Analysis of Two Phosphoproteins Related to pp60src from Schmidt–Ruppin D Virus Particles

(Accepted 5 October 1982)

SUMMARY

During endogenous phosphorylation of partially purified pp60src from virus particles, besides pp60src two additional phosphoproteins, 45K and 42K, were found. These proteins copurify with pp60src. They were shown to be proteolytic degradation products due to the action of the virus-associated protease p15. All three phosphoproteins were present in particles of two different sarcoma virus strains, Schmidt–Ruppin D and Prague C, indicating that this phenomenon is general rather than strain-specific. The degradation rate of pp60src was reduced by the presence of 3 mM-ZnCl₂, which acts as a protease inhibitor.

Virus particles of the Schmidt–Ruppin strain, subgroup D (SR-D), contain the sarcoma virus-encoded transformation-specific phosphoprotein pp60src and its associated protein kinase (Owada et al., 1981; Bunte et al., 1981). This transforming protein can be identified in three different ways: (i) as a 60000 mol. wt. (60K) polypeptide labelled with [³⁵S]methionine and precipitated with tumour-bearing rabbit (TBR) serum (Brugge & Erikson, 1977); (ii) by IgG-phosphorylation in an immune complex-bound kinase assay (Collett & Erikson, 1978) and (iii) by endogenous phosphorylation and subsequent immunoprecipitation with TBR serum (Bunte et al., 1981).

During immunoprecipitation of pp60src from SR-D virus particles, Bunte et al. (1981) observed a coprecipitation of two smaller phosphoproteins of 45K and 42K. The nature of the two predominant phosphoproteins besides pp60src was unclear. Coprecipitation and the phosphorylation at a tyrosine residue, which is specific for the pp60src-associated protein kinase (Hunter & Sefton, 1980), suggested that the proteins were either related to, or target proteins for, pp60src. In a previous analysis, antiserum raised against a synthetic peptide directed against the C-terminus of pp60src precipitated pp60src and the 45K but not the 42K phosphoprotein (Bunte et al., 1981). Also, partial proteolytic cleavage analysis indicated a relationship between pp60src and the 45K phosphoproteins. Furthermore, purification of pp60src by ion-exchange chromatography and gel filtration showed that the protein kinase activity was associated with the 45K and 42K and not with the 60K molecules (Donner et al., 1981).

These observations pointed to the possibility that the phosphoproteins were generated by proteolytic degradation. Since avian RNA tumour viruses harbour a protease which is closely associated with the structural protein p15 (von der Helm, 1977; Dittmar & Moelling, 1978) and which is also responsible for proteolytic cleavage of the viral reverse transcriptase polypeptide β to α (Moelling et al., 1980), this protease was considered a likely candidate also for degrading pp60src during the isolation procedure. The p15-associated protein kinase is inhibited by 3 mM-ZnCl₂ (Dittmar & Moelling, 1978).

To find the effect of ZnCl₂ on the degradation of pp60src, disrupted virus particles were incubated for the endogenous phosphorylation reaction in the absence or presence of ZnCl₂. During this reaction, pp60src and in addition a 45K and a 42K protein were phosphorylated (Bunte et al., 1981). Virus used for this experiment originated from a cloned Schmidt–Ruppin D strain isolated in this laboratory (Owada et al., 1979). To rule out the possibility that the pp60src associated with this virus is unique to this strain, a second uncloned sarcoma virus, Prague C (PR-C) obtained from the National Cancer Institute, was included. The result is shown in Fig. 1(a). The products of the endogenous reaction were either applied to the gel directly (right) or after an immunoprecipitation with TBR serum (left). pp60src, the 45K and 42K phosphorylation proteins were found in both virus strains even though their amounts appeared to be higher in the
(a) Endogenous phosphorylation of virus particles from two different strains. Virus particles of the SR-D and PR-C strains were purified from tissue culture supernatants through a 20 to 70% sucrose gradient as described (Owada et al., 1981). The virus was lysed (each 0-6 mg) in the presence of 2% Nonidet P40 for 10 min at 0 °C and endogenously phosphorylated in a total of 100 μl containing 20 mM Tris-HCl pH 8.2, 15 mM-MgCl2, 5 mM-dithiothreitol, 20 μCi of [γ-32P]ATP in the absence (−) or presence (+) of 3 mM-ZnCl2. Incubation was for 30 min at 30 °C. The reaction was terminated by addition of EDTA to 10 mM. The reaction mixture was diluted with 500 μl of RIPA buffer (50 mM-Tris-HCl pH 7.2, 150 mM-NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate and processed for radioimmunoprecipitation with TBR serum (5 μl) as described (Owada & Moelling, 1980). The precipitates were analysed by electrophoresis on 10% polyacrylamide gels (Laemmli, 1970) and autoradiography. Aliquots (5 μl) of the reaction products were processed directly for gel electrophoresis (right), the residual volume (95 μl) was diluted with RIPA buffer and processed for radioimmunoprecipitation, gel electrophoresis and autoradiography (left). (b) Cleavage of pp60src by the p15-associated protease. A 25 μl volume of pp60src-containing viral membrane vesicles (Bunte et al., 1981) were incubated for endogenous phosphorylation and subsequently an immunoprecipitation with TBR serum was performed as described in (a). The washed immune complexes were treated with 0, 1, 3, and 5 μl of p15 (0-1 mg/ml) in 10 mM-BES buffer [N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid pH 6.5, 0.1 mM-EDTA] at 37 °C for 18 h. The reaction products were processed for gel electrophoresis and
cloned SR-D virus. Phosphorylation of the IgG of TBR serum was also higher with the cloned SR-D than with this PR-C virus (unpublished observation). Probably due to protection from protease activity, no 45K or 42K proteins were detected in the presence of 3 mM-ZnCl₂, but instead some fragments of intermediate molecular weight appeared.

If the protease inhibitor ZnCl₂ prevents the generation of 42K and 45K proteins, the reverse effect should also be possible, namely their production after treatment of pp60 with p15. For this, membrane vesicles containing only intact pp60 src molecules and no degradation products were prepared from virus particles according to a published procedure (van de Ven et al., 1978) and treated with p15 in vitro as described (Dittmar & Moelling, 1978). The result is shown in Fig. 1 (b). pp60 src is degraded by p15 treatment in vitro and the 45K and 42K proteins are generated as proteolytic degradation products. Proteolysis in vitro continues with increasing amount of p15 to even smaller fragments (Fig. 1 b).

To rule out completely the possibility that the 42K protein was a potential target protein and not a proteolytic fragment of pp60 src, a fingerprint analysis of the 42K protein was performed. SR-D virus (1 mg) was lysed and endogenously phosphorylated as described. The reaction product was processed by gel electrophoresis and then autoradiography. The pp60 src and 42K proteins were identified and cut out of the dried gel. The gel pieces were rehydrated, the proteins eluted, supplemented with bovine serum albumin and digested with trypsin (1 mg/ml) for 5 h at room temperature. The two-dimensional separation was performed using high voltage electrophoresis in the first dimension and ascending chromatography in the second dimension (Collett et al., 1979). Both peptides were analysed separately and also after mixing. The result is shown in Fig. 1 (c), a, b and c. pp60 src and the 42K protein exhibit a single phosphopeptide which comigrates after mixing. Therefore, it can be concluded that the 42K phosphoprotein is related to pp60 src. This was demonstrated for the 45K phosphoprotein in a previous report (Donner et al., 1981).

We have shown that two phosphoproteins of 45K and 42K are proteolytic cleavage products of pp60 src. Proteolytic degradation seems to be a general phenomenon for pp60 src from viral sources, since the same degradation products found in SR-D were also generated in PR-C virus particles. The virus-associated protease p15 was identified as being responsible for the degradation. It remains to be investigated whether the protease is already active in the virus particle or becomes active during lysis and endogenous phosphorylation. Preliminary data with [³⁵S]methionine-labelled virus indicate that proteolysis occurs mainly after virus lysis and subsequent release of pp60 src from the virus membrane. This may be due to a different localization of pp60 src and the p15-associated protease in the virus.

In spite of the identification of the protease, which degrades pp60 src, use of the known protease inhibitor for further studies or purification of pp60 src is not very useful since it inhibits the kinase activity as well. Recently, ZnCl₂ had been found to enhance the pp60 src protein kinase. This effect, however, was observed at much lower concentrations of ZnCl₂ (10 μM) than the one applied here, and was attributed to the inhibition of phosphatases (Gallis et al., 1981). The proteolytic degradation products observed from cell-derived pp60 src preparations are of different size and probably due to the action of other unknown proteases (Erikson et al., 1979; Krueger et al., 1980). The nature of the kinase that phosphorylates the 45K and 42K proteins was not identified. It is, however, a tyrosine-specific protein kinase. Tyrosine-specific phosphorylation is attributed to the pp60 src-associated kinase (Hunter & Sefton, 1980).

Max-Planck-Institut für Molekulare Genetik
Innestrasse 63–73, D-1000 Berlin 33, F.R.G.

THOMAS BUNTE
KARIN MOELLING*
REFERENCES


*(Received 25 May 1982)*