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p59$^{v\text{-}rel}$, the Transforming Protein of Reticuloendotheliosis Virus, Is Complexed with at Least Four Other Proteins in Transformed Chicken Lymphoid Cells

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Previous studies have identified the protein product of v-*rel*, the oncogene carried by reticuloendotheliosis virus (REV), as a 59,000-dalton phosphoprotein located predominantly in the cytosol of transformed chicken lymphoid cells. In immune precipitates of p59$^{v\text{-}rel}$, there is a closely associated protein kinase activity. In chicken lymphoid cells that do not contain REV, p68$^{c\text{-}rel}$ is found free in the cytosol not associated with other proteins and not detectably phosphorylated. In this study, we found that immune precipitates of 59$^{v\text{-}rel}$ from REV-transformed cells contain at least four other proteins, of approximate molecular weights 124, 115, 68, and 36 kilodaltons (kDa). The 124-, 115-, and 36-kDa proteins are apparently unrelated to p59$^{v\text{-}rel}$ in sequence, and their coprecipitation suggests that they are complexed with p59$^{v\text{-}rel}$. The coprecipitating 68-kDa protein was found to be p68$^{c\text{-}rel}$, which, like the other three proteins, precipitates by virtue of its association with p59$^{v\text{-}rel}$. Glycerol gradient analysis suggested the presence of more than one type of complex: one containing p115, p68$^{c\text{-}rel}$, p59$^{v\text{-}rel}$, and p36, and another containing p124, p115, p59$^{v\text{-}rel}$, and possibly p68$^{c\text{-}rel}$. In vitro kinase activity was found in all size classes, coinciding with the distribution of p115 and p59$^{v\text{-}rel}$. The complex(es) was stable under a variety of conditions, including a wide range of ionic strengths, chelators, and detergents, and through multiple cycles of immune precipitation and elution. This suggests a specific and functionally significant interaction among the members that may be of direct relevance to the mechanism of REV-induced transformation.

Reticuloendotheliosis virus (REV) strain T is an acute leukemia virus of young chickens (for a review, see N. R. Rice and R. V. Gilden, *in* P. Reddy, T. Curran, and A. M. Skalka (ed.), *The oncogene handbook*, in press). The virus consists of a replication-competent helper genome (REV-A) and a replication-defective transforming genome (REV-T) (11). In addition to portions of the helper virus *gag*, *pol*, and *env* genes, REV-T contains the 1.4-kilobase oncogene v-*rel* (3, 22). Nucleotide sequence analysis has shown that v-*rel* encodes a 503-amino-acid protein whose N- and C-termini are derived from the REV-A *env* gene (28, 31). Antibodies to synthetic peptides (21, 26) and to fusion proteins containing portions of the v-*rel* sequence (9, 10, 27, 30) have allowed the detection of p59$^{v\text{-}rel}$. It is a phosphoprotein (phosphorylated mainly on serine [9, 21, 30]) that is located primarily in the soluble cytoplasmic fraction of the leukemic cell (9, 27, 30), and it is essentially unrelated to any of the other oncogene products. Immune precipitates of p59$^{v\text{-}rel}$ contain a closely associated protein kinase activity, and during the in vitro kinase assay p59$^{v\text{-}rel}$ becomes phosphorylated mainly on serine (21, 27, 30; S. Simek, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1987).

Hybridization studies showed that v-*rel* is highly related to DNA sequences found in normal galliform birds (13, 25, 32) and led to the suggestion that v-*rel* originated from the turkey c-*rel* gene (31). A large portion of that gene, corresponding to all the regions found in v-*rel*, has been molecularly cloned and sequenced (31). These studies showed that at the amino acid level, more than 95% of v-*rel* residues are identical in turkey c-*rel*. On the basis of this similarity, we used v-*rel* antisera to detect the protein encoded by chicken c-*rel* (26). It has a molecular weight of 68,000 and is found in the cytosol of lymphoid cells and, to a lesser degree, in fibroblasts. In contrast to p59$^{v\text{-}rel}$, we have not observed phosphorylation of p68$^{c\text{-}rel}$ either in vivo or in vitro (26). Recently, we have also been able to detect a human c-*rel* protein (p82$^{hc\text{-}rel}$) with two v-*rel* antisera (E. Brownell, F. W. Ruscetti, R. G. Smith, and N. R. Rice, Oncogene, in press).

To date, the observation of protein kinase activity in p59$^{v\text{-}rel}$ immune precipitates is the only clue to the mechanism of cellular transformation by v-*rel*. In this study, we extended our comparison of the physical properties of p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ with the goal of defining differences between them that may account for the transforming activity of v-*rel*. We found that p59$^{v\text{-}rel}$ is capable of forming complexes with at least four other proteins, one of which is p68$^{c\text{-}rel}$. All size classes of the complex(es) exhibited protein kinase activity in vitro.

MATERIALS AND METHODS

Cells. The REV-T-transformed chicken lymphoid cell line S3D6 was established by Hoelzer et al. (12). MSB-1 cells are chicken T lymphocytes transformed by the Marek's disease agent. Both cell lines were grown in RPMI 1640 plus 10% fetal calf serum at 37°C.

Metabolic labeling. Cells were washed twice in phosphate-buffered saline and incubated in methionine-free medium for about 10 min at 37°C. [^{35}S]methionine (Amersham, 1,000 Ci/mmol) was added to a final level of 50 to 100 μCi/ml, and the cells were incubated for 2 to 6 h.

Immune precipitation. Our procedure has been described in detail elsewhere (21). Briefly, cells were lysed in either TNT buffer (20 mM Tris [pH 7.5], 200 mM NaCl, 1% Triton

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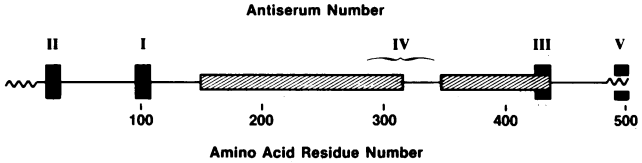

FIG. 1. p59$^{v\text{-}rel}$ antisera. p59$^{v\text{-}rel}$ is a 503-amino-acid protein, including 12 N-terminal and 19 C-terminal residues derived from the REV-A *env* gene. These are designated by the wavy lines. Antisera were raised to synthetic peptides I, II, III, and V (▮) (21, 26) and to a portion of p59$^{v\text{-}rel}$ (▨) expressed in bacteria (27). This figure is reproduced from reference 26 with the permission of Harwood Academic Publishers GmbH.

X-100] or RIPA buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.25% sodium dodecyl sulfate [SDS]) containing 1 mM phenylmethylsulfonyl fluoride and 200 Kallikrein units of aprotinin per ml. Lysates were centrifuged for 10 min at 10,000 × *g* in an Eppendorf microfuge, and supernatants were incubated with antisera and protein A Sepharose (Pharmacia, Inc.). Immune precipitates were collected, washed in either RIPA or TNT, and applied to SDS-polyacrylamide gels.

In vitro kinase assay. S3D6 cells were lysed in TNT and immune precipitated as described above. Washed precipitates were incubated for 10 min at 30°C in 30 μl of TNT containing 10 mM manganese chloride, 1 mM phenylmethylsulfonyl fluoride, 200 Kallikrein units of aprotinin per ml, 10 μM ATP, and 5 to 10 μCi of [γ-^{32}P]ATP (Amersham Corp.; 3,000 Ci/mmol). Samples were washed once in TNT and applied to a 10% gel after being boiled in loading dye.

RESULTS

Proteins that coprecipitate with p59$^{v\text{-}rel}$. In the experiments described below, we used five p59$^{v\text{-}rel}$-specific antisera (Fig. 1). Four of the antisera were raised against synthetic peptides whose sequences are contained in p59$^{v\text{-}rel}$. The remaining serum was raised against a 262-amino-acid portion of p59$^{v\text{-}rel}$ expressed in bacteria. All five antisera are able to precipitate p59$^{v\text{-}rel}$, and all have been described previously (21, 26, 27). The predicted turkey c-*rel* protein contains sequences highly related but not identical to peptides I, II, and III and to the fusion protein IV (31), and we have shown that antisera II, III, and IV are able to precipitate chicken p68$^{c\text{-}rel}$ from lysates of REV-T-transformed cells and of MSB-1 cells, which do not contain REV-T (26). Antiserum V was raised against a peptide found at the C-terminus of p59$^{v\text{-}rel}$ but which was not present in the turkey c-*rel* sequence. As expected, this antiserum does not recognize chicken p68$^{c\text{-}rel}$ (26).

In the first experiment, three antisera (III, IV, and V) were used to immune precipitate p59$^{v\text{-}rel}$ from lysates of REV-transformed chicken lymphoid cells (the S3D6 cell line). In addition to p59$^{v\text{-}rel}$, the precipitates contained several other proteins (Fig. 2). One of these had a molecular weight of 68,000 and is the chicken c-*rel* protein (26). Its apparent precipitation by antiserum V, which recognizes p59$^{v\text{-}rel}$ but not the c-*rel* protein, was a surprise, and we will discuss this result more fully later. All three antisera also precipitated proteins of molecular weights 124,000, 115,000, and 36,000. An additional protein of about 50,000 daltons was observed in this experiment but was not seen reproducibly in others. Its significance is unknown.

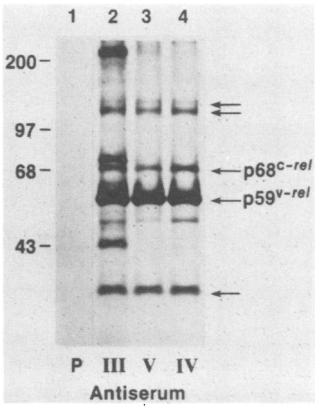

FIG. 2. Immune precipitation with three v-*rel* antisera (III, V, and IV). S3D6 cells were grown in [^{35}S]methionine, lysed in TNT buffer, immune precipitated with the indicated antiserum, and electrophoresed on a 10% SDS-polyacrylamide gel. Molecular masses (in kilodaltons) of protein size standards are shown on the left. Arrows indicate proteins coprecipitating with p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$. Several additional proteins precipitate with antiserum III, but not with antisera IV or V. These proteins also precipitate from uninfected cells and presumably represent cross-reacting cellular proteins. P, Preimmune antiserum.

To test whether the 124-, 115-, and 36-kilodalton (kDa) proteins are individually precipitable by the v-*rel* antisera, the cell lysate was boiled in 1% SDS and 1% 2-mercaptoethanol (or, in some experiments, in 1% SDS alone) before immune precipitation. This treatment destroyed precipitability of these proteins by each of the three antisera (Fig. 3A). In contrast, p59$^{v\text{-}rel}$ was precipitable by all three antisera after boiling, and p68$^{c\text{-}rel}$ was precipitable by antisera III and IV, as expected. One-dimensional V8 protease maps also

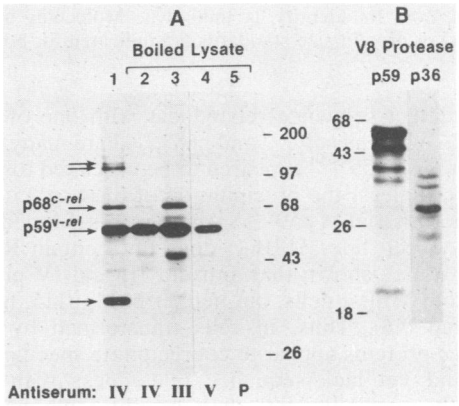

FIG. 3. p36, p115, and p124 do not appear to be related to p59$^{v\text{-}rel}$. (A) Immune precipitation of boiled lysate. S3D6 cells were grown in [^{35}S]methionine and lysed in TNT buffer. SDS was added to a portion of the lysate to a final concentration of 1%, and the mixture was boiled for 5 min. Aliquots of boiled and unboiled lysate were immune precipitated as indicated and electrophoresed on a 10% SDS-polyacrylamide gel. P, Pre-immune antiserum. Arrows indicate proteins coprecipitating with p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$. (B) V8 protease digestion of p59 and p36. S3D6 cells were grown in [^{35}S]methionine, lysed in TNT, immune precipitated with antiserum IV, and electrophoresed on a 10% SDS-polyacrylamide gel. p59 and p36 were eluted from the dried gel, digested with V8 protease (Miles Scientific), and fractionated on a 12% SDS-polyacrylamide gel (4). Molecular masses (in kilodaltons) of protein size standards are indicated for both panels.

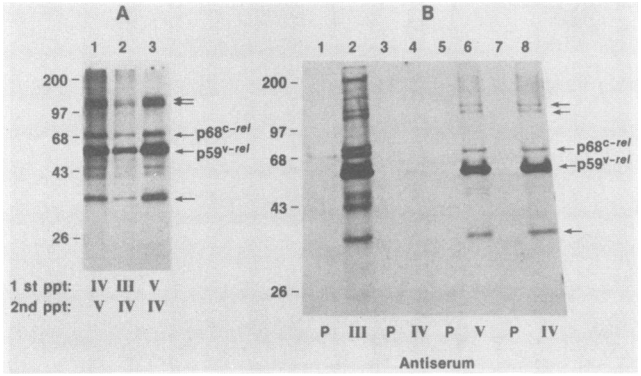

FIG. 4. Elution and reprecipitation of the complex. (A) S3D6 cells were grown in [^{35}S]methionine, lysed in TNT, and immune precipitated with antiserum III, IV, or V. After the precipitates were washed in TNT, each was incubated overnight in the presence of excess competing homologous protein or peptide (peptide III, bacterial *rel* protein IV, or peptide V, respectively). Eluates were collected, reprecipitated with a different antiserum, and electrophoresed on a 10-to-20% sucrose gradient gel. For example, the sample in lane 1 was first precipitated with antiserum IV, then eluted with protein IV, and finally reprecipitated with antiserum V. (B) S3D6 cells were grown in [^{35}S]methionine and lysed in TNT. Samples (each 5% of the total) were immune precipitated with preimmune serum (lane 1) or antiserum III (lane 2). The rest was precipitated with antiserum III, washed in TNT, and divided in two. Twenty percent of the total was incubated with excess peptide V, and the resulting eluate was reprecipitated with preimmune serum (lane 3) or antiserum IV (lane 4). Eighty percent of the total was incubated with excess peptide III. Samples of this eluate were immune precipitated with preimmune serum (lane 5) or antiserum V (lane 6). The remainder was precipitated with antiserum V and then eluted with peptide V. The resulting eluate was precipitated with preimmune serum (lane 7) or antiserum IV (lane 8). Electrophoresis of the final precipitates was carried out on a 10% SDS-polyacrylamide gel. The 68-kDa protein seen in lane 1 is not p68$^{c\text{-}rel}$. It is occasionally seen with various sera, whether preimmune or immune, whether v-*rel*-specific or not. Its identity is unknown. Molecular masses (in kilodaltons) of protein size standards are indicated for both panels.

failed to detect sequence relatedness with one of the proteins, p36. Whereas p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ share many common fragments (26), p59$^{v\text{-}rel}$ appeared to be unrelated to p36 (Fig. 3B). Nevertheless, the precipitation of p124, p115, and p36 correlates with that of p59$^{v\text{-}rel}$. Another transformed chicken lymphocyte cell line, MSB-1, does not contain REV. We have previously shown that antisera III and IV precipitate p68$^{c\text{-}rel}$ from MSB-1 cells, but neither p124, p115, nor p36 is precipitated (26). Thus, in cells transformed by REV-T, these three proteins appear to coprecipitate specifically with p59$^{v\text{-}rel}$ and yet lack sequence relatedness with it. This suggests the possibility that they are physically complexed with p59$^{v\text{-}rel}$.

If such a complex exists, the members would be expected to remain together through multiple immune precipitation steps. For example, the immune precipitate collected with antiserum III could be eluted from the antibody by excess peptide III. The p59$^{v\text{-}rel}$-containing eluate could then be precipitated with antiserum IV. Any proteins specifically bound to p59 would be expected to reprecipitate along with p59. When this experiment was performed, we found that in addition to p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$, p124, p115, and p36 all appeared in the second immune precipitate (Fig. 4A, lane 2). Furthermore, this result was not dependent on the order in which the precipitations were performed. The same proteins were seen when the first precipitation was done with antiserum IV and the second was done with antiserum V (Fig. 4A, lane 1), or when the first was done with antiserum V and the second was done with antiserum IV (Fig. 4A, lane 3).

The same result was obtained after an additional cycle of elution and reprecipitation. Figure 4B, lane 2 shows the initial precipitation of lysate by antiserum III, and lane 6 shows its reprecipitation by antiserum V after elution with peptide III. As before, p36, p59$^{v\text{-}rel}$, p68$^{c\text{-}rel}$, p115, and p124 all appear in the second precipitate. When this precipitate was eluted with excess peptide V and reprecipitated with antiserum IV, the same proteins appeared (Fig. 4B, lane 8). Thus, p124, p115, p68, and p36 copurify with p59$^{v\text{-}rel}$ through three cycles of precipitation with three different antisera. Furthermore, both the precipitation and elution steps showed specificity for p59$^{v\text{-}rel}$. For example, neither p59$^{v\text{-}rel}$ nor the associated proteins were precipitated by preimmune sera (Fig. 4, lanes 1, 5, and 7). In addition, elution required the appropriate peptide. To demonstrate this, we attempted to elute the antiserum III precipitate with peptide V instead of peptide III. The result was that there was no precipitable p59$^{v\text{-}rel}$, p124, p115, p68, or p36 in the eluate (Fig. 4, lane 4).

p68$^{c\text{-}rel}$ is a member of the complex. The preceding experiments have shown that p124, p115, and p36 show no sequence relatedness to p59$^{v\text{-}rel}$ and yet coprecipitate with it, suggesting the presence of a complex involving these four proteins. What about p68$^{c\text{-}rel}$? Since p68$^{c\text{-}rel}$ is precipitable by antisera III and IV in MSB-1 cells, which do not contain REV, its presence in immune precipitates of S3D6 cell lysate is to be expected whether it is part of a complex or not. However, the apparent precipitation of p68$^{c\text{-}rel}$ by antiserum V (Fig. 2 and 4), which does not recognize p68$^{c\text{-}rel}$ in MSB-1 cells, can only be explained if p68$^{c\text{-}rel}$ is a member of the complex. To learn the identity of the 68-kDa protein precipitated by antiserum V, we tested whether it could be reprecipitated by antisera III and IV after disruption of the complex by boiling. Under these circumstances, only proteins with sequence relatedness to p59$^{v\text{-}rel}$ were expected to precipitate. The result of this experiment was that antisera III and IV were able to precipitate not only p59$^{v\text{-}rel}$, but also the 68-kDa protein (Fig. 5, lanes 6 and 7). This is consistent with the hypothesis that the 68-kDa protein is p68$^{c\text{-}rel}$ and that it is precipitated by antiserum V by virtue of its association with p59$^{v\text{-}rel}$.

Stability of the complex. The experiments described above demonstrate that in addition to p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$, three p59$^{v\text{-}rel}$-specific antisera precipitate proteins of 124, 115, and 36 kDa from REV-transformed cells. Two additional p59$^{v\text{-}rel}$ antisera (antisera I and II) were also tested, and we found that the results depended on the buffer used to lyse the [^{35}S]methionine-labeled cells. In the experiments described above, the lysis buffer was TNT. When TNT was used with antisera I and II, antiserum II precipitated some p59$^{v\text{-}rel}$ (though less than antisera III, IV, and V) and p68$^{c\text{-}rel}$, but little or no p124, p115, or p36 (Fig. 6, lane 9). Antiserum I precipitated little or none of any of the five proteins (Fig. 6, lane 8). When the cells were lysed in RIPA buffer, both antisera I and II efficiently precipitated p59$^{v\text{-}rel}$, p68$^{c\text{-}rel}$, p124, and p115 (Fig. 6, lanes 2 and 3). Antisera III, IV, and V precipitated these four proteins as well (Fig. 6, lanes 4, 5, and 6).

The simplest explanation of these results is that in the Triton X-100-based buffer, the epitopes in p59$^{v\text{-}rel}$ recognized by antisera I and II are relatively inaccessible to antibody. Little or none of the protein was precipitated, and therefore little or none of the associated proteins was pre-

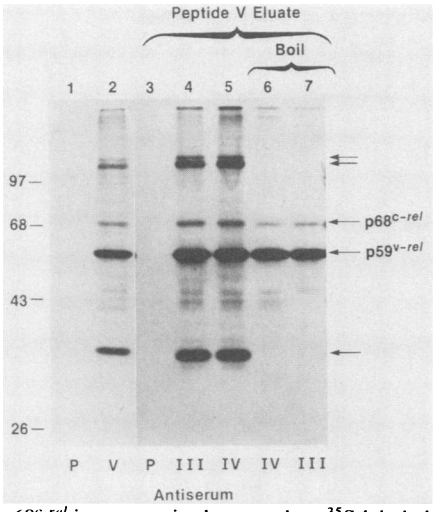

FIG. 5. p68$^{c\text{-}rel}$ is present in the complex. ^{35}S-labeled S3D6 cells were lysed in TNT, and small samples were immune precipitated with preimmune serum (lane 1) or antiserum V (lane 2). Most of the lysate was incubated with antiserum V (which does not recognize p68$^{c\text{-}rel}$), and the immune precipitate was collected as usual on protein A-Sepharose. After washing the precipitate, it was incubated overnight with excess peptide V. The eluate was collected, and samples were immune precipitated with preimmune serum (lane 3), antiserum III (lane 4), or antiserum IV (lane 5). Additional samples were placed in 1% SDS and 1% 2-mercaptoethanol and were boiled for 5 min before immune precipitation with antiserum IV (lane 6) or antiserum III (lane 7). Molecular masses (in kilodaltons) of protein size standards are indicated.

cipitated. Lysis in RIPA buffer resulted in exposure of the antisera I- and II-binding sites. Then not only did p59$^{v\text{-}rel}$ precipitate, but so did p124 and p115. However, none of the five antisera precipitated p36 when the cells were lysed in RIPA. (The 37-kDa protein precipitated by antiserum I [Fig. 6, lane 2] is not p36; it is present in uninfected chicken cells and is presumably a cross-reacting cellular protein.) One possible explanation is that in RIPA, p36 was displaced from the complex, and it is this displacement that rendered the antisera I- and II-binding sites accessible to antibody.

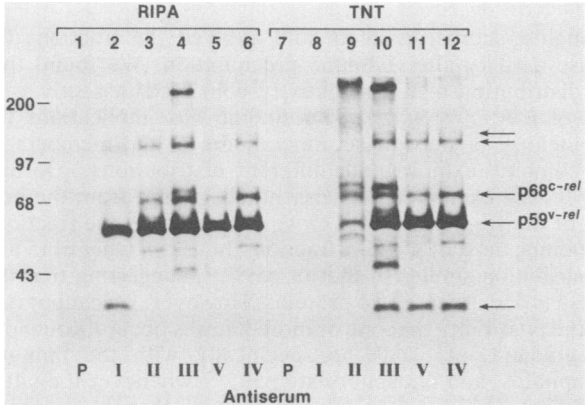

FIG. 6. The effect of lysis buffer on immune precipitation of p59$^{v\text{-}rel}$. S3D6 cells were grown in [^{35}S]methionine and lysed in RIPA or TNT as indicated at top. Immune precipitation with antisera I through V and washing of the precipitates were performed in the lysis buffer. Products were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Molecular masses (in kilodaltons) of protein size standards are indicated. P, Preimmune serum.

To determine which of the ingredients in RIPA caused dissociation of p36, ^{35}S-labeled cells were lysed in TNT to which EDTA, sodium deoxycholate, or SDS had been added. p36 was not dissociated from the complex by 2 mM EDTA or by 0.5% deoxycholate but was almost completely liberated by 0.25% SDS (data not shown).

To further probe the interaction among members of the complex, we tried several additional lysis buffers. We found that the complex was not affected by low salt (20 mM Tris [pH 7], 10 mM potassium chloride), or by TNT containing high salt (1 M NaCl), 10 mM EDTA, 1 mM EGTA, or 3% 2-mercaptoethanol (data not shown). It appears, therefore, that the complex is stabilized by factors other than divalent cations or ionic interactions. It is possible that disulfide bonds contribute to the complex. In most experiments the complex was completely dissociated by being boiled in 1% SDS (see Fig. 3A), but in others the addition of 1% 2-mercaptoethanol was required. Whether disulfide bonds occur in the native complex or form during its isolation and handling is not known.

Sedimentation of the complex. If p59$^{v\text{-}rel}$ is physically complexed with other proteins, this association should be reflected in its sedimentation behavior. Specifically, p59$^{v\text{-}rel}$ should sediment not as a protein of 59,000 molecular weight but as part of a larger entity. We have tested this hypothesis by glycerol gradient analysis of ^{35}S-labeled cell lysates. REV-T-transformed cells were grown in [^{35}S]methionine, lysed in buffer containing 10 mM Tris (pH 7.5), 200 mM NaCl, and 1% Triton X-100, and applied to 10-to-30% glycerol gradients made up in the same buffer plus 1 mM EDTA and 1 mM EGTA. After centrifugation, the gradient was fractionated and alternate fractions were immune precipitated with antiserum IV. The immune precipitates were then analyzed as usual on an SDS-polyacrylamide gel. Proteins of known molecular weight were sedimented in a parallel gradient, fractions of which were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining.

As shown in Fig. 7A, p59$^{v\text{-}rel}$ sedimented with an apparent molecular weight higher than 59,000. In addition, in those fractions containing the most p59$^{v\text{-}rel}$ (fractions 10 and 12), prominent coprecipitating proteins of 115, 68, and 36 kDa were seen. There was little or no monomeric p59$^{v\text{-}rel}$, but a significant fraction of the total sedimented even faster than that in fractions 10 and 12. This very-high-molecular-weight material differed in two respects: it (but not fractions 10 and 12) contained p124, and it contained little or no p36. These were completely reproducible results in the many gradients we have analyzed, and they suggest that there are at least two types of complexes. One apparently contains p115, p68$^{c\text{-}rel}$, p59$^{v\text{-}rel}$, and p36 (with a minimum molecular weight of 278,000), and the second contains p124, p115, p59$^{v\text{-}rel}$, and possibly p68$^{c\text{-}rel}$ (with a minimum molecular weight of 298,000).

The results of various control experiments assured us that we were not observing trapping and aggregation caused by overloading of the gradients. First, we have previously shown that in cells that do not contain REV, p68$^{c\text{-}rel}$ sediments with an apparent molecular weight of 68,000 (26). Second, purified radioactive human immunodeficiency virus p24 sedimented with an apparent molecular weight of 24,000 even after being mixed with lysate from the REV-transformed cells (data not shown). Finally, boiling of the ^{35}S-labeled lysate before centrifugation resulted in p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ sedimenting in apparent monomeric form (Fig. 7B) and in the absence of p124, p115, and p36, as expected.

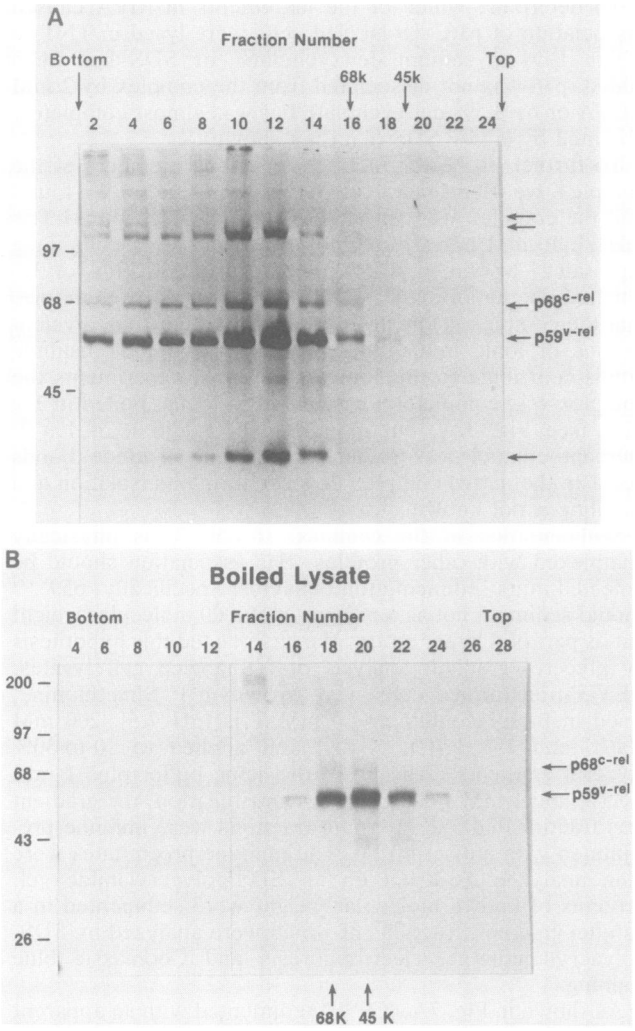

FIG. 7. Sedimentation of the complex. (A) S3D6 cells were grown in [^{35}S]methionine, lysed in TNT, and sedimented through 10-to-30% glycerol gradients in the SW 50.1 rotor at 48,000 rpm for 16 h at 4°C. Fractions were immune precipitated with antiserum IV and electrophoresed on 10% SDS-polyacrylamide gels. (B) SDS was added to the cleared lysate to a final concentration of 1%, and the mixture was boiled for 5 min prior to being centrifuged. Molecular mass standards were centrifuged in a parallel gradient. They were bovine albumin (68 kDa) and chick ovalbumin (45 kDa).

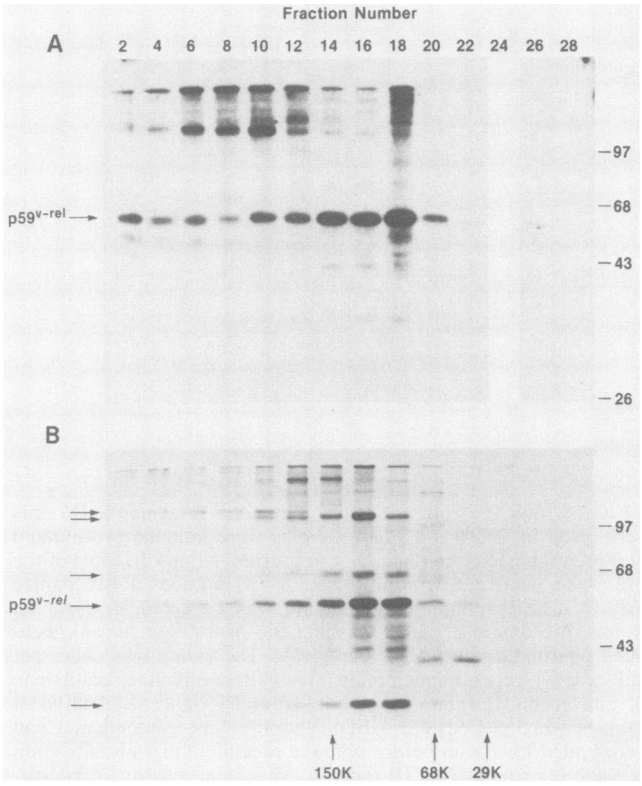

FIG. 8. In vitro protein kinase activity in immune precipitates of sucrose gradient fractions. (A) S3D6 cells were lysed in TNT, and 0.5 ml of lysate was centrifuged for 24 h at 38,000 rpm in an SW41 rotor through a 5-to-20% sucrose gradient made up in TNT. The gradient was collected in 30 fractions, and alternate fractions were immune precipitated with antiserum IV. The washed precipitates were assayed for in vitro kinase activity and the products were electrophoresed on a 10% SDS-polyacrylamide gel. Fraction 24 was lost. (B) S3D6 cells were grown in [^{35}S]methionine for 3 h, lysed in TNT, centrifuged in parallel with the sample described for panel A, and immune precipitated with antiserum IV. Precipitates were analyzed on a 10% SDS-polyacrylamide gel. Molecular weight markers (yeast alcohol dehydrogenase, 150 kDa; bovine albumin, 58 kDa; and carbonic anhydrase, 29 kDa) were centrifuged in a parallel gradient.

Sedimentation analysis clearly does not provide an accurate molecular weight of the complexes. The complex containing p36 consistently sediments significantly faster than the 68-kDa marker and the REV-A *gag* precursor but slightly slower than the REV-A *gag-pol* precursor (about 170 kDa) and yeast alcohol dehydrogenase (150 kDa). Since the calculated minimum molecular weight of this complex is 278 kDa, other factors, such as a highly nonspherical shape, must be involved.

Kinase activity. We have previously shown that immune precipitates of p59$^{v\text{-}rel}$ contain a manganese-preferring protein kinase activity that is stable to extremes of salt and to detergent washes (0.5% deoxycholate or 0.1% SDS) (21, 27). During the in vitro kinase assay, p59$^{v\text{-}rel}$ is phosphorylated predominantly on serine residues (30; Simek, Ph.D. thesis). To determine whether the kinase activity is an integral part of the p59$^{v\text{-}rel}$ complex, we sedimented S3D6 cell lysate through a sucrose gradient and assayed the fractions for kinase activity after immune precipitation. We found that the distribution of kinase activity coincided perfectly with that of p59$^{v\text{-}rel}$ (Fig. 8). This finding has implications for the identity of the kinase, since some of the members of the complex exhibit quite different distributions. p36 and p68$^{c\text{-}rel}$, for example, were essentially absent from the bottom of the gradient, and p124 was absent from the fractions containing most of p59$^{v\text{-}rel}$ (fractions 14 to 18). Only p115 had a distribution similar to that of p59$^{v\text{-}rel}$, suggesting that the kinase is one of these two proteins. However, we cannot rule out the possibility that one or more kinases occur throughout the gradient, associate nonspecifically with the immune precipitates, and phosphorylate p59$^{v\text{-}rel}$ whenever the latter is present. Our previous studies showing that the kinase is tightly associated with p59$^{v\text{-}rel}$ (21) argue against such nonspecificity, but it remains a formal possibility.

In addition to p59$^{v\text{-}rel}$, a protein of about 36 kDa was phosphorylated during the in vitro kinase reaction (Fig. 8A). We showed that it is identical in size to p36 by coelectrophoresing ^{35}S-labeled S3D6 immune precipitates with ^{32}P-

labeled kinase products in adjacent lanes on the same gel (data not shown). This is consistent with the similar distributions of ^{35}S-labeled p36 and ^{32}P-labeled p36 in the sucrose gradients (Fig. 8). A similar analysis of the high-molecular-weight phosphoprotein found predominantly in fractions 6 to 10 (Fig. 8A) suggests that it may be p124 (data not shown).

DISCUSSION

We have shown that at least three other proteins coprecipitate with p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ from lysates of REV-transformed chicken lymphoid cells. One possible explanation for this finding is that these proteins have no relatedness or functional interaction with p59$^{v\text{-}rel}$ but merely cross-react with our antisera. We think this is very unlikely because five different antisera, each raised against a distinct region of p59$^{v\text{-}rel}$, all precipitated p115 and p124, and three of the sera precipitated p36. While it is not unusual for a peptide antiserum to cross-react with several cellular proteins, it is extremely unlikely that sera raised against four different peptides would all cross-react with the same proteins.

A second possible explanation of the data is that p115 and p124 are dimers or precursors of p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ and that p36 is a proteolytic fragment of one or both of them. We believe this explanation is also unlikely for the following reasons. First, the hypothetical dimer linkage would have to be of unprecedented strength, for it survived boiling in gel loading buffer containing 2% SDS and up to 20% 2-mercaptoethanol. The duration of boiling (1 to 10 min) made no difference in the amount of p115 and p124 detected (N. Rice, unpublished observations). Second, both p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ were precipitable even after the lysate was boiled in 1% SDS. Under these conditions, p124, p115, and p36 were not precipitable, indicating that their sequences are not related to that of p59$^{v\text{-}rel}$. Third, the one-dimensional V8 protease map of p36 is unrelated to that of p59$^{v\text{-}rel}$. To date we have not been able to obtain sufficient ^{35}S-labeled p115 or p124 to be able to map them, but efforts to do so are continuing. Fourth, no high-molecular-weight forms were precipitated from MSB-1 cells, which contain p68$^{c\text{-}rel}$ but not p59$^{v\text{-}rel}$, suggesting that p124 is not simply a dimer of p68$^{c\text{-}rel}$. Fifth, sedimentation analysis demonstrated that p124, p115, and p36 cosediment with p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$, suggesting that the high-molecular-weight proteins exist within a heterogeneous complex rather than as independent homodimers.

A third possibility is that the interaction between members of the complex has no functional significance and merely represents nonspecific binding of "sticky" proteins. However, the stability of the complex argues against this interpretation. We have shown that the complex is unaffected by very low, moderate, or high salt concentrations, by chelators, or by 1% Triton X-100 or 1% sodium deoxycholate. Lysis in RIPA, which contains 0.25% SDS, resulted in dissociation of p36, but the remainder of the complex was intact. This stability approaches that of the complex between simian virus 40 large T antigen and p53 (17) and of the polyoma middle T-pp60$^{c\text{-}src}$-p81 complex (5, 6) and suggests a specific and functionally significant interaction among the members.

We therefore believe that the most plausible explanation of the data is that p115, p124, and p36 are proteins unrelated to p59$^{v\text{-}rel}$ that are specifically complexed with it in REV-transformed chicken lymphoid cells. An interesting feature of this complex is that it also contains p68$^{c\text{-}rel}$. To our knowledge, there is no other known example of a viral oncogene product interacting with its normal cellular homolog.

While immune precipitation always showed the same five proteins to be present (though occasionally the p115 and p124 bands were not well resolved), the composition of the complex is not clear. In fact, glycerol gradient analysis suggested that there may be at least two different complexes; one composed of p115, p68, p59, and p36, and the other composed of p124, p115, p59, and possibly p68. Though we have no data on this point, it is also possible that oligomeric p59, in the absence of the other proteins, contributes to the sedimentation profile. Occasionally there is a suggestion that there are additional proteins involved as well (e.g., in the range of 40 to 50 kDa), but to date these findings have not been reproducible. What is clear is that there is little or no free p59$^{v\text{-}rel}$ in the transformed cell lysates. Neither is there free p68$^{c\text{-}rel}$. This is in contrast to results obtained with MSB-1 cells, which do not contain REV, in which p68$^{c\text{-}rel}$ sediments with an apparent molecular weight of 68 kDa (26).

The size of the complex(es) cannot be deduced from sedimentation analysis. Whereas the minimum weight for the smallest complex is 115 + 68 + 59 + 36 = 278 kDa, it sediments more slowly than the 170-kDa REV-A *gag-pol* precursor or yeast alcohol dehydrogenase (150 kDa). This suggests a high frictional coefficient ratio and an elongated shape. Precedents include the herpes simplex virus regulatory protein ICP4, whose dimer form has a molecular weight of 342,000 but sediments at about the same rate as phosphorylase *b* (molecular weight, 185,000) (18, 24). Recent results of Tung et al. (29) are consistent with this interpretation. These authors fractionated lysate from REV-transformed cells on Sephacryl S-300 and found that p59$^{v\text{-}rel}$ eluted over a broad size range, with a peak at about 400 kDa. Their results differ from ours in finding only two members of the complex (p59$^{v\text{-}rel}$ and p36), but this may be due to simple factors such as quantity of lysate or conditions of ^{35}S labeling.

The identities of the proteins complexed with p59 and p68 are unknown. We do know that p36 is unlikely to be the 36-kDa pp60$^{v\text{-}src}$ substrate (8, 19). Antibodies to the latter detected a 36-kDa protein in S3D6 cells, but they did not precipitate the p59$^{v\text{-}rel}$ complex, nor did they recognize ^{35}S-labeled p36 excised from a gel (N. R. Rice, unpublished observations). Thus, all that is known of p36 is that it is a phosphate acceptor in vitro (Fig. 8) and is phosphorylated in vivo (29). Nothing is known of the two high-molecular-weight proteins, except that p124 also appears to act as a phosphate acceptor in vitro. It will be of great interest to learn whether the protein kinase activity observed in immune precipitates of p59$^{v\text{-}rel}$ can be attributed to any of the five proteins in the complex.

Knowledge of the interaction of oncogene products with cellular proteins can illuminate mechanisms of oncogenesis. The v-*sis* product, for example, is closely related to platelet-derived growth factor (23). It binds to the platelet-derived growth factor receptor, resulting in the permanent activation of the latter (15). Transformation by polyomavirus middle T antigen involves binding to and activation of pp60$^{c\text{-}src}$ (2, 6) or p62-$^{c\text{-}yes}$ (14) and apparently also binding to an 81-kDa phosphatidylinositol kinase (5). The v-*fos* product forms a complex with a 39-kDa protein (7) that has recently been shown to be the c-*jun* product (20) and to have transcriptional regulatory activity (1, 16). We are therefore hopeful that discovery of the identities of the proteins that complex with p59$^{v\text{-}rel}$ will be directly relevant to an understanding of the mechanism of transformation by REV-T. Consistent with this hope is the lack of complex formation in REV-T-infected chicken embryo fibroblasts, in which transforma-

tion does not occur (S. Simek and N. Rice, manuscript in preparation).

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