeling was comigrating on the gel. In a subsequent experiment (Fig. 4), very puzzling results were obtained for R1 preparations at different stages of purification; whereas in the tube assays the extent of labeling corresponded to the degree of R1 purification, in the blot assay the most purified protein (lane 1) incorporated as much 32P as the less purified R1 (lane 2). These results led us to explore as second alternative that the R1 labeling on the blot was produced by a PK that is able to move from its location on the membrane and to interact with R1 during the PK assay.

To test this hypothesis, duplicate samples of the four R1 preparations used in the left panel of Fig. 4 were blotted again. Instead of being further processed as a whole piece containing the four lanes of migration, the membrane was cut either in strips corresponding to each lane (Fig. 4, middle panel) or in small squares around the R1 (Fig. 4, right panel). Thereafter,
each piece of membrane was treated in individual containers. The complete disappearance of R1 labeling when the protein was isolated on small squares and, labelings corresponding conversely to the degree of the R1 purification when the lanes were individually treated, strongly indicated that indeed a movable protein could be involved. Positive proof of this was obtained in experiments where the membrane corresponding to a gel lane containing 50 μg of Ad5ΔE1ΔE3 extract was cut into 10 1-cm pieces, which were each individually incubated with pieces of membrane containing only R1 (Fig. 5A). A strong R1 signal was generated by only one piece of paper which contained proteins having M<sub>r</sub> between 40,000 and 45,000. In a complementary experiment, the excision of the 40–45-kDa area of a lane containing an aliquot of the same Ad5ΔE1ΔE3 extract produced a complete disappearance of the labeling of the BM5-R1 protein loaded in an adjacent lane of the gel (Fig. 6). These results clearly showed that only protein(s) in that M<sub>r</sub> range was responsible for the R1 labeling on blot. Phosphoamino acid analysis of BM5-R1 protein <sup>32</sup>P-labeled on blot detected phosphorylation only on serine residues (data not shown).

CKII Co-purifies with R1—Several observations suggested that CKII could be the major PK co-purifying with R1, which phosphorylated R1 on blot. (i) The catalytic subunit of the human CKII has a M<sub>r</sub> of 42,000. (ii) The N-terminal domain of R1 possesses several potential phosphorylation sites for CKII. (iii) This PK has been reported to co-purify with a large number of diverse proteins. Using pure CKII from two sources (sea urchin and human recombinant), we observed that pET-R1 was indeed a good substrate for this PK (Fig. 3, lane 4). Similar K<sub>m</sub> values of 6 and 7 μM were measured for ATP in the presence of 5 mM MgCl<sub>2</sub> for the phosphorylation of BM5-R1 by its co-purifying PK and that of pET-R1 by CKII, respectively. A hallmark of CKII is that GTP can serve nearly as well as ATP as the phosphate donor. We found that GTP inhibited similarly the phosphorylation of pET-R1 by CKII and that of BM5-R1 by its co-purifying PK (Fig. 7A). These results indicated that CKII could be the major PK co-purifying with R1. This was substantiated by the obtainment of similar inhibition curves with heparin (Fig. 7B), a well known potent inhibitor of CKII. Furthermore, polyclonal antibodies against the CKII catalytic subunit detected on different preparations of BM5-R1, a 44-kDa protein, in amounts that correlated well with the levels of R1.
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FIG. 7. The BM5-R1 co-purifying PK shows CKII-like properties. A, effect of GTP on R1 phosphorylation. B, effect of heparin on R1 phosphorylation. C, detection of the 44-kDa CKII α protein by immunoblotting with anti-CKII α. The detection of the 44-kDa CKII α protein demonstrated that BM5-R1 contains RK and PK(s). Unfortunately, we were unable to localize in which

phosphorylation measured in our standard PK assay (Fig. 7C, lanes 2 and 3). We were unable to detect CKII α in the NaCl-washed preparation (lane 1); from a value of 5 pmol/min/mg for the CKII specific activity (27), the 10 μg of protein loaded on the gel should contained about 2 pg of CKII α, an amount that falls 5-fold below the limit of detection of our immunoblot assay.

Another argument in favor of CKII being the only PK co-purifying with BM5-R1 came from experiments performed to map the phosphorylation sites. With 2 units of pure human CKII for the phosphorylation of 2 μg of pET-R1 or with 2 μg of a maximally phosphorylated preparation of BM5-R1 containing a high amount of co-purifying CKII, 1.75 and 1.8 mol of phosphate, respectively, could be incorporated by 1 mol of R1, suggesting that at least two CKII phosphorylation sites are present on the protein. After CNBr digestion of either pET-R1 phosphorylated by CKII or BM5-R1 phosphorylated by its associated PK, we observed that the major sites of phosphorylation were located only on one large fragment migrating on SDS-PAGE at a position corresponding to 45 kDa. By N-termin-

al sequencing, the beginning of that fragment was localized at position 72. As the next methionine in the amino acid sequence is located at position 340, the M of this largest CNBr fragment (72–340) is predicted to be 25,000. The anomalous behavior of the fragment on SDS-PAGE is probably associated to two stretches of charged residues (191–203 and 224–236). Such stretches are known to retard protein mobility on SDS-PAGE (28). As, during the separation of the CNBr fragments on gel, small labeled fragments could have been lost, deleted R1 was also used to localize the phosphorylation sites. The first mutant protein (∆2–340) used was produced in 293 cells by a recombinant adenovirus (12). When tested in the PK assay, the immunoprecipitated protein was unlabeled (data not shown). To narrow down the localization, we used the HSV-1 R1 (DN247) protein. This protein alone or in combination with either the Ad5ΔE1ΔE3 extract or pure CKII was also unlabeled. Altogether, these results strongly indicate that CKII is the major PK that phosphorylated R1 in vitro and that the phosphorylation sites are located between amino acids 72 and 245, a segment that contains nine potential CKII phosphorylation sites.

Identification of CKII as the PK Responsible for the R1 Labeling on Blot—By doing an experiment similar to the one shown in Fig. 5A for the localization of the movable PK, we demonstrated that the pure recombinant CKII was able to move in the PK assay on blot and to give a strong signal at the BM5-R1 position (Fig. 5B). To verify that this labeling represented R1 phosphorylation and not autophosphorylated CKII bound to R1, we eluted the labeled protein from the membrane and resubmitted it to SDS-PAGE. All the radioactivity migrated at the R1 position showing that CKII autophosphorylation did not contribute significantly to the radioactive signal seen in those experiments. Furthermore, by deleting different parts of lanes containing samples of crude extract as illustrated in the left and middle panels of Fig. 6, we found that only proteins having M, between 40,000 and 44,000 were necessary to produce R1 labeling on blot. The deletion removing proteins below 30,000 that did not affect the level of labeling clearly showed that the regulatory β subunit of CKII (M, 26,000) was unessential to R1 phosphorylation by the CKII α.

As neither pure CKII nor the PK co-purifying with BM5-R1 were able to give a positive signal with pET-R1 on blot (Figs. 3 and 6), we suspected that a post-translational modification not occurring in bacteria was necessary for the R1-CKII interaction. We first tested the most obvious possibility that the interaction required phosphorylation by CKII. As can be seen in Fig. 6 (right panel, lane 2), pET-R1 phosphorylated by CKII with unlabeled ATP in tube prior to the blot assay yielded a strong signal. This result suggested that the R1 phosphorylation by CKII is necessary to increase the affinity between the two proteins to a level sufficient to maintain CKII-R1 binding during the washes after the renaturation step. This conclusion is also supported by the observation that a >80% dephosphorylation of BM5-R1 by alkaline phosphatase treatment before the PK assay on blot nearly completely abolished its capacity to be labeled in an assay similar to the one depicted in the right panel of Fig. 6.

Elimination of pET-R1 and NaCl-washed BM5-R1 Phosphorylation by Velocity Sedimentation—To further examine the origin of the low level of phosphorylation either of pET-R1 in the presence of MnCl₂ (2 × 10⁻⁷ mol of ³²P/min/mol) or of the NaCl-washed BM5-R1 preparation, both proteins were submitted to glycerol gradient centrifugation in 250 mM NaCl. Following this procedure, pET-R1 (Fig. 8A) and histones (not shown) were no longer phosphorylated using the conditions described by Cooper et al. (10), suggesting separation of the contaminating PK(s). Unfortunately, we were unable to localize in which
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Analysis of the BM5-R1 gradient revealed a nearly complete separation of the residual co-purifying kinase CKII, which was excluded by the observation that the sedimentation procedure caused only a slight reduction of the R1 reductase activity. The purification by our standard peptidoaffinity method of the HSV-2 R1 from recombinant adenovirus-infected cells described here or of the HSV-1 R1 from HSV-infected cells (18) yielded proteins that were phosphorylated at rates previously observed for the autophosphorylation of several PKs. In addition, the purified HSV-1 R1 appeared to be able to phosphorylate casein at a rate equal to 1/100 of the one of CKII for the same substrate, suggesting that the protein had an intrinsic PK activity (18). However, after additional purification either by a NaCl wash followed by velocity sedimentation on glycerol gradient for BM5-R1 or by immunoprecipitation with R1 specific antibodies for the HSV-1 R1, both proteins lost in parallel their putative autophosphorylation potential and their capacity to phosphorylate casein and histones. These observations are more compatible with the hypothesis that contaminant PK(s) had been separated from R1, but the possibility that the more extensive purification has eliminated a factor involved in R1 activation cannot be completely ruled out.

Our search of an explanation for the R1 labeling on blot led to the finding that CKII was co-purifying with BM5-R1. Subsequent analyses strongly suggested that it was the major PK responsible of BM5-R1 labeling in solution. (i) The amount of CKII detected by immunoblot in different R1 preparations correlated well with the level of R1 phosphorylation. (ii) The amount of R1 protein (if detectable, >0.05 μg) was quantified by Coomassie Blue staining ( ). Inset, the BM5-R1 phosphorylation ( ) was also measured in presence of heparin ( ). Closed symbols on the x axis represent the phosphorylation obtained with control samples: 2 μg for pET-R1 and 1 μg for BM5-R1. The arrow indicates the position of catalase.

From studies done on limited amounts of purified R1 obtained from HSV-2-infected cells, we and others had proposed that this protein possessed an intrinsic PK activity (18). When the HSV-2 R1 became available in larger amounts as a recombinant protein produced either in adenovirus-infected cells or in E. coli, we searched to totally exclude the involvement of contaminating PKs before undertaking a detailed analysis of this unusual PK. The results of these works presented here suggest that the HSV-2 R1 protein does not by itself possess a PK activity but is instead a good substrate for host cell PKs. With this conclusion is based mainly on the finding that an extensive purification of both types of recombinant protein led to a parallel decrease of their putative autophosphorylation potential and their putative capacity to phosphorylate exogenous substrates. As the ribonucleotide reductase activity of the R1 protein was not altered by the purification procedure, the disappearance of these enzymatic phosphorylations is not attributable to R1 instability. Moreover, the finding that the protein does not significantly bind ATP, which is strong evidence against an intrinsic PK activity, also argues against any other intrinsic enzymatic activity involving ATP as phosphate donor.

The most likely explanation for the weak phosphorylation seen with our recombinant protein produced in E. coli is that it contains a trace amount of one or several bacterial PK(s). This is supported by the complete elimination of phosphorylation by glycerol gradient centrifugation. Unfortunately, we have been unable to identify any E. coli PK involved in the pET-R1 labeling probably because it was present in too low of an amount. With a method of purification that differs markedly from ours, others have obtained higher levels of phosphorylation for either the full-length HSV-1 or HSV-2 R1 subunits or for a truncated HSV-2 R1 consisting of amino acids 1 to 270 (10, 20). In an attempt to explain these differences, we have purified by peptidoaffinity the recombinant HSV-1 R1 from crude bacterial extracts kindly provided to us by J. Conner. This purified protein exhibited a level of phosphorylation (5 fmol/min/mg) similar to the one obtained with pET-R1 (2 fmol/min/mg), suggesting that our method of purification is more effective to eliminate bacterial PK(s).2 We considered it unlikely that the autophosphorylation potential of pET-R1 would have been inactivated following efficient in vivo autophosphorylation because we were unable to detect the presence of phosphate on this protein using a high performance liquid chromatography method that detected 1.8 mol of phosphate/mol of maximally phosphorylated BM5-R1.

The purification by our standard peptidoaffinity method of either the HSV-2 R1 from recombinant adenovirus-infected cells described here or of the HSV-1 R1 from HSV-infected cells (18) yielded proteins that were phosphorylated at rates previously observed for the autophosphorylation of several PKs. In addition, the purified HSV-1 R1 appeared to be able to phosphorylate casein at a rate equal to 1/100 of the one of CKII for the same substrate, suggesting that the protein had an intrinsic PK activity (18). However, after additional purification either by a NaCl wash followed by velocity sedimentation on glycerol gradient for BM5-R1 or by immunoprecipitation with R1 specific antibodies for the HSV-1 R1, both proteins lost in parallel their putative autophosphorylation potential and their capacity to phosphorylate casein and histones. These observations are more compatible with the hypothesis that contaminant PK(s) had been separated from R1, but the possibility that the more extensive purification has eliminated a factor involved in R1 activation cannot be completely ruled out.

Our search of an explanation for the R1 labeling on blot led to the finding that CKII was co-purifying with BM5-R1. Subsequent analyses strongly suggested that it was the major PK responsible of BM5-R1 labeling in solution. (i) The amount of CKII detected by immunoblot in different R1 preparations correlated well with the level of R1 phosphorylation. (ii) The co-purifying PK exhibited several standard criteria used to distinguish CKII from other serine/threonine PK. (iii) Similar phosphopeptide maps were obtained following trypsin diges-
tion of either pET-R1 phosphorylated by CKII or of two BM5-R1 preparations exhibiting low or high rate of R1 phosphorylation.\(^4\) Copurification of CKII with a protein substrate is not peculiar to HSV-2 R1; it has been reported for a large number of cytoplasmic or nuclear proteins exhibiting diverse functions (29–38). Some of these CKII substrates, for example DNA topoisomerase II and Grp94, were, like the HSV-R1, reported to possess autophosphorylation potential until improvements in their purification clearly demonstrated their substrate nature (30, 36). Interestingly, it has been shown that proteins containing serine- and glutamic acid-rich cluster of amino acids such as the PK, p130PTSLRE, could upon phosphorylation by CKII bind specifically with Src homology 2 domains in a phosphotyrosine-independent manner (39). We have observed that the HSV-2 R1 exhibited a similar property in \textit{in vitro} binding assays done with glutathione S-transferase fused Src homology 2 domains.\(^5\)

The most astonishing demonstration of the present work is that phosphorylation of a protein on blot as proof of autophosphorylation could be misleading. Proving intrinsic autophosphorylation has often been a difficult task mainly because eukaryotic PKs exhibit a strong tendency to co-purify with their protein substrates. The PK assay on blot was thought to give irrefutable evidence of the autophosphorylation potential of a protein if care was taken to demonstrate that the \(^{32}\text{P}\) was incorporated in the protein of interest and not in the protein used to block the membrane (40). Our finding that a PK can detach from its position on a membrane, bind with high affinity to one of its protein substrates located elsewhere, and phosphorylate it is an artifact that could be easily prevented by the isolation of the putative PK by an appropriate cutting of the membrane. Is this phenomenon a frequent cause of \(^{32}\text{P}\) labeling on blot? We observed that the labeling on blot of most of the proteins present in a crude extract was not affected either by the removal of the part of the membrane containing the CKII \(\alpha\) subunit or by isolating them on small pieces of membrane. However, a positive PK assay on blot reported for the BCR gene product (41) could be another case of artificial phosphorylation by CKII. As the HSV R1, the BCR gene product contains potential CKII phosphorylation sites in an area of the protein rich in serine and in acidic amino acids shown to be essential for the protein phosphorylation in solution (41).

CKII is not the only eukaryotic PK that is able to phosphorylate R1. Using pure recombinant PKA, we have recently detected a weak phosphorylation of pET-R1. PKC could be another PK able to modify the R1 protein as five potential phosphorylation sites are detected in the protein by the program Prosite. Using antibodies against these two PKs, we have been unable to detect their presence in any of our preparations after the standard peptidoaffinity step.\(^5\) However, as for CKII, we cannot exclude the possibility that they are present in amounts below the detection limits of our immunoblots. Our difficulty in obtaining preparations of both types of recombinant R1 that did not exhibit significant level of phosphorylation illustrates how important it is to use purification procedures that effectively separate the protein of interest from co-purifying PK(s). A close examination of all the other studies on R1 phosphorylation revealed that the presence of contaminating PK was never completely ruled out (8, 10, 14, 20, 42–46). In most cases, immunoprecipitation was used to purify the R1 protein. Such a procedure has been used many times to show \textit{in vitro} interactions between proteins and is well known for its propensity to falsely identify PKs. It is surprising that, in several of these studies, coprecipitation of a great number of proteins with the R1 has been noticed without seriously addressing the possibility that \textit{bona fide} PKs could be present in the precipitates and responsible for R1 phosphorylation (20, 46). In recent experiments,\(^1\) we have detected the coprecipitation with HSV-2 R1 of tyrosine PKs of the family of growth factor receptors and of the Src family, and also of CKII and PKC. Others have reported that the HSV-2 R1 possesses Src homology 3 binding sites that could be involved in interactions with tyrosine PKs of the Src family (45). We believe that the tyrosine PKs that we have found in complexes with R1 could have been responsible for the reported phosphorylation of calmodulin and IgGs by the HSV-2 R1 (20). These proteins are well known substrates of tyrosine PKs (47–49). CKII also could have contributed to calmodulin phosphorylation, whereas R2 phosphorylation could be the result of PKA activity, as we have observed that this protein is an \textit{in vitro} substrate of this enzyme. Unfortunately, the utilization of immunoprecipitation to purify mutated R1 proteins in several studies casts doubts about the value of previous conclusions that certain mutated residues could be important for the autophosphorylation potential of the protein (10, 20, 45, 46). It is important to realize that the level of phosphorylation of a mutated protein such as R1 could be affected by changes in either its affinity for the coprecipitating PKs or conformation-dependent availability of phosphorylation sites. The importance of the latter possibility has been underlined in a recent phosphorylation-related analysis of several mutated forms of p53, where it was shown that the conformation of p53 is of central importance not only for its availability as a substrate for different PKs but also for the phosphorylation pattern generated by the same PK (50).

In conclusion, our present results suggest that the N-terminal domain of HSV-R1 is not a PK domain. However, we cannot completely rule out that an interaction with a cellular factor is necessary to activate the HSV-R1 cryptic PK activity or that it can phosphorylate only certain specific substrates. Even if the N-terminal domain of HSV-R1 is not a PK domain, its capacity to bind with high affinity to cellular proteins could be important in viral pathogenesis. In this respect, it is important to recall that the HSV-R1 is synthesized during the lytic cycle in amounts large enough to produce dominant negative effects (1–2% of total cellular proteins).

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