Supplementary Material

Nuclear import of purified HIV-1 Integrase .

Integrase remains associated to the RTC throughout the infection process until provirus integration occurs and is therefore one likely candidate to mediate HIV DNA nuclear import. To elucidate the mechanisms of IN nuclear import, labelled, un-tagged recombinant IN was used in the nuclear import assay in HeLa cells in the presence of Ran mix, energy mix and the same import factors tested previously with RTCs (Supplementary Figure 1). Some IN nuclear import was observed in the absence of added import receptors, suggesting either that importins were not absolutely required or that some importins remained in the permeabilised cells. However, imp7 and the imp7/impβ heterodimer clearly stimulated nuclear accumulation of IN up to 100 fold above background while impβ alone had a weaker activity (Supplementary Figure 1). Purified IN, unlike RTCs, was also imported by the impα/impβ heterodimer as previously reported (Gallay et al. 1997) (Supplementary Figure 1).

These effects were specific and import was abolished if the impα/impβ heterodimer was blocked with a classical NLS peptide or if imp7 or the impβ/imp7 heterodimer were blocked with the BIB domain, the domain of rpL23a recognised by these importins (Jakel and Gorlich 1998) (Supplementary Figure 2). A modest inhibitory effect on impβ-dependent import was observed with the IBB-domain, which competes impα-binding to impβ, but leaves the binding of ribosomal proteins largely unaffected (Jäkel et al., 1999) (Supplementary Figure 2).

The components of the Ran system and energy mix were required for maximal stimulation of nuclear import by imp7, impβ and the imp7/impβ and impα/impβ heterodimers (Supplementary Figure 3). When the mutant imp7 K61D was used instead of imp7, either alone or in combination with impβ, IN nuclear import was clearly inhibited, providing additional support for the role of the Ran system (Supplementary Figure 3). Similar results were observed in macrophages (not shown).

The specific, RanGTP-sensitive interaction of IN with various import receptors was also examined (Supplementary Figure 4). Impα, impβ and imp7 bound to IN and the interaction was inhibited by RanGTP. Imp9 did not bind to IN, showing the specificity of the assay. Interestingly, transportin also showed an ability to bind IN. Such interaction is likely to be non-functional since transportin alone had little or no effect in the nuclear import assay (Supplementary Figure 1).The reasons for these multiple interactions of IN with these importins are unclear but it is not unprecedented that basic proteins (like integrase) can bind to and be imported by more than one factor (Jäkel and Görlich, 1998). Moreover, some importins that bind to such basic proteins are not active in the nuclear import assay (Jäkel and Görlich 1998; Jäkel et al. 1999). This can be explained by the finding that importins fulfil two distinct functions as nuclear import factors and cytoplasmic chaperones for basic proteins (Jäkel et al. 2002). Since the impα/impβ heterodimer was not active in the nuclear import assay using purified RTCs, we suspect that this NLS is masked upon IN binding to the viral nucleic acids. A similar masking of a NLS upon RNA binding has been described for the HIV-1 Rev protein (Henderson and Percipalle, 1997).
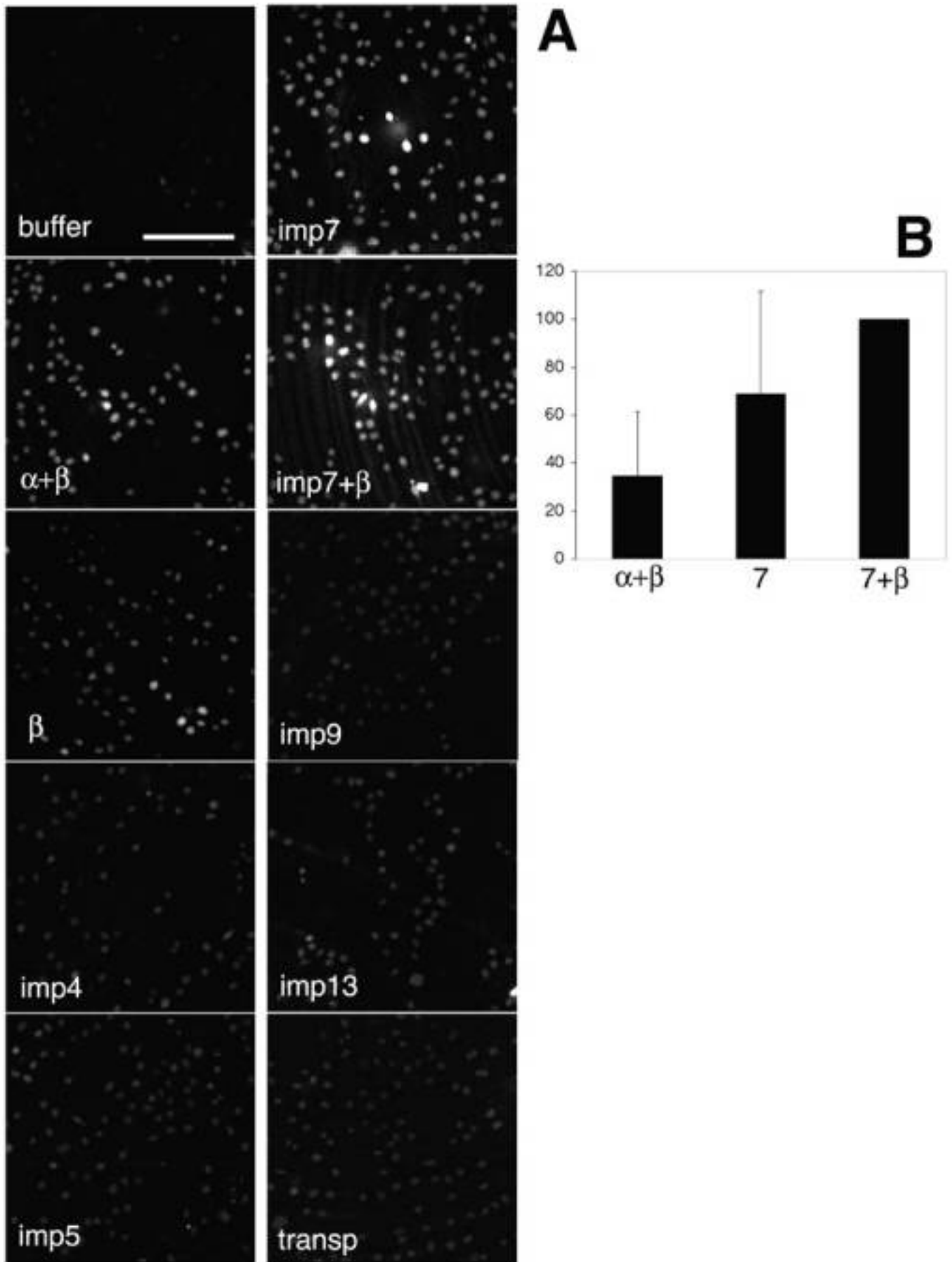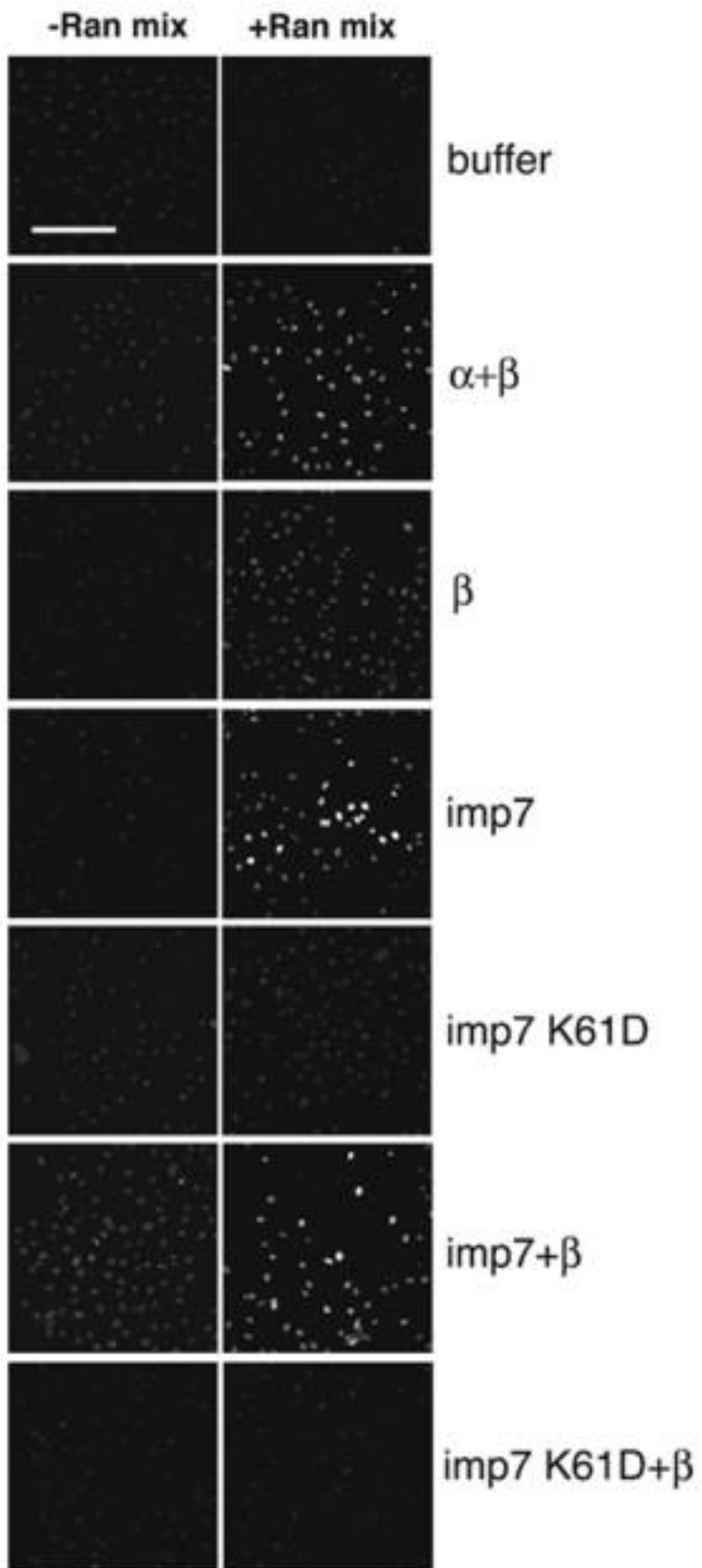
Supplementary Figure 1. *Nuclear import of IN is stimulated by imp7 and the*

imp7/impβ and impα/impβ heterodimers. (A) Import assays were performed in permeabilised HeLa cells for 15 minutes at 25^0C in the presence of 0.5μM labelled recombinant IN and 0.75μM of the indicated nuclear import receptors in the presence of 1x Ran mix and energy mix. Scale bar 150μm. (B) quantification of IN accumulation in HeLa nuclei following the import assay. Images acquired by confocal microscopy were analysed using the MetaMorph software version 4.5r4 (Universal Imaging Corp.). Samples were blanked on the control (containing buffer only) and the total fluorescence divided by the number of cells per field (counted in the transmission mode). At least 300 cells were counted per experiment. Bars represent the mean fluorescence per cell ± standard deviation of four independent experiments. The samples containing imp7+β were given an arbitrary value of 100.
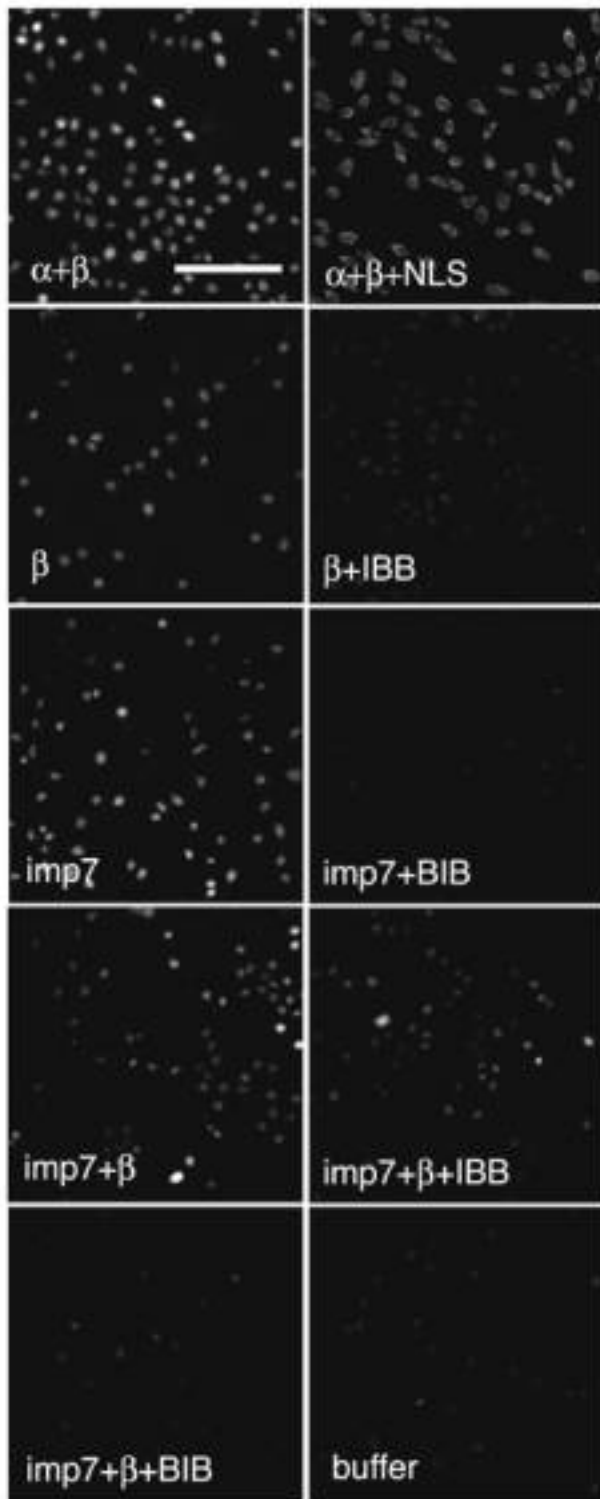
Supplementary Figure 2. *Nuclear import of integrase is inhibited by specific competitors. Import assays were performed in permeabilised HeLa cells for 15 minutes at 25^0C in the presence of $0.5\mu M$ labelled recombinant IN and $0.75\mu M$ of the indicated nuclear import receptors in the presence of 1x Ran and energy mix and the following competitors: $1.5\mu M$ BSA-NLS (NLS), $3\mu M$ IBB or BIB domains fused to the maltose binding protein (MBP). BSA and MBP alone did not inhibit*

IN nuclear import at the same concentration tested for the fusion proteins (not shown). Scale bar, 150μm.

Supplementary Figure 3. *Nuclear import of integrase is stimulated by Ran mix and specific importins. Import assays were performed in permeabilised HeLa cells for 15 minutes at 25^0C in the presence of $0.5\mu M$ labelled recombinant IN and $1\mu M$ of the indicated nuclear import receptors with or without 1x Ran and energy mixes. The mutant imp7 K61D cannot bind Ran. Scale bar, $150\mu m$.*
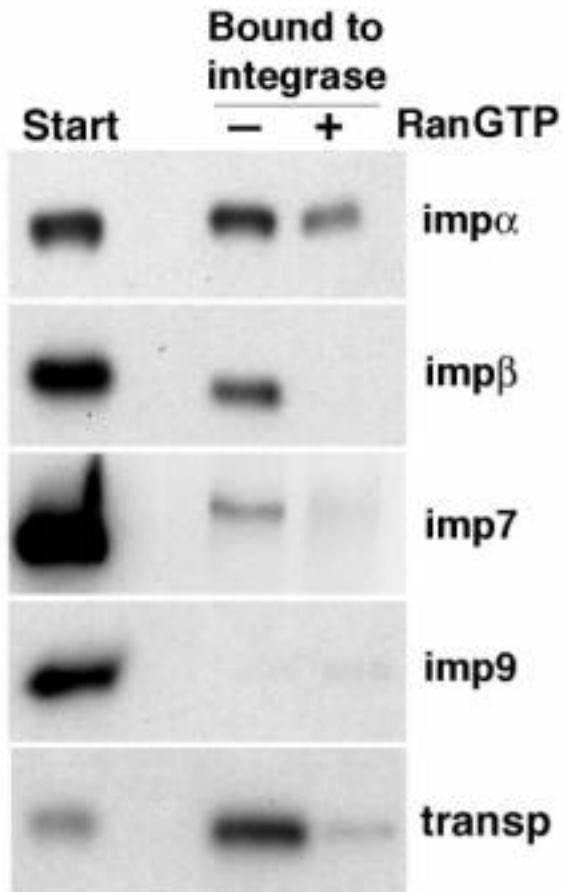
Supplementary Figure 4. *RanGTP-sensitive binding of IN to impα, impβ and imp7. Recombinant zz-tagged integrase was immobilised onto IgG-sepharose and incubated in the presence of a HeLa cytosolic extract with or without $5\mu M$ recombinant RanQ69L (GTP). RanQ69L lacks GTPase activity. Bound proteins were eluted and analysed by SDS-PAGE and Western-blotting using antibodies against the indicated importins*

Preparation of viral stocks

To make HIV-1 vector stocks, 5×10^6 293T cells were seeded in 100 mm dishes and transfected the following day by calcium phosphate with 20 µg/100mm dish of plasmid pHR' or pHR'SINcPPT (expressing either the green fluorescent protein cDNA or the puromycin-N-acetyl transferase gene), 15 µg/100 mm dish of plasmid pCMV•R8.2, (expressing viral core proteins) or pCMVΔR8.9 (expressing viral core proteins but no Vpr and accessory proteins) and 5µg/100mm dish of plasmid pMD.G, expressing VSV-G (Naldini et al., 1996; Zufferey et al., 1997; Demaison et al. 2002) or pSV3gp120, expressing the gp120 envelope glycoprotein. Culture medium was replaced after 24 hours; the viral-containing supernatant was collected 48 hours after transfection and filtered through a 0.45 µm filter. The filtered supernatant was incubated at 37^0C for 1 hour in the presence of 70 U/ml DNAseI (Boheringer) and 10 mM $MgCl_2$. Virus was purified by centrifugation through a 25-45% sucrose cushion at 23,000 rpm in a Beckman SW28 rotor for 2 hours at 4^0C. The sucrose interphase containing purified virions was frozen at -80^0C. Viral titres were determined by infecting HeLa cells with serial dilution of virus containing supernatant in the presence of 8 µg/ml polybrene. Approximately 48 hours after infection, cells were harvested in phosphate-buffered saline containing 10mM EDTA and analysed by FACS to detect green fluorescent protein expression. To prepare stocks of SF-162 HIV-1 primary isolate, peripheral blood mononucleated cells (PBMC) (approximately 10^6 cells/5 ml) were grown for 48 hours in RPMI media supplemented with 10% FCS and 0.5µg/ml phytohemagglutinin. Cells were infected with 10^5 cfu in the presence of 10U IL-2/ml and cultured for a further 72 hours. Infected cells were mixed with 5×10^6 fresh PBMC in a total volume of 15 ml and the virus harvested at the peak of infection as monitored by reverse transcriptase assay. Virus stocks were snap-frozen in liquid nitrogen.

Binding assay for integrase

pINSD plasmid containing HIV-1 integrase was obtained through the AIDS Research and reference Reagent Program, Division of AIDS, NIAID, NIH from

Dr. Alan Engelman and Dr. Robert Craigie (Engelman and Craigie, 1992). Integrase was amplified from pINSD-IN by PCR as an NcoI-BamHI fragment and cloned into zzTevpQE80 (N-zz-tagged; C-His-tagged construct). ZzTev-integrase was expressed in *E.Coli*, the bacterial pellet resuspended in 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, sonicated and cleared by ultracentrifugation at 100,000g. N-His tagged RanQ69L was expressed in *E.Coli* from a pQE32 vector (Quiagen) and purified by nickel-NTA-agarose followed by cation exchange chromatography using SP Sepharose (Amersham Biosciences). For the binding assays, zzTev-integrase was immobilised onto IgG-sepharose (1-2 µg of protein/µl matrix) and 25 µl of the immobilized protein were incubated for 4 hours with 0,8 ml of a HeLa cytosolic extract (adjusted to 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) with or without 5 µM purified RanQ69L. Bound proteins were eluted with 1,5 M $MgCl_2$, 50 mM Tris-HCl pH 7.5, precipitated with 95% isopropanol, separated by SDS-PAGE and analysed by Western-blotting.