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pIRS1 and pTRS1 Are Present in Human Cytomegalovirus Virions

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The virus-coded proteins pIRS1 and pTRS1 were found associated with purified human cytomegalovirus virions. The proteins were not degraded when intact virions were treated with trypsin, which suggests that they are localized inside the viral particle. In transfection experiments pIRS1 and pTRS1 modestly activated expression from a reporter plasmid containing the viral major immediate-early promoter but did not influence the activity of a reporter carrying the *irs1/trs1* immediate-early promoter. Both reporters were activated by the combination of pIRS1 or pTRS1 and pUL69, which is also present in virions.

The *irs1* and *trs1* genes belong to the immediate-early class of human cytomegalovirus (HCMV) (reviewed in references 2 and 8) genes and are expressed throughout the entire replicative cycle of the virus (9, 10). The steady-state levels of their corresponding protein products accumulate and are highest during the late phase of infection (9). In addition, the $irs1^{263}$ gene, whose promoter and coding region are located entirely within the *irs1* open reading frame, is also expressed throughout infection, but the highest steady-state levels of the $pIRS1^{263}$ protein can be found toward the end of the early phase of infection. Although they exhibit little activity on their own, pIRS1 and pTRS1 can activate transcription from viral promoters in cooperation with the immediate-early 1 and 2 proteins (4, 5, 9, 10). $pIRS1^{263}$ appears to antagonize the activation function of pIRS1 and pTRS1 (9). Among the proteins residing in the tegument of the mature virion (1), two have been shown to function as activators of immediate-early viral gene expression: pUL82 (pp71), also known as the upper matrix protein (6), and pUL69 (12, 13). In this communication, we demonstrate that both the pIRS1 and pTRS1 proteins, but not $pIRS1^{263}$, are present in mature HCMV virions, noninfectious viral particles, and dense bodies. Either pIRS1 or pTRS1 can cooperate with pUL69, but not pUL82, to enhance the activity of reporter plasmids containing the major immediate-early promoter, the *irs1/trs1* immediate-early promoter, or a non-HCMV promoter.

We previously showed that pIRS1 and pTRS1 could be detected in infected cells as early as 2 hours postinfection (9). Since other immediate-early proteins were previously shown to be expressed at detectable levels no sooner than 4 to 6 h postinfection, we wondered if the relatively early detection of pIRS1 or pTRS1 resulted from proteins released from virions. We purified mature virions, noninfectious enveloped particles, and dense bodies by rate-velocity sedimentation in glycerol-tartrate gradients (1, 3, 11) and tested each particle fraction for the presence of pIRS1, pTRS1, and $pIRS1^{263}$ by Western blot assay using a cocktail of three different monoclonal antibodies specific for either pIRS1 and $pIRS1^{263}$ or pTRS1 (9). All three particle fractions contained both pIRS1 and pTRS1, but $pIRS1^{263}$ was not detected (Fig. 1).

To determine whether pIRS1 and pTRS1 are localized on the outer surface of the viral envelope or reside within the virion, we performed a protease digestion assay. Initially, a control experiment was performed to demonstrate that treatment for 3 h at 37°C with trypsin (90 μg/ml) degrades proteins outside the virion envelope. Exposed carbohydrate residues on the membrane proteins of gradient-purified mature virions (18 μg of total protein), transferrin (1 μg), or bovine muscle actin (1 μg) were labeled with biotin hydrazide, and the labeled proteins were detected by reaction with streptavidin conjugated to horseradish peroxidase (glycoprotein detection kit; Amersham Life Sciences). The reactions with actin and transferrin confirmed that the labeling reagent was specific for glycoproteins, since the glycoprotein transferrin was labeled while actin was not (Fig. 2A, lanes 1 and 2). Virion membrane proteins were labeled and appeared as a broad smear in the 45- to 60-kDa range as seen previously (1), and this labeled material was substantially degraded by treatment with trypsin (Fig. 2A, lanes 3 and 4), demonstrating that proteins residing outside of the virion envelope are susceptible to proteinase digestion.

Next, gradient-purified virus particles (18 μg of total protein) were digested for 3 h at 37°C with trypsin (90 μg/ml) and a Western blot assay was performed to assess the effect of the treatment on virion-associated pIRS1 and pTRS1. The product of UL99, pp28, which is a known internal constituent of the virion (1, 7), was also monitored. In intact virions, essentially all of the pIRS1, pTRS1, and pp28 was resistant to trypsin digestion (Fig. 2B, lanes 1, 2, 5, and 6), but when virions were disrupted by treatment with heat or 1% Triton X-100, the proteins became sensitive to protease treatment (Fig. 2B, lanes 3, 4, 7, and 8). Since pIRS1 and pTRS1 are not degraded when intact virions are treated with trypsin, we conclude that they are located within the particles and are not exposed to the outer surface of the viral envelope.

We have previously shown (9) that while pIRS1 and pTRS1 do not markedly activate transcription on their own, they do enhance transcription from a variety of viral promoters in the presence of the HCMV-coded IE1 and IE2 transcriptional regulatory proteins. Accordingly, we asked if pIRS1 or pTRS1 could activate transcription together with either of the other two known transcriptional regulatory proteins in virions, pUL69 or pUL82 (pp71). We performed a series of transfec-

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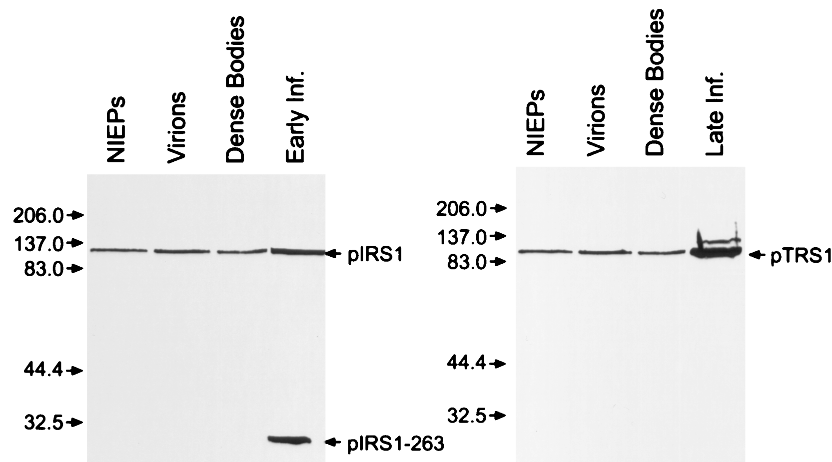

FIG. 1. Detection of pIRS1 and pTRS1 in gradient-purified HCMV virion fractions. Virus particles were purified by sedimentation in glycerol-tartrate gradients. Proteins contained in fractions of noninfectious enveloped particles (NIEPs), mature virions, and dense bodies were separated on 10% polyacrylamide gels containing sodium dodecyl sulfate, transferred onto nitrocellulose membranes, reacted with a pIRS1- or pTRS1-specific monoclonal antibody cocktail (9), and visualized with an alkaline phosphatase-conjugated secondary antibody. Extracts from primary human foreskin fibroblasts prepared at either the early or the late stage of infection (early or late inf., respectively) served as controls. The positions to which molecular mass markers migrated are indicated to the left of the gels. Data was scanned with Photoshop software, and figures were prepared with Freehand software.

tion experiments in which a luciferase reporter plasmid carrying the HCMV major immediate-early promoter (pGL3-MIEP [9]) was cotransfected with one or several effector plasmids expressing pIRS1 or pTRS1 (pCEP4-IRS1-His or pCEP4-TRS1-His [9]), pUL69 (pHM160 [12]), or pUL82 (pCMV71 [6]). pIRS1 or pTRS1 activated the major immediate-early promoter by a factor of about 2 to 3 when it was transfected alone with the promoter (Fig. 3A, experiments 3 and 4), and pUL69 or pUL82 (pp71) alone activated the major immediate-early promoter by a factor of 4 to 5 (Fig. 3A, experiments 5 and 6). Either pIRS1 or pTRS1 cooperated with pUL69 to activate transcription by factors of about 13 and 10, respectively (Fig. 3A, experiments 7 and 8), but they did not enhance reporter activity in cooperation with pUL82 (pp71) (Fig. 3A, experiments 9 and 10). pUL69 and pUL82 (pp71) exerted an additive effect on the major immediate-early promoter, inducing its activity by a factor of 9, and inclusion of pIRS1 or pTRS1 increased the induction to a factor of 17 to 19 (Fig. 3A, experiments 11 to 13). Presumably, the increase observed in the presence of pUL69, pUL82 (pp71), and either pIRS1 or pTRS1 reflects pUL69-pIRS1 or -pTRS1 cooperation.

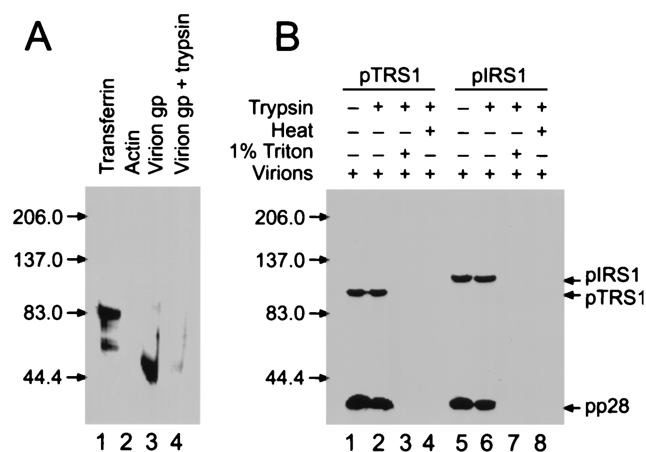

FIG. 2. Demonstration that virus-associated pIRS1 and pTRS1 are resistant to digestion with trypsin. (A) Proteins outside the virion envelope are degraded by trypsin treatment. Glycoproteins exposed on the surfaces of virions were labeled with biotin hydrazide, separated on a 5-to-12% polyacrylamide gel containing sodium dodecyl sulfate, transferred onto a nitrocellulose membrane, and visualized with streptavidin conjugated to horseradish peroxidase. gp, glycoprotein. (B) Trypsin resistance of pIRS1, pTRS1, and pp28 during a 3-h digestion at 37°C (lanes 2 and 6) and trypsin sensitivity after boiling for 5 min at 96°C (lanes 3 and 7) or in the presence of 1% Triton X-100 (lanes 4 and 8). Proteins were separated as indicated for panel A, detected with pIRS1-, pTRS1-, and pp28-specific monoclonal antibodies, and visualized with alkaline phosphatase-conjugated secondary antibody in a chemiluminescent reaction. The positions to which molecular mass markers migrated are indicated to the left of the gels.

Similar experiments were performed with a reporter containing the HCMV *irs1*/*trs1* immediate-early promoter (Fig. 3B) (pGL3-I/TRS1 was prepared by cloning a PCR-amplified 458-bp segment from sequence positions 189295 to 189752 of the AD169 genome into the *Nhe*I and *Hin*dIII sites of pGL3-Basic [Promega Biotec]) or a non-HCMV promoter from Rous sarcoma virus (Fig. 3C) (pGL3-RSVLTR was constructed by inserting the *Sal*I-*Hin*dIII fragment of pREP4 [Invitrogen] into the *Xho*I and *Hin*dIII sites of pGL3-Basic). The results were qualitatively similar to those obtained for the reporter containing the major immediate-early promoter, except that neither pIRS1 nor pTRS1 alone influenced the *irs1*/*trs1* promoter (Fig. 3B, experiments 3 and 4). Each of them did, however, cooperate with pUL69 to activate the reporter by a factor of about 10 (Fig. 3B, experiments 7 and 8).

Since expression of the effector proteins is controlled by the HCMV immediate-early promoter and this promoter can be activated by some of the effectors (Fig. 3A), it is difficult to know what portion of the luciferase activity is due to a direct effect of the effectors on the reporter promoter. The enhancement observed in our assay likely results from elevated activity of the major immediate-early promoter in effector plasmids in addition to direct effects of the viral activators on the major immediate-early promoter in the reporter plasmid. In spite of this ambiguity, it is clear that pIRS1 and pTRS1 act in concert with pUL69 to induce the accumulation of products encoded by plasmids containing the major immediate-early promoter. We have previously demonstrated (9) that pIRS1 and pTRS1 do not affect the levels of transfected DNAs and that they cooperate with the HCMV IE2 protein to enhance expression from a luciferase reporter at the level of mRNA accumulation.

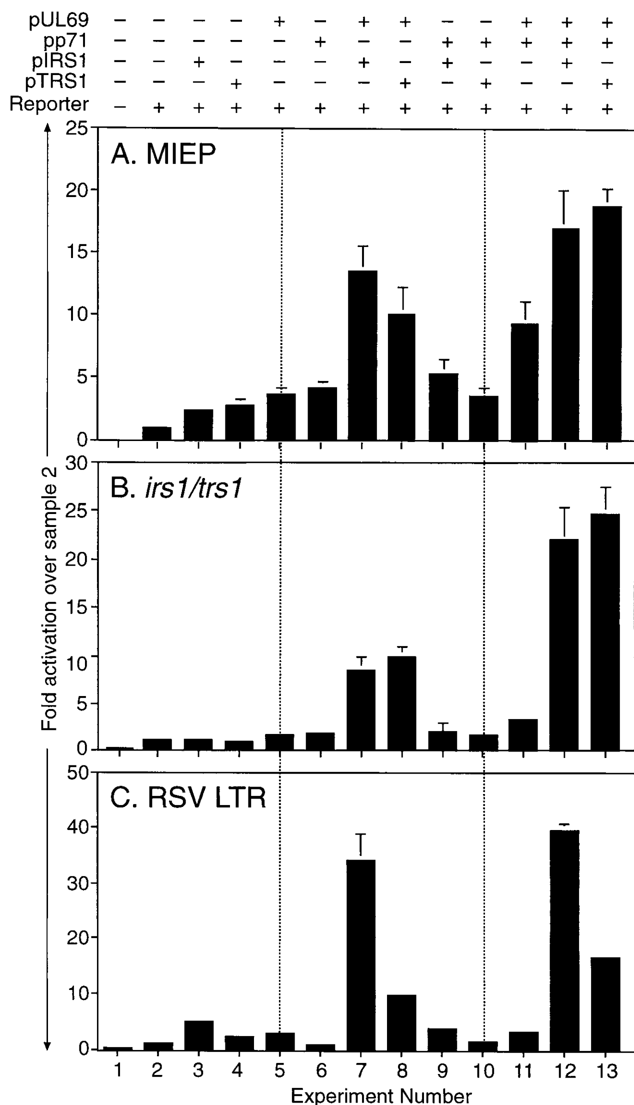

FIG. 3. Demonstration that pIRS1 and pTRS1 activate expression of a reporter plasmid in cooperation with pUL69. HeLa cells (approximately 70% confluent) were transfected in triplicate with the pGL3-MIEP (A), pGL3-I/TRS1 (B), or pGL3-RSVLTR (C) luciferase reporter plasmid plus effector plasmids expressing pp71, pUL69, pIRS1, or pTRS1, as indicated at the top of the figure. Transfection mixtures contained 2.5 μg of total DNA (0.5 μg of the reporter plasmid DNA and 0.5 μg of each effector plasmid DNA; reactions were balanced with the pCEP4 DNA) and 5 μl of Lipofectamine (Gibco/BRL). The transfection mixture was incubated with cells for 12 h at 37°C in Opti-MEM I medium (Gibco/BRL). Luciferase activity was measured at 40 h posttransfection, and the average of experimental results plus the standard deviation is reported for each experiment. RSV LTR, Rous sarcoma virus long terminal repeat.

Thus, we interpret the results of the luciferase assay to demonstrate that pIRS1 or pTRS1 can cooperate with pUL69 to enhance the accumulation of RNA whose transcription is directed by the HCMV major immediate-early promoter.

In sum, we have shown that pIRS1 and pTRS1 are present inside the envelope of HCMV virions (Fig. 1 and 2). Since the newly identified $pIRS1^{263}$ (9), which consists of the C-terminal domain of pIRS1, was not detected in virions, it seems likely that the N-terminal domain shared by pIRS1 and pTRS1 contains a signal for virion localization. Since pIRS1 and pTRS1 can activate expression from reporters containing viral immediate-early promoters in cooperation with another virion constituent, pUL69 (Fig. 3A and B), we propose that one function of these proteins is to assist in the activation of HCMV promoters as the viral DNA first reaches the nucleus.

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